UiT
THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Science and Technology

Department of Chemistry

Iron dependent gene regulation and siderophore systems in *Vibrionaceae*

Transcriptomics, comparative genomics and phylogenetics.

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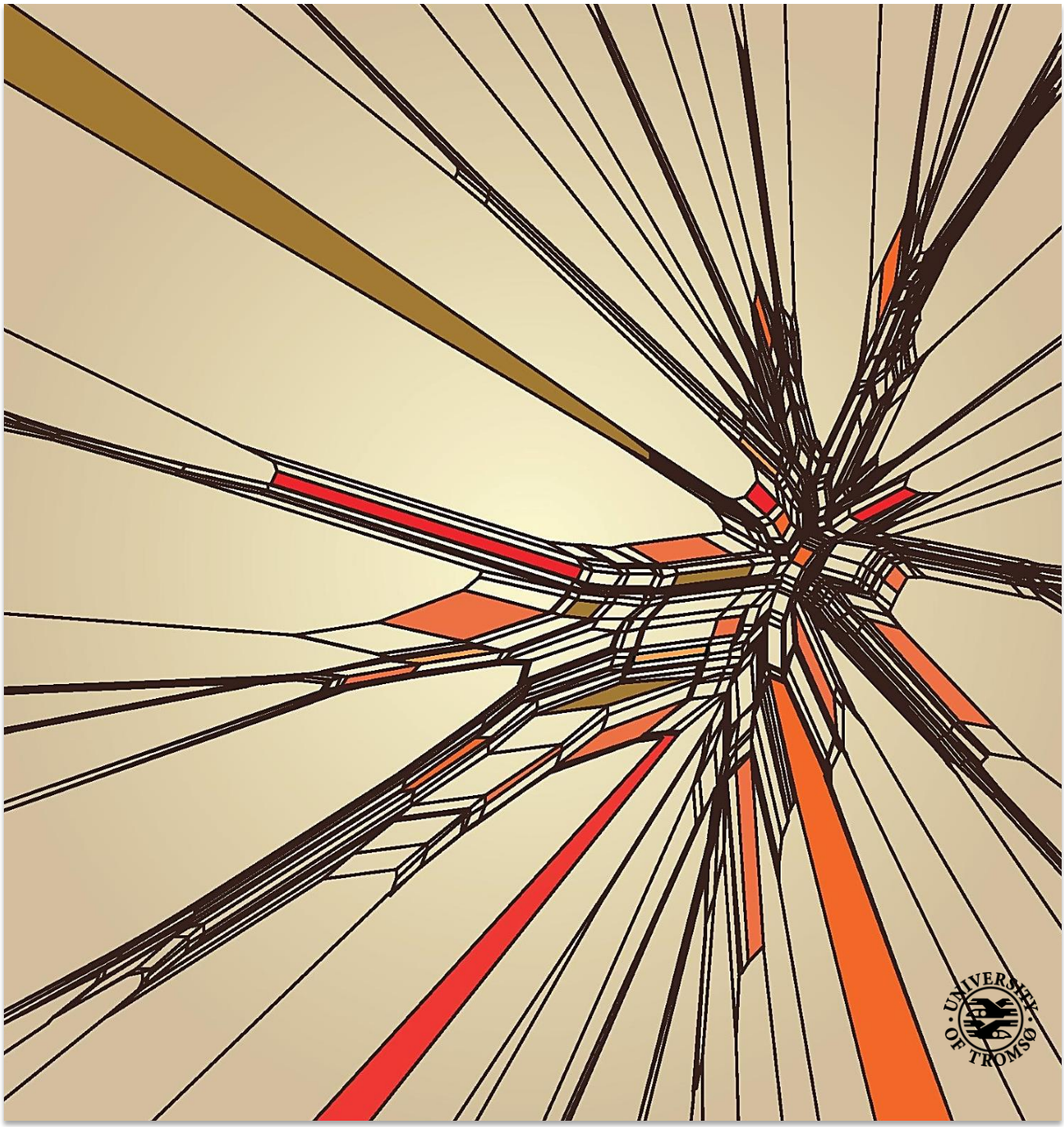
Sunniva Katharina Thode

A dissertation for the degree of Philosophiae Doctor – December 2016



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Du har din egen väg att gå, skit samma vilket håll

Det kanske blir en liten omväg, men vad spelar det för roll

Alltid lär man sig väl nåt, det var väl så det skulle va

- Lars Winnerbäck

Til Odin

Contents

Acknowledgments

The work leading up to this thesis was carried out at the Molecular Biosystems Research Group, Norstruct, Department of Chemistry, UiT – The Arctic University of Norway.

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Sunniva Katharina Thode

December 2016, Tromsø, Norway

None of the following work could have been performed without music

Abstract

Bacteria live in fluctuating environments, which they sense and respond to through gene regulation. Availably of iron is an example of an element fluctuating and that can Bacteria sense. Iron is an essential micronutrient that is scares in several environments, especially in a host. The ferric uptake regulator, Fur, is the major iron level sensing protein and regulator of iron acquisition systems in Bacteria. Fur act in a repressive manner. Under low iron conditions, Fur is inactive and iron acquisition systems are expressed. Within the *Vibrionaceae* bacterial family, there are several pathogen representatives with sophisticated iron acquisition systems. The iron acquisition systems are essential for survival and the virulence of the pathogen colonizing a host.

In the present work, the cold-adapted fish pathogen *Vibrionaceae* representative, *Aliivibrio salmonicida,* has been used as a model for understanding the Fur regulon and the immediate responses of iron limitations. Also, the siderophore-based iron acquisition systems of the *Vibrionaceae* family have been investigated. In **Paper 1**, transcriptomic studies resulted in identification of the first transcriptional responses of *A. salmonicida* to iron limitations. Pan-transcriptome studies identified shared and unique strategies to iron limitations between the *Vibrionaceae* representatives, *A. salmonicida, Vibrio vulnificus,* and *Vibrio cholerae.* In **Paper 2**, transcriptomic studies of an *A. salmonicida fur* null mutant provide deeper and more fine-grained data of the Fur regulon in *A. salmonicida.* Also, targets are predicted for the sRNA RyhB and novel small RNA predictions are provided. **Paper 3**, provide deeper understanding of the patterns, origin, distribution, and evolution of *Vibrionaceae* siderophore biosynthesis systems and receptors.

Overall, results from these studies have increased our understanding of the *A. salmonicida* Fur regulon and strategies of surviving iron limitations. For the *Vibrionaceae* siderophore based iron acquisition systems, we have broadened the knowledge of distribution of the siderophore biosynthesis systems, receptors and the evolution within the family.

List of Papers

Paper 1

Sunniva Katharina Thode, Tim Kahlke, Espen Mikal Robertsen, Hilde Hansen and Peik Haugen (2015). **The immediate global responses of *Aliivibrio salmonicida* to iron limitations.** *BMC Microbiol* 15:9

Paper 2

Sunniva Katharina Thode, Cecilie Bækkedal, Jenny Johansson Söderberg, Erik Hjerde, Hilde Hansen and Peik Haugen (2016). **Construction of a fur null mutant and RNA-sequencing provide deeper global understanding of the *Aliivibrio salmonicida* Fur regulon**. To be submitted to *PeerJ*.

Paper 3

Sunniva Katharina Thode, Mikolaj Kozlowski, Ewelina Rojek, Rafi Ahmad, Peik Haugen (2016). **Distribution, origin and evolution of siderophore systems in *Vibrionaceae*.** Manuscript

Abbreviations

aa	amino acid
ABC transporter	ATP binding cassette transporter
as	*Aliivibrio salmonicida*
ATP	adenosine triphosphate
Bp	base pair
blastP	Protein BLAST
CDS	Coding sequence
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
e.g.	for example
ENA	European Nucleotide Archive
etc.	et cetera
Feo system	ferrous iron transport system
Fur	the ferric uptake regulator
G+/-	Gram positive/negative
i.e.	that is
IM	inner membrane
Kb	kilo bases
LB	Luria Bertani broth/ Lysogen Broth
Lf	Lactoferrin
M	Molar
Mb	Mega bases
ML	Maximum Likelihood
MLSA	Multilocus Sequence Alignment
mRNA	messenger RNA
ncRNA	non coding RNA
NRPS	Non-ribosomal peptide synthase
nt	nucleotide
OM	outer membrane
RNA	ribonucleic acid
sRNA	small regulatory RNA
Tf	Transferrin
vc	*Vibrio cholerae*
wt	wild type
γ	gamma

BACKGROUND

Bacteria typically live in fluctuating surroundings, which they sense through sensing proteins and molecules, and respond to through gene regulation [2]. The response time from sensing the environmental changes to the alteration of gene expression can be crucial for survival. Minor adaptive alterations in gene expression can result from e.g. minor temperature changes and small changes in the nutrition composition. Larger changes of environment, e.g. oxidative stress (like transition from anaerobic to aerobic conditions), nutritional starvation (e.g. low iron), transition from environment into a host etc., lead to stress responses in the bacteria. Bacterial stress responses that are deployed are e.g. superoxidases, iron acquisition systems, flagella, tumbling, and swarming. Examples of factors in a bacterium's surroundings that can effect gene expression are shown in Figure 1.

