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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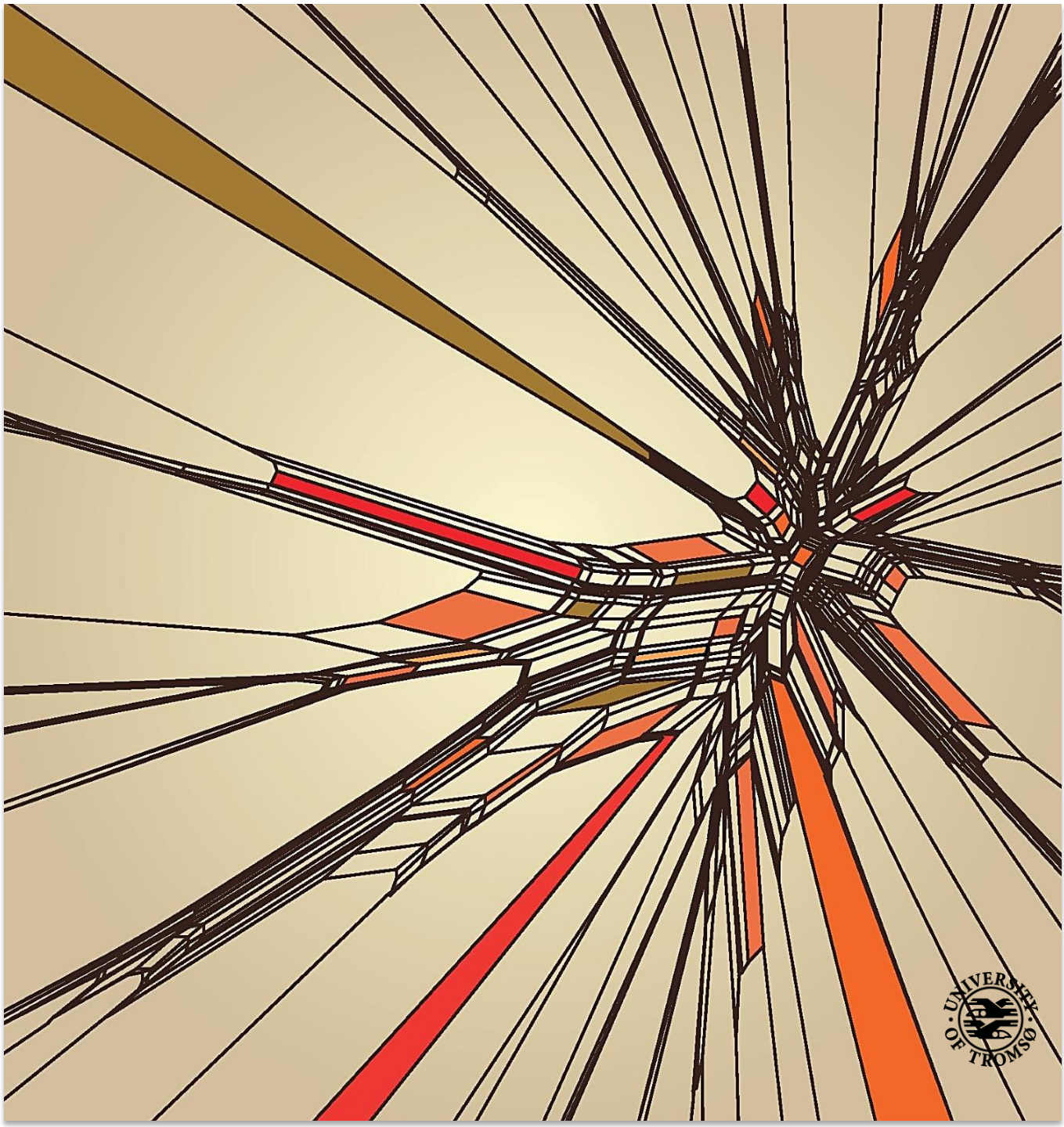
Sunniva Katharina Thode

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.

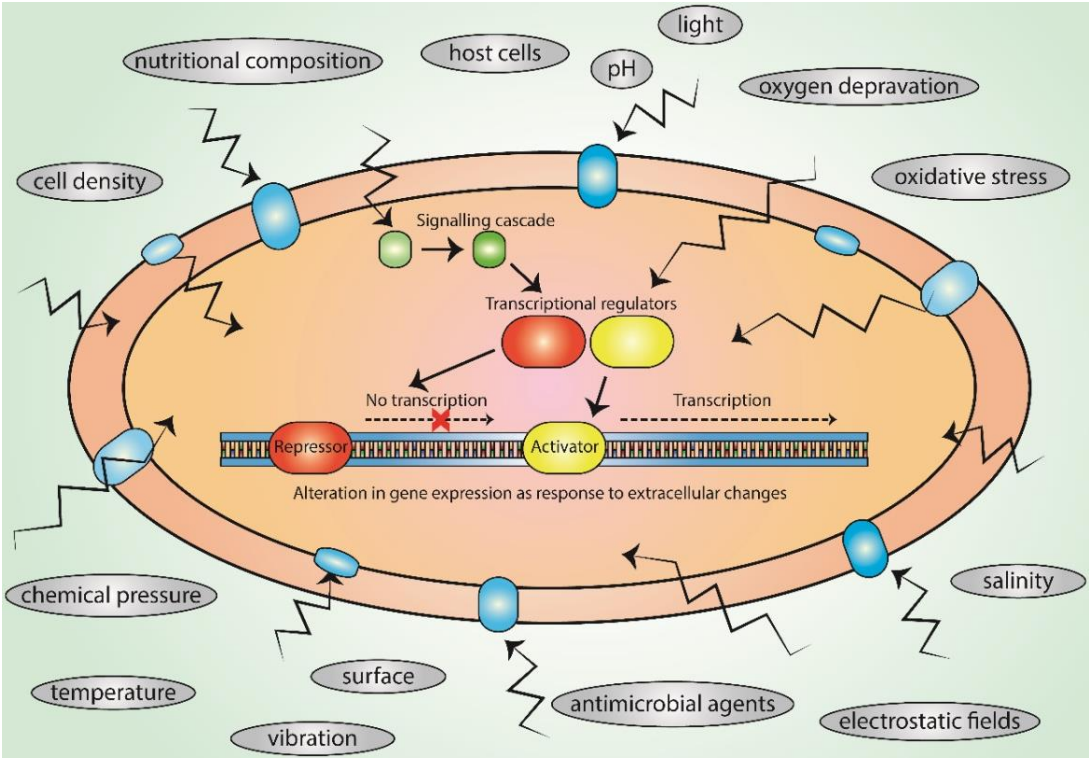

Figure 1. Environmental changes in a bacterium's surroundings is sensed by the bacterium and responded to by e.g. repression or activation of genes. Sensing can be performed by membrane proteins or by proteins within the cytoplasm.

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.

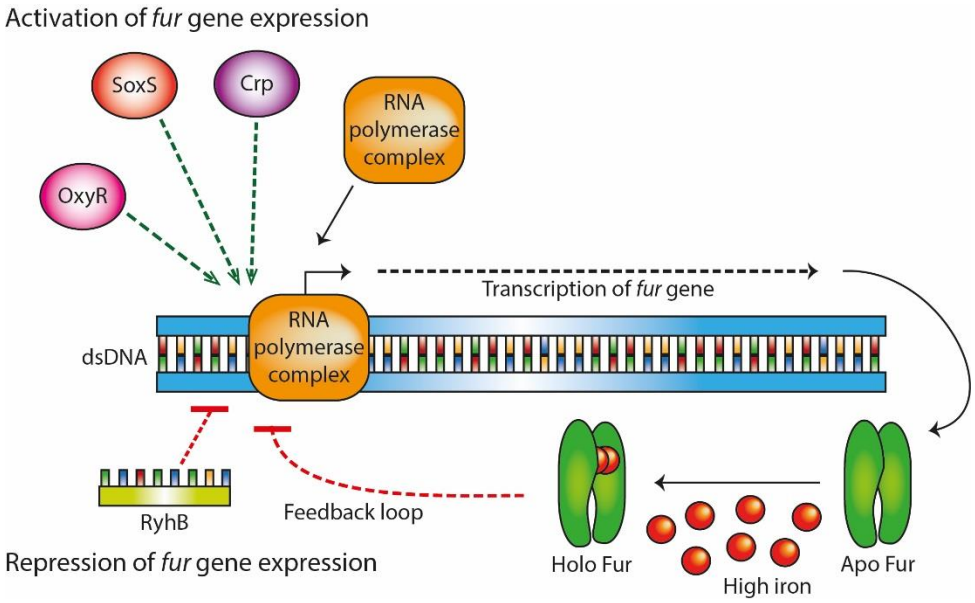

Figure 2 In *E.coli* transcription of the fur gene is activated by SoxS, OxyR and Crp. The activation promotes RNA polymerase binding to the DNA and the *fur* gene is transcribed. The *fur* gene is repressed in a feedback loop by active Fur, blocking the RNA polymerase. The small RNA RyhB targets *fur*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

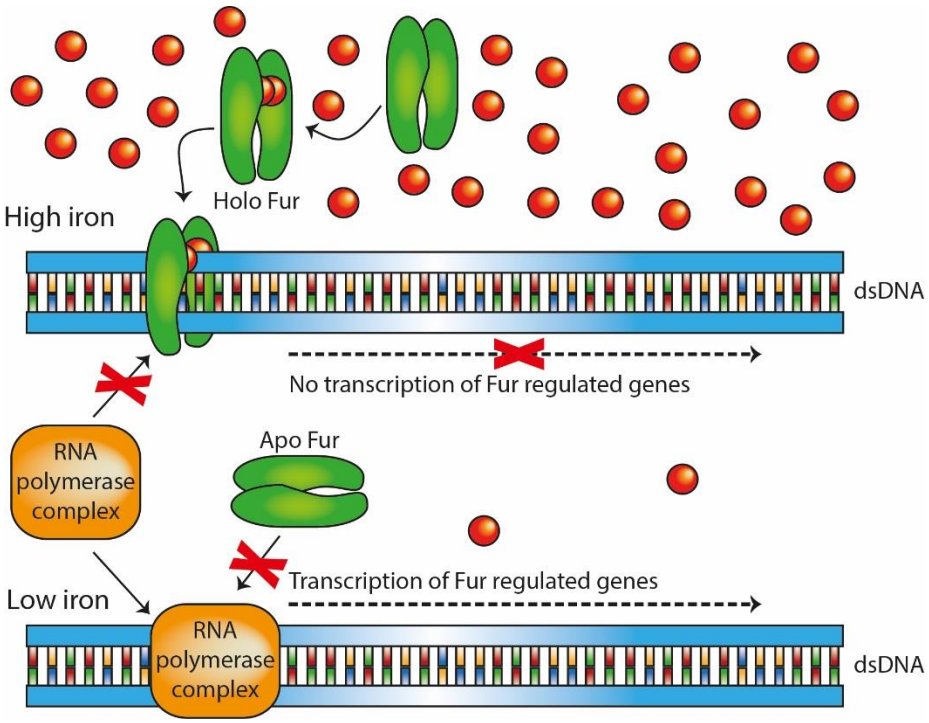

Figure 3. The Fur repression mechanism. In high iron conditions, Fur is activated by iron and bind to the Fur-box upstream of the Fur regulated genes, blocking the RNA polymerase binding site and Fur regulated genes are not expressed. Under low iron conditions, Fur is inactive and can not bind the Fur-box,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

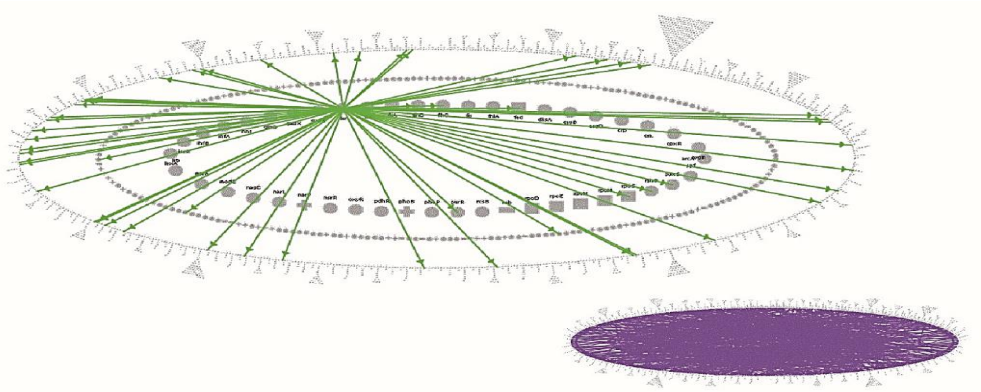

Figure 4. Regulatory network of Fur in *E. coli*. Green lines mark the 131 direct targets for Fur regulation, including 7 other master regulators (*flhD, flhC, felC, soxS, ryhB, rpoS, purR*). The small network figure with purple lines show the 3158 direct and indirect targets of Fur in *E. coli* K-12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

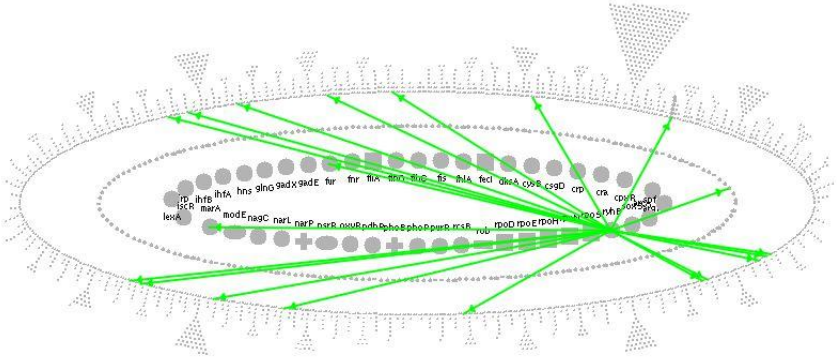

Figure 5. Regulatory network of RyhB in *E. coli*. RyhB regulates the mRNA of 28 genes including the mRNA for 2 master regulators [1].

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.

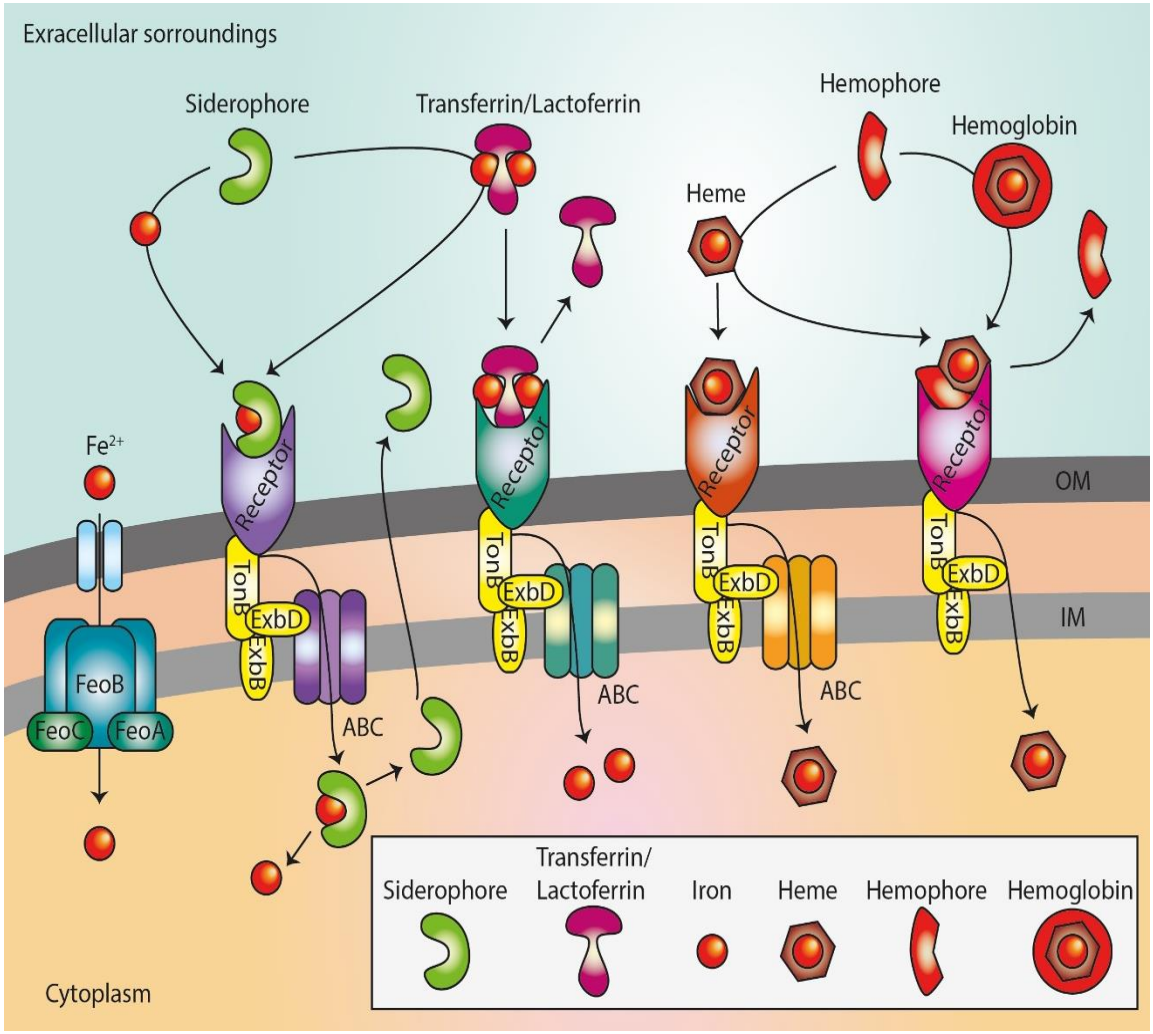

Figure 6. Simplified model of iron acquisition systems in Gram- bacteria. Details of the systems are described in the background (periplasmic shuttle proteins are not included in the figure). Ferrous iron is transported through the Feo-system. Siderophores are synthesized, secreted extracellularly to 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].

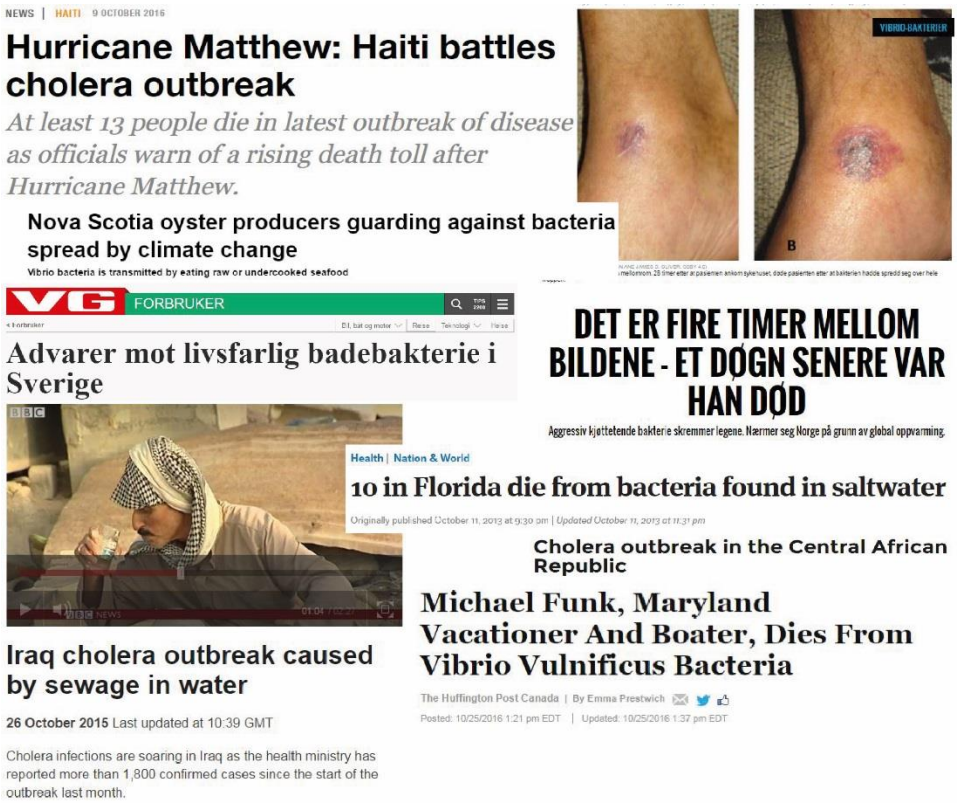

Figure 7. Headlines from recent news relating to the *Vibrionaceae* representatives *V. cholerae, V. vulnificus* and *V. parahaemolyticus.*

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.