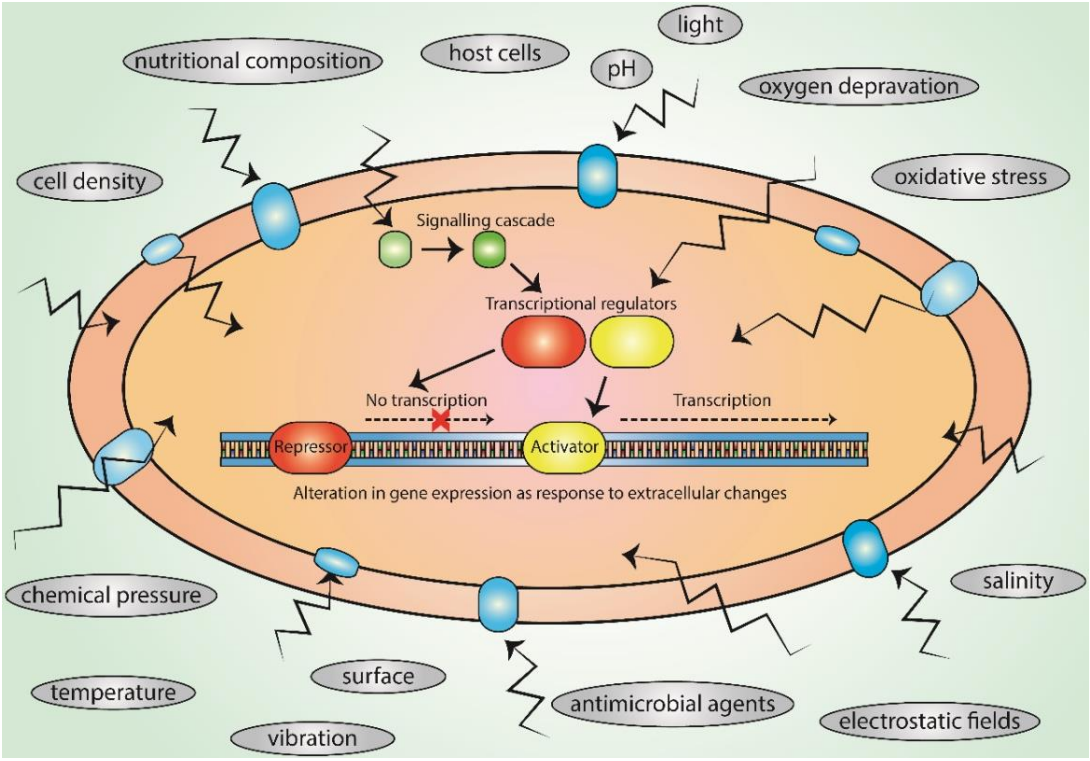

Figure 1. Environmental changes in a bacterium's surroundings is sensed by the bacterium and responded to by e.g. repression or activation of genes. Sensing can be performed by membrane proteins or by proteins within the cytoplasm.

For pathogens, the transition from being free-living to entering a host is a dramatic change of environment. In this travel, salt concentration, iron levels, nutritional composition, oxidative reagent, etc., might change dramatically. Iron is one of the most limiting factors for a pathogenic bacterium entering a host and deployment of iron acquisition systems are crucial.

The work leading up to this thesis has had focus within the Gram-negative γ-proteobacteria family *Vibrionaceae*. Two of the papers are on the transcriptional responses regulating iron homeostasis in the fish pathogen *Aliivibrio salmonicida*, and the third paper is on the siderophore based iron acquisition within the *Vibrionaceae* family. Thus, iron, iron sensing, and iron homeostasis strategies of Gram-negative bacteria will be the focus of this background. In addition, previous research on iron homeostasis in *Vibrionaceae*, with a special focus on siderophore biosynthesis and utilization, will be introduced. As the pathogenic *Vibrionaceae* are in focus, examples of low iron conditions in vertebrate hosts will be used as recurring examples to explain the sophisticated iron acquiring systems of Bacteria.

1. Iron – a necessary, but potentially harmful agent

Iron is an essential micronutrient to most microorganisms [3]. The metal plays an important role in several biological processes like electron transport, glycolysis, as cofactor for proteins, ATP synthesis, DNA synthesis, oxygen transport, and defense against toxic reactive oxygen intermediates [4, 5]. The iron concentration threshold needed to keep up such biological processes usually range from 10^{-5} to 10^{-7} M [3, 4]. The supply of free iron varies according to habitat. Iron is abundant in the earth's crust, but scares in aerobic habitats due to poor solubility at physical pH [3, 5]. The bioavailability of iron is as low as 10^{-9} to 10^{-18} M in many habitats [5]. Though iron is a key element for life sustainability, it is potentially harmful as it produces reactive radicals in aerobic conditions through the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\circ$ and $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + OOH^\circ$ [6]. The radicals can cause damages to DNA, RNA, proteins, lipids, and other cellular

components [6]. Due to irons potential harmful qualities, the uptake and intracellular processing of iron must be tightly regulated and monitored in Bacteria [6].

2. Iron homeostasis in Bacteria

For pathogens, iron availability in a host is extremely low, as restriction of free iron is one of the innate immune defense mechanisms [7]. The free iron levels in vertebrate serum are about 10^{-24} M, far below the concentration needed for the biological processes in Bacteria [8]. To overcome the challenges of low iron conditions the bacteria need iron sensing, iron acquisition, and iron holding machinery. Upon infection, the pathogens iron acquisition systems need have high affinity for iron in order to outcompete a hosts iron binding complexes. Also, to avoid toxic intracellular levels of iron, the iron uptake must also be tightly regulated. The ferric uptake regulator (Fur) is the major iron sensing and iron responding regulator in Bacteria [9].

2.1 The ferric uptake regulator (Fur)

Fur is a repressive regulator in Bacteria, acting according to intracellular iron levels. Fur is the main regulator in iron homeostasis [10, 11], but also regulates genes involved in DNA metabolism, energy metabolism, redox-stress resistance, chemotaxis, metabolic pathways, swarming, bioluminescence, production of toxins, and other virulence factors [1, 11, 12]. Thus, Fur is a so-called master regulator. Expression of *fur* is regulated by several factors, as shown in Figure 2. In *Escherichia coli* the oxidative stress response proteins OxyR and SoxS activates the transcription of the *fur* gene [13]. Down-regulation of iron acquisition systems under oxidative stress is crucial to prevent harmful oxidative radicals being formed [10, 11]. The expression of *fur* is also activated by the global regulator Crp [14]. In addition, Fur represses expression of *fur* itself in a feedback loop, and the *fur* mRNA is a target for the small RNA RyhB.

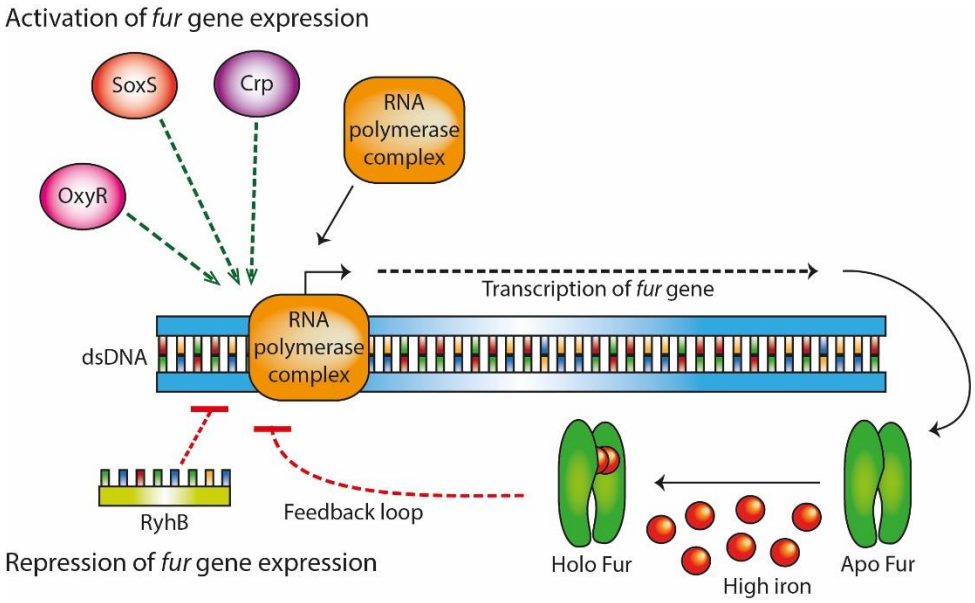

Figure 2 In *E.coli* transcription of the fur gene is activated by SoxS, OxyR and Crp. The activation promotes RNA polymerase binding to the DNA and the *fur* gene is transcribed. The *fur* gene is repressed in a feedback loop by active Fur, blocking the RNA polymerase. The small RNA RyhB targets *fur* mRNA, blocking translation.

Currently, five 3D crystal structures of Fur from *Pseudomonas aeruginosa*, *E. coli*, *Vibrio cholerae*, *Helicobacter pylori,* and *Campylobacter jejuni* are available [15-19]. These structures reveal that Fur acts as a homodimer, with zinc and iron binding sites in each monomer. The N-terminal domain of each monomer is the DNA binding domain, while the C-terminal is the dimerization domain and Fur acts as a homodimer both in apo and holo form.

2.2 The Fur regulon

Fur acts mainly as a repressor and is activated by iron as shown in Figure 3. Binding of the corepressor iron causes conformational changes to Fur, making it able to bind DNA [10]. Activated Fur recognizes and binds specific DNA codes, blocking the transcription of the associated genes. The DNA sequences recognized by Fur are called the Fur-box, or the Fur binding site. Several suggestions on the organization of the Fur-box have been proposed e.g., a palindromic 19 bp site, a sequence of three 6 bp repeats, and a 7-1-7 motif [20-23]. Fur covers a longer stretch of DNA than the binding site (suggesting interaction with DNA flanking the Fur-box) and have a tendency

to polymerize along the DNA [22]. As shown in Figure 3, the Fur-iron homodimer complex binds to the DNA in promotor regions, blocking the transcriptions of Fur regulated genes, e.g. iron acquisition genes, ensuring that the intracellular iron levels do not reach toxic levels. Under low iron conditions Fur is inactive and do not repress the expression of e.g. iron acquisition systems.

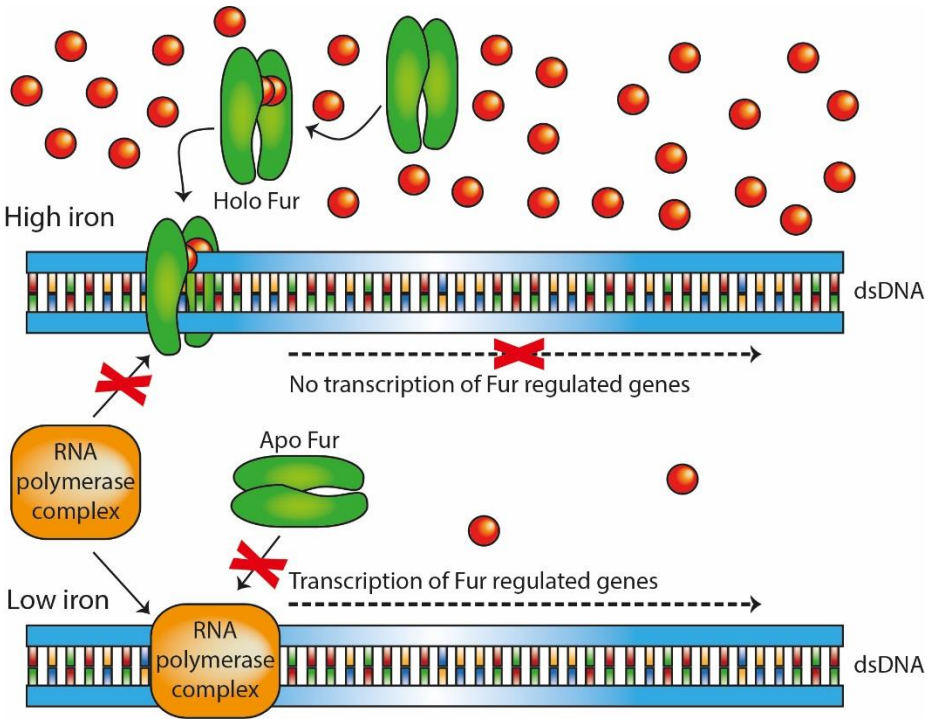

Figure 3. The Fur repression mechanism. In high iron conditions, Fur is activated by iron and bind to the Fur-box upstream of the Fur regulated genes, blocking the RNA polymerase binding site and Fur regulated genes are not expressed. Under low iron conditions, Fur is inactive and can not bind the Fur-box, The RNA polymerase binds to the transcription start binding sites and the Fur regulated genes are expressed.

In *E. coli* K-12, Fur directly regulates 131 genes including the transcription of 7 other master regulator genes, i.e., *flhD, flhC, felc, soxS, ryhB, rpoS, purR* [1]. The indirect and direct effect of Fur regulation involves 3158 genes in *E. coli*, according to ecocyc.org [1]. Thus, Fur affect approximately 70% of the *E. coli* K-12 genome, demonstrating the complexity and importance of the ferric uptake regulator. A schematic overview of direct and indirect regulation by Fur is shown in Figure 4.

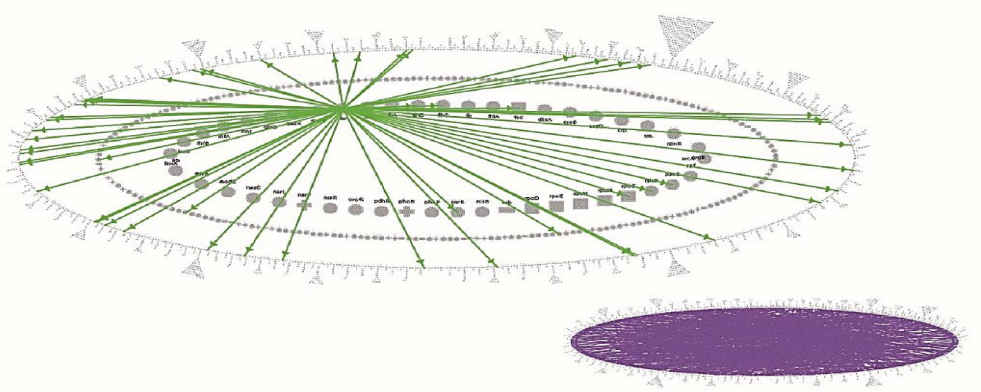

Figure 4. Regulatory network of Fur in *E. coli*. Green lines mark the 131 direct targets for Fur regulation, including 7 other master regulators (*flhD, flhC, felC, soxS, ryhB, rpoS, purR*). The small network figure with purple lines show the 3158 direct and indirect targets of Fur in *E. coli* K-12 [1].

2.3 Fur regulated small RNAs

Fur regulates the expression of small regulatory RNA (sRNA) genes in addition to protein coding genes [24]. sRNAs typically function in a post-transcriptional manner. sRNAs target mRNA, regulating its stability, targeting it for degradation and/or blocks the translation of the mRNA [25]. The best studied Fur regulated sRNA is RyhB [26]. Active Fur repress expression of *ryhB*, RyhB targets are not destabilized or degraded, and the net-effect is appearance of genes being activated by Fur. Typical targets for RyhB are the transcripts for iron using and iron storing proteins [24, 26]. According to ecocyc.org, *E.coli* K-12 RyhB directly targets the mRNA from 28 genes (shown in Figure 5), including regulation of the mRNA for the master regulators MarA and Fur [1]. Thus, resulting in an indirect regulation of 3153 genes [1].

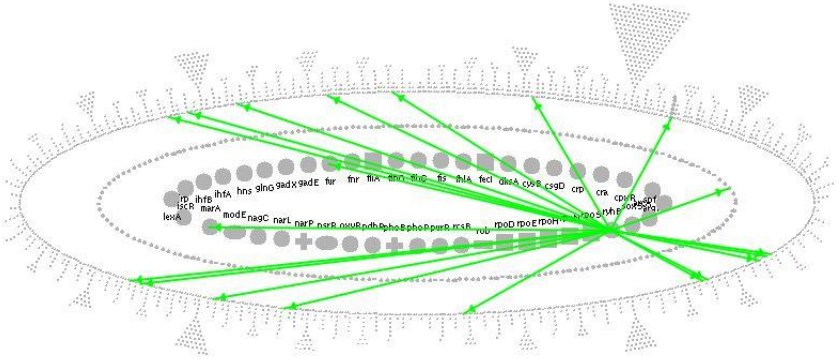

Figure 5. Regulatory network of RyhB in *E. coli*. RyhB regulates the mRNA of 28 genes including the mRNA for 2 master regulators [1].

Examples of other RyhB targets in *E. coli* are the mRNAs for the iron storage protein bacterioferrin (Bfr), bacterioferritin-associated ferredoxin (Bfd), the iron storage protein cytoplasmic ferritin (Ftn), the iron-containing superoxide dismutase (SodB), and the fumarase FumA [24-26]. In *Shigella dysenteriae*, RyhB target mRNA of VirB, a transcriptional activator of several virulence-associated genes [25, 27]. In *P. aeruginosa*, sRNAs with similar functions as RyhB are named PrrF1 and PrrF2. PrrF1&2 mutation studies showed defects in iron and heme homeostasis, alterations in biofilm formation, and that PrrF1 and PrrF2 affect virulence gene expression in the bacteria [28]. The examples illustrate the diversity and importance of RyhB, and similar sRNAs, for Bacteria. The role of RyhB is particularly important under low iron conditions, as it typically targets iron storing and iron using proteins, ensuring the iron usage being kept to a minimum.

2.4 Iron acquisition systems in Gram-negative Bacteria

Under low iron conditions, e.g. in the ocean or inside a host [7, 8, 29, 30], iron-sparing and iron-acquiring systems are necessary for survival. Fur and RyhB regulate these systems. Under low iron conditions, Fur is inactive and Fur regulated genes, e.g., iron acquisition systems and *ryhB* are expressed. Secondly, RyhB targets mRNAs for e.g., iron storing and iron using proteins. Thus, making the bacterial cell able to survive under the low iron conditions. Low concentrations of free iron in vertebrates is an innate defense mechanism against bacterial infection [7]. Vertebrates bind iron in complexes as heme, hemoglobin, transferrin (Tf) and lactoferrin (Lf), keeping the free iron levels as low as 10^{-24} M [8]. Pathogens invading vertebrates need a concentration of 10^{-5} to 10^{-7} M [3, 4] for the essential biological processes. Thus, in order to survive inside a vertebrate host, the pathogen must encode several sophisticated iron acquisition and iron sequestering systems. The different iron acquisition systems a bacterial genome may encode are described below and in Figure 6.