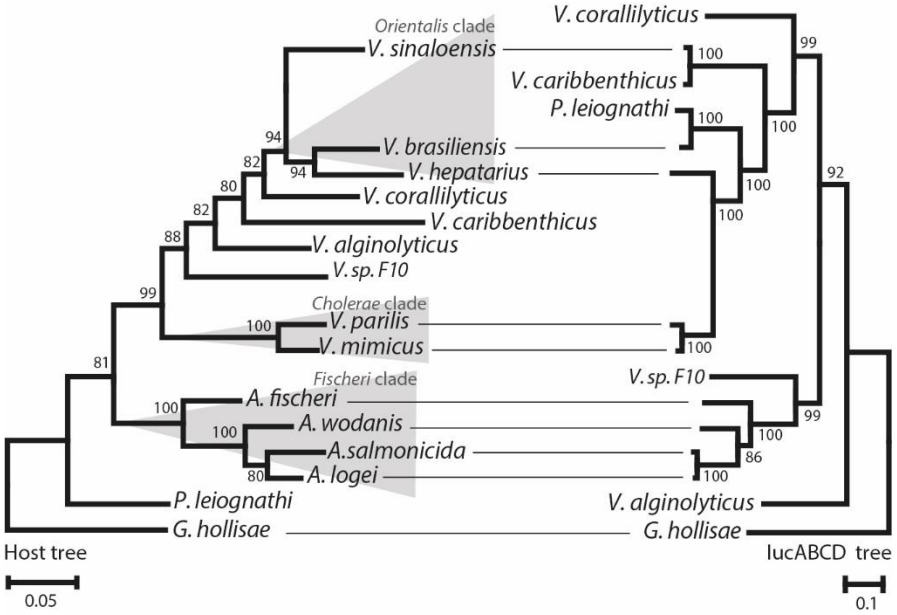

Figure 8. Inheritance of the aerobactin biosynthesis cluster (IucABCD) in *Vibrionaceae.* Host phylogeny on the left and aerobactin phylogeny on the right. Analysis were conducted in Mega6 and trees were generated using the ML method. Bootstrap values are shown at the nodes (JTT model, 2000 replicates).

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.

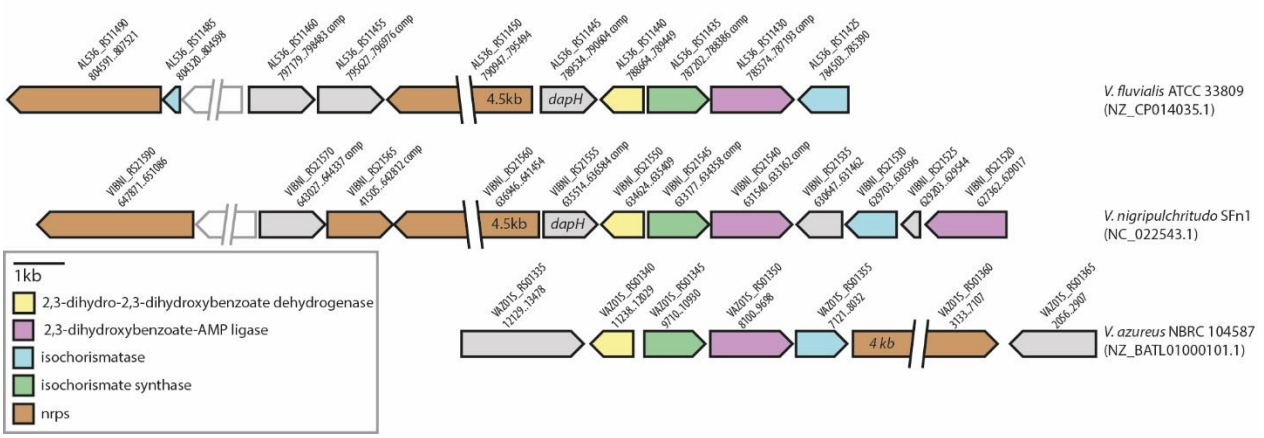

Figure 9. Predictions of previously undescribed catechol siderophore biosynthesis gene clusters in *V. fluvialis*, *V. nigripulchritudo* and *V. azureus*. Genome accession numbers are shown below strain number in parenthesis, locus tag and nucleotide range for each gene are shown above their corresponding 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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Paper 1

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

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Abstract

Background: Iron is an essential micronutrient for all living organisms, and virulence and sequestration of iron in pathogenic bacteria are believed to be correlated. As a defence mechanism, potential hosts therefore keep the level of free iron inside the body to a minimum. In general, iron metabolism is well studied for some bacteria (mostly human or animal pathogens). However, this area is still under-investigated for a number of important bacterial pathogens. *Aliivibrio salmonicida* is a fish pathogen, and previous studies of this bacterium have shown that production of siderophores is temperature regulated and dependent on low iron conditions. In this work we studied the immediate changes in transcription in response to a sudden decrease in iron levels in cultures of *A. salmonicida*. In addition, we compared our results to studies performed with *Vibrio cholerae* and *Vibrio vulnificus* using a pan-genomic approach.

Results: Microarray technology was used to monitor global changes in transcriptional levels. 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 of growth. Using our statistical cut-off values, we retrieved thirty-two differentially expressed genes where the most up-regulated genes belong to an operon encoding proteins responsible for producing 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 using *V. cholerae* and *V. vulnificus*, thus indicating that these genes are involved in a shared strategy to mitigate low iron conditions.

Conclusions: The present work highlights the effect of iron limitation on the gene regulatory network of the fish pathogen *A. salmonicida*, and provides insights into common and unique strategies of *Vibrionaceae* species to mitigate low iron conditions.

Keywords: *Aliivibrio salmonicida*, Iron homeostasis, Ferric uptake regulator, Siderophore, Bisucaberin, Microarray

Background

Iron is an essential micronutrient for all living organisms [1-3], and withholding of iron is recognized as a first line of defence against microorganisms (e.g., bacteria) [4,5]. Extremely low iron concentrations create an efficient barrier against potential invading pathogens that may have entered the organism through, for example, an open wound on the skin surface. This defence strategy puts extraordinary pressure on invading pathogens to carry extremely efficient mechanisms to sequester iron from within the host [3,6]. Iron acquisition systems are therefore regarded as important virulence factors. Low iron conditions force pathogens into a stress mode, which results in the down-regulation of genes that encode iron-using and iron-storage proteins, and up-regulation of genes involved in iron acquisition [4,7,8]. Consequently, pathogenic bacteria often express and utilize multiple iron sequestering systems ranging from siderophore based systems, heme uptake systems and systems for uptake of free iron [8].

Although iron is an essential micronutrient, high concentrations of iron in the presence of oxygen are potentially harmful due to formation of oxidative radicals [9]; thus, influx and intracellular processing of iron must be tightly regulated. The ferric uptake regulator (Fur) is the main regulator of genes involved in iron uptake, storage and metabolism, and acts in an iron-dependent manner

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[10-13]. In *E. coli,* Fur acts mainly as a transcriptional repressor: at high iron concentrations it binds iron and forms homodimers which suppress the transcription of genes involved in a wide range of metabolic functions. Genes regulated by Fur not only include genes directly involved in iron homeostasis, but also DNA and energy metabolism, redox stress resistance, chemotaxis, bioluminscence and production of toxins and other virulence factors [2,10,14,15]. Fur is therefore regarded as a global regulator. Finally, it is also well established that Fur can indirectly activate gene expression by blocking the expression of the small RNA named RyhB, which typically targets mRNA for degradation. For example, RyhB targets the *fur* mRNA in a feedback regulation loop, and also targets mRNA that encode iron-using or iron-storing proteins like Bfr, SodB and FumA [16].

Fur recognizes and binds to a site on the DNA known as the Fur-box. Several alternative hypotheses for Fur-boxes have been proposed; for example a palindromic 19 bp site, three 6 bp repeats, and 7–17 motif [17-20]. In 2009, Ahmad and co-workers suggested a *Vibrio* Fur binding site consensus to be 5′-AATGATAAT NATTTCATT-3′ [21]. This *Vibrio* consensus is similar to the suggested Fur box in other bacteria like *Bacillus subtilis*, *Yersinia pestis*, *E. coli* and *Pseudomonas aeruginosa* [22-25].

The importance of iron, and the elaborate regulation of iron uptake and homeostasis in bacterial cells in general, has prompted a number of researchers to study the roles of iron with regard to bacterial virulence and pathogenicity. In two recent studies, global responses to low iron conditions in cultures of *Vibrio vulnificus* and *Vibrio cholerae* (both human pathogens from the diverse family *Vibrionaceae*) were studied [11,26]. Here, cultures of *V. cholerae* and *V. vulnificus* were grown to mid log phase with iron chelators included in the growth medium from the beginning of the experiments. The results from these two experiments showed up-regulation of genes involved in siderophore biosynthesis and transport: TonB systems, heme transport and utilization, ferrous iron transport, and superoxide dismutase. In addition, the *V. vulnificus* experiment showed an up-regulation of a Tad-1 cluster.

We are studying the roles of iron in another *Vibrionaceae* representative, *Aliivibrio salmonicida. A. salmonicida* is the causative agent of cold-water vibriosis, and possesses several iron acquisition systems that may be important for its pathogenicity [27]. This assumption is based on the observation that the bacterium only produces significant amounts of siderophores when grown at or below 10°C [28,29], which coincides with the observation that outbreaks of cold-water vibriosis are normally associated with temperatures below 10°C [28]. Another intriguing feature of *A. salmonicida* is that it produces the dihydroxamate siderophore bisucaberin that has not yet been found in other *Vibrionaceae* representatives [30,31]. The bisucaberin biosynthesis genes (VSAL_I0134-I0136) in *A. salmonicida* strain LFI1238 are located on a genomic island that has likely been acquired by horizontal gene transfer from an unknown source [27]. The genome of the LFI1238 strain also harbors another siderophore biosynthesis system (VSAL_II0273-VSAL_II0278), which is commonly found in *Vibrionaceae*. However these latter genes are assumed to be disrupted and are annotated as pseudogenes [27]. Also, the transport of siderophores is carried out through siderophore receptors, and the energy for transport of the iron-siderophore complex across the membrane is provided by TonB systems. *Vibrionaceae* genomes usually contain 2–3 TonB systems [32-34]. The *A. salmonicida* genome encodes three TonB systems [27], where the TonB1 gene VSAL_I751 (*tonB1*) contains a frame-shift mutation and likely produce a non-functional protein.

Here, we have studied the immediate global responses in cultures of *A. salmonicida* to low iron conditions using microarray, and compared the results with comparable studies in *V. cholerae* [11] and *V. vulnificus* [26] using a pan-genome approach. In the two latter studies long-term responses to low iron was monitored (using microarray). We hypothesize that it is the immediate phase that is most critical for bacterial survival during iron starvation. Hence, we wanted to identify the genes that are first affected by low iron conditions, and avoid secondary effects such as unrelated stress responses. Our results provide new insights into how *A. salmonicida* responds to low iron conditions.

Methods

Bacterial strains, culture conditions and sampling

A. salmonicida strain LFI1238 [27] was cultured in LB medium containing 1% NaCl (Luria-Bertani broth Miller, Difco) at 8°C with 200 rpm in all experiments. To determine the optimal concentration of iron chelator 2,2'-dipyridyl (Sigma-Aldrich), *A. salmonicida* was grown to an optical density (600 nm) of 0.4 before the culture was split into 6 separate flasks. One flask was kept as control whereas 10–500 μM 2,2'-dipyridyl was added to the remaining cultures.

For Northern blots and microarray analysis (see below), six individual colonies (i.e. biological replicates) of *A. salmonicida* LFI1238 were grown until they reached an OD_{600} of approximately 0.5. The replicates were then split into two sub samples: one of these parallels was kept as the control, while 50 μM of the iron chelator 2,2'-dipyridyl was added to the other half. Samples were harvested after 15 min, spun down and frozen for later use.

Total RNA purifications

For microarray analysis and Northern blotting total RNA was extracted from the cell pellets using IsolRNA (5prime) and DNA was removed using the DNA-*free* kit (Applied Biosystems). DNase-treated total RNA was subsequently run through RNeasy MinElute Cleanup columns (Qiagen) to remove any remaining contaminants, and to further concentrate the RNA. The RNA was finally dissolved in 16 μl RNase free water.

Microarray analysis

cDNA was made by using the Amino Allyl Labeling cDNA Kit as described by the manufacturer protocol (Applied Biosystems). Reactions contained 18 μg total RNA. cDNA samples were labeled with the CyDye™ Post-Labelling Reactive Dye Pack (GE healthcare). Control samples (i.e. untreated samples) were labeled with Cy3, which produces green fluorescent light when scanned (at 532 nm), and treated samples were labeled with Cy5, which produces red light (at 635 nm). Two of the six slides were labeled in the opposite manner, and were used as dye-swap controls to adjust for unequal labeling efficiencies between the fluorescent dyes.

The labelled samples were hybridized to "*Vibrio salmonicida* V1.0.1 AROS" slides (Eurofins MWG Operon) at 42°C for 20 hours on a TECAN HS4800 hybridisation station, and microarray slides were subsequently washed, once in 0.1 × SSC/0.1% SDS for 5 min at 42°C, then once in 0.1 × SSC/0.1% SDS for 10 min at room temperature, and finally four times in 0.1 × SSC for 1 min at room temperature. Slides were scanned using a GenePix 4000D scanner (Axon Instruments Inc.) at 532 and 635 nm. Images were explored and initial data analyses were performed by using the GenePix Pro v6.1 software. The final analysis of expression data was done using the R-based Limma software.

Northern blot analysis

Northern blot analysis was used to validate the microarray expression data. Treated and untreated RNA samples from each of the six biological replicates were pooled. Ten μg total RNA was separated on 1.2% denaturing formamide agarose gels, and run at 90 V for four hours in 1× MOPS buffer at 4°C. RNA was next transferred to a Hybond-N+ nylon membrane (Amersham) by capillary transfer. Selected gene specific dsDNA was amplified using PCR and labelled with [α-32P] and was used as probes according to the Amersham Megaprime DNA labeling system kit (GE healthcare). Hybridizations were performed over-night at 42°C using ULTRAhyb hybridization solution (Applied biosystems) and signals were acquired on phosphoimaging screens (Fujifilm) and scanned using a BAS-5000 phosphoimager (Fujifilm). Quantification of signals was done using the ImageGauge software v4.0 (Fujifilm) in profile mode. The intensities of the different bands were normalized to 16S rRNA probes.

Computation of core, accessory and unique transcripts

To compare our microarray results with data from similar experiments using *V. vulnificus* strain CMCP6 [26] and *V. cholerae* strain 0395 [11], the protein sequences of differentially expressed genes were downloaded from GenBank (using geneID numbers). For *V. cholerae* the geneIDs are from the genome sequence of *V. cholerae* O1 N16961 and not strain O395 that was used in the experiment. Accession numbers for the *V. cholerae* O1 N16961 are AE003852 and AE003853, accession numbers for *V. vulnificus* CMCP6 genome are AE016795 and AE016796, and finally accession numbers for the *A. salmonicida* LFI1238 are FM178379 and FM178380. OrthoMCL [35] was used to identify core genes, i.e. genes present in all three genomes. Genes up-regulated in two datasets were denoted as accessory transcripts and unique transcripts were up-regulated in one dataset. Percent identity cut-off and percent match cut-offs were set to 50%. The inflation parameter was set to 0.

Ethics statement

The research presented in this paper do not involve human subjects, and we see no ethical issues.

Results and discussion

Microarray analysis of iron depleted *A. salmonicida*

Using our model organism *A. salmonicida* we tested the immediate global changes in gene expression in response to low-iron stress conditions using a microarray approach. The iron chelator 2,2'-dipyridyl was used to create iron-limited conditions, and the appropriate chelator concentration was found by comparing the growth of *A. salmonicida* in the absence or presence of various 2,2'-dipyridyl concentrations. As shown in Figure 1, the growth of the bacterium was clearly affected when the growth medium contained 100 or 500 μM 2,2'-dipyridyl. Based on this result we decided to use 50 μM 2,2'-dipyridyl because it resulted in only a slight growth reduction, and we assumed that a strong growth inhibition would induce broader and less relevant stress responses.

Samples for microarray analysis were prepared by growing *A. salmonicida* in LB with 1% NaCl at 8°C. *A. salmonicida* requires NaCl for growth, and the NaCl concentration of the medium is known to affect growth, motility and other activities [36]. In our experiment we used a NaCl concentration close to the physiological conditions the bacterium would experience inside its natural host (Atlantic salmon) [37], as well as temperature where up-regulation of iron sequestration systems is known to occur, and the bacterium is known to develop cold water vibriosis [28,29]. The cultures were

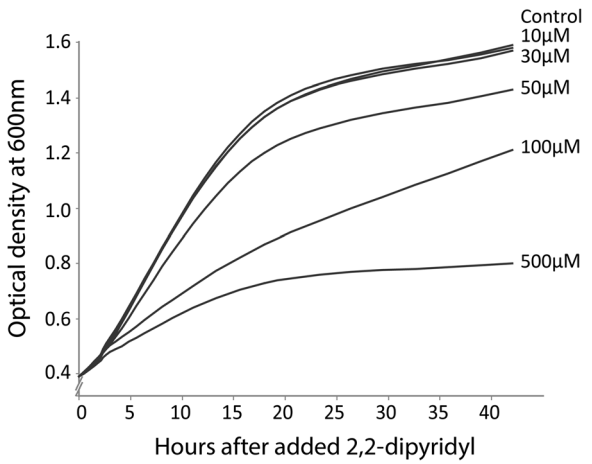

Figure 1 Titration of 2,2′-dipyridyl concentration. *A. salmonicida* strain LFI1238 was grown in LB containing 1% NaCl to an optical density at 0.4 (600 nm). The culture was split into six individual flasks and supplemented with different concentrations of 2,2'-dipyridyl before growth was monitored for 44 hours. Culture treated with 50 μM 2,2'–dipyridyl showed a slight reduction in growth and this concentration was therefore used in all subsequent experiments.

grown to mid log phase (OD_{600nm} 0.5) before 2,2'-dipyridyl was added to a final concentration of 50 μM, and samples were collected after 15 minutes to monitor immediate responses.

Table 1 lists 32 differentially expressed genes that fulfilled our criteria (fold change values ≥ 1.5 and p-values ≤ 0.10). These threshold values were chosen after evaluating alternative combinations of cut-off values, and evaluating the biological soundness of the resulting data (i.e. keep maximum valuable data while minimizing the introduction of noise). Also, biological replicates tend to create more variation between samples compared to technical replicates, and too strict cut-off values can therefore exclude biologically sound data. In our analysis, all differentially expressed genes (Table 1) are up-regulated (in treated sample) and associated with predicted Fur-boxes [21]. Moreover, based on the current annotations, the majority (at least 21 of 32) of genes have predicted functions in iron homeostasis. The operon associated with the highest fold change values contains three genes (*bibABC*) for biosynthesis of the siderophore bisucaberin. Interestingly, of all sequenced bacteria in the relatively large *Vibrionaceae* family, *A. salmonicida* was until recently the only representative with this system. Using the amino acid sequences for the *bibABC* genes in a Blast search we identified homologous genes (99% identity) in the very closely related *Aliivibrio logei* [38]. This observation favours that the system was acquired by horizontal gene transfer in the most recent common ancestor of the two *Aliivibrio* species. This scenario is more parsimonious than the alternative, which is that the system was lost in all other *Vibrionaceae* representatives. Other genes on the list with functions in iron metabolism include siderophore receptors, heme receptors and the associated ATP-binding cassette (ABC) and TonB transport systems. The gene encoding ferrioxamine B receptor (BfrH) is possibly encoding a siderophore receptor (ferrioxamines are siderohores). The same operon encodes a TonB3 system. The operon encoding FhuC and FhuD (associated with siderophore-iron transport) is also up-regulated under iron limited conditions. The CDSs encoding TolR2, a TonB dependent receptor and a putatively exported protein are located in the same operon and are all up-regulated. A recent publication suggests that TolR is likely TtpC, which is necessary for stabilisation of TonB2 binding in *Vibrio anguillarum* and *V. cholerae* [39]. TonB1 is the only TonB, which appears to be up-regulated. Apparently, this TonB is most likely non-functional due to a frameshift in *A. salmonicida*.

None of the differentially expressed genes on our lists were down-regulated (i.e. did not fulfil the cut-off criteria). This is surprising since *ryhB* is moderately up-regulated (4.6 fold) under low iron conditions, and down-regulation of known RyhB targets is expected based on evidence from other species. There are two possible explanations for this finding: the data is valid and all significantly differentially expressed genes are up-regulated, however we cannot rule out that unknown technical issues have affected our data leading to this result. Although we have not validated any potential RyhB targets by Northern blot analysis, the overall agreement between fold change values in our microarray and Northern blot data (see below) support the conclusion that the microarray data is valid and we have no reason to suspect serious technical issues. Known RyhB targets that are identified in both *E. coli* and *V. cholerae* include *sodB*, *sdhC*, *fumA* and *gltB1* [40,41]. All these are present in our dataset, but are not differentially expressed (fold change −1.03, −1.16, −1.10 and −1.27, respectively). In *V. cholerae,* 31 genes are up-regulated in a *ryhB* null mutant; however, the fold change values for these potential RyhB targets are very moderate (majority varies between 1.6 – 3.3 fold) [41]. Similarly, when RyhB is over-expressed in *E. coli,* fold change values for the majority of down-regulated genes vary between 1.5 – 6 [40]. Based on this information, it may not be surprising that secondary effects such as for example RyhB regulation is not detected in our experiment considering that; i) RyhB is only moderately up-regulated, ii) *A. salmonicida* has a relatively long doubling time (6–8 hours) at 8°C, and iii) we measured effects after a very short exposure time (15 min) to the iron chelator.

Northern blot analysis

Northern blot analysis was used to validate the microarray expression data of 5 up-regulated genes; VSAL_I0134,

Table 1 Differentially expressed genes in *A. salmonicida* LFI1238 stimulated with 50 μM 2,2'-dipyridyl

CDS	Gene	Product[1]	Fold change[2]	p-value[2]
Transport/binding proteins				
VSAL_I1734		heme receptor (pseudogene)	1.5	0.03
VSAL_I1751	*tonB1*	TonB protein (pseudogene)	5.0	0
VSAL_I1752	*exbB1*	TonB system transport protein	2.4	0.01
VSAL_I1754	*hmuT*	heme transporter protein, putative periplasmic binding protein	4.3	0
VSAL_I2257	*feoA*	ferrous iron transport protein FeoA	1.8	0.06
VSAL_I2258	*feoB*	ferrous iron transport protein FeoB	1.8	0.07
VSAL_I2259	*feoC*	ferrous iron transport protein FeoC	1.8	0
VSAL_I2588	*fbpA*	iron(III) ABC transporter, periplasmic iron-compound-binding protein	2.1	0.08
VSAL_II0110		TonB dependent receptor	3.4	0
VSAL_II0112	*tolR2*	biopolymer transport protein	2.0	0
VSAL_II0150	*fhuC*	ferrichrome transport ATP-binding protein	3.2	0
VSAL_II0151	*fhuD*	ferrichrome-binding periplasmic protein	3.2	0.01
VSAL_II0909	*bfrH*	ferrioxamine B receptor	3.3	0
VSAL_p320_27		iron ion ABC transporter, periplasmic component	2.4	0.01
VSAL_p320_29		iron ion ABC transporter ATP-binding protein	1.7	0.07
Adaptation				
VSAL_I1749	*huvX*	heme uptake and utilization protein	1.7	0
Biosynthesis of cofactors, carriers				
VSAL_I0134	*bibA**	Bisucaberin siderophore biosynthesis protein A	7.6	0
VSAL_I0135	*bibB**	Bisucaberin siderophore biosynthesis protein B	5.8	0.01
VSAL_I0136	*bibC**	Bisucaberin siderophore biosynthesis protein C	1.9	0.06
VSAL_I1750	*phuW*	putative coproporphyrinogen oxidase	2.2	0
Cell envelope				
VSAL_I1248		membrane protein	2.9	0
VSAL_I1785		putative exported protein	2.2	0
VSAL_I1786		putative iron-regulated protein	2.8	0
VSAL_I1864		putative outer membrane protein	4.2	0
VSAL_II0074		membrane protein	3.4	0
VSAL_II0111		putative exported protein	2.3	0
VSAL_II0717		putative membrane protein	1.6	0.02
VSAL_II0868		putative lipoprotein	3.4	0
sRNA				
VSAL_I3102s		VSsrna22 small RNA RyhB	4.6	0
Unknown function, no known homologues				
VSAL_I2980		hypothetical protein	1.5	0.1
VSAL_I2892		hypothetical protein	3.7	0
VSAL_II0148		hypothetical protein	3.8	0

[1]Annotated product of CDS [2]Fold change values are shown for ≥ 1.5 differentially expressed genes with p-values ≤ 0.1. Positive fold change value indicate up-regulation compared to untreated control. *_bibA_ is annotated as L-2,4-diaminobutyrate decarboxylase in the *A. salmonicida* genome annotations, and *bibB and bibC* are annotated as iucD and iucC, respectively [26,30].

VSAL_I0135, VSAL_I0448, VSAL_II0148, VSAL_II0110. The intensities of the different bands were normalized to 16S rRNA. Autoradiogram pictures are shown in Figure 2, and show that the Northern blot data are in good overall agreement with the microarray analysis. For example, for VSAL_I0135 (*bibB*) the microarray and Northern blot analyses show fold change values of 5.8 and 5.2, respectively, and for VSAL_II0110 the respectively fold change values

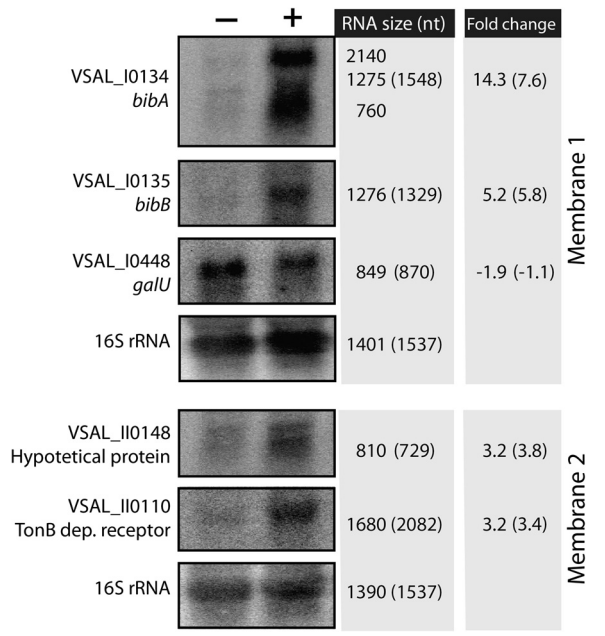

Figure 2 Validation of selected microarray results using Northern blot analysis. RNAs from six replicates of *A. salmonicida* were pooled and separated on denaturating 1.2% formamide agarose gels, transferred to two membranes and tested for presence of specific RNAs using radio-labeled probes. Plus (+) indicates addition of 50 μM of the iron chelator 2,2'-dipyridyl to the cultures 15 min prior to sampling, whereas minus (–) indicates the untreated control. Numbers in the left column indicate the size of the RNA as measured from the gel, and numbers in parentheses indicate the theoretical size. The right column indicate normalized fold change values calculated from the Northern blot autoradiograms, while numbers in parentheses show the corresponding microarray fold change values.

were 4.5 and 3.2. The microarray fold change value that differs most in magnitude from the Northern Blot result is for VSAL_I0134 (*bibA*). Here, the microarray and Northern Blot values were 7.6 and 14.7, respectively.

Comparison of results with other global expression profiling studies from *Vibrionaceae*

Next, we wanted to compare our result to similar global expression profiling studies (microarray) where the response of other representatives of the *Vibrionaceae* family to low-iron conditions was studied. By uncovering responses that are shared between bacteria belonging to the *Vibrionaceae* family, or that are unique to one species, we may eventually provide a deeper understanding of mechanisms involved in virulence. Two such datasets are currently available: Crosa and co-workers [26] tested responses of *V. vulnificus* strain CMCP6 to iron-limiting conditions by adding 50 μM ethylenediamine-di-(o-hydroxyphenylacetic) acid (EDDA) (iron-depleted conditions) to TSBS medium cultures from the beginning, in addition to untreated controls, and harvested cells at mid-log phase (i.e. OD_{600nm} 0.6-0.8). Three biological replicates were pooled before cDNA synthesis to avoid culture variations in microarray analysis. In their analysis they were able to identify 49 genes that are differentially up-regulated during iron-depleted conditions. In another study by Mey et al. [11], *V. cholerae* strain 0395 was grown in EZ RDM defined medium with no added iron (i.e. iron-depleted conditions), or EZ RDM with 40 μM of ferrous sulfate (iron-replete conditions) to OD_{650nm} 0.3. In their study, they identified 84 differentially expressed genes during iron-depleted conditions.

In our comparative analysis we adopted the Pan genome concept and organized the differentially-expressed transcripts into core, accessory and unique transcripts. A Venn-diagram representation of the comparative analysis are shown in Figure 3. More detailed information about the comparative analysis results are found in Additional file 1. Core transcripts are differentially expressed in all datasets, unique transcripts are differentially expressed in one dataset, whereas accessory transcripts are differentially expressed in two datasets. Although the three experiments were performed differently (e.g., different growth media, different iron chelators/iron-deplete medium, different sampling time/cell densities, etc.), we believe they could identify potentially interesting common or unique responses to low iron conditions among the three bacteria.

We used the software OrthoMCL [35] with percent identity and percent match cut-off set to 50 and the inflation value set to 0 to identify potential homologs. Subsequently, we curated the generated homology clusters manually and identified 9 core transcripts (10 in *V. vulnificus* as VV1_1660 and VV1_1661 are paralogs, and

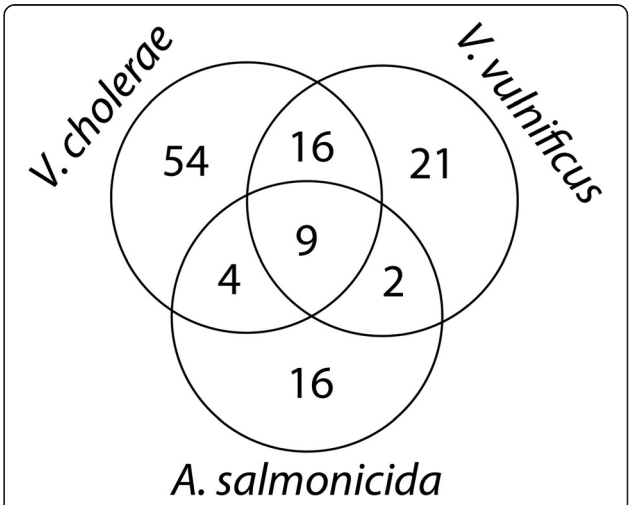

Figure 3 Venn diagram summarizing numbers of transcripts that are differentially expressed in *A. salmonicida*, *V. cholerae* and *V. vulnificus* under low-iron conditions. Numbers are based on this study, and the microarray studies using *V. cholerae* [11] and *V. vulnificus* [26].

both cluster together with VC0608 in *V. cholerae* and VSAL_I2588 in *A. salmonicida*). Three of the core transcripts belonging to a ferrous iron transport system (*feoA*, *feoB* and *feoC*), one transcript belonging to a TonB system (*exbB1*), two transcripts encode proteins that are potentially involved in heme uptake/utilization, two transcripts which may belong to a TonB2 system, and finally one transcript encoding a ferric iron ABC transporter periplasmic iron-compound-binding protein. Therefore, all differentially expressed core transcripts encode proteins involved in iron homeostasis.

Sixteen accessory transcripts are shared between *V. vulnificus* and *V. cholerae*. These encode products involved in siderophore biosynthesis, siderophore and heme transport and utilization, iron storage (*bfd* and *bfr*), and oxidative stress response (*sodA*). Of the 16 accessory genes shared between *V. cholerae* and *V. vulnificus,* ten are not present in the *A. salmonicida* genome (e.g. *bfr*, *bfd* and the different *vtc* component genes). Moreover, the finding that three siderophore biosynthesis sequences (VC0771/VV2_0838, VC0773/VV2_0835 and VC0774/VV2_0834) are shared only between *V. vulnificus* and *V. cholerae* does not seem reasonable, and may reflect the fact that some siderophore biosynthesis proteins are more related between *V. vulnificus* and *V. cholerae* than they are to the bisucaberin biosynthesis system in *A. salmonicida*. Four accessory transcripts are shared between *V. cholerae* and *A. salmonicida* (VSAL_I2892/VC0091, VSAL_I1786/VC1264, VSAL_I1785/VC1265 and VSAL_II0074/VC1588), and finally two accessory transcripts are shared between *A. salmonicida* and *V. vulnificus* (i.e. TonB1 VSAL_I1751 and VV21614, and a periplasmic heme binding protein encoded by VSAL_I1754 and VV21611). The fact that *V. cholerae* and *V. vulnificus* share the highest number (i.e. sixteen) of common up-regulated transcripts is reasonable since they are more closely related to each other than to *A. salmonicida*. In addition, the experimental conditions used for *V. cholerae* and *V. vulnificus* are more similar.

Fifty-four, twenty-one and sixteen transcripts are unique to *V. cholerae, V. vulnificus* and *A. salmonicida*, respectively, and of these at least eighteen, eight and ten transcripts are directly associated with iron homeostasis. In *V. cholerae* the unique transcripts encode proteins with functions in vibriobactin biosynthesis (*vibD-H*), siderophore transport (*viuA, viuC, viuD, viuG, viuP, irgA, vctA, fhuA* and *fhuC*), heme transport (*hutA* and *hutD*), iron transport (*tonB1, tonB2* and *exbD2*), transcription regulation (*irgB* and *vctR*), various enzymatic catalysis (e.g. *ligA-2*, *fumC*, *ptrB*, *napA-D*, *napF*, *menB*), and finally hypothetical functions. In *V. vulnificus* half of the eight unique transcripts encode proteins that are involved in vulnibactin biosynthesis and transport (VV20839, VV20840, VV20841 and VV20844), and the remaining four have functions in iron transport, i.e. TonB systems (VV21618, VV20359 and VV20360) and an ABC-type Fe^{3+} transport protein (VV11662). In *A. salmonicida* unique transcripts are directly associated with bisucaberin biosynthesis genes (*bibA*, *bibB* and *bibC*), ferrioxamine B receptor (*bfrH*), ferrichrome binding (*fhuC* and *fhuD*), iron transport, i.e. an ABC transport system (VSAL_p320_27 and VSAL_p320_29), a TonB2 dependent receptor (VSAL_II0110), and a heme receptor (VSAL_I1734).

Siderophore biosynthesis in *Vibrionaceae*

The approximately 150 different *Vibrionaceae* species (157 species in the NCBI taxonomy database when excluding unclassified sp. and subspecies) [42] have the potential to synthesize various siderophore iron chelators. For example, *V. cholerae* encodes the system *VibABCDEFH* that is responsible for the production of vibriobactin. Similarly, *A. salmonicida* contains the *bibABC* genes, which encode enzymes involved in production of the bisucaberin siderophore [30]. *V. vulnificus* produces the species specific siderophore vulnibactin [43]. Vulnibactin is structurally similar to vibriobactin, but its biosynthesis pathway is not fully understood [44]. The genes *venB*, *vvsA* and *vvsB* are involved in the biosynthesis, but their roles are unclear. *V. vulnificus* also synthesizes a hydroxamate-type siderophore. Unfortunately, neither its structure nor its biosynthetic pathway have been identified [45].

In our analysis the three genes involved in bisucaberin biosynthesis in *A. salmonicida* top our list of differentially up-regulated genes/operons. The result resembles expression data from both *V. cholerae* [11] and *V. vulnificus* [26] where siderophore biosynthesis genes were highly up-regulated (although they did not top the list of up-regulated genes) after being grown in low iron medium. Together these results strongly support the idea that siderophore production and utilization represent one of the first and probably most important responses to mitigate low iron conditions. It is however still unclear why different *Vibrio/Aliivibrio* species use different siderophores. One possible explanation is that the utilization of multiple siderophores represents an advantage in the competition for scarce resources. However, some vibrios can partly mitigate such strategies by utilizing siderophores produced by other bacteria.

TonB systems

In Gram negative bacteria the transport of ferri-siderophores and heme across the membrane requires energy. The energy is provided by TonB systems, which consist of the TonB, ExbB and ExbD proteins. In vibrios TonB2 systems also include the TtpC protein [1,39]. Vibrio genomes typically contain two or three TonB systems [32,46]. Interestingly, in our analysis *tonB1*, *tonB2* and

exbD2 from *V. cholerae,* and *tonB2* from *V. vulnificus,* are considered unique, whereas the remaining TonB transcripts (*exbB1*, *exbB2* and *exbD1*) are either core or accessory transcripts. *tonB1* is shared between *A. salmonicida* and *V. vulnificus* even though the *A. salmonicida tonB1* gene is a pseudogene (contains one frameshift mutation). *tonB2* transcripts were not identified in the *A. salmonicida* microarray dataset. Intriguingly the *V. cholerae tonB1* transcript was considered unique, but after further examinations we realized that this transcript was excluded from the results because it was just below the cut-off settings for identities, while the TonB1 transcripts from *V. vulnificus* and *A. salmonicida* were just above the cut-off setting. This show the weaknesses of a small dataset and the problems of setting specific cut-offs.

Conclusions

We studied the immediate effect of low iron conditions, and compared this to similar studies where effects were examined after prolonged growth in low iron conditions. We identified 32 up-regulated genes, whereas no genes were found to be down-regulated. Although caution should be taken in extrapolating *in vitro* results to what may occur *in vivo*, it is our belief that studies such as those performed here will provide a better understanding of iron uptake and metabolism in bacteria, and eventually provide us with some insights into their virulence and survival mechanisms, their ability to adapt to changing environmental conditions, and finally their evolution. We have studied expression of genes that are essential for iron homeostasis in a single species, but by studying a collection of species from a broader spectrum of bacteria e.g., from the same family (i.e. *Vibrionaceae*), unique and common strategies for mitigating low iron conditions can be identified. A future goal for us is to use such knowledge to compare environmental isolates with known pathogens to better understand the relevance of iron homeostasis in virulence. Finally, increased knowledge on iron uptake systems and regulation is highly relevant to on-going efforts where such systems are used as targets for potential drugs with the goal to control pathogenic bacteria.

Availability of supporting data

Microarray data are available in the ArrayExpress database under accession number GSE57996.

Additional file

Additional file 1: Table S1. The table lists differentially expressed (up-regulated) core, accessory and unique transcripts in *A. salmonicida*, *V. vulnificus* and *V. cholerae*. Amino acid sequences of corresponding genes were retrieved from ENA, and used as input for clustering of orthologs using OrthoMCL with amino acid percent match and percent identity cut-offs set to 50%.

Abbreviations

PCR: Polymerase chain reaction; LB: Luria Bertani broth; Fur: Ferric uptake regulator; dsDNA: Double-stranded DNA; rpm: Rounds per minute; OD: Optical density; min: minutes; MOPS: 3-(N-morpholino)propanesulfonic acid; SSC: Saline-sodium citrate; SDS: Sodium dodecyl sulfate; CDS: Coding sequence; rRNA: Ribosomal RNA; mRNA: Messenger RNA; sRNA: Small RNA; *AS*: *Aliivibrio salmonicida*; ABC: ATP-binding cassette.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PH and HH conceived of the study and designed experiments. HH supervised the microarray analysis, and PH supervised the study and helped drafting the manuscript. SKT participated in the design of experiments, performed the wet-lab experiments, initial microarray data analysis and Northern data analysis, and drafted the manuscript. TK performed the pan-genome analysis, and EMR performed the Limma based microarray data analysis. All authors read and approved the final manuscript.