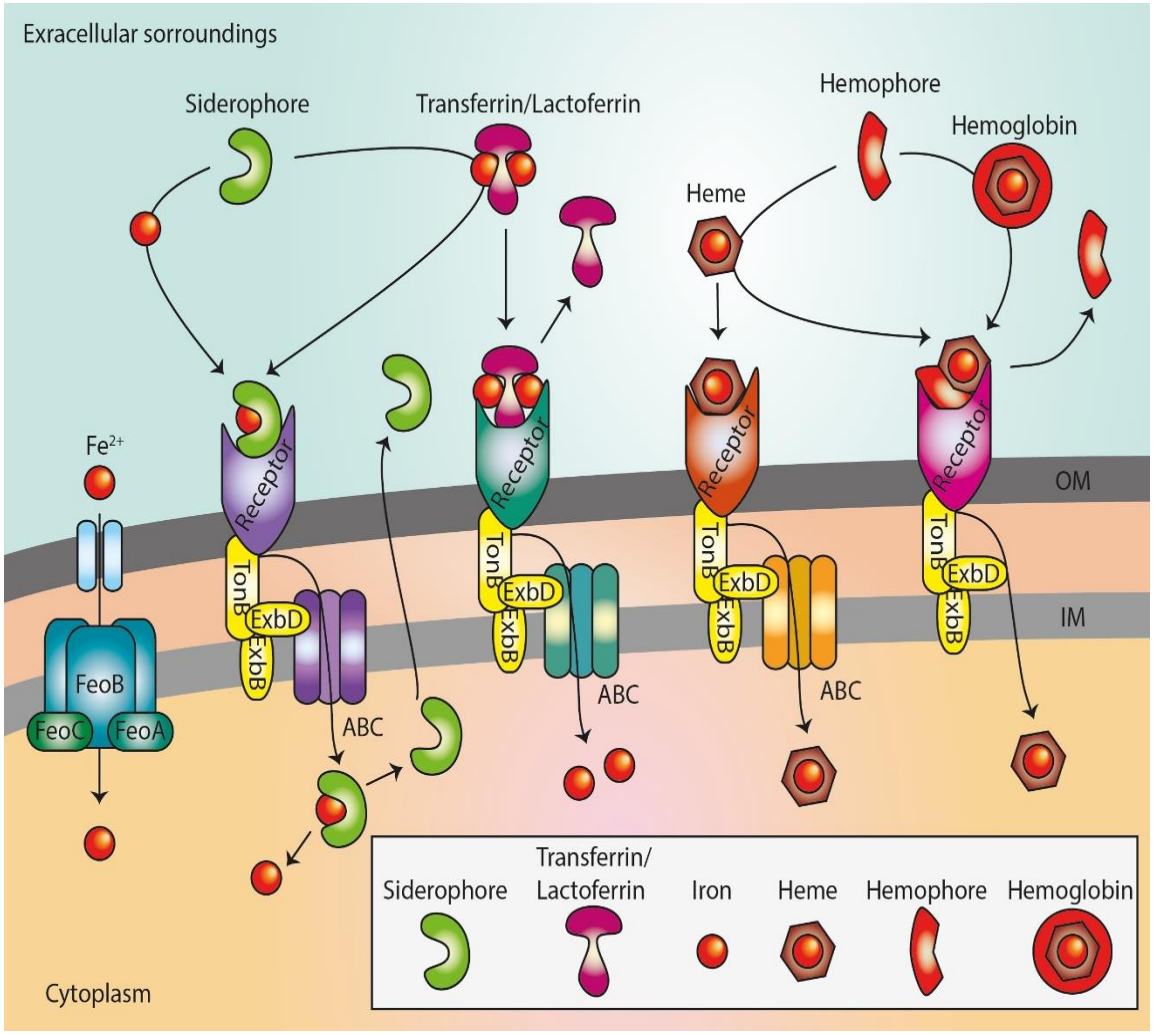

Figure 6. Simplified model of iron acquisition systems in Gram- bacteria. Details of the systems are described in the background (periplasmic shuttle proteins are not included in the figure). Ferrous iron is transported through the Feo-system. Siderophores are synthesized, secreted extracellularly to bind iron, or steal iron from host molecules like Tf and Lf. Ferri-siderophores receptors recognize and transport it over the membranes. Iron is released from the siderophore and the siderophore is re-used or degraded. Tf and Lf receptors bind Tf and Lf and transports the iron atoms over the membranes, Tf or Lf is released from receptor. Heme is recognized by heme receptors and transported over the membranes. Hemophores are produced by some bacteria, secreted extracellularly to bind heme or steal heme from heme-binding complexes. Heme-hemophores are recognized by hemophore receptors and heme is transported over the membranes. The hemophore is released from the receptor.

Ferrous iron transport. Ferrous iron transport is a simple form of iron transport. In Gram-negative bacteria, ferrous iron is believed to cross the outer membrane to the periplasmic space through undefined porins [31]. From the periplasmic space, the iron is transported over the inner membrane using the FeoA, FeoB, and FeoC proteins [31, 32]. The largest of these proteins is FeoB,

which forms a trimer channel of FeoB trimers [32]. Ferrous iron is transported through the FeoB channel over the inner membrane and into the cytosol. FeoA and FeoC are cytosolic proteins stabilizing the FeoB channel [32, 33].

Siderophore based iron acquisition. Siderophores are low molecular weight, ferric ion binding agents produced and utilized by bacteria (and fungi) under low iron conditions [34]. The molecular weight ranges from 400 to 1000 kDa, and the Fe^{3+} association constant ranges from 10^{12} to 10^{52} [5]. The biological role of siderophores is to scavenge iron from the environment, and make it available to the bacterium [34]. Siderophores are synthesized intracellular through a cascade of siderophore biosynthesis proteins [34]. The siderophores are secreted by pumps or transport proteins to the environment where it binds ferric iron [5]. Cognate siderophore receptors on the outer membrane recognize the siderophore-iron complex [34]. In Gram-negative bacteria, the siderophore-iron complex is shuttled over the membranes using the TonB complex as an energy transducer. A periplasmic shuttle protein leads the ferric-siderophore through the periplasmic space and through an ABC transporter over the inner membrane [5]. In the cytosol, iron is release from the siderophore by e.g., siderophore ferric reductases and transferred to iron using or iron storage proteins [5, 8, 35]. The siderophore is reused by secretion or degraded [8]. For vertebrate pathogens; siderophores with iron affinity equal to or higher than the iron affinity of lactoferrin or transferrin (association constant for $Fe^{3+} \approx 10^{20}$ [36] and $\approx 10^{36}$ [37], respectively) are able to outcompete these complexes [30].

Direct heme transport. Heme is a prosthetic group often bound to larger proteins like hemoglobin, myoglobin or hemopexin [30, 38]. Several bacteria have the ability to use heme as an iron source [38]. Heme is recognized by heme-binding outer membrane receptors and transported over the membranes through the receptor and ABC transporter, using the TonB system as an energy provider and a periplasmic binding protein as a shuttle protein [38]. Once inside the cytoplasm, iron is released by heme oxygenases or reverse ferrochelatase activity, or the hemegroup is used as a cofactor [30, 39, 40].

Hemophore based heme transport. Bacteria may have the ability to secrete specific heme acquisition molecules called hemophores [41]. Hemophores are extracellular proteins synthesized and secreted by some bacteria [41]. Extracellularly, they bind free heme or steal heme from hemoproteins [41]. Heme-hemophores are recognized by hemophore receptors, and heme is released from the hemophore and transported over the membranes utilizing the TonB complex as energy provider [41]. The empty hemophore is released from the receptor to the extracellular space.

Transferrin/Lactoferrin. Some pathogenic bacteria can utilize the hosts' transferrin and lactoferrin [42]. Different strategies in stealing the iron from Tf and Lf are utilized. One is where the siderophore have a similar or higher affinity for iron than Tf or Lf, thus stealing iron directly from the complexes [30, 42]. Another strategy is binding Tf or Lf to specific receptors on the outer membrane [42]. Bound to the receptor, iron is removed from Tf or Lf, shuttled over the outer membrane and periplasmic space using a periplasmic ferric ion binding protein, and through an ABC transporter over the inner membrane [42]. Tf/Lf is released from the receptor using the TonB system [42].

The TonB systems. The TonB system is needed for energy-dependent active transport of iron-bound substrates [43, 44]. The TonB systems consist of the TonB, ExbB, ExbD proteins, and sometimes a TtpC protein. The TonB systems are bound to the inner membrane of Gram-negative Bacteria [44, 45]. The TonB protein interact with TonB-dependent receptors in the periplasm and ExbB stabilize TonB to the inner membrane [45]. ExbD has three transmembrane domains that interact with TonB and ExbB. In some cases, the TonB protein is small and the addition of a TtpC protein to the system is necessary for stabilization and contact in the periplasmic space [44, 46]. Two models of the energy transducer mechanism of TonB have been proposed [45]. One model suggests TonB shuttling from the inner membrane to the periplasmic space to interact with the TonB-box of the TonB-dependent receptor. The other model suggests that the TonB protein is embedded in the inner membrane, spanning into the periplasmic space where it interact with the TonB-plug for conformational change of the receptor.

Iron storage proteins. To overcome the challenges of fluctuating iron supplies, the poor solubility of iron and irons potential toxicity, Bacteria can store iron in iron storage proteins [3]. The known bacterial iron storage proteins are ferritins, bacterioferritins and dodecameric ferritin [47]. Bacterioferritins binds iron in heme and form large 12- or 24-meric complexes with one heme group in each mer facing the inner pocket of the complex [3]. Ferritin studies have shown that expression of ferritins are induced by iron and post-exponential growth and that it can detoxify iron as well as store it. In *E. coli* the FtnA subunits form a 465 kDa complex with an inner cavity that can hold up to 2500 iron atoms [47].

Bacteria may encode some or all iron acquisition and sequestering systems described above. For pathogens, low concentration of free iron and presence of heme molecules are markers of vertebrate tissue [30]. For a pathogen to survive and colonize in a host, expression of several iron acquisition systems are crucial [7, 30]. Thus, the iron acquisition systems play an important role in the virulence of different pathogens.

3. The *Vibrionaceae* family as model organisms for studying iron homeostasis

Within the *Vibrionaceae* family, there are several well-studied pathogens with sophisticated iron acquisition systems. In November 2016, genome sequences from 110 classified Vibrionaceae species were available, with additional 14 unclassified strains [48]. The large number of genome sequences available together with several well-studied pathogen representatives makes the *Vibrionaceae* family a good target for studying iron acquisition systems. In June 2016, the family was divided in 11 genera, comprising 189 classified species [49]. The eleven genera (with number of classified species in parenthesis) are; *Vibrio* (132), *Photobacterium* (29), *Aliivibrio* (7), *Salinivibrio* (6), *Enterovibrio* (5), *Grimontia* (4), *Candidatus Photodesmus* (2), *Allomonas* (1), *Catenococcus* (1), *Echinimonas* (1) and *Photococcus* (1). Phenotypically, *Vibrionaceae* are Gram-negative γ-proteobacteria, curved or straight rods, facultative anaerobes and most of the

representatives are motile due to one or more polar flagella [50]. Their genomes are organized in two chromosomes [51] and extrachromosomal DNA is often present in the form of one or several plasmids. Representatives of *Vibrionaceae* are found in aquatic environments all around the world, where iron availability typically is low [29, 52]. Their habitats range from the freezing oceans of the Arctic to the hydrothermal vents in the deep seas, open waters, estuaries, marine sediments, rivers and other fresh water environment [50]. The majority of the *Vibrionaceae* species identified are harmless to humans and marine organisms, but the pathogens are the most studied.

3.1 *Vibrionaceae* human pathogens

Vibrionaceae human pathogens are usually transmitted by consumption of contaminated seafood or water, or it is transmitted through wounds. In humans, the pathogens may cause gastrointestinal infections, sepsis, skin and soft tissue infections [53]. Examples of human pathogen *Vibrionaceae* are; *V. cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio fluvialis*, *Photobacterium damseleae*, *Vibrio mimicus* and *Grimontia hollisae* [53]. Examples of recent news relating to *Vibrionaceae* human pathogens are shown in Figure 7. The most famous *Vibrionaceae* representative is the human pathogen *V. cholerae*, which is the causative agent of the disease cholera causing 1.4 - 4.3 million cases of disease, and 28,000 - 143,000 deaths in humans each year [54]. *V. cholerae* is transmitted through contaminated waters and is a problem in disaster-areas caused by war, natural disasters or overpopulated poverty areas. Dependent on strain, *V. cholerae* can also cause primary sepsis, wound infections, necrotizing fasciitis (flesh-eating disease), etc. [53]. *V. vulnificus* is another severe human pathogen. It causes gastroenteritis, primary sepsis, wound infections and necrotizing fasciitis [53]. It has been proposed that *Vibrionaceae* related human diseases will increase due to climate changes rising the water temperatures [55].