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S1. Table of results from pan-transcriptome comparison of microarray results from *A. salmonicida LFI1238* , *V. cholerae O395* and *V. vulnificus CMCP6* .

Core

A. salmonicida [1]	*V. cholerae* [2]	*V. vulnificus* [3]	annotation
VSAL_I2588	VC0608	VV1_1660/VV1_1661	iron (III) ABC transporter periplasmic iron-compund-binding protein/ *fbpA*
VSAL_II0111	VC1548	VV2_0364	hypotetical protein linked to TonB/TonB system biopolymer transport protein
VSAL_I2259	VC2076	VV1_0147	hypotetical protein linked to *FeoAB/feoC*
VSAL_I2258	VC2077	VV1_0148	*feoB*
VSAL_I2257	VC2078	VV1_0149	*feoA*
VSAL_I1749	VCA0908	VV2_1616	heme uptake and utilization protein *huvX* / *hutX* / putative heme utilization protein #*hutX*
VSAL_I1750	VCA0909	VV2_1615	coproporphyrinogen III oxidase *phuW* / *hutW*
VSAL_I1752	VCA0911	VV2_1613	*exbB1/ exbB*
VSAL_II0112	VC1547	VV2_0363	*tolR/ exbB* -like linked to *TonB2/MotA/TolQ/ExbB proton channel family protein*

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Accessory

A. salmonicida	*V. cholerae*	*V. vulnificus*
VSAL_I2892	VC0091	
VSAL_I1786	VC1264	
VSAL_I1785	VC1265	
VSAL_II0074	VC1688	

VSAL_I1751	VV2_1614
VSAL_I1754	VV2_1611

VC0364	VV1_1340
VC0365	VV1_1341
VC0771	VV2_0838
VC0773	VV2_0835
VC0774	VV2_0834
VC1546	VV2_0362
VC2210	VV2_0837
VC2694	VV1_1252
VCA0227	VV2_0842
VCA0228	VV2_0110
VCA0229	VV2_0111
VCA0230	VV2_0112
VCA0907	VV2_1617
VCA0912	VV2_1612
VCA0914	VV2_1610
VCA0915	VV2_1609

Unique *V. vulnificus*

Gene_ID
VV1_0454
VV1_1662
VV1_2329
VV1_2330
VV1_2331
VV1_2332
VV1_2333
VV1_2334
VV1_2335
VV1_2336
VV1_2337
VV1_2682
VV1_2683
VV1_2684
VV2_0359
VV2_0360
VV2_0839
VV2_0840
VV2_0841
VV2_0844
VV2_1618

Unique *A. salmonicida*

Gene_ID
VSAL_I0134
VSAL_I0135
VSAL_I0136
VSAL_I1248
VSAL_I1734
VSAL_I1864
VSAL_I2980
VSAL_II0110
VSAL_II0150
VSAL_II0151
VSAL_II0717
VSAL_II0854
VSAL_II0868
VSAL_II0909
VSAL_p320_27
VSAL_p320_29

Unique *V. cholerae*

Gene_ID
VC0200
VC0201
VC0474
VC0475
VC0772
VC0775
VC0776
VC0777
VC0778
VC0779
VC0780
VC1112
VC1216
VC1266
VC1267
VC1343
VC1371
VC1514
VC1515
VC1516
VC1542
VC1543
VC1544
VC1545
VC1572
VC1573
VC1973
VC2209
VC2211
VC2227
VC2415
VC2695
VCA0043
VCA0063
VCA0215
VCA0216
VCA0217
VCA0231
VCA0232
VCA0233
VCA0234
VCA0262
VCA0576
VCA0676
VCA0677
VCA0678
VCA0679
VCA0680
VCA0784
VCA0910
VCA0916
VCA0976
VCA0977
VCA1041

Paper 2

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

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Abstract

Background. The ferric uptake regulator (Fur) is a transcription factor and the main regulator of iron acquisition in prokaryotes. When bound to ferric iron, Fur recognizes its DNA binding site and generally executes its function by repressing transcription of its target genes. Due to its importance in virulence, the Fur regulon is well studied for several pathogenic model bacteria. In our previous work, we used computational predictions and microarray to gain insights into Fur-regulation in *A. salmonicida*, and have identified a number of genes and operons that appear to be under direct Fur-regulation. To provide an even more accurate and deeper global understanding of the Fur regulon we now 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.

Results. An *A. salmonicida fur* null mutant strain was constructed. 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 the RyhB sRNA 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.

Conclusion. The present work provides by far the most comprehensive and deepest understanding of the Fur regulon in *A. salmonicida* to date. Our data will also contribute to a better understanding of how Fur plays a key role in iron homeostasis in bacteria in general, and help to show how Fur orchestrates iron uptake when iron levels are extremely low, e.g., during the critical early phases of infections.

Keywords: *Aliivibrio salmonicida*, Fur, the ferric uptake regulator, iron homeostasis, RNA-sequencing, RyhB, gene dosage effect, small regulatory RNAs, sRNAs

Introduction

The ferric uptake regulator, Fur, represents the main regulator of iron levels in prokaryotic microorganisms (reviewed in Fillat 2014). In addition to regulating iron acquisition genes, Fur also regulate genes involved in e.g., the TCA cycle, DNA metabolism, energy metabolism, redox-stress resistance, chemotaxis, swarming, metabolic pathways, toxin production and other virulence factors, and is therefore considered as a so-called master regulator (Escolar et al. 1999; Hantke 2001; McHugh et al. 2003; Mey et al. 2005a; Pajuelo et al. 2016). Transcriptomic studies on *fur* null mutants of *Vibrio cholerae* (Mey et al. 2005a) and *Vibrio vulnificus* (Pajuelo et al. 2016) have shown that Fur represses expression of siderophore biosynthesis and transport genes, heme transport and utilization genes, ferric and ferrous iron transport genes, stress response and biofilm genes amongst others. The same studies have shown that Fur have an activating effect on genes involved in stress responses, chemotaxis, motility and toxin production. In *Escherichia coli* K-12, Fur directly regulates 131 genes including those of seven other master regulators, i.e., *flhD, flhC, felc, soxS, ryhB, rpoS* and *purR* (Keseler et al. 2013; McHugh et al. 2003), which subsequently results in regulation of 3158 genes in total (incl. direct and indirect effects) (Keseler et al. 2013). This huge number of genes translates to 70% of the total number of genes in *E. coli* K-12 (EcoCyc), and illustrates the central role of Fur in cellular processes far beyond iron homeostasis.

The 3D-structure of Fur from *Pseudomonas aeruginosa*, *E. coli*, *V. cholerae*, *Helicobacter pyroli* and *Campylobacter jejuni* is known (Butcher et al. 2012; Dian et al. 2011; Pecqueur et al. 2006; Pohl et al. 2003; Sheikh & Taylor 2009). These structures show that Fur mainly acts as a homodimer in both apo and holo forms, where at least two zinc ligands per monomer stabilize the dimer (Fillat 2014). The iron binding sites are located in a DNA binding domain of each monomer. Here, iron binding causes conformational changes that enable Fur to bind to its DNA target (known as the Fur-box) (Fillat 2014). Although several different Fur-box motifs have been proposed over the years, the current literature seems to have converged on that the Fur-box is a 19 bp palindromic sequence centered around a non-conserved nucleotide (Baichoo & Helmann 2002; Davies et al. 2011; De Lorenzo et al. 1988; Escolar et al. 1998). Once bound to its DNA target Fur mainly acts as a repressive regulator by blocking the transcription of downstream genes.

Fur activating activity was observed during early investigations of the Fur regulon and was proposed to be due to post-transcriptional regulation (Hantke 2001). The activating effect was later discovered to be due to the Fur-regulated small regulatory RNA (sRNA) named RyhB (Masse et al. 2003; Massé & Gottesman 2002; Masse et al. 2005). The apparent activating activity of Fur was found to be due to, at least in part, a secondary effects caused by *ryhB*. The RyhB sRNA is responsible for destabilizing mRNAs of its target, and repression of *ryhB* by holo-Fur was therefore interpreted as activation by Fur. RyhB typically targets iron-using or iron-binding proteins as a way of preserving the iron levels in the cell at low iron conditions (Davis et al. 2005; Masse et al. 2005; Murphy & Payne 2007). In *E. coli* RyhB directly targets 28 mRNAs (of which two encodes master regulators MarA and Fur) (EcoCyc). Other examples of RyhB targets in *E. coli* are the mRNA of *bfr, cysE, sodAB, fumA, sucBCD, icsRSUA* and *sdhABCD* (Massé & Gottesman 2002). In *V. cholerae* RyhB targets mRNAs of *sodB, sdhC, gltB1* and *fumA* and not mRNAs of iron storage genes like *bfr* and *ftn* (Davis et al. 2005).

The aim of this study was to investigate the Fur regulon in *A. salmonicida*, the causative agent of cold-water vibriosis in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*) at sea-water temperatures below 10°C (Colquhoun & Sorum 2001; Enger et al. 1991). In a previous study we identified a *Vibrionaceae*-specific Fur-box consensus as 5'- AATGANAATNATTNTCATT-3', and used computational methods to predict Fur-regulated genes and operons in four *Vibrionaceae* genomes, including *A. salmonicida* (Ahmad et al. 2009). Fur-binding motifs were associated with 60 single genes and 20 operons (89 genes). Later we used molecular dynamics (MD) simulations and binding free energy calculations to gain more insights into the interactions between *A. salmonicida* Fur (asFur) and proposed Fur-binding sites (Pedersen et al. 2010). Here, Fur-binding to promoters was dependent on the number of Fur-boxes, and the predicted "strength" (i.e., calculated similarity to Fur-box consensus) of the individual Fur-boxes. Finally, we studied Fur-regulation in *A. salmonicida* using iron-depletion experiments in combination with custom whole-genome microarray chips (Ahmad et al. 2012; Thode et al. 2015). Thirty-two genes were found to be significantly up-regulated 15 min after exposure to low-iron conditions (suggesting Fur-regulation), and interestingly, the *bibABC* genes responsible for producing the siderophore bisucaberin were identified as being most highly up-regulated (Thode et al. 2015). We have now constructed an *A. salmonicida fur* null mutant and used Illumina RNA-sequencing (RNA-seq) to compare the

transcriptomes of the wild-type strain and the *fur* null mutant. Overall, we find that the RNA-seq data overlap remarkably well with our previous findings when using microarray. However, we also show that high-throughput RNA-sequencing provide us with a much more accurate and fine-grained global understanding of the Fur regulon in *A. salmonicida*, compared to what we knew from our previous microarray work.

Material and methods

Bacterial strains, culture conditions, and sampling for RNA sequencing

A. salmonicida LFI1238 (Hjerde et al. 2008) was used as parental strain for the construction of the *A. salmonicida fur* null mutant. Parental and mutant strains were cultured in LB medium [Luria-Bertani broth Miller, Difco (later corrected to Lysogeny Broth (Bertani 2004))] containing 2.5% NaCl at 12°C and 200 rpm unless otherwise indicated. For *E. coli* strain S17-1 the growth conditions were 37°C and 200 rpm in LB medium with 1% NaCl. The suicide plasmid pDM4 was propagated in *E. coli* S17-1 cells. For selection of *E. coli* S17-1 transformants and *A. salmonicida* transconjugants, 25 µg or 2 µg of chloramphenicol/ml was added to the medium, respectively.

For RNA sequencing three biological replicates of *A. salmonicida* LFI1238 and *A.salmonicida fur* null mutant were grown in LB medium with 1% NaCl at 8°C and 200 rpm to mid log growth phase, i.e., at optical density (600 nm) of approximately 0.5. Ten mL samples were harvested, spun down and the cell pellets were then stored at -80°C for later processing.

Construction of an A. salmonicida fur *null mutant*

The *A. salmonicida fur* null mutant was constructed using the suicidal plasmid pDM4 and allelic exchange, as described by others (Milton et al. 1996). First we constructed the plasmid pDM4Δ*fur*, consisting of merged flanking regions of the *A. salmonicida fur* gene. The upstream flanking region of the *fur* gene was amplified by PCR using primers FurA forward (5'-CTACTCGAGATATTTATTTCCCTTTAATTC-3') and FurB reverse (5'-CACGTAAACTAAATATGACTTTTCCTGTATTGG-3'). For amplification of the downstream

flanking region primers FurC forward (5'-TATTTAGTTTACGTGCATAAAAAA-3') and FurD reverse (5'-CCCACTAGTATAACAAAGACTCTACTCCAG-3') were used. The resulting upstream and downstream PCR products were fused together using an overlap PCR, cut with restriction enzymes *XhoI* and *SpeI*, and ligated into the corresponding sites of pDM4. The resulting pDM4Δ*fur* construct was transformed into *E.coli* S17-1 and used as donor cells in conjugation experiments with *A. salmonicida* as described elsewhere (Bjelland et al. 2011). The allelic exchange was performed on LB agar containing 5% sucrose. The resulting *fur* null mutant was verified using PCR and DNA sequencing.

Total RNA purifications

For RNA sequencing, total RNA was purified from cell pellets using the Masterpure complete DNA & RNA purification kit (Epicentre) following the manufacturer's protocol, followed by an additional DNA removal step using the DNA-free kit (Applied Biosystems). DNase-treated total RNA was subsequently purified using the RNA cleanup RNeasy MinElute kit (Quiagen). The quality of total RNA preps was determined using a Bioanalyzer and a Prokaryote Total RNA Pico Chip (Agilent Technologies). Finally, ribosomal (r) RNA was removed from each sample (5µg total RNA) using the Ribo-Zero rRNA Removal Kit (bacteria) (Epicentre) according to the manufacturer's instructions. rRNA-depleted RNA samples were ethanol precipitated (to recover small RNAs), and analyzed on a Bioanalyzer using mRNA Pico Chips (Agilent Technologies).

RNA sequencing and data analysis

RNA-sequencing libraries were generated from purified rRNA-depleted RNA samples using the strand-specific TruSeq stranded mRNA library prep kit (Illumina), and sequenced at the Norwegian Sequencing Centre using the Illumina NextSeq 500 with mid output reagents with a read length of 75 bp and paired end reads, giving an average output of approximately 54 million reads per sample. The reads were quality checked using FastQC. Further analysis of the RNA-Seq data was performed using a Galaxy pipeline consisting of EDGE-pro v1.0.1 (Estimated Degree of Gene Expression in Prokaryotes) and DESeq. EDGE-pro was used to align the reads to

the *A. salmonicida* LFI1238 genome (Hjerde et al. 2008), and to estimate gene expression. Differences in gene expression between wild-type and *fur* null mutant were determined using DESeq. Log_2 fold changes of the genes were recalculated to × differential expression values (i.e., Δ*fur*/wt) and genes were defined as significantly differentially expressed based on a p-value ≤0.05 and differentially expression values of Δ*fur*/wt ≥2× and ≤-2×.

sRNA and mRNA target predictions

The Rockhopper software (McClure et al. 2013) was used to identify sRNA from the RNA-seq data. Input files in the analysis were fastaq files from the RNA-seq data of wild type and *fur* null mutant strains, a protein coding gene position file (.ptt), a non-coding RNA position file (.rnt), and finally genome files from *A. salmonicida* LFI1238 [NC_011312.1 (Chr I), NC_011313.1 (ChrII), NC_011311.1 (pVSAL840), NC_011314.1 (pVSAL320), NC_011315.1 (pVSAL54) and NC_011316.1 (pVSAL43)]. sRNAs identified by Rockhopper were visualized in Artemis and manually curated based on a set of criteria. To be accepted as a potential sRNA, its gene should be (i) located in an intergenic region, (ii) between 30−350 nt in length, (iii) located 30 nt or more from the nearest CDS if on the same strand, and 10 nt if on the complementary strand (based on the method of Toffano-Nioche et. al. (Toffano-Nioche et al. 2012)). RNAs fulfilling the criteria described above were further examined for presence of small open reading frames (sORF) using a method adopted from Meulen et. al. (van der Meulen et al. 2016), since there is an increasing awareness of their presence in bacterial genomes although their significance is not fully understood (Hobbs et al. 2011). Finally, EDGE-pro and DESeq was used to estimate differential gene expression levels for the sRNAs/sORFs.

TargetRNA2 and IntaRNA were used to identify potential sRNAs targets (Busch et al. 2008; Kery et al. 2014). Using sRNA sequences as queries, the programs searches for complementary regions in 5' regions of mRNAs in the *A. salmonicida* LFI1238 genome. Only targets predicted by both programs were accepted. Moreover, we also searched for mRNA targets for up-regulated sRNAs (ten sRNAs with folds Δ*fur*/wt ≥2× in the RNA-seq dataset), including RyhB, among the 34 most down-regulated genes in our RNA-seq data set. This was done to identify sRNAs with critical roles in iron homeostasis (similar to RyhB). In addition, we

predicted binding between RyhB and its known targets (*sodB, gltB, sdhC* and *fumA*) verified experimentally in *E. coli* and *V. cholerae.* Nucleotide sequences of RyhB targets from *E. coli* and *V. cholerae* were extracted from ENA. The nucleotide sequences were aligned with corresponding sequences in *A. salmonicida* and examined using Jalview.

Biological characterization of A. salmonicida fur *null mutant*

A. salmonicida LFI1238 wt and *fur* null mutant (Δ*fur*) were cultured in LB (Difco) at 8°C and 200 rpm in all experiments. Growth of cultures was monitored with optical density measured at 600nm (OD_{600nm}). To determine growth effects of *fur* null mutation, four replicates of *A. salmonicida* LF1238 wt and Δ*fur* were cultured from lag phase until stationary phase. To determine *fur* null mutation growth effects to low iron conditions, wt and Δ*fur* cultures were first grown to OD_{600nm} of 0.38 and 0.33 (mid log phase), respectively. The cultures were then split into 5 separate flasks. One culture was kept as control whereas 25−500 μM of the iron chelator 2,2'-dipyridyl was added to the remaining cultures. To determine *fur* null mutation growth effects to oxidative conditions, wt and Δ*fur* cultures were first grown OD_{600nm} of 0.4 and 0.35 (mid log phase), respectively. The cultures were then split into 5 separate flasks. One culture was kept as control whereas 50−1000 μM of hydrogen peroxide was added to the remaining cultures.

Results and discussion

Construction and basic characterization of an A. salmonicida fur *null mutant*

To better understand the Fur regulon in *A. salmonicida*, a *fur* null mutant was constructed using the genetic system described by Milton et. al. (Milton et al. 1996). Briefly, approximately 250 bp of upstream and 250 bp downstream sequences flanking the *fur* gene were merged and inserted into the pDM4 suicide vector (contains *sacBR*), then transformed into *E. coli* S17-1 cells, and finally conjugated into *A. salmonicida* LFI1238 to trigger recombination and deletion of *fur*.

Basic characterization of the *fur* null mutant was done to examine the physiological and morphological effects of the *fur* deletion. Because Fur is a global regulator, we expected the *fur*

null mutant to loose fitness due to loss of control of central cellular processes. For example, loss of Fur is expected to reduce the growth rate, and result in reduced ability to respond to external chemical stress, such as presence of H_2O_2 and iron chelators (Becerra et al. 2014; Fillat 2014; Hassett et al. 1996; Touati 2000; Yang et al. 2013). Effects on growth was monitored by comparing the growth rates of the wild-type and the *fur* null mutant in LB with 1% NaCl at 8°C and 200 rpm shaking. The OD_{600nm} of the starting cultures were set to 0.01 and then monitored until cultures reached stationary phase (typically OD_{600nm} 1.2−1.4). The lag phase for the wt and *fur* null mutant lasted approximately 10 and 35 hours, respectively, and doubling times were approximately 6 and 12 hours during mid log phase (Figure S1A and S1B). To test the ability to respond to chemical stress the *fur* null mutant and the wild-type strain were exposed to increasing concentrations of hydrogen peroxide (H_2O_2) and the iron chelator 2,2'-dipyridyl. The minimum inhibitory concentration of H_2O_2 on growth for the wild-type and *fur* null mutant were 500µM and 50µM, respectively (Figure S2A and S2B). In a similar experimental setup with 2,2'-dipyridyl the effects were less dramatic (Figure S2C and S2D). The minimum inhibitory of 2,2'-dipyridyl concentrations were similar (approx. 100µM) for both wild-type and mutant strain. However, whereas the wild-type strain grows well in the presence of 1mM 2,2'-dipyridyl, the *fur* null mutant cannot grow in the presence of 500µM.

In summary, deletion of the *fur* gene results in longer lag phase during growth, longer cell doubling time and reduced ability to respond to oxidative reagents and iron chelators. This is in agreement with results from other γ-proteobacteria model organisms (e.g., *V. vulnificus* Δ*fur* shows higher sensitivity to oxidative stress, reduced fitness and growth (Pajuelo et al. 2016) and *V. cholerae* Δ*fur* shows reduction in logarithmic growth (Mey et al. 2005a)) and support the validity of the *fur* mutant.

RNA-sequencing identifies 296 differentially expressed genes in the A. salmonicida fur *null mutant*

To provide accurate data on the Fur regulon we next compared the transcriptome of the *A. salmonicida fur* null mutant and the wild-type using an RNA sequencing approach. RNA samples (from three biological replicates) were prepared from *A. salmonicida* LFI1238 wild-type

and *fur* null mutant cells grown in LB containing 1% NaCl at 8°C to mid log phase (OD_{600nm}≈0.5). The given temperature and salt concentration were chosen because *A. salmonicida* is responsible for development of cold-water vibriosis in Atlantic salmon (i.e., at physiological salt conditions) at temperatures below 10 °C (Bergheim et al. 1990; Colquhoun & Sorum 2001). We realize that the environmental conditions the bacterium encounters inside its natural host cannot be reproduced in our experimental setup, but salt concentration and temperature are highly relevant parameters for studying factors important for development of cold-water vibriosis. RNA samples from biological replicates were subjected separately to paired-end RNA sequencing using Illumina NextSeq 500 with 75 bp read length. Sequencing generated an average output of approximately 54 million reads per sample. RNA-seq data was analyzed using a Galaxy pipeline running EDGE-pro v1.0.1 and DESeq. EDGE-pro was used to align reads to the *A. salmonicida* LFI1238 genome, and estimate gene expression. Comparison of gene expression between wild-type and *fur* null mutant were done using DESeq. Reads originating from rRNA and tRNA genes were excluded from the data analysis. Threshold values for differential expression were set to ≥2× difference (equal to Log_2=1), and with p-value≤ 0.05.

Figure 1 shows how a total of 296 differentially expressed genes are distributed among functional gene classes (functional classes based adapted from MultiFun (Serres and Riley 2000)). One hundred sixty-two and 134 genes are up-regulated and down-regulated, respectively. All functional classes, except "ribosome constituents", "nucleotide biosynthesis" and "cell division", are represented, and the two classes "cell envelope" and "transport/binding proteins" contain the highest number of genes. Considerable up-regulation of the two latter classes is expected since Fur generally regulates genes as a repressor (Fillat 2014), and loss of Fur is therefore expected to result in up-regulation (in *fur* null mutant) of genes involved in iron binding and transport over the membranes. Down-regulated genes are more evenly distributed among 18 of the 21 functional classes, including central processes such as "energy metabolism", "central metabolism", "amino acid biosynthesis" and "cell processes". Although there is no clear pattern, the combined data of up-regulated and down-regulated genes support that *as*Fur is a master regulator with functions similar to that of Fur in *E. coli* (*ec*Fur) (McHugh et al. 2003).

285 *Chromosomal distribution of differentially expressed genes*

286 Table 1 and Table 2 summarize details of genes and operons that are up- or down-regulated, and
287 Figure 2 shows the chromosomal distribution and the position of these differentially expressed
288 genes. Previous studies have shown a strong correlation between the distance of genes from *oriC*
289 (Chr I), and their general transcription level (also known as the *gene dosage effect*) (Dryselius et
290 al. 2008; Toffano-Nioche et al. 2012). That is, genes located close to *oriC* are, statistically, more
291 likely to be transcribed at higher levels than genes located further away from *oriC*, and we were
292 curious to see if *as*Fur-related genes are found clustered at specific regions of Chr I, perhaps with
293 relevance to their expression levels due to gene dosage.

294 In our experimental setup the average RPKM value for the upper half of Chr I (i.e., the region
295 closest to *ori*C) is significantly higher compared to that of the lower half (660/330 for wild-type
296 and 560/397 for *fur* null mutant). Gene dosage effects have yet to be demonstrated for Chr II
297 (Dryselius et al. 2008; Toffano-Nioche et al. 2012), which is in agreement with the RPKM values
298 in our experiment (RPKM values are similar for the upper and lower halves of the chromosome).
299 Differentially expressed genes appear to be relatively evenly distributed on the chromosome,
300 except for some clustering of genes between Chr I pos. 1.85−2.01 Mb. They represent a TonB1
301 system, heme transport and utilization, and cell envelope genes (up-regulated genes), and
302 oxidative stress response, metabolism and sRNAs (down-regulated genes). In other words, there
303 is apparently no clear pattern with respect to *as*Fur-regulated genes and their genomic position. It
304 is interesting to note, however, that the bisucaberin biosynthesis gene cluster and *ryhB* (encodes
305 the RyhB sRNA) are both located close to *oriC*. We have previously reported that the bisucaberin
306 biosynthesis system is included in the immediate response to iron limitations in *A. salmonicida*
307 (Thode et al. 2015), and its genomic location may contribute to the high level of expression and
308 fast response to iron starvation.

309

310 as*Fur regulates iron acquisition systems*

311 As expected, a high proportion of up-regulated genes (28 of 64) are directly associated with iron
312 metabolism, e.g., siderophore biosynthesis and transport, TonB systems (delivery of energy to
313 iron transport), and heme uptake and utilization. The most up-regulated (92×) gene is *bibA*,

which together with the two downstream genes *bibBC* (48× and 11× up-regulated in the *fur* null mutant, respectively) are responsible for producing the siderophore bisucaberin. Interestingly, within the large *Vibrionaceae* family *bibABC* are restricted to *A. salmonicida* and *Aliivibrio logei* (Kadi et al. 2008; Thode et al. 2015), and are in *A. salmonicida* (together with a siderophore transport system, *bitABCDE*) flanked by transposable elements (i.e., a genomic island; see (Hjerde et al. 2008)). Homology search with the BibABC amino acid sequences from *A. salmonicida*, identified that the close relative *Aliivibrio wodanis* also possess the bisucaberin biosynthesis system. The coverage and identity percentage from blastP (with *A.salmonicida* sequences used as query) were 87% identity over 100% coverage for BibA, 90% identity over 99% coverage for BibB and 89% identity over 100% coverage for BibC.

Other siderophore receptors and iron-related transport systems that are significantly up-regulated in the *fur* null mutant include the ferrichrome transport system [VSAL_II0150−0152 (6.7−12.5×)], the ferrioaxamine B receptor [VSAL_II0909 (18.8×)] and its associated ABC transporters [VSAL_II0907 (5.9×) and II0908 (18.8×)]. A siderophore ferric reductase [VSAL_II0148 (8×)] responsible for removing iron from the siderophore, the TonB1 system [VSAL_I1751−1753 (18.8−28.4×)], and finally *huvB*, *huvC* and *huvD* [VSAL_I1754−I1756 (5.8−39.7×)] responsible for heme transport, are up-regulated in the *fur* null mutant. The heme uptake and utilization gene *huvX* [VSAL_I1749 (20.2×)] and *phuW* [VSAL_I1750 (39.7×)], which encode a putative coproporphyrinogen oxidase believed to be responsible for removing iron from heme, are highly up-regulated in the *fur* null mutant. The TonB2 system [VSAL_II0110−II0116 (55.8−17.3× up-regulated)], iron(III) ABC transporters [VSAL_II0907 (5.9×) and II0908 (11.2×)] and a siderophore receptor gene *desA* [VSAL_II0909 (18.8×)] are all highly up-regulated. Interestingly, *feoABC* (VSAL_I2257−I2259) that encode the ferrous iron transport system, are apparently not strongly regulated by Fur, as only *feoC* from this system has a up-regulation ≥2× (i.e., 2.3×).

In summary, removal of the *fur* gene results in up-regulation of 28 genes directly associated with iron homeostasis (siderophore biosynthesis, transport and utilization, heme transport and utilization, ABC transporters and TonB1 and TonB2 systems) in *A. salmonicida*. *bibA* is by far the most up-regulated (92×) gene, whereas the remaining iron-relevant genes are up-regulated 55−5×.

344

345 as*Fur regulates several metal transports systems*

346 As shown in Figure 1 and Table 1, several transport systems are up-regulated in the *fur* null
347 mutant. *as*Fur may be involved in the homeostasis of other metals than iron, as multi metal
348 resistance protein genes, a multidrug efflux pump and nickel and zinc transporter genes are up-
349 regulated in the *fur* null mutant. In detail; the multi metal resistance genes *zntA* (VSAL_I2067)
350 and VSAL_II0143 are up-regulated 8.5× and 5.7×, respectively. The multidrug efflux pump
351 encoded by *vcmD* (VSAL_ I2891) is 8.5× up-regulated. A large operon (VSAL_II0118-II0125)
352 with annotated nickel and zink transporters is also up-regulated 4.1−25.7× in the *fur* null mutant.
353 Also, the outer membrane protein A gene (VSAL_I1819), a MFS transporter gene
354 (VSAL_II0149) and *potE* (VSAL_II1067) are up-regulated 5.9×, 5.6× and 5.0×, respectively.

355

356 *Down-regulated genes in asFur null mutant*

357 Fur primarily functions as a repressor. The down-regulated genes in our study (i.e., in the *fur* null
358 mutant) are expected to be positively regulated by *as*Fur in the wild-type, either via the
359 repression of *ryhB* (or other sRNAs with similar function), which typically destabilizes its mRNA
360 targets (Oglesby-Sherrouse & Murphy 2013), or by direct stimulation of expression by *as*Fur
361 itself. In this study we cannot conclusively distinguish between these two possibilities, although
362 we have predicted potential targets of RyhB and other up-regulated sRNAs (see below).

363 Table 2 shows 34 down-regulated genes in the *fur* null mutant compared to wild type.
364 Overall, the Δ*fur*/wt values for down-regulated genes are significantly lower than that of up-
365 regulated genes (the strongest down-regulation is -8.6×, when excluding *fur* that has been deleted
366 from the genome). In Table 2 we therefore present genes that are ≤ -3× down-regulated. The
367 majority of the genes are categorized as "motility/chemotaxis" or "metabolism". "Metabolism"
368 genes are involved in different pathways such as amino acid, energy, nucleotide, carbon etc.
369 Moreover, several motility and chemotaxis genes are down-regulated between -3.5× and -6.3× in
370 the *fur* null mutant. Of these, four encode flagellin subunits [*flaC-flaE* (VSAL_I2317- I2319) and
371 (*flaF* VSAL_I2517)], one encodes a sodium-type polar flagellar protein (*motX* VSAL_2771) and

two encodes methyl-accepting chemotaxis proteins (VSAL_I0799 and VSAL_I2193). Three heat shock proteins encoded by *groL1* (VSAL_I0017), *groS1* (VSAL_I0018) and *htpG* (VSAL_I0814) are also down-regulated. Heat shock proteins are involved in protein folding and unfolding, cell cycle control, transport and stress responses amongst others. Transcriptome studies of a Δ*fur* mutant in *V. vulnificus* have also shown a down-regulation of heat-shock protein genes, chemotaxis protein genes and motility-associated genes (Pajuelo et al. 2016). Two oxidative stress response protein encoding genes, *sodB* and *catA* (VSAL_I1858 and VSAL_II0215), are down-regulated in the *fur* null mutant. SodB is an iron binding protein and a RyhB target in other organisms, and CatA is a heme-binding protein.

In summary, differentially down-regulated genes in the *A. salmonicida fur* null mutant have significantly lower differential expression values (i.e., ×) than the up-regulated genes possibly due to, in part, secondary regulatory effects rather than being directly regulated by Fur. The majority of down-regulated genes have functions in chemotaxis, motility, heat shock and oxidative stress response.

Identification of sRNAs with roles in iron homeostasis

ncRNAs represent an important part of regulons in bacteria, often controlling critical and early steps in regulatory pathways (Gottesman 2005). We therefore set out to explore the presence and function of sRNAs in our RNA-seq dataset. Table 1 already showed us that *ryhB* is up-regulated 43× in the *fur* null mutant, which strongly supports that RyhB in *A. salmonicida* has a similar role in iron homeostasis as what was established for its homologs in e.g., *E. coli* (Masse et al. 2005) and *V. cholerae* (Davis et al. 2005). Here, RyhB is produced under low-iron conditions and stops production of iron-using/storing proteins, and therefore contributes to a lowered demand for iron.

To search for other sRNAs with potential roles in iron homeostasis we re-analyzed the RNA-seq dataset. The rational was that any Fur-regulated sRNA gene are likely candidates to have roles in iron metabolism by targeting specific mRNAs for degradation. One sRNA gene (VSAL_II2005s) that fulfilled this criterion was identified among 252 sRNA genes that we predicted in a previous work (Ahmad et al. 2012). VSAL_II2005s was up-regulated 4×. Furthermore, we analyzed the RNA-seq data using Rockhopper. Rockhopper predicts ncRNAs from RNA-seq data. The sRNA predicted by Rockhopper were manually curated using the

Artemis software. Briefly, to be accepted as a true sRNA, its gene had to be (i) located in an intergenic region, (ii) between 30−350 nt in length, (iii) located 30 nt or more from the nearest CDS if on the same strand, and 10 nt if on the complementary strand.

Ninety-three potential sRNA were predicted using Rockhopper, including predictions of sRNAs in pseudogene regions. Seventeen were kept after manual curation, eight of which overlapped or were complementary to previously predicted sRNAs in *A. salmonicida* (Ahmad et al. 2012). These eight sRNAs were VSAL_I4057s, VSAL_I4069s and VSAL_I4164s (overlapping), and VSAL_I4107s, VSAL_I4164s, VSAL_I4189s, VSAL_II2008s and VSAL_II2050s (complementary). Of the remaining nine new sRNAs identified by Rockhopper and manual curation, six are located on Chr I and three on Chr II (see Table 3). New sRNAs 4 and 7 both contain sORFs, which potentially encode small proteins (see Material and methods) (Hobbs et al. 2011). The nine new sRNA were added to the *A. salmonicida* genome annotation using Artemis, and the RNA-seq data was re-analyzed for differentially expressed genes using EDGE-pro and DESeq. Two of the new sRNAs, i.e., number 1 and 9, were up-regulated 2.2× and 2.5× in the *fur* null mutant, respectively. Homology searches did not give significant hits.

In summary, RyhB and a previously predicted sRNA (VSAL_II2005s) were up-regulated in the *A. salmonicida fur* null mutant. Nine new sRNAs were predicted using Rockhopper and manual curation, of which two were differentially expressed (i.e., number 1 and 9 in Table 3).

sRNA target predictions

Next, we used the TargetRNA2 and IntaRNA softwares to test if the up-regulated sRNAs identified above can explain some of the down-regulated protein-coding genes. The up-regulated sRNAs *ryhB*, VSAL_II2005s and new sRNAs 1 and 9 (Table 3) were tested for target binding towards the 34 down-regulated genes presented in Table 2. *ryhB* is up-regulated 43.7×, and typically targets mRNA for iron using and iron storage proteins (Davis et al. 2005; Masse et al. 2005; Mey et al. 2005b; Murphy & Payne 2007; Oglesby-Sherrouse & Murphy 2013). We expected to find same/ similar targets in our datasets. RyhB target predictions suggests that seven of the mRNAs listed in Table 2 have significant complementarity to RyhB. Two of the corresponding genes, i.e., *sodB* and *fur*, represent known targets from other organisms (Davis et al. 2005; Masse et al. 2005; Mey et al. 2005b). The other identified targets are *cysN*

(VSAL_I0421), VSAL_I0422, *tcyP* (VSAL_I1813), VSAL_II1026 and VSAL_I0424. Furthermore, we tested other known targets for complementarity to RyhB. Matches were found to *gltB* and *sdhC*, which were down-regulated 2.1× and 1.3×, respectively. We therefore consider *gltB* as a potential RyhB target in *A. salmonicida*, while *sdhC* is probably not due to the weak regulation. In *E. coli* K-12, GltB is an iron-sulfur binding protein. Thus, down-regulation of *gltB* is an iron sparing strategy.

Our target predictions for VSAL_II2005s (which was 4× up-regulated) suggest significant complementarity to *tcyP* (VSAL_I1813). Interestingly, *tcyP* was also identified as a RyhB target, which may explain why *tcyP* has a relative strong down-regulation of -8.6× (when compared to the other down-regulated genes) in the *fur* null mutant. No potential targets were identified for the new sRNAs 1 and 9.

In summary, *as*RyhB appears to have similar regulatory functions as its known homologs from other model organisms, and may account for the down-regulation of seven of the 34 genes in Table 2. We also identified *tcyP* as a potential target for both RyhB and VSAL_II2005s. No complementatrity was found between the new sRNAs 1 and 9 and mRNAs corresponding to the down-regulated genes listed in Table 2.

Concluding remarks

We have studied the Fur regulon of *A. salmonicida* using gene knock out technology and compared the transcriptome of the *fur* null mutant with its isogeneic wild type using RNA sequencing. Our results show that *as*Fur acts as a master regulator in *A. salmonicida* affecting ~7% of the CDSs, when threshold values were set to 2× differential expression and with p-values ≤ 0.05. We also demonstrate that *as*Fur acts mainly as a repressor. This conclusion is based on that Δ*fur*/wt differential expression values of up-regulated genes in the *fur* null mutant are significantly higher than that of down-regulated genes. Furthermore, we demonstrated a strong *gene dosage effect* for Chr I. This result adds to the growing list of *Vibrionaceae* bacteria where the transcription level is, statistically, highest in the chromosomal regions surrounding the *oriC*, and weaker for genes located further away from *Ori*C. Finally, we identify sRNAs with potential roles in iron homeostasis. The role for RyhB is well established, and in addition we identify VSAL_II2005s, which was 4× up-regulated in a fur null mutant, as another sRNA that contains significant complementarity to *tcyP* (VSAL_I1813).

Our current data is in good overall agreement with our previous work (Ahmad et al. 2012; Ahmad et al. 2009; Pedersen et al. 2010; Thode et al. 2015). As expected, we found a large overlap in data between experiments where *A. salmonicida* was subjected to low-iron conditions and global changes in gene expression was monitored using microarray (Thode et al. 2015), and this work where the global gene expression data (using RNA-seq) of a *fur* null mutant and wild type strain were compared. Of the 32 genes identified by microarray, 4 are not ≥2x up-regulated in the *fur* null mutant (i.e., *feoA, feoB,* VSAL_II0717 and VSAL_I2980), while the remaining 28 are ≥2× up-regulated. With the latest data we conclude that we today have a more accurate and fine-grained global understanding of how *A. salmonicida* regulates gene expression under low-iron conditions, which is a highly relevant setting that the bacterium is expected to confront during initial phases of infection of Atlantic salmon. In the future, we will shift our focus from basic knowledge of bacterial iron metabolism towards how this can be applied to the benefit of society. Possible directions could be using microbial iron metabolism systems as targets for new antimicrobial drugs and/or production of iron scavenging molecules that could be useful in medicine and agriculture (Saha et al. 2013, Gorska et al. 2014).

Availability of supporting data

RNA sequencing data are available in the European Nucleotide Archive (ENA) under accession number PRJEB17700.

Additional files

Figure S1. Linear and logarithmic growth curves of *A. salmonicida* LFI1238 wt and *fur* null mutant.

Figure S2. Chemical titration test of *A. salmonicida* LFI1238 wt and *fur* null mutant.