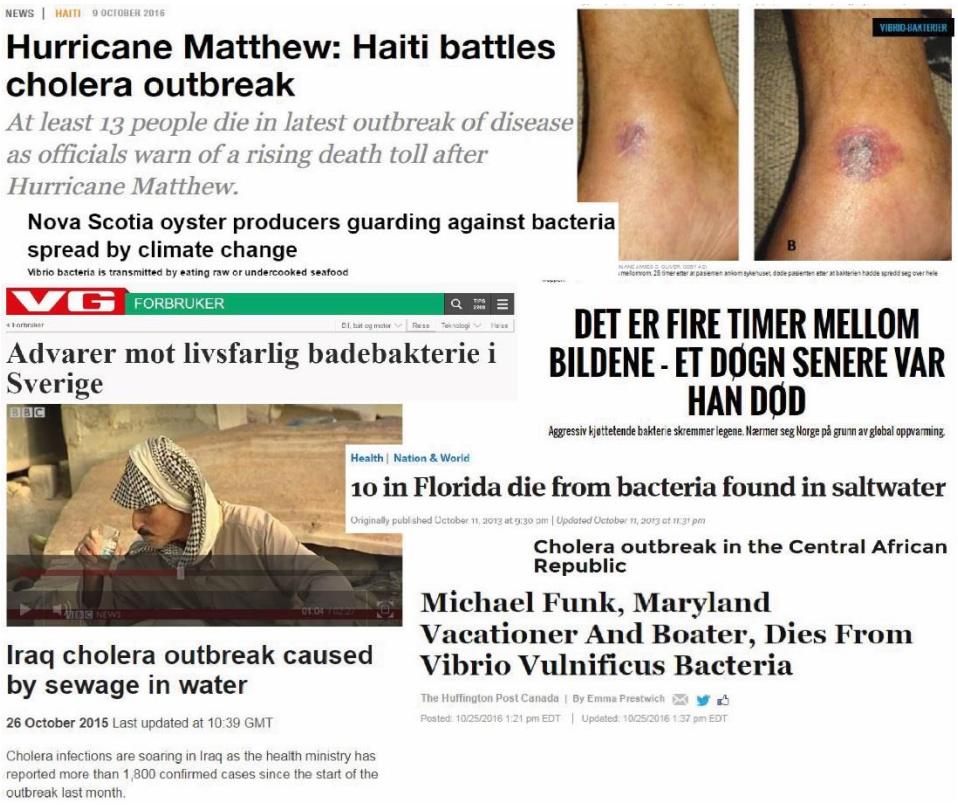

Figure 7. Headlines from recent news relating to the *Vibrionaceae* representatives *V. cholerae, V. vulnificus* and *V. parahaemolyticus.*

3.2 *Vibrionaceae* pathogens of aquatic animals

Several of the human pathogens are transmitted through contaminated seafood. *Vibrionaceae* representatives are found in aquatic environments all over the globe, and the opportunistic pathogens have different niches that are sometimes overlapping. In the aquatic environment, *Vibrionaceae* pathogens can cause disease in fish, corals, zooplankton, molluscs, shrimp, squid etc. *Vibrio shilonii*, *Vibro mediterranei* and *Vibrio coralliilyticus* can cause bleaching of corals [50]. *Vibrio anguillarum*, *A. salmonicida* and *V. vulnificus* are pathogens of several fish species and *Vibrio harveyi* is a shrimp pathogen [50].

In this work, two of the papers are focused on the *Vibrionaceae* representative *A. salmonicida,* which is a cold adapted bacterium belonging to the *Vibrionaceae* family. It is the causative agent of cold-water vibriosis (or "Hitra disease") and was first discovered in Norwegian salmonoid cultures

in the late 1970's [56, 57]. *A. salmonicida* can cause disease in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*). Characteristics of cold-water vibriosis are tissue degradation, hemolysis and sepsis [56]. Characteristics of the bacteria are that they are halophilic, psychrophilic, rod shaped, Gram-negative and can possess up to ten polar flagella [58]. The optimum temperatures for growth in liquid culture is between 10°C and 16°C, with a maximum growth temperature of 22°C [58, 59]. Outbreaks of cold-water vibriosis caused by *A. salmonicida* at temperatures above 10°C has not been reported, suggesting temperature dependent virulence [60]. Interestingly, *A. salmonicida* only produce significant amounts of siderophores at temperatures below 10°C [61]. In 2008, the complete genome of *A. salmonicida* LFI1238 was published [62]. The genome sequence revealed an organization of genes into two chromosomes (3.3Mb and 1.2 Mb) and four plasmids (85.5 Kb, 30.8 Kb, 5.4 Kb and 4.3 Kb). Several iron acquisition systems are encoded in the *A. salmonicida* LFI1238 genome, making it interesting as a cold-adapted model for studying iron homeostasis related gene regulation.

3.3 Iron homeostasis, Fur and Fur regulon in *Vibrionaceae*

It is established that iron acquisition systems are important for the virulence of pathogens. The iron homeostasis, Fur and the Fur regulon have been studied for years for several representatives of the *Vibrionaceae* family. The Ferric uptake regulator crystal structure from *V. cholerae* is solved and revealed a homodimer protein with two metal binding sites in each monomer. The metal binding site located in the DNA binding domain (Zn2 in reference) were assigned as the iron binding sites, causing the necessary conformational change for DNA binding. Fur binds to the Fur-box, blocking transcription of the downstream genes. In 2009, Ahmad et al. [63] proposed the *Vibrionaceae* Fur binding site consensus (Fur-box) as a palindromic 19 bp sequence (5'-AATGATAATNATTTCATT-3'). The consensus was used to predict the Fur regulon of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Aliivibrio fischeri* and *A. salmonicida*. They predicted 50-61 single genes and 17-20 operons (harboring 68-89 genes) to be part of the Fur regulon.

In *V. cholerae*, Fur regulate the expression of proteins of iron acquisition systems like the production of the siderophore vibriobactin, siderophore receptors and transporters, heme transport and utilization, TonB systems, iron storage proteins, and ferrous iron transport [64]. In addition to regulating expression of iron homeostasis proteins, *vc*Fur regulate regulatory proteins IrgB and VctR, enzymes FumC and SodB and different hypothetical proteins. ChIP-seq analysis in *V. cholerae* revealed that *vc*Fur regulate expression of proteins involved in multidrug resistance, sodium/dicarboxylate transporters, chemotaxis, the sigma factor RpoS, and possibly have roles in nickel and zinc regulation [21]. Also, *vc*RyhB target mRNA for proteins involved in motility, chemotaxis and biofilm formation in addition to iron using and iron storing proteins [65].

In *V. vulnificus*, Fur repress iron acquisition systems, attachment and biofilm proteins, serum resistance proteins, and various stress response proteins to cope with oxidative stress, cold/heat-shock, and acid-shock [66]. Motility, chemotaxis and toxins are directly or indirectly activated by Fur in *V. vulnificus* [66].

The TonB system, as previously described, is essential in transport of particularly siderophores and heme into the cells. *Vibrionaceae* typically encode two TonB systems, and in some cases three [45]. Both TonB1 and TonB2 systems function as energy transducers for heme transport in several vibrios, with higher efficiency using the TonB1. The TonB1 and TonB2 systems are equally important in a virulence perspective in *V. alginolyticus* and *V. vulnificus* [45]. In *V. anguillarum,* the TonB2 is "promiscuous" to siderophore transport and essential for virulence [67], indicating the importance of siderophore utilization for virulence. Siderophore biosynthesis and utilization in *Vibrionaceae* is a focus of this thesis and described in the next chapter.

3.4 Siderophore based iron acquisition in *Vibrionaceae*

Representatives of *Vibrionaceae* typically synthesize one or more siderophores. As previously mentioned, siderophores are secreted to the environment where it binds iron and ferri-

siderophores are recognized and bound by cognate receptors for translocation into the cytosol. Within the cytosol, iron is released from the siderophore and the siderophore is degraded or reused.

Hydroxamate siderophores are a class of siderophore with a hydroxamic acid as the iron-chelating moiety. *A. salmonicida* produce the di-hydroxamate siderophore bisucaberin [68]. Bisucaberin synthesis has not been reported in other representatives of the *Vibrionaceae*. The bisucaberin biosynthesis genes *bibABC* are encoded on a proposed horizontally transferred island together with a siderophore transport system where *bitA* encode the ferri-bisucaberin receptor [62, 69]. Aerobactin is a hydroxamate siderophore synthesized by *V. mimicus* [70, 71], *G. hollisae* [70, 72] and *Vibrio* sp. DS40M5 [73]. Biosynthesis proteins are encoded by *iucABCD* and the ferri-aerobactin receptor is encoded by *iutA* [71, 72]. The genome of *A. salmonicida* LFI1238 also encode an aerobactin biosynthesis operon, the operon is however of degenerate nature [62].

Catechol siderophores are siderophores with 2,3-dihydroxybenzoic acid (DHBA) as the iron-chelating moiety [74]. Vibriobactin is a catechol siderophore produced and utilized by *V. cholerae* [75]. The gene clusters, *vibABCDEFH,* encoding the vibriobactin biosynthesis proteins, are organized in two locations on chromosome I [76, 77]. The receptor ViuA bind ferri-vibriobactin to the outer membrane for translocation to the cytosol [78]. In *V. cholerae*, ViuA can also transport ferri-fluvibactin [79]. Fluvibactin and nigribactin are catechol siderophores synthesized by *V. fluvialis* and *Vibrio nigripulchritudo*, respectively [80, 81]. The genes encoding the biosynthesis for fluvibactin and nigribactin are not known. Vulnibactin is also a catechol siderophore, produced by *V. vulnificus* through proteins encoded by the gene cluster VV2_0830 - VV2_0844 [82]. The receptor VuuA bind ferri-vulnibactin for transport over the membranes [83]. *V. anguillarum* synthesize two different siderophores dependent on serotype. *V. anguillarum* serotype O2 produce the catechol siderophore vanchrobactin using VabABCEFH and DapH proteins [84]. The receptor for ferri-vanchrobactin transport is FvtA [85]. *V. anguillarum* serotype O1, containing the virulence plasmid pJM1 [86], synthesize the mixed catechol and hydroxamate siderophore anguibactin [87]. Anguibactin synthesis is encoded by *angB/GCDEHMNRTU* on the

pJM1 plasmid, with additional *angABCE* encoded in the chromosome [88]. The chromosomal *angABCE* are homologs of the *vabABCE* genes in the vanchrobactin gene cluster. In *V. anguillarum* strains containing pJM1, the vanchrobactin biosynthesis gene cluster that has been disrupted in *vabF* by a IS element, making the strains unable to produce vanchrobactin in addition to anguibactin [89]. FatA is the ferri-anguibactin receptor [90]. *V. harveyi* also synthesize anguibactin and the anguibactin biosynthesis proteins are encoded in the chromosome [91, 92].