Abbreviations

ABC transporter: ATP-binding cassette; Fur: Ferric Uptake Regulator; *ec*Fur: *Escherichia coli* Fur; *as*Fur: *Aliivibrio salmonicida* Fur; sRNA: small regulatory RNA; ORF: Open reading frame; mRNA: messenger RNA; TCA: tricarboxylic acid; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; bp: base pair; nt: nucleotide; LB: Luria Bertani broth/ Lysogen Broth; tRNA: transfer RNA; rRNA: ribosomal RNA; Chr: Chromosome; MFS transporter: major facilitator superfamily transporter; h: hours; PCR: Polymerase Chain Reaction; OD: optical density; wt: wild type; RPKM: reads per kilo base per million reads; RNA-seq: RNA sequencing; rpm: rounds per minute; *AS*: *Aliivibrio salmonicida*; sORF: small open reading frame; ncRNA: non coding RNA; Δ*fur*: *fur* null mutant.

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Competing interests

The authors declare that they have no competing interests.

Ethics statement

The research presented in this paper do not involve human subjects, and we see no ethical issues.

Authors` contributions

PH, HH and SKT conceived the study and designed experiments. HH supervised and participated in the construction of the *fur* null mutant, and PH supervised the studies and helped draft the manuscript. SKT performed the construction of the *fur* null mutant, biological characterizations,

cultured samples for RNA sequencing, total RNA purifications, post analysis of the RNA-seq data and drafted the manuscript. CB performed the RNA-seq data analysis, expression analysis, sRNA and mRNA target predictions and drafted the manuscript. EH supervised the data analysis, provided and helped adjust scripts, and deposited the data to ENA. JS performed quality testing and rRNA depletion of the RNA. All authors read and approved the final manuscript.

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Tables

Table 1: Up-regulated (≥4×) genes in *A. salmonicida fur* null mutant compared to wild type.

VSAL_nr	*gene*	Annotation	*Δfur*/ wt	Fur-box**
Siderophore biosynthesis and transport				
VSAL_I0134 *	*bibA*	Bisucaberin siderophore biosynteshis protein A	92.6	x
VSAL_I0135	*bibB*	Bisucaberin siderophore biosynteshis protein B	48.2	x
VSAL_I0136	*bibC*	Bisucaberin siderophore biosynteshis protein C	11.1	x
VSAL_I0137	*bitA*	TonB-dependent iron-siderophore receptor precursor	9.3	x
VSAL_II0148		2Fe-2S binding protein, siderophore ferric reductase	8.0	x
VSAL_II0150	*fhuC*	ferrichrome transport ATP-binding protein FhuC	7.0	x
VSAL_II0151	*fhuD*	ferrichrome-binding periplasmic protein	12.5	x
VSAL_II0152	*fhuB*	ferrichrome transport protein FhuB	6.7	x
VSAL_II0907		iron(III) ABC transporter, periplasmic iron-compound-binding (pseudo)	5.9	x
VSAL_II0908	*hatC*	iron(III) ABC transporter, ATP-binding protein	11.2	x
VSAL_II0909	*desA*	ferrioxamine B receptor	18.8	x
TonB systems				
VSAL_I1751	*tonB1*	TonB protein (pseudogene)	18.8	x
VSAL_I1752	*exbB1*	TonB system transport protein ExbB1	25.2	x
VSAL_I1753	*exbD1*	TonB system transport protein ExbD1	28.4	x
VSAL_II0110		TonB dependent receptor	55.8	x
VSAL_II0111		putative exported protein	35.3	x
VSAL_II0112	*tolR2*	biopolymer transport protein TolR	25.7	x
VSAL_II0113	*exbB2*	TonB system transport protein ExbB2	17.3	x
VSAL_II0114	*exbD2*	TonB system transport protein ExbD2	27.6	x
VSAL_II0115	*tonB2*	TonB protein	30.1	x
VSAL_II0116		putative exported protein	23.4	x
Heme uptake and utilization				
VSAL_I1734		heme receptor (pseudogene)	6.6	x
VSAL_I1749	*huvX*	heme uptake and utilization protein HuvX	20.2	x
VSAL_I1750	*phuW*	putative coproporphyrinogen oxidase PhuW	39.7	x
VSAL_I1754	*huvB*	heme transporter protein HuvB, periplasmic binding protein	39.7	x
VSAL_I1755	*huvC*	heme transporter protein HuvC, transmembrane permease component	13.5	x
VSAL_I1756	*huvD*	heme transporter protein HuvD, ATP-binding component	5.8	x
small RNA				
VSAL_I3102s	*ryhB*	small RNA RyhB	43.7	x
VSAL_II2005s		VSAsRNA006	4.0	
Other transport				

VSAL_I1819		outer membrane protein A	5.9	
VSAL_I2067	*zntA*	lead, cadmium, zinc and mercury-transporting ATPase	8.5	
VSAL_I2891	*vcmD*	multidrug efflux pump	8.5	x
VSAL_II0118		membrane protein	16.9	
VSAL_II0119		putative exported protein	25.7	
VSAL_II0120		nickel transporter	16.7	
VSAL_II0121		putative exported protein	16.7	
VSAL_II0122		putative membrane protein	8.7	
VSAL_II0123		zinc ABC transporter periplasmic substrate binding protein	7.4	
VSAL_II0124		zinc ABC transporter ATP binding protein	6.3	
VSAL_II0125		zinc ABC transporter permease	4.1	
VSAL_II0149		MFS transporter	5.6	
VSAL_II1043		cation efflux pump, cobalt-zinc-cadmium resistance protein	5.7	
VSAL_II1067	*potE*	putrescine-ornithine antiporter	5.0	
Metabolism				
VSAL_I1785		thiol oxioreductase	5.7	
VSAL_I1786		peptidase, putative iron-regulated	8.2	x
VSAL_I2892		methyltransferase	12.4	x
VSAL_II0932	*bcsA*	cellulose synthase catalytic subunit	6.1	
VSAL_II1066	*speF*	ornithine decarboxylase, inducible	7.4	
Cell envelope				
VSAL_I1328		putative membrane associated peptidase	4.4	
VSAL_I1783		putative lipoprotein	4.4	
VSAL_I1784		putative lipoprotein	5.0	
VSAL_I1820		putative lipoprotein	4.0	
VSAL_I1864		putative membrane protein	20.1	x
VSAL_II0074		membrane protein	67.3	x
VSAL_II0868		putative lipoprotein	8.0	x
VSAL_II0931		membrane protein (fragment)	4.8	
VSAL_II0933		putative exported protein	6.2	
VSAL_II0937		membrane protein	4.0	
Unknown function				
VSAL_I0881		putative exported protein	15.7	x
VSAL_I0882		putative exported protein	14.1	x
VSAL_I0883		putative exported protein	14.4	x
VSAL_I0884		putative exported protein	5.0	x
VSAL_II0469		hypothetical protein	4.5	
VSAL_II0934		hypothetical protein	4.0	

* p-value not analyzed

** fur-box predictions from Ahmad et.al. (Ahmad et al. 2009)

649 **Table 2:** Down-regulated (≤ -3×) genes in *A. salmonicida fur* null mutant compared to wild type.

VSAL_nr	*gene*	annotation	*Δfur/* wt	sRNA target
Motility/ chemotaxis				
VSAL_I0799		methyl-accepting chemotaxis protein	-3.5	
VSAL_I2193*		methyl-accepting chemotaxis protein	-3.6	
VSAL_I2317	*flaE*	flaggelin subunit E	-5.1	
VSAL_I2318	*flaD*	flaggelin subunit D	-4.3	
VSAL_I2319	*flaC*	flaggelin subunit C	-6.2	
VSAL_I2517	*flaF*	flaggelin subunit F	-3.9	
VSAL_I2771	*motX*	sodium-type polar flagellar protein MotX	-5.0	
Oxidative stress response				
VSAL_I1858	*sodB*	superoxide dismutase [Fe]	-3.1	RyhB
VSAL_II0215	*catA*	catalase	-3.4	
Metabolism				
VSAL_I0122	*prlC*	oligopeptidase A	-3.2	
VSAL_I0421	*cysN*	sulfate adenylyltransferase subunit 1	-3.4	RyhB
VSAL_I0422		ion transporter superfamily protein	-3.8	RyhB
VSAL_I0423	*cysC*	adenylylsulfate kinase	-4.0	
VSAL_I1133	*hisG*	ATP phosphoribosyltransferase	-3.4	
VSAL_I1769	*nrdA*	ribonucleoside-diphosphate reductase 1 alpha chain	-3.8	
VSAL_I1857	*queD*	queuosine biosynthesis protein	-4.0	
VSAL_II0666	*idnK*	thermosensitive gluconokinase	-4.4	
VSAL_II0846		putative acetyltransferase	-3.4	
VSAL_II1026		putative tryptophanyl-tRNA synthetase	-6.4	RyhB
small RNA				
VSAL_I4000s		VSsRNA001	-4.1	
VSAL_I4069s		VSsRNA070	-3.4	
VSAL_I4100s		VSsRNA 101	-4.1	
VSAL_I4139s		VSsRNA140	-3.9	
Chaperones/ heat shock proteins				
VSAL_I0017	*groL1*	60 kda chaperonin 1	-3.2	
VSAL_I0018	*groS1*	10 kDa chaperonin 1	-3.9	
VSAL_I0814	*htpG*	chaperone protein HtpG (heat shock protein HtpG)	-3.2	
Cell envelope/ transport				
VSAL_I1813	*tcyP*	L-cystine transporter	-8.6	RyhB, VSAL_II2005s
VSAL_II0853		MFS transporter	-4.0	
VSAL_II0854		secretion protein, HlyD family	-3.9	
VSAL_II1062		membrane protein	-3.3	
Unknown function				

VSAL_I0424		hypothetical protein	-3.2	RyhB
VSAL_I2064		conserved hypothetical protein	-4.0	
VSAL_II0168		putative exported protein	-7.9	
Mutated gene/ control gene				
VSAL_I0833	*fur*	ferric uptake regulator protein	-128.7	RyhB

*fur-box predicted in Ahmad et. al. (Ahmad et al. 2009)

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652 **Table 3:** sRNAs identified by Rockhopper.

New sRNA	Start bp	Stop bp	Length	Flanking upstream	Flanking downstream	Strand	Possible sORF	*Δfur*/wt RNA seq	p-value RNA seq
1	51134	51393	259	VSAL_I0047	VSAL_I0048	+	no	2.22	0
2	776673	776837	164	VSAL_I0690	VSAL_I0691	+	no	-1.27	0.41
3	2343220	2343291	71	VSAL_I2181	VSAL_I2182	+	no	1.21	0.15
4	2405357	2405638	281	VSAL_I2233	VSAL_I2234	+	yes	1.06	0.66
5	2812966	2813103	137	VSAL_I3191r	VSAL_I2601	+	no	-1.52	0.18
6	3259173	3259344	171	VSAL_I3008	VSAL_I3009	-	no	-1.05	0.69
7	692443	692539	96	VSAL_II0641	VSAL_II0642	+	yes	1.97	0.01
8	814013	814056	43	VSAL_II2035s	VSAL_II0738	-	no	-1.05	0.85
9	1141984	1142209	225	VSAL_II1046	VSAL_II1047	+	no	2.53	0.00

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Figure legends

Figure 1. Functional distribution of genes that are ≥2× differentially expressed between *A. salmonicida* wild type and the *fur* null mutant strain. The number in parenthesis represent the percentage of the total number of genes within the genome in each functional class.

Figure 2. Schematic circular diagrams of the *A. salmonicida* chromosomes I and II (ChrI and ChrII). Circles indicate from outside to inside differentially expressed genes ≥4× (indicated with light blue filled circles) and ≤ -3× (indicated with orange filled circles), the scale in base-pairs, CDSs on leading strand (green), CDSs on lagging strand (blue), non-coding RNA genes [sRNAs (red), tRNAs and rRNAs (grey)], differential expression in *fur* null mutant compared to wild-type strain (up-regulation is shown in green bars, down-regulation in red bars), amount of RNA-seq reads mapped to the chromosome in *fur* null mutant (blue bars) and wild-type (red bars) strain. Figure is not to scale.

Figure S1. Linear (A) and logarithmic (B) growth curves of *A. salmonicida* LFI1238 wt and *fur* null mutant grown in LB containing 1% NaCl, at 8°C with 200 rpm agitation. Four biological replicates were used. Grey area indicate the measured span and dotted line indicate the average curve.

Figure S2. Chemical titration test of *A. salmonicida* LFI1238 wt and *fur* null mutant. Growth conditions were LB containing 1% NaCl, at 8°C with 200 rpm agitation. The cultures were grown to mid-log phase, split to smaller cultures and added increasing amounts of H_2O_2 and 2,2'-dipyridyl. A) *AS* wt grown with increasing concentrations of H_2O_2. B) *AS* Δ*fur* grown with increasing concentrations of H_2O_2. C) *AS* wt grown with increasing concentrations of 2,2'-dipyridyl. D) *AS* Δ*fur* grown with increasing concentrations of 2,2'-dipyridyl.

Figure 1

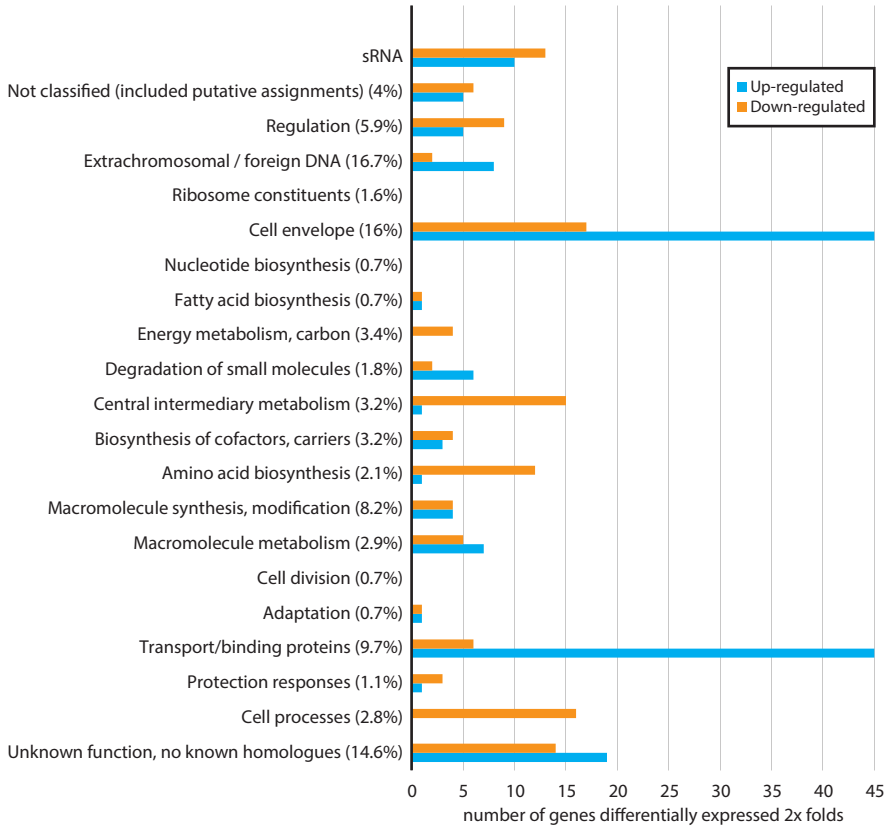

sRNA
Not classified (included putative assignments) (4%)
Regulation (5.9%)
Extrachromosomal / foreign DNA (16.7%)
Ribosome constituents (1.6%)
Cell envelope (16%)
Nucleotide biosynthesis (0.7%)
Fatty acid biosynthesis (0.7%)
Energy metabolism, carbon (3.4%)
Degradation of small molecules (1.8%)
Central intermediary metabolism (3.2%)
Biosynthesis of cofactors, carriers (3.2%)
Amino acid biosynthesis (2.1%)
Macromolecule synthesis, modification (8.2%)
Macromolecule metabolism (2.9%)
Cell division (0.7%)
Adaptation (0.7%)
Transport/binding proteins (9.7%)
Protection responses (1.1%)
Cell processes (2.8%)
Unknown function, no known homologues (14.6%)
Up-regulated
Down-regulated
0 5 10 15 20 25 30 35 40 45
number of genes differentially expressed 2x folds

Figure 2

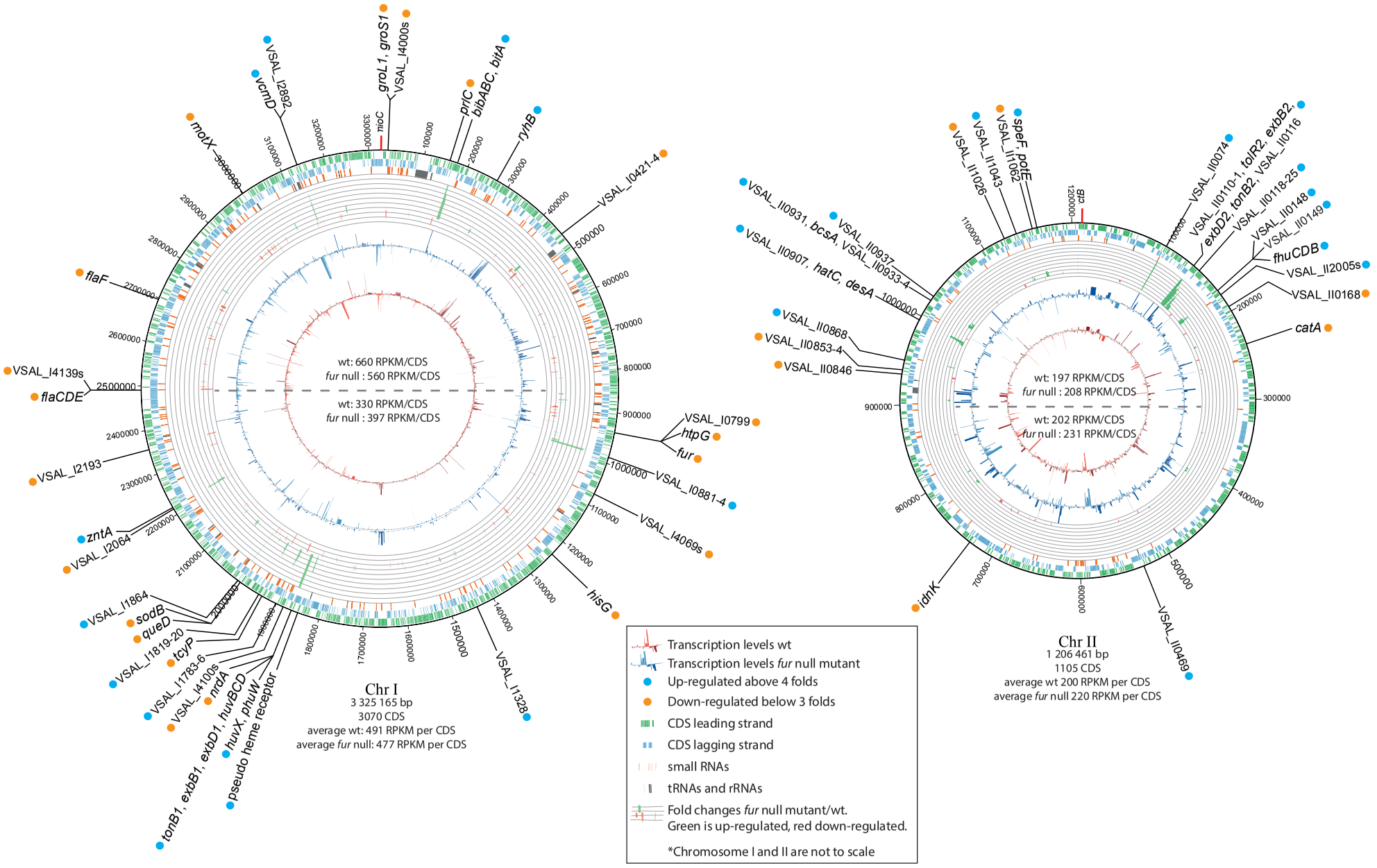

wt: 660 RPKM/CDS
fur null : 560 RPKM/CDS
wt: 330 RPKM/CDS
fur null : 397 RPKM/CDS
Chr I
3 325 165 bp
3070 CDS
average wt: 491 RPKM per CDS
average fur null: 477 RPKM per CDS
wt: 197 RPKM/CDS
fur null : 208 RPKM/CDS
wt: 202 RPKM/CDS
fur null : 231 RPKM/CDS
Chr II
1 206 461 bp
1105 CDS
average wt 200 RPKM per CDS
average fur null 220 RPKM per CDS
Transcription levels wt
Transcription levels fur null mutant
Up-regulated above 4 folds
Down-regulated below 3 folds
CDS leading strand
CDS lagging strand
small RNAs
tRNAs and rRNAs
Fold changes fur null mutant/wt.
Green is up-regulated, red down-regulated.
*Chromosome I and II are not to scale
groL1, groS1
VSAL_I4000s
prlC
bibABC, bitA
ryhB
VSAL_I0421-4
VSAL_I0799
htpG
fur
VSAL_I0881-4
VSAL_I4069s
hisG
VSAL_I1328
VSAL_I2892
vcmD
motX
flaF
VSAL_I4139s
flaCDE
VSAL_I2193
zntA
VSAL_I2064
VSAL_I1864
sodB
queD
VSAL_I1819-20
tcyP
VSAL_I1783-6
VSAL_I4100s
nrdA
tonB1, exbB1, exbD1, huvBCD
huvX, phuW
pseudo heme receptor
VSAL_II0931, bcsA, VSAL_II0933-4
VSAL_II0907, hatC, desA
VSAL_II0868
VSAL_II0853-4
VSAL_II0846
idnK
VSAL_II0469
VSAL_II1026
VSAL_II1043
VSAL_II1062
speF, potE
gdh
VSAL_II0074
VSAL_II0110-1, tolR2, exbB2, exbD2, tonB2, VSAL_II0116
VSAL_II0118-25
VSAL_II0148
VSAL_II0149
fhuCDB
VSAL_II2005s
VSAL_II0168
catA