Carboxylate siderophores are siderophores with carboxylic acid as the iron-binding moiety. Vibrioferrin is a hydrophilic carboxylate siderophore produced by *V. parahaemolyticus* [93]. The biosynthesis gene cluster for vibrioferrin is *pvsABCDE* and ferri-vibrioferrin is transported by PvuA [94, 95]. Piscibactin is a mixed carboxylate and hydroxamate siderophore synthesized by *P. damseleae subsp. piscicida* [96, 97]. Piscibactin biosynthesis is encoded by *dapH* and *Irp123459*, and the cognate receptor is encoded by *frpA* [96, 97].

Several of the *Vibrionaceae* representatives have the ability to cheat on siderophores produced by other species by encoding receptors for exogenous siderophores (also known as xenosiderophores). Production of species-specific siderophores is a form of kin discrimination, which is circumvented by the acquirement of exogenous siderophore receptors by the other species [74]. Also, if different siderophores have high similarities in the structures, they may be transported by the same receptor. Studies have shown that siderophores from one species can inhibit growth or functions of other species. The growth of *V. anguillarum* is inhibited by a siderophore secreted by *Pseudomonas fluorescens* [98] and *V. alginolyticus* swarming is inhibited by low concentrations of the siderophore avaroferrin [99]. Inhibitory effect from other siderophores may also be bypassed by the acquisition of receptors for the inhibitory siderophore. *V. alginolyticus* is potentially an excellent cheater as the genome encodes several putative siderophore receptors [99]. The genome of *A. salmonicida* encodes receptors for aerobactin and deferoxamine B as potential exogenous siderophore receptors [62]. As mentioned, *V. cholerae* can transport fluvibactin. In *V. cholerae*, fluvibactin can be

transported by the receptors ViuA, VctA, and IrgA [79]. *V. cholerae* can also transport derivatives of enterobactin through IrgA and VctA and ferrichrome by FhuA [79, 100, 101]. Ferrichrome can also be utilized by *V. parahaemolyticus* and *V. anguillarum* [74]. Aerobactin can be transported by *V. vulnificus* and *V. parahaemolyticus* [74]. Also, *V. vulnificus* can utilize vibriobactin and deferoxamine B, and *V. anguillarum* can utilize rhodotorulic acid and citrate [74].

AIMS OF STUDY

Main objective:

The main objective of this study was to use transcriptome and comparative approaches to achieve a better understanding of key pathways for iron acquisition systems of the *Vibrionaceae* family. Secondary objectives have changed along with new results.

Secondary objectives:

1) Identification of the early gene expression changes in *A. salmonicida* in the transition to low iron stress conditions
2) Using gene knock out technology and transcriptome analysis to give a detailed understanding of the Fur regulon of *A. salmonicida*
3) Provide an overview of the *Vibrionaceae* siderophore biosynthesis systems and siderophore receptors, and investigate distribution and evolution of these within the family.

SUMMARY OF PAPERS

Paper 1

The immediate global responses of *Aliivibrio salmonicida* to iron limitations

Sunniva Katharina Thode, Tim Kahlke, Espen Mikal Robertsen, Hilde Hansen and Peik Haugen

BMC Microbiology (2015) 15:9

In this paper, we studied the immediate changes in transcription as response to a sudden decrease in iron levels in cultures of *A. salmonicida*. In addition, we compared our results to similar studies in *V. cholerae* and *V. vulnificus* using a pan-genomic approach. Cultures of *A. salmonicida* were grown to mid log phase before the iron chelator 2,2'-dipyridyl was added and samples were collected after 15 minutes exposure to the low iron conditions. Microarray technology was used to monitor global changes in transcriptional levels. Using our statistical cut-off values, we retrieved thirty-two differentially expressed genes. The highest up-regulated genes belong to an operon encoding proteins for biosynthesis of the siderophore bisucaberin. A subsequent pan-transcriptome analysis revealed that nine of the up-regulated genes from our dataset were also up-regulated in datasets from similar experiments in *V. cholerae* and *V. vulnificus*, thus indicating that these genes are involved in a shared strategy to mitigate low iron conditions.

Paper 2

Construction of a *fur* null mutant and RNA-sequencing provide deeper global understanding of the *Aliivibrio salmonicida* Fur regulon

Sunniva Katharina Thode, Cecilie Bækkedal, Jenny Johansson Söderberg, Erik Hjerde, Hilde Hansen and Peik Haugen

To be submitted to *PeerJ*

In this work, we generated an *A. salmonicida fur* knock-out strain and used RNA-sequencing to compare gene expression between the wild-type and *fur* null mutant strains to provide a more accurate and deeper global understanding of the Fur regulon. In addition, we predicted novel small RNAs and predicted targets for the small regulatory RNA RyhB in *A. salmonicida*. Biological assays demonstrate that deletion of *fur* results in loss of fitness, with reduced growth rates and ability to withstand low-iron conditions, and oxidative stress. When comparing expression levels in the wild-type and the *fur* null mutants we retrieved 296 differentially expressed genes distributed among 18 of 21 functional classes of genes. A gene cluster encoding biosynthesis of the siderophore bisucaberin represented the highest up-regulated genes in the *fur* null mutant. Other highly up-regulated genes all encoded proteins important for iron acquisition. Potential targets for RyhB was predicted from the list of down-regulated genes, and significant complementarities were found between RyhB and mRNAs of the *fur*, *sodB*, *cysN* and VSAL_I0422 genes. Other sRNAs with potential functions in iron homeostasis were identified.

Paper 3

Distribution, origin and evolution of siderophore systems in *Vibrionaceae*

Sunniva Katharina Thode, Mikolaj Kozlowski, Ewelina Rojek, Rafi Ahmad, Peik Haugen

Manuscript, 2016

In this work, we performed a comprehensive literature study of *Vibrionaceae* siderophores, siderophore biosynthesis and siderophore receptors. We predicted homologs of the known systems within the family and investigated distribution, origin, and evolution of the different biosynthetic systems within the family. Firstly, we compiled the existing knowledge on *Vibrionaceae* siderophores, the corresponding siderophore biosynthesis gene systems and the siderophore receptors encoded in the genomes. We identified 8 different *Vibrionaceae* siderophore biosynthesis systems and 12 siderophore receptors. Homologous systems were identified by blast searches, and the result was then mapped onto a *Vibrionaceae* phylogeny. We identified 60 biosynthetic clusters distributed in 42 *Vibrionaceae* species and 14 unclassified *Vibrionaceae* strains, and 330 siderophore receptors in 78 *Vibrionaceae* species and 40 unclassified *Vibrionaceae* strains. The majority of taxa are associated with at least one type of siderophore biosynthesis system, some (e.g., aerobactin and vibrioferrin) of which are widely distributed, whereas others (i.e., bisucaberin and vibriobactin) are found in only one single lineage. Cognate receptors are even more widespread into many taxa. A phylogenetic analysis of two siderophore systems (piscibactin and vibrioferrin) show that the present-day distribution can be explained by an old insertion into *Vibrionaceae*, followed mainly by stable vertical evolution and extensive loss.

RESULTS AND DISCUSSION

This project started out with the main goal to investigate the regulatory mechanisms of *A. salmonicida* to maintain iron homeostasis, as part of a larger effort to understand the virulence mechanisms of the fish pathogen. As the project progressed, it became apparent that siderophore systems likely play key roles in virulence, and therefore represent a very interesting research field. Siderophore systems will consequently be the focus of this discussion. In **Paper 1**, entitled "*The immediate global responses of* Aliivibrio salmonicida *to iron limitations*", we show that expression of the siderophore biosynthesis gene cluster, responsible for production of bisucaberin, is one of the first responses to iron limitation. In **Paper 2**, entitled "*Construction of a* fur *null mutant and RNA-sequencing provide deeper global understanding of the* Aliivibrio salmonicida *Fur regulon*", we report a strong Fur regulation of the bisucaberin gene cluster. In **Paper 3**, entitled "*Distribution, origin and evolution of siderophore systems in* Vibrionaceae" we provide an overview of the known *Vibrionaceae* siderophore systems, distribution of homologs, and analysis of the evolution of some of the systems.

Here, the following topics will be discussed; relevance of the bisucaberin system to the pathogenicity of *A. salmonicida*, if acquisition of the bisucaberin lead to loss of function of the aerobactin system in *A. salmonicida*, the development of a bioinformatics workflow for prediction of catechol siderophore systems. In addition, development of a siderophore production pipeline for siderophores that can be utilized in agriculture, medical drug, and/or life sciences research will be discussed as future perspectives.

4. The siderophore bisucaberin is a potent virulence factor

The bisucaberin siderophore production system is possibly the most potent virulence factor of the iron acquisition systems in *A. salmonicida*. Interestingly, *A. salmonicida* only produce bisucaberin in significant amounts at temperatures below 10°C [61], which coincides with the temperature at

which the associated disease breaks out [60]. With this knowledge in mind, we therefore cultured the bacterium at 8°C so that results should be relevant for the associated disease (i.e., cold-water vibriosis). Also, salt concentration of 1% NaCl in growth media was chosen to mimic physiological conditions the bacterium would experience inside its natural host [102]. Caution should be taken when interpreting the relevance of *in vitro* studies to mechanisms of disease in the wild. However, with experiments under controlled conditions, we can only do our best to ensure that some of the experimental parameters are as relevant as possible. In Paper 1, cultures of *A. salmonicida* LFI1238 were grown in LB media containing 1% NaCl at 8°C to mid-log phase. The iron chelator 2,2'-dypyridyl was then added (final concentration 50 μM) to create low iron conditions. Samples were harvested after 15 minutes to monitor the immediate responses. Microarray technology was used to monitor the immediate transcriptional responses to the low iron conditions. We identified 32 differentially expressed genes, where the bisucaberin biosynthesis operon (*bibABC*) were associated with the highest fold change values (*bibA* 7.6×, *bibB* 5.8×, and *bibC* 2.2×). This shows that bisucaberin is one of the fastest responses to low iron conditions. Similarly, in Paper 2, an *A. salmonicida fur* null mutant was obtained, cultures of the *fur* null mutant and wild type were grown in LB media containing 1% NaCl at 8°C, cells were harvested from mid-log phase, and RNA-sequencing technology was used to monitor the global response of *fur* deletion. We identified 296 differentially expressed genes as a response to deletion of the *fur* gene. Again, the bisucaberin operon represented the most differentially up-regulated genes, with up-regulation of *bibA* 92.6×, *bibB* 48.2×, and *bibC* 11.1×. Taken together, we show in two independent studies that the bisucaberin system in *A. salmonicida* is fast responding to iron levels and tightly regulated by the iron-sensing Fur.

In other pathogenic bacteria, siderophore production and/or siderophore transport are important for virulence. One striking example is the human pathogen *Staphylococcus aureus*, where siderophore biosynthesis of staphylobactin is encoded by *sbnABCDEFGHI* and mutation of the biosynthesis *sbnE* gene eliminates the ability to produce siderophores [103]. An infection study

using the murine kidney abscess model showed decrease in virulence of the *S. aureus sbnE* mutant compared to wild type [103]. Virulence of *V. vulnificus* has been examined in an infant mouse model using a strain unable to produce catechol siderophores and a strain producing the catechol siderophore (now known as vulnibactin) [104]. The non-catechol producing strain showed decrease in virulence, indicating that the catechol siderophore vulnibactin is important for the pathogenicity of *V. vulnificus*. In *V. anguillarum* 775, the TonB2 system is essential for transport of the siderophore anguibactin, and virulence of the strain is decreased more than 100-folds in *tonb2* mutants [67]. In contrast, synthesis and transport of the siderophore vibriobactin is not essential for the pathogenesis of *V. cholerae* [105]. One should keep in mind, however, that *V. cholerae* encode homologs of several exogenous siderophore receptors (i.e., IutA, VctA, IrgA, and FhuA) and the piscibactin siderophore system (Figure 2, Paper 3) and can therefore potentially utilize the piscibaction siderophore or siderophores produced by other organisms.

It is interesting that the bisucaberin system is one of fastest responses to iron limitations, tightly regulated by Fur (Paper 1 and Paper 2), and that bisucaberin only is expressed at temperatures which the associated disease breaks out [60, 61]. Thus, bisucaberin is possibly the most potent virulence factor in the iron acquisition systems in *A. salmonicida.* However, further studies are required to determine the definite role of bisucaberin in the virulence of *A. salmonicida.* For example, knockout deletion strains of both the biosynthesis system and the receptor could be obtained. Subsequently, resulting mutant and wild type could be compared in an Atlantic salmon infection challenge study. If the bisucaberin system deletion strains are less virulent, or do not survive at all, the bisucaberin dependent virulence of *A. salmonicida* could finally be answered. The drawback of such a study would be, i) the considerable cost of buying and rearing live animals, and ii) the ethical implications of having a relatively large number of salmon suffering through the disease, and finally be sacrificed. We have therefore, in vain tried to develop an alternative infection model for the disease (i.e., a fish cell culture model). The temperature dependent mechanism of the bisucaberin production could also be investigated. For example, the system substituted with a

different siderophore system in the same genomic location (i.e., under the control of the same promoter). Subsequent siderophore assays could answer if the temperature dependent production is due to regulatory mechanisms, or possibly other factors (e.g., protein stability, molecule stability).

5. Did acquisition of a bisucaberin system lead to loss of function of the aerobactin system in *A. salmonicida*?

Preliminary data indicate that the bisucaberin system in *A. salmonicida* originates from another order of Bacteria. Perhaps the best argument to support this statement is that the bisucaberin system (*bibABC* and *bitA*) is located on a proposed horizontally transferred island in the *A. salmonicida* LFI1238 genome [62], and that bisucaberin synthesis is not reported in other *Vibrionaceae* representatives. During this project, we identified homologues bisucaberin systems in *Aliivibrio logei* (Paper 1) and *Aliivibrio wodanis* (Paper 2)), both of which are very closely related to *A. salmonicida.* We therefore purpose that the bisucaberin system has been introduced into *Vibrionaceae* through horizontal gene transfer into a recent common ancestor of *A. salmonicida*, *A. wodanis* and *A. logei* (transfer indicated by red arrow in the *Fischeri* clade in Figure 2, Paper 3). The bisucaberin system was not identified in other *Vibrionaceae* representatives (Paper 3), further supporting a recent introduction of the system. We have tried to identify the origin of the bisucaberin system. Using the amino acid sequences of BibABC as queries in blastP (Protein BLAST) we identified homologs in the *Shewanellaceae* family (Paper 3). Using BibA and BibB as queries, we get hits in e.g. *Shewanella algae* with scores 57% and 60% identity (98% and 97% coverage), respectively. Using BibC as query, we get hits in e.g., *Shewanella baltica* and *Shewanella putrefaciens* with 60% identity over 74% coverage. In addition to these hits, we get significant hits in several other representatives of *Shewanellaceae* (e.g, *Shewanella oneidensis*, *Shewanella xiamenensis,* and unclassified *Shewanella* strains). For each individual search we get hits in other bacterial families, however when we consider the system as one (i.e., BibABC), we only get hits in the *Shewanellaceae* family. In addition, comparison of chemical structures show that bisucaberin

is structurally similar to the siderophores putrebactin and avarofferin, produced by *S. putrefaciens* and *S. algae*, respectively [99, 106]. Interestingly, *S. putrefaciens* can simuntanolusly produce bisucaberin, avaroferrin, and putrebactin with precursor directed siderophore biosynthesis, [107]. Taken together, using the amino acid sequences of bisucaberin system we find homologs in several *Shewanella* representatives, there are similarities in structures of bisucaberin and *Shewanella* siderophores, and *S. putrefaciens* can produce bisucaberin with precursor direction. Thus, a *Shewanellaceae* strain is a possible origin of the bisucaberin system. However, the hypothesis cannot be confirmed or discarded, until the genome sequence of a more convincing donor for the bisucaberin system is available.

The *A. salmonicida* LFI1238 genome contains an aerobactin siderophore system (*iucABCD* and *iutA*) [62]. However, the operon is degenerated, and two of the biosynthesis genes are annotated as pseudogenes. The aerobactin biosynthesis genes and aerobactin receptor are not differentially expressed under low iron conditions (Paper 1), nor as a response to deletion of *fur* (Paper 2). Thus, the entire system (including its promoter) is most likely non-functional and the set of genes in the operon should be annotated as pseudogenes. The chain of events that resulted in the loss of function of the aerobactin system in *A. salmonicida,* and the acquisition of the bisucaberin system, is at best unclear. One possibility is; that the bisucaberin system was acquired due to loss of a functional aerobactin system. Alternatively; the loss of function of the aerobactin system was due to redundancy, after the introduction of the bisucaberin system. To distinguish between the two possibilities we need to look at the facts we have available. *A. wodanis* and *A. logei* also encode the aerobactin system (Paper 3), and none of the aerobactin genes are annotated as pseudogenes. This supports that the loss of function happened after the evolutionary splits between these species. In other words, mutations in the aerobactin system accumulated after the speciation of *A. salmonicida.* To further investigate the chain of events, we investigated the evolutionary history of the aerobactin system itself. E.g., is the presence of this system in aliivibrios due to one or more insertion events? I.e., has the aerobactin been introduced once and been followed by stable

inheritance? Evolutionary history was investigated as follows; First, (i) we used bioinformatics to repair the amino acid sequences of the aerobactin biosynthesis proteins IucA and IucC, using the corresponding pseudogenes as templates in *A. salmonicida.* Then, (ii) we constructed an IucABCD Maximum Likelihood (ML) tree from sequences retrieved in Paper 3 (see Supplementary file S1, Paper 3). Finally, (iii) we juxtaposed the IucABCD tree to a corresponding host tree based on multilocus sequence alignment (MLSA) files (kindly provided by Dr. Sawabe [108]). Briefly, sequences were aligned using ClustalW and concatenated using Splitstree4. Next, Mega6 was used to generate ML trees from concatenated alignments (for more details, see Material and Methods in Paper 3). Figure 8 show the juxtaposed host and IucABCD phylogenies.

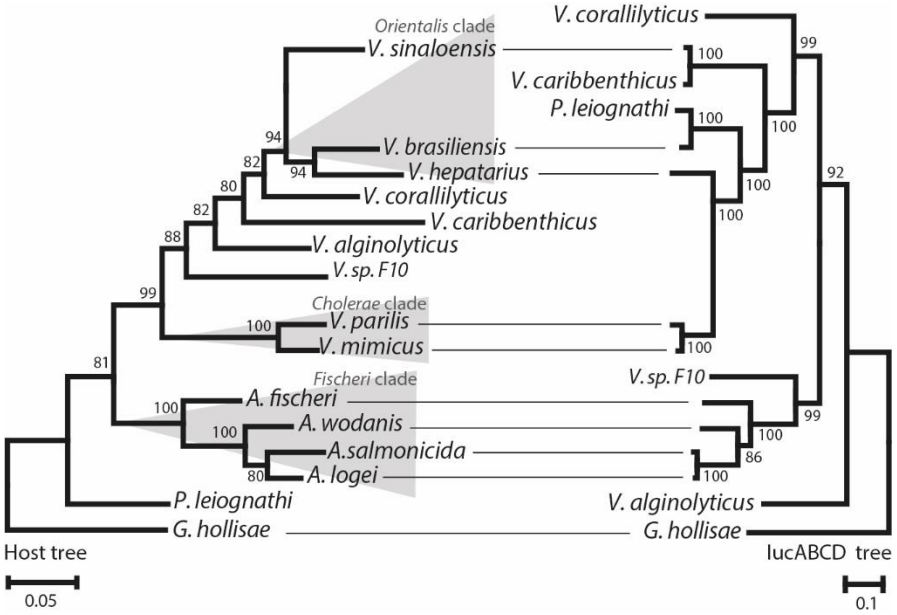

Figure 8. Inheritance of the aerobactin biosynthesis cluster (IucABCD) in *Vibrionaceae.* Host phylogeny on the left and aerobactin phylogeny on the right. Analysis were conducted in Mega6 and trees were generated using the ML method. Bootstrap values are shown at the nodes (JTT model, 2000 replicates).

Nodes in the trees are highly supported by 80-100% bootstrap values, and the tree topologies of the MLSA based host phylogeny and the concatenated IucABCD phylogeny are similar for the *Fischeri* clade. Thus, indicating that the aerobactin biosynthesis system was introduced to a common ancestor of the *Fischeri* clade, followed by stable inheritance. This further supports the hypothesis

that loss of function of the *A. salmonicida* aerobactin system occurred after acquisition of the bisucaberin system, and after speciation of *A. salmonicida*.