Figure S1

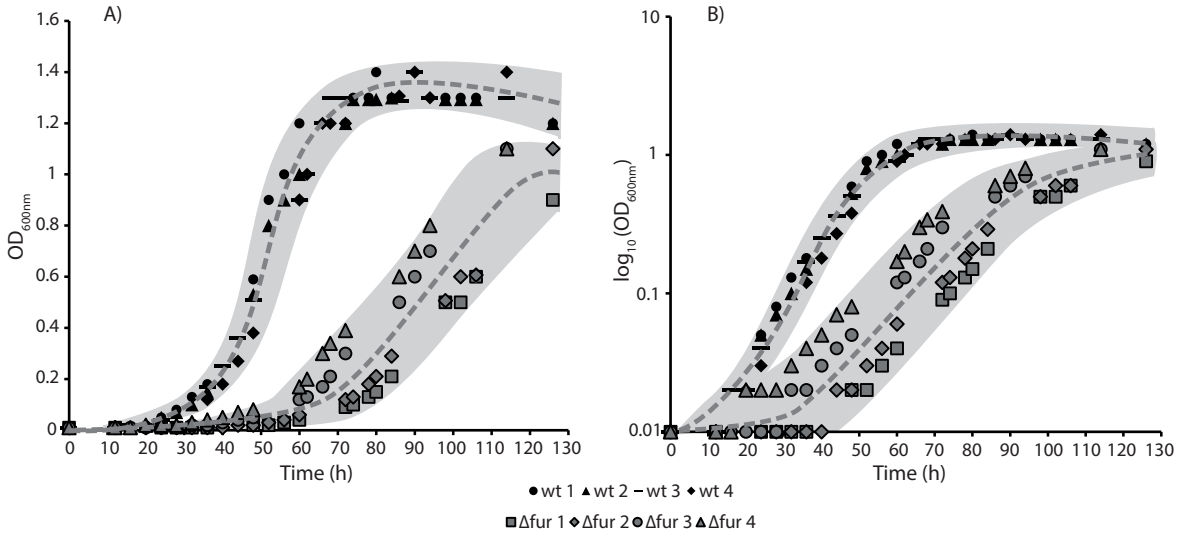
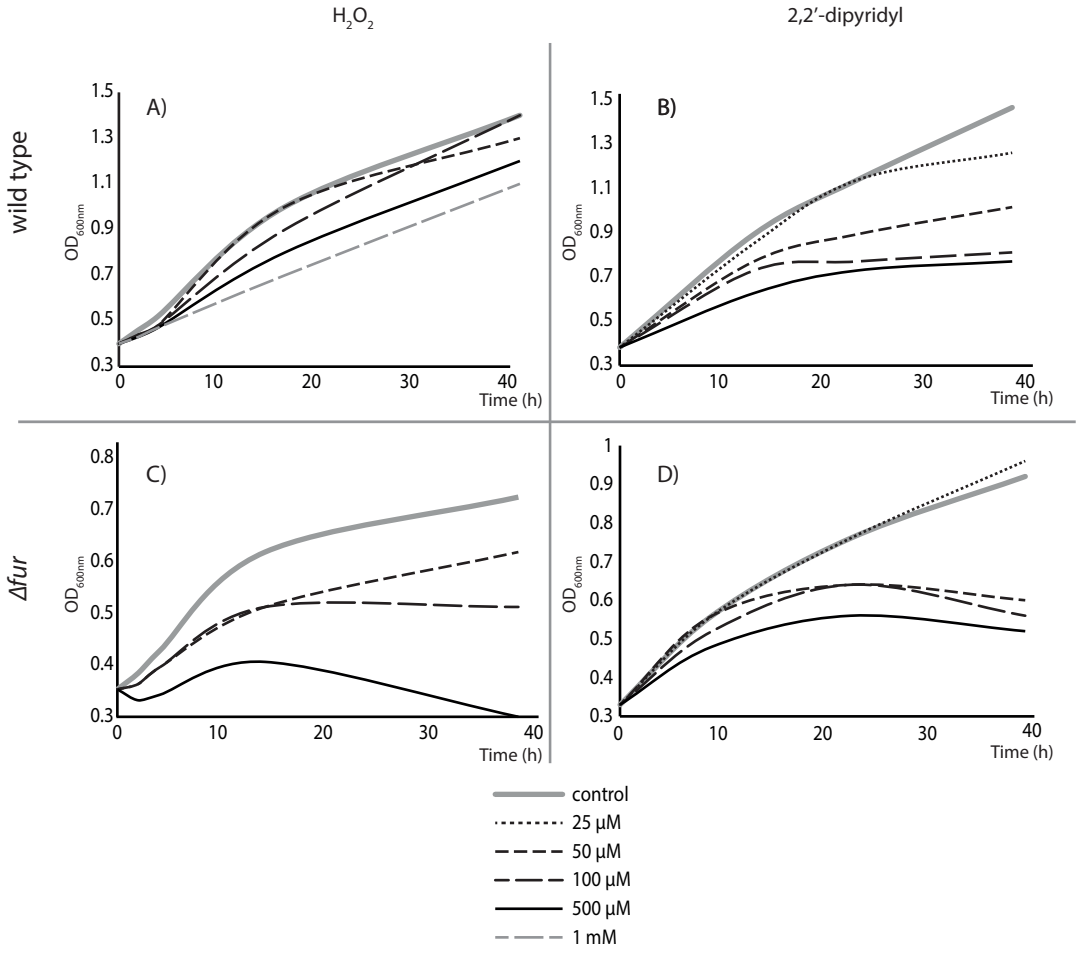

A)
B)
OD600nm
log10 (OD600nm)
Time (h)
Time (h)
wt 1 wt 2 wt 3 wt 4
Δfur 1 Δfur 2 Δfur 3 Δfur 4

Figure S2


H2O2
2,2′-dipyridyl
wild type
Δfur
A)
B)
C)
D)
OD600nm
Time (h)
control
25 µM
50 µM
100 µM
500 µM
1 mM

Paper 3

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

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Keywords

Siderophore biosynthesis, iron metabolism, bisucaberin, vibrioferrin, piscibactin, catechols, hydroxamates, carboxylates, *Vibrionaceae*.

Abstract

Introduction: Siderophores are low molecular weight compounds synthesized and secreted by e.g., bacteria and fungi to bind and scavenge iron. Extracellular ferri-siderophore complexes are recognized by cognate receptors on the cell surface for transport over membranes. Several siderophore systems found in model bacteria from *Vibrionaceae* are known and well understood, e.g., the molecular structure of the siderophore, the biosynthesis gene cluster and pathway, and the gene expression pattern. Less is however known about how these systems are distributed in the approximately 140 *Vibrionaceae* species, and which evolutionary processes contributed to the present-day distribution. In this work, we compiled existing knowledge on siderophore biosynthesis systems and siderophore receptors from *Vibrionaceae* and used phylogenetic analyses to investigate their distribution, origin and evolution.

Results: A comprehensive literature study identified eight different siderophore biosynthesis systems and twelve siderophore receptors in *Vibrionaceae.* 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.

Conclusions: The present work provides the most comprehensive overview of the distribution of siderophore-based iron acquisition systems in *Vibrionaceae*, and presents the first phylogenetic analyses of these systems. The results suggests that the present-day distribution is a result of several evolutionary processes, such as old and new gene acquisitions, gene loss, and both vertical and horizontal gene transfers.

Introduction

Siderophores represent a group of relatively small and low molecular weight secondary metabolites with high-affinity binding potential to ferric iron [1]. They are produced and secreted by a broad range of microorganisms, e.g., bacteria and fungi, and some plants. Under low iron conditions, such as in aquatic environments or inside a vertebrate host, bacteria must use highly specific strategies to acquire iron and other essential micronutrients [2, 3]. To overcome the iron starvation, siderophores are synthesized and secreted to their surroundings where they chelate ferric iron. Once bound, the ferric iron-siderohphore complexes are recognized by cognate siderophore receptors, and transported over the membrane by ABC transporters using TonB complexes as energy transducers.

Interestingly, bacteria produce siderophores of several major classes, each of which can have a diverse set of molecular structures, presumably because production of unique siderophores can provide individual bacterial species with an advantage in the competition with others [4]. For example, polymicrobial studies have shown that siderophores from one species can inhibit growth or functions of other species, e.g. low concentrations of avaroferrin from *Shewanella algae* inhibit swarming of *Vibrio alginolyticus* and a siderophore from *Pseudomonas fluorescens* inhibit growth of *Vibrio anguillarum* [5, 6]. Such kin discrimination strategy can however be bypassed by

"cheaters", i.e., bacteria expressing receptors on their surface with affinity to siderophores produced by others [7]. This mechanism is also known as exogenous or xeno-siderophore utilization. Evidently, there must be a constant battle between microorganisms for available iron; they can produce (i) own siderophores and the respective receptors, and/or (ii) "cheating" receptors for utilization of siderophores produced by others.

In this work, we have studied siderophore biosynthesis systems and their respective receptors from the *Vibrionaceae* family. *Vibrionaceae* represents a large and diverse group of Gram-negative Gamma Proteobacteria, and the evolutionary relationships between many of the approximately 140 different species were recently updated by Sawabe and coworkers [8]. Representatives of this family have been heavily studied, in most cases due to their ability to cause serious disease in humans or animals. The majority of species are however harmless and represent no threat, but instead play important roles in the environment e.g., in recycling of nutrients.

The causative agent of the human disease cholera, *Vibrio cholerae*, is the most famous *Vibrionaceae* representative. *V. cholerae* produces the catechol siderophore vibriobactin using proteins encoded by *vibABCDEFH* [9, 10]. Here, ferric iron-vibriobactin complexes are recognized by the receptor ViuA [11]. Moreover, *V. cholerae* can cheat on derivatives of enterobactin (produced by e.g., *Escherichia coli*) using the receptors IrgA and VctA [12], fluvibactin (synthesized by *Vibrio fluvialis*) using the ViuA, VctA and IrgA receptors, and finally ferrichrome by using the FhuA receptor [12-14]. *Vibrio vulnificus* represents another significant human pathogen [15]. This bacterium produces the catechol siderophores vulnibactin by using proteins encoded by the gene cluster VV2_0830 - VV2_0844 [16], and recognizes ferri-vulnibactin via the VuuA receptor [17]. It has also been proposed that *V. vulnificus* produces an uncharacterized hydroxamate siderophore, and an uncharacterized catechol siderophore using, in part, same genes as for vulnibactin [16, 18]. Finally, *V. vulnificus* can transport and utilize aerobactin (IutA receptor) [19], deferoxamine B (DesA receptor) [20, 21] and vibriobactin [22]. The human pathogen *Vibrio parahaemolyticus* [23] produces the carboxylate siderophore named vibrioferrin (encoded by *pvsABDE*) [24]. Vibrioferrin is sensitive to photolysis and has a lower affinity for iron compared to other catechol-type siderophores in vibrios. Ferri-vibrioferrin is recognized and transported over the membranes using the receptor PvuA [25]. *V. parahaemolyticus* can "cheat" using the exogenous siderophores enterobactin, aerobactin, ferrichrome and possibly vibriobactin and fluvibactin [22, 26-29].

Several *Vibrionaceae* fish pathogens have been studied with respect to siderophore production and utilization, e.g., *V. anguillarum*, a pathogen causing haemorrhagic septicaemia in fish, bivalves and crustaceans [30], *Aliivibrio salmonicida*, causing cold-water vibriosis in Atlantic salmon at low seawater temperatures [31, 32], *Photobacterium damselae subsp. piscicida* [33, 34], and *V. alginolyticus* [35]. Dependent on the strains, *V. anguillarum* can synthesize and utilize the mixed catechol/hydroxamate siderophore anguibactin (serotype O1 strain; biosynthesis encoded by *angABCE*^{B}/$_{G}$*MTHRNUD* and recognized by FatA receptor) [36, 37]. Intriguingly, anguibactin biosynthesis genes are located both on a virulence plasmid named pJM1, and on chromosomes (*angABC* and *angE*) [37]. In contrast, serotype O2 strains produce and utilize the catechol siderophore vanchrobactin (biosynthesis encoded by *dapH* and *vabABCEFH* [38], and recognized by the receptor encoded by *fvtA* [39]). *V. anguillarum* utilize exogenous siderophores like enterobactin, ferrichrome and citrate [40, 41]. *A. salmonicida* synthesizes and utilizes the di-hydroxamate siderophore bisucaberin (biosynthesis encoded by *bibABC* and recognized by the BitA receptor) [42, 43]. It has been postulated that the siderophore production is vital for the virulence of *A. salmonicida*. This assumption is based on that production of significant amounts of bisucaberin is restricted to low temperature conditions (bacterium only causes disease at low temperatures) [42]. Also, we recently showed that the genes responsible for bisucaberin are highly up-regulated under low iron conditions [44]. A system for aerobactin synthesis is in contrast not expressed, probably because the cluster is non-functional due to frameshift mutations and loss of the promotor [45]. The genome of *A. salmonicida* also encode the deferoxamine B receptor DesA and the aerobactin receptor IutA [45]. The fish pathogen *P. damselae subsp. piscicida* produces the mixed carboxylate and hydroxamate siderophore piscibactin (encoded by *dapH* and *irp123459*) and is probably transported by FrpA [33, 34]. *V. alginolyticus* is an emerging foodborne pathogen that causes gastroenteritis and peritonitis in humans [35]. The *V. alginolyticus* B522 strain contains the vibrioferrin biosynthesis cluster [5, 46], and can also utilize siderophores synthesized by *V. cholerae*, *V. fluvialis* and *V. parahaemolyticus* and ferrichrome [47, 48].

Payne and co-workers recently reviewed siderophore biosynthesis and utilization in *Vibrionaceae* [7]. This inspired us to use the existing knowledge to investigate the distribution and evolution of the different siderophore systems further. In this work, we first performed a comprehensive literature study on *Vibrionaceae* siderophore systems, and compared the gene synteny of the corresponding siderophore gene clusters. Furthermore, we searched the databases

for siderophore systems in all available *Vibrionaceae* genomes, and mapped the result onto a *Vibrionaceae* phylogenetic network. The evolution of individual siderophore biosynthesis systems and receptors was subsequently studied by constructing phylogenetic trees based on amino acids datasets, and by comparing the resulting tree topologies to host trees. Through the presented work we wish to broaden the perspective and existing knowledge on siderophore synthesis and utilization within the *Vibrionaceae* family.

Material and Methods

Data retrieval

Siderophore biosynthesis gene clusters and associated siderophore receptor genes in *Vibrionaceae* were identified by a literature search, and the corresponding protein sequences were retrieved from ENA/GenBank. Updated RefSeq accession numbers for identified proteins with the 'WP' prefix (replaced the 'YP', 'NP' and 'ZP' prefixes) are presented in Table 1 and 2. These sequences were next used as queries in BLASTP searches to find homologous sequences. BLASTP was run using the non-redundant protein database while restricted to the *Vibrionaceae* family (NCBI taxid:641). Only hits with ≥80% coverage and ≥50% identity were considered, and one representative sequence per species was kept (i.e., presence/absence variations within species were not considered). Hits labelled "low quality protein" in the databases were excluded.

Mapping of siderophore systems onto a Vibrionaceae *phylogenetic network*

A *Vibrionaceae* host phylogeny was inferred based on sequence alignments of the genes *ftsZ, gap, gyrB, mreB, pyrH, recA, rpoA* and *topA,* provided by Dr.Sawabe [8]. Splitstree4 [49] was used to concatenate the sequences to construct a multi locus sequence alignment (MLSA), and to generate an unrooten phylogenetic network. Settings were set to 'NeighbourNet' method with 'uncorrected P' distance. Presence/absence of siderophore biosynthesis and receptor genes were mapped onto the phylogenetic network (only complete siderophore biosynthesis clusters are shown). The siderophore receptors were considered separately, and mapped onto the same network. Species with positive hits, but not included in the MLSA dataset, were placed onto the network based on the literature.

Phylogeny analysis of siderophore biosynthesis systems and receptors

Amino acid sequences of proteins involved in siderophore biosynthesis were aligned individually using ClustalW [50]. Proteins belonging to same clusters were concatenated using SplitsTree4 [49] and exported to Fasta format, thus generating the final datasets. Next, Mega6 [51] was used to generate Maximum Likelihood (ML) trees based on the individual siderophore biosynthesis datasets. The robustness of nodes in the resulting phylogenies was tested by running Bootstrap analyses, using the ML method (2000 replicates, JTT substitution model, uniform rates, and 'Complete deletion' in gap handling).

To address inheritance of the siderophore biosynthesis systems, we next constructed host phylogenies of same taxa as those containing the siderophore systems. Host trees were based on concatenated datasets of the same eight genes as described above. ML-trees were constructed using the Tamura-Nei model [52], and all gaps and missing data were removed. Phylogenies of the vibrioferrin (PvsABCDE) and piscibactin (Irp123459) systems, and their corresponding MLSA host trees, were rooted on *Aliivibrio wodanis* and *Photobacterium profundum*, respectively. The phylogeny of siderophore receptors was constructed essentially as described above. Briefly, amino acid sequences of homologous receptor sequences were aligned using ClustalW, and Mega6 [51] was then used to make ML-trees. Bootstrap analysis was done using the ML method, 2000 pseudoreplicates, the JTT model, uniform rates, and complete deletion of gaps. Corresponding MLSA trees were constructed as described for the cluster. The receptor phylogenies were compared to host trees, which were constructed as described above.