To conclude, the bisucaberin system is located on a proposed horizontally transferred island in the *A. salmonicida* LFI1238 genome. We also find homologs of the system in *A. logei* and *A. wodanis*, and the system was probably acquired by a common ancestor of *A. salmonicida*, *A. logei*, and *A. wodanis*. Origin of the bisucaberin system is still unclear, but homology searches point to a *Shewanellaceae* representative as donor. The *A. salmonicida* LFI1238 contain a non-functional aerobactin system. The operon and promotor is degenerated by several mutations, at the mutations probably accumulated as a result of redundancy after the acquisition of the bisucaberin system.

6. A bioinformatics workflow for discovery of catechol siderophore systems

We have used a bioinformatics approach to discover and identify previously unknown/undescribed catechol siderophore biosynthesis gene clusters. The method is based on efforts presented in Paper 3, where we use a combination of database searches and manual curation to find siderophore systems. Known *Vibrionaceae* clusters encoding the biosynthesis system for catechol siderophores consist of 7-11 genes, the genes are located on both strands, and not necessarily in immediate proximity to each other (see Figure 1B, Paper 3). All catechol clusters contain four genes encoding isochorismate synthase (e.g., *vabC*, *vibC* and *angC*), isochorismatase (e.g., *vabB*, *vibB* and *angB*), 2,3-dihydro-2,3-dihydrobenzoate dehydrogenase (e.g., *vabA*, *vibA* and *angA*), and 2,3-dihydroxybenzoate-AMP ligase (e.g., *vabE*, *vibE* and *angE*). This pattern is also reported by Fischbach and co-workers [109]. In addition, the *Vibrionaceae* catechol siderophore gene clusters encode at least one non-ribosomal peptide synthase (NRPS), and siderophore receptor or transport genes are located in close proximity to the synthesis cluster. In our work, homology searches using the amino acid sequences corresponding to the known *Vibrionaceae* catechol siderophore biosynthesis clusters revealed that a number of *Vibrionaceae* representatives encode the four "core"

catechol proteins, i.e., isochorismate synthase, isochorismatase, 2,3-dihydro-2,3-dihydrobenzoate dehydrogenase, and 2,3-dihydroxybenzoate-AMP ligase, without encoding the remaining genes of the known *Vibrionaceae* siderophore systems. We set out to investigate if we could utilize this common gene pattern of known *Vibrionaceae* catechol siderophore gene clusters, to predict unknown or undescribed catechol siderophore gene clusters.

Currently, there is no dedicated computer software available that can consistently predict previously undetected biosynthetic clusters. Our workflow to mine genomic sequences for siderophore systems is described here. First, we retrieved genome sequences of *Vibrionaceae* representatives from ENA (European Nucleotide Archive), and subjected them to an antiSMASH [110] analysis to identify NRPS clusters. Next, genomic regions surrounding positive hits for NRPSs were examined manually for presence of the four "core" enzymes (isochorismatase, isochorismate synthase, 2,3-dihydroxybenzoate-AMP ligase, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase) found in *Vibrionaceae* catechol siderophore biosynthesis gene clusters [109]. This was specifically done by blastP queries, using the amino acid sequences belonging to the genes in the NRPS clusters as queries. Finally, in order to be considered as a complete system, a gene encoding siderophore transport, or siderophore utilization proteins, had to be located in close proximity to the biosynthesis genes. *V. fluvialis* and *V. nigripulchritudo* produce the catechol siderophores fluvibactin and nigribactin (Figure 1C, Paper 3), respectively [80, 81]. However, the genes responsible for their production are unknown. Thus, genome sequences of *V. fluvialis* and *V. nigripulchritudo* were subjected to the workflow described above. In addition, the genome sequences of *Vibrio furnissii*, *Vibrio auzerus and Vibrio natriegens* were analyzed because homology hits of catechol biosynthesis gene cluster indicated that they potentially produce catechol siderophores that are not described. Figure 9 shows the predicted catechol siderophore biosynthesis gene clusters found in *V. fluvialis* ATCC33809, *V. nigripulchritudo* SFn1, and *V. azureus* NBRC 104587. For *V. furnissii* NCTC11218 (NCBI ref seq: NC_016602.1), a cluster highly similar to that in *V. fluvialis* (see Figure 9) were predicted in chromosomal region 15709 – 27395. In addition, for

V. natriegens NBRC15636 (NCBI ref. seq.: NZ_ATFJ01000040.1) we predict a catechol siderophore biosynthesis cluster in the nucleotide region 1,517,745–1,540,877.

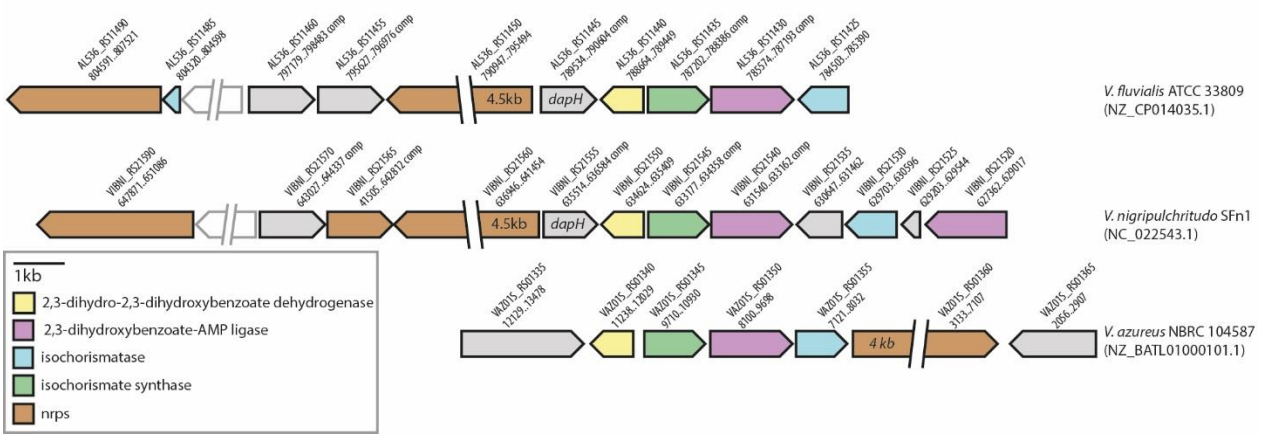

Figure 9. Predictions of previously undescribed catechol siderophore biosynthesis gene clusters in *V. fluvialis*, *V. nigripulchritudo* and *V. azureus*. Genome accession numbers are shown below strain number in parenthesis, locus tag and nucleotide range for each gene are shown above their corresponding gene.

Fluvibactin and nigribactin (produced by *V. fluvialis* and *V. nigripulchritudo*, respectively) are two siderophores with structures similar to vulnibactin and vibriobactin (see Figure 1C, Paper 3). The clusters predicted in *V. fluvialis* ATCC33809 and *V. nigripulchritudo* SFn1 (Figure 9) are similar, but still different, when compared to the clusters encoding the vulnibactin and vibriobactin clusters. Thus, we have possibly identified the biosynthetic clusters for fluvibactin and nigribactin biosynthesis. The cluster we predicted in *V. furnissii* NCTC11218 is organized identical to that of *V. fluvialis*. Because *V. furnissii* and *V. fluvialis* are phylogenetically related, the system has most likely been introduced into a relatively recent common ancestor. Our results also indicate that *V. natriegens* NBRC15636 and *V. azureus* NBRC 104587 produce catechol siderophores. In order to verify the results, strains presented here should be experimentally tested for catechol siderophore production by e.g. colorimetric determination as described by Riuoux et al. [111]. The molecular structures of fluvibactin and nigribactin are known, thus structure-specific determination of the compounds is possible, and strains *V. fluvialis* ATCC33809, *V. furnissii* NCTC11218 should therefore be examined for fluvibactin production, and strain *V. nigripulchritudo* SFn1 should be examined for nigribactin production. Knockout deletion studies can further verify if the genes are

in fact responsible for catechol siderophore synthesis. Suspected gene cluster can also be cloned and expressed heterologous in e.g. a non-catechol producing *E. coli* strain, and subsequently examine that strain for catechol siderophore production.

Our future goal is to identify all catechol siderophore biosynthesis gene clusters within *Vibrionaceae*, then use this knowledge to investigate other bacterial families, and experimentally verify our predictions. Similar approaches for hydroxamate and carboxylate siderophore biosynthesis gene clusters might be possible, but with our selection presented in Paper 3, we could not see clear patterns. Therefore, we will collect and compare hydroxamate and carboxylate siderophore biosynthesis gene clusters originating from other bacterial families to investigate if we can set up a similar workflow for prediction of these types of clusters.

7. Future perspectives

One of our future goals is to develop a cost-efficient siderophore production pipeline. The idea is to use a community of marine bacteria collected from environmental samples from the Arctic region as microbial factories, to convert rest-raw material from the fish industry to valuable bacterial secondary metabolites, such as siderophores, pigments, terpents, lipids, etc. Siderophores have a relatively wide range of potential uses, ranging from research in life sciences to agriculture and medicinal drugs. For example, only 0.1-1% of the worlds bacteria are estimated as culturable, using traditional laboratory culturing approaches. Siderophore based culturing approaches has proven useful, to promote, e.g., growth, cultivation, and purification, of otherwise unculturable microorganisms [112]. In agriculture, siderophores can be utilized to promote plant growth, as a biocontrol against plant pathogens (phyto-pathogens) and an agent of heavy metal bioremediation [112]. In medicine, siderophore based drugs can be used in the treatment of iron overload, aluminum overload, cancer, and malaria [112]. Desferrioxamine B, marketed under the name Desferal, is an example of a siderophore in use. Desferal is used to remove excess iron in thalassemia patients suffering iron overload as a result of treatments [34]. In addition, utilizing siderophores

and their cognate receptors to deliver drugs intracellularly, in a Trojan-horse strategy, is a sophisticated and elegant approach for both drug delivery and siderophore application [113].

For development of the siderophore producing pipeline, we aim to use microbial communities comprising of sequenced strains that are available to us. In our libraries, we have approximately 100 fully sequenced marine bacterial strains and trial studies of siderophore production by these strains are promising. We will set out to identify the siderophores produced, predict the siderophore biosynthesis gene clusters, and investigate the best composition of strains for siderophore production. We will also investigate the optimum rest-raw material for siderophore production, and use stable continuous cultures (method is under development) for siderophore production.

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