Results and discussion

Compilation of siderophore biosynthesis gene cluster sequences from Vibrionaceae

In this work we set out to do a comprehensive search, both in the literature and the global sequence databases, to identify gene clusters for biosynthesis of siderophores in *Vibrionaceae*, and compile and visualize the result in a simple and comprehensible manner. Figure 1 and Table 1 summarizes our findings. Based on the literature we identified eight siderophore biosynthesis clusters responsible for producing aerobactin, bisucaberin, vibrioferrin, vibriobactin, vanchrobactin,

178 piscibactin, anguibactin and vulnibactin. Figure 1 shows that genes belonging to the individual
179 siderophore biosynthetic pathways are typically found clustered "bumper-to-bumper" on the
180 chromosome (or as in *V. anguillarum*, on a plasmid). Pathways for hydroxamate or carboxylate
181 type siderophores are encoded by 3−5 genes, all encoded on the same DNA strand, whereas
182 catechol or mixed siderophores pathways are typically encoded by 6−11 genes, including one or
183 more NRPS gene(s), located on both strands and not necessarily in immediate proximity to each
184 other. The synteny and general organization of the latter siderophore biosynthetic gene cluster types
185 therefore appear more complex. *V. fluvialis* and *Vibrio nigripulchritudo* produce the catechol
186 siderophores fluvibactin and nigribactin (Figure 1C), respectively [53, 54]. However, the genes
187 encoding the biosynthesis systems are unknown.
188 Next, we used the known *Vibrionaceae* amino acids sequences (see Figure 1A and 1B) as
189 queries in blastP searches to identify homologous siderophore gene clusters in all available
190 *Vibrionaceae* genomes/ sequences in European Nucleotide Archive (ENA). Threshold values were
191 set to ≥80% coverage and ≥50% identity. Only complete siderophore biosynthesis clusters were
192 kept (i.e., all genes needed for biosynthesis must be present). Our search identified 60 biosynthetic
193 clusters in total, distributed among 42 species and 4 genera, and 14 unclassified *Vibrionaceae*
194 strains (i.e., *Vibrio* sp.), (see Supplementary file S1 for details). The majority of species can
195 potentially produce 1−3 of the known *Vibrionaceae* siderophores, with zero being the minimum
196 and four the maximum.
197 Bacteria must encode and express siderophore receptors on their surface in order to take up
198 and utilize siderophore-Fe^{3+} complexes. It is therefore of equal importance to identify and map the
199 existence of siderophore-associated receptors. In a similar approach as described above, we
200 identified and used siderophore receptor amino acid sequences in blastP searches. Accession
201 numbers of siderophore receptors that were used as queries are presented in Table 2. The receptor
202 searches identified 330 siderophore receptors in 78 classified *Vibrionaceae* species (and 40
203 unclassified *Vibrionaceae* strains), representing 5 genera (when using the same cut-off values as
204 described above). The complete list of identified siderophore receptors are presented in
205 Supplementary file S2. We found homologs of known *Vibrionaceae* siderophore receptors in
206 almost all *Vibrionaceae* species (Twenty-nine of the representatives in the split network do not
207 encode homologs of known *Vibrionaceae* siderophore biosynthesis clusters or receptor. Of the 29,

only 14 are fully sequenced), and the maximum number of different siderophore receptors found in a single genome was eight (i.e., in *V. alginolyticus*).

In summary, we searched the literature for known siderophore biosynthetic gene cluster from the *Vibrionaceae* family and identified eight different. The amino acids sequences corresponding to the known *Vibrionaceae* siderophore biosynthetic clusters and siderophore receptor were used as queries in blastP to identify homologs within the same family. A total of 60 biosynthetic clusters distributed among 42 species and 14 unclassified *Vibrionaceae* strains were identified. Using a similar approach we identified 330 siderophore receptor genes in 78 *Vibrionaceae* species and 40 unclassified *Vibrionaceae* strains.

Distribution of siderophore biosynthesis clusters and siderophore receptors in the Vibrionaceae family

Figure 2 shows the distribution of siderophore biosynthetic systems and receptor genes on a phylogenetic network containing 86 representative species and unclassified strains from *Vibrionaceae*. Overall, the figure shows that the vast majority of species are associated with at least one type of siderophore system. We have however not examined to what extent each of the siderophore system are present in each species. In other words, individual isolates may or may not contain siderophore systems associated with that species, as indicated on the splits network. Moreover, some siderophore systems are restricted to a very narrow phylogenetic lineage, whereas others have a wide but sporadic presence. For example, the aerobactin, vanchrobactin and piscibactin biosynthesis clusters appears to be scattered across multiple phylogenetic lineages, and the anguibactin clusters are found in *V. anguillarum* as well as in the *Splendius* and *Harveyi* clades. Similarly, vibrioferrin biosynthesis clusters are found in *A. wodanis*, *Vibrio navarrensis*, and inside the *Harveyi* and *Splendidus* clades.

In contrast to the widespread, but sporadic distribution of the siderophore biosynthetic genes described above, bisucaberin biosynthesis clusters are narrowly distributed into one lineage, i.e., in three species from the *Fischeri* clade. This finding suggest that bisucaberin was introduced into *Vibrionaceae* through horizontal gene transfer into the most recent common ancestor of *A. wodanis*, *A. logei*, and *A. salmonicida* (indicated by a red arrow in Figure 2). Similarly, vulnibactin is restricted to *V. vulnificus*, and vibriobactin is only found in the closely related species *Vibrio*

albensis and *V. cholerae*. Interestingly, no siderophore biosynthesis clusters were identified in the *Halioticoli* clade.

In addition to showing presence/absence of siderophore biosynthetic gene clusters, Figure 2 also displays how the respective siderophore receptors are distributed in *Vibrionaceae*. Some main findings are that (i) the presence of biosynthetic genes for individual siderophores is accompanied by the presence of the corresponding receptor, (ii) the number of different types of receptors typically exceeds (and in some cases by far) the number of biosynthetic cluster types, and (iii) similar to the biosynthetic clusters the receptors are widely distributed in *Vibrionaceae*. E.g., *iutA* (aerobactin receptor gene) and *desA* (deferroxamine B receptor gene) are found in nearly all clades. Also, the receptor genes *viuA* (for vibriobactin), *vuuA* (for vulnibactin), *pvuA* (for vibrioferrin), *vctA* and *irgA* (both for enterobactin), and finally *fhuA* (for ferrichrome) are widely distributed. In contrast, other receptors are more narrowly distributed, e.g., the bisucaberin receptor gene *bitA*, which is restricted to the *Fischeri* clade, more specifically to the same three *Aliivibrio* species that contain corresponding bisucaberin biosynthesis clusters.

Interestingly, (iv) known pathogens are conspicuously rich in siderophore receptors. E.g., *V. cholerae, V. alginolyticus* and *V. parahaemolyticus* encode seven, eight and five different receptor types, respectively. It is tempting to speculate that this richness likely reflects the lifestyle of these bacteria, where iron acquisition would be critical, especially during the initial phases of infections. Also, having multiple siderophore receptors would make them efficient "cheaters", i.e., they can use siderophores produced by other species rather from themselves. The receptors IrgA, VctA, FhuA and DesA are found in many "cheaters" throughout *Vibrionaceae*. Another explanation for the apparent richness in receptor types is that these species have been characterized in more detail than environmental isolates, but at least pathogens still encode a higher number of known siderophore receptor types.

Evolution siderophore systems

To evaluate the evolutionary history of siderophore systems (biosynthesis and receptors) in *Vibrionaceae*, and to better understand their present-day distribution, we concatenated the protein sequences from the most abundant types of biosynthetic clusters separately, and aligned the resulting sequences using ClustalW. Only species included in Figure 2 were investigated.

Maximum likelihood (ML) trees were generated from PvsABCDE (vibrioferrin cluster) and Irp123459 (piscibactin cluster) datasets. Similarly, datasets and ML-tree were made for siderophore receptors. The rationale for treating receptor sequences separate from biosynthesis genes was that receptor genes are often located elsewhere in the genome, and are much more widely distributed than the biosynthesis genes. ML-trees of the concatenated biosynthesis proteins and receptors were juxtaposed a host phylogeny based on same dataset as that used in Figure 2. Similar tree topologies (congruence) were interpreted as same evolutionary trajectories (i.e., vertical evolution), whereas conflicting topologies would suggest horizontal gene transfer events.

Figure 3 shows the genetic organization and phylogeny of the piscibactin system. Nodes in the trees are highly supported by 95−100% bootstrap values. Although there are some discrepancies in the phylogenies, the overall tree topologies are very similar. Based on the criteria described above the data thus suggest that the piscibactin biosynthesis pathway was introduced early into *Vibrionaceae* and then stably inherited in a few lineages, and lost in the majority of lineages. Similarly, the overall topology for the proposed piscibactin receptor FrpA and the corresponding host tree are in good overall agreement, except for one clear case of misplacement, i.e., *Vibrio harveyi* and *Vibrio rotiferianus* (*Harveyi* clade). Interestingly, these two species only contain the receptor, and not the biosynthesis system. This strongly suggests one horizontal gene transfer event of the FrpA receptor into the common ancestor of these two close related species.

Figure 4 shows the genic organization and phylogeny of the vibrioferrin system. Intriguingly, the result is strikingly similar to that of the piscibactin system. The overall tree topologies for the biosynthesis system and the host phylogenies are very similar, except that *V. harveyi* and *V. rotiferianus* are clearly misplaced (strongly supported by high bootstrap values). The evolution of the associated receptor (PvuA) appears to be more influenced by horizontal gene transfer events. The PvuA and host trees are mostly congruent within the *Splendidus* clade, whereas the remaining branches have multiple clear, highly supported, misplacements in the PvuA protein (compared to the host tree). Therefore, the evolution of the biosynthesis and receptor genes are, in part, different with partly vertical and horizontal gene transfers.

The very narrow distribution of the bisucaberin cluster (in the *Fischeri* clade) suggest a different evolutionary history, i.e., a recent insertion event into a common ancestor of *A. salmonicida*, *A. wodanis* and *Aliivibrio logei* (indicated by an arrow in Fig. 3). Until recently, the

bisucaberin biosynthesis genes (*bibABC*) were found exclusively in *A. salmonicida* (within *Vibrionaceae*) [44]. Here, the system is located on a genomic location (island) flanked by transposable elements. Our current blastP searches show that similar clusters are also found in *A.logei* and *A. wodanis*, together with the corresponding receptor gene *bitA*. Origin of the system is still unclear. We have in vain tried to identify the donor organism by running blastP and PSI-blast searches. The best database hits point to *Shewanella* as a possible source (BibA and BibB has 57% and 60 identity over 98 % and 97% coverage, respectively, to *S. algae*. BibC 60% identity over 74% coverage to *Shewanella baltica* and *Shewanella putrefaciens*), but this needs to be addressed again as more genomic data from environmental marine bacterial strains are added to the databases.

In summary, the present-day distribution of siderophore systems in *Vibrionaceae* appears to be, perhaps as can be expected, a result of a combination of events: both old and new gene acquisitions, extensive gene loss, and both vertical and horizontal gene transfers. We realize that we have only started to scratch the surface of understanding the origin and evolution of siderophore systems in *Vibrionaceae* (and other families). It is our intention to expand our analyses to more siderophore systems and to go even deeper into their gene organization, distribution and evolution. Our preliminary results indicate that the story is far from complete and will likely reveal more surprises and fascinating examples of rampant gene transfers.

Concluding remarks

We have used existing knowledge on siderophore systems to search for homologs in the databases, and mapped the result on a *Vibrionaceae* phylogenetic network. Overall, the result shows that the vast majority of species are associated with at least one type of siderophore biosynthesis system. Some systems (e.g., aerobactin and vibrioferrin) are seemingly scattered throughout the family, with a wide, but sporadic distribution, whereas others are presence in one lineage only (e.g., bisucaberin and vibriobactin). Cognate receptors are generally associated with its biosynthesis system, but are in addition spread into many other taxa (that does not encode the siderophore biosynthesis). A limited analysis of the origin and evolution of a few of the siderophore systems show that the present-day distribution can be explained by a combination of events, i.e., old and new gene acquisitions, extensive gene loss, and vertical and horizontal gene transfers. We realize

that we have only started to scratch the surface of understanding the origin and evolution of siderophore systems in *Vibrionaceae* (and other families), and it is our intention to expand our analyses to more siderophore systems and go deeper into their gene organization, distribution and evolution. Our preliminary results on more system indicate that the story is far from complete, and the future will likely reveal more surprises and fascinating examples of stable, as well as rampant, gene transfers.

Availability of supporting data

Supplementary file S1: Complete lists of homology hits from the blastP query of eight *Vibrionaceae* siderophore biosynthesis clusters.

Supplementary file S2: Complete lists of homology hits from the blastP query of twelve *Vibrionaceae* siderophore receptors.

Abbreviations

aa: Amino acid; MLSA: Multilocus sequence alignment; NRPS: Non-Ribosomal Peptide Synthase; ML: Maximum Liklihood; nt: nucleotide; ABC transporter: ATP-binding cassette transporter; *V.sp*: *Vibrio* species; *A. sp*: *Aliivibrio* species; *G. sp*: *Grimontia* species; *P. sp*: *Photobacterium* species; blastP: Protein Blast; ENA; European Nucleotide Archive.

Competing interests

The authors declare that they have no competing interests.

Ethics statement

The research presented in this paper do not involve human subjects, and we see no ethical issues.

Authors` contributions

PH and SKT conceived the study and drafted the manuscript. PH constructed the *Vibrionaceae* MLSA tree, and supervised the study. SKT performed literature studies, data collections, mapped the data to the MLSA tree, studied and visualized the clusters, developed the method for finding catechol siderophore clusters, constructed the MLSA and cladograms of siderophore biosynthesis clusters and receptors. MK, ER and RA performed data collections and initial trial studies.

Figure and table legends

Table 1. Accession number of known *Vibrionaceae* siderophore biosynthetic proteins used as query for homolog searches using blastP. The table gives an overview of the accession numbers for each protein sequence corresponding to a gene in the siderophore biosynthesis cluster. Provided are also the origin species, references and what siderophore the cluster produce.

Table 2. Accession number of known *Vibrionaceae* siderophore receptor proteins used as query for homolog searches using blastP. The table gives an overview of the accession numbers for protein sequence of the receptors. Provided are also the origin species, references and what siderophore the receptors can transport.

Figure 1. Organization of *Vibrionaceae* siderophore biosynthesis clusters and schematic structure of the known *Vibrionaceae* siderophores. A) *Vibrionaceae* hydroxamate, carboxylate and mixed hydroxamate/carboxylate siderophore biosynthesis clusters. B) *Vibrionaceae* catechol and mixed catechol/hydroxamate siderophore biosynthesis cluster. C) Schematic structure representation of known *Vibrionaceae* siderophores (chirality and lengths of binding are not exact).

Figure 2. Distribution of homologs of known *Vibrionaceae* siderophore biosynthesis clusters and receptors mapped to a phylogenetic split network based on a dataset from Sawabe and co-workers [8], consisting of the genes *ftsZ*, *gap*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA* for each species. The

tree was constructed using Splitstree4 to concatenate alignments and settings for network was were uncorrected_P and NeighborNet [49]. Branch lengths are to scale and species located outside grey arches were not included in the MLSA files and have been approximately placed according to literature [55-70].

Figure 3. Inheritance of the piscibactin biosynthesis cluster and receptor within the *Vibrionaceae* family. A) The cluster organization of the biosynthesis cluster and the cognate receptor. B) Host phylogeny on the left and piscibactin biosynthesis system (Irp123459) phylogeny on the right. C) Host phylogeny on the left and piscibactin receptor (FrpA) phylogeny on the right. *species that do not encode the piscibactin biosynthesis system, i.e., the FrpA homolog is an exogenous siderophore receptor. Evolutionary analyses were conducted in MEGA6 [51]. The host trees were generated using the ML method and the TM model [52]. The siderophore biosynthesis cluster and receptor trees were generated using the ML method and the JTT model [71]. Bootstrap values are shown at the nodes (JTT model, 2000 replicates) [72]. Branch lengths are measured substitutions per site. Bootstrap values are shown at the nodes (JTT model, 2000 replicates) [72].

Figure 4. Inheritance of the vibrioferrin biosynthesis cluster and receptor within the *Vibrionaceae* family. A) The cluster organization of the biosynthesis cluster and the cognate receptor. B) Host phylogeny on the left and vibrioferrin biosynthesis system (PvsABCDE) phylogeny on the right. C) Host phylogeny on the left and vibrioferrin receptor (PuvA) phylogeny on the right. *species that do not encode the vibrioferrin biosynthesis system, i.e., the PuvA homolog is an exogenous siderophore receptor. Evolutionary analyses were conducted in MEGA6 [51]. The host trees were generated using the ML method and the TM model [52]. The siderophore biosynthesis cluster and receptor trees were generated using the ML method and the JTT model [71]. Bootstrap values are shown at the nodes (JTT model, 2000 replicates) [72]. Branch lengths are measured substitutions per site. Bootstrap values are shown at the nodes (JTT model, 2000 replicates) [72].

Supplementary file S1. Complete lists of homology hits from the blastP query of eight *Vibrionaceae* siderophore biosynthesis clusters with accession numbers and coverage/ identity/ e-value scores.

Supplementary file S2. Complete lists of homology hits from the blastP query of twelve *Vibrionaceae* siderophore receptor with accession numbers and coverage/ identity/ e-value scores.

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Table 1

Siderophore	Organism	Siderophore biosynthesis protein accession numbers	Ref
Aerobactin	*V. mimicus*	IucA(WP_000554936.1) IucB(WP_000033134.1) IucC(WP_000372426.1) IucD(WP_000401386.1)	[73]
Bisucaberin	*A. salmonicida*	BibA(WP_012549025.1) BibB(WP_012549026.1) BibC(WP_012549027.1)	[43]
Vibrioferrin	*V. parahaemolyticus*	PvsA(WP_015313675.1) PvsB(WP_015313676.1) PvsC(WP_015313677.1) PvsD(WP_015313678.1) PvsE(WP_015313679.1)	[24]
Vibriobactin	*V. cholerae*	VibA (WP_000654285.1) VibB (WP_000997093.1) VibC(WP_000245175.1) VibD(WP_000874996.1) VibE (WP_000205544.1) VibF (WP_000523394.1) VibH(WP_001880577.1)	[9, 10]
Vanchrobactin	*V. anguillarum*	DapH(WP_011154675.1) VabA(WP_064624836.1) VabB(WP_064624831.1) VabC(WP_043004165.1) VabE(WP_019281788.1) VabF (WP_019281791.1) VabH (WP_019281793.1)	[38]
Piscibactin	*P. damselae subsp. Piscicida*	DapH (AKQ52526.1)Irp1(AKQ52532.1) Irp2(AKQ52531.1) Irp3(AKQ52533.1) Irp4(AKQ52534.1) Irp5(AKQ52536.1)	[33]
Anguibactin	*V. anguillarum*	AngA(WP_013857267.1) AngB(WP_013857270.1) AngC(WP_043004165.1) AngE(WP_013857269.1) AngB/G(WP_011154672.1) AngM(WP_011154633.1) AngT(WP_011154640.1) AngH(WP_011154645.1) AngR(WP_011154639.1) AngN(WP_011154642.1) AngU(WP_011154641.1) AngD(WP_011154670.1)	[37]
Vulnibactin	*V. vulnificus*	VV2_0830(WP_011081748.1) VV2_0831(AAO07755.1) VV2_0834(WP_011081751.1) VV2_0835(WP_011081752.1) VV2_0836(WP_011081753.1) VV2_0838/VenB(WP_011081755.1) VV2_0839(WP_011081756.1) VV2_0840(WP_011081757.1) VV2_0844(AAO07767.2)	[16]

Table 2

Organism	Receptor	Transport	Ref
V. mimicus	IutA (WP_000843157.1)	Aerobactin	[73]
A. salmonicida	BitA (WP_012549028.1)	Bisucaberin	[43]
V. parahaemolyticus	PvuA (WP_057620147.1)	Vibrioferrin	[25]
V. cholerae	ViuA (WP_000279435.1)	Vibriobactin Fluvibactin	[11]
V. anguillarum	FvtA (WP_019281795.1)	Vanchrobactin	[39]
V. anguillarum	FatA (WP_011154638.1)	Anguibactin	[74]
V.cholerae	VctA (WP_000350325.1)	Enterobactin Fluvibactin	[13]
V. cholerae	IrgA (WP_000086048.1)	Enterobactin Fluvibactin	[13]
V.vulnificus	VvuA (WP_015728225.1)	Vulnibactin	[17]
V. cholerae	FhuA (WP_053043596.1)	Ferrichrome	[14]
V. furnissii	DesA (WP_004725209.1)	Deferoxamine B	[75]
P. damselae subsp. Piscicida	FrpA (AKQ52529.1)	Piscibactin	[33]

Figure 1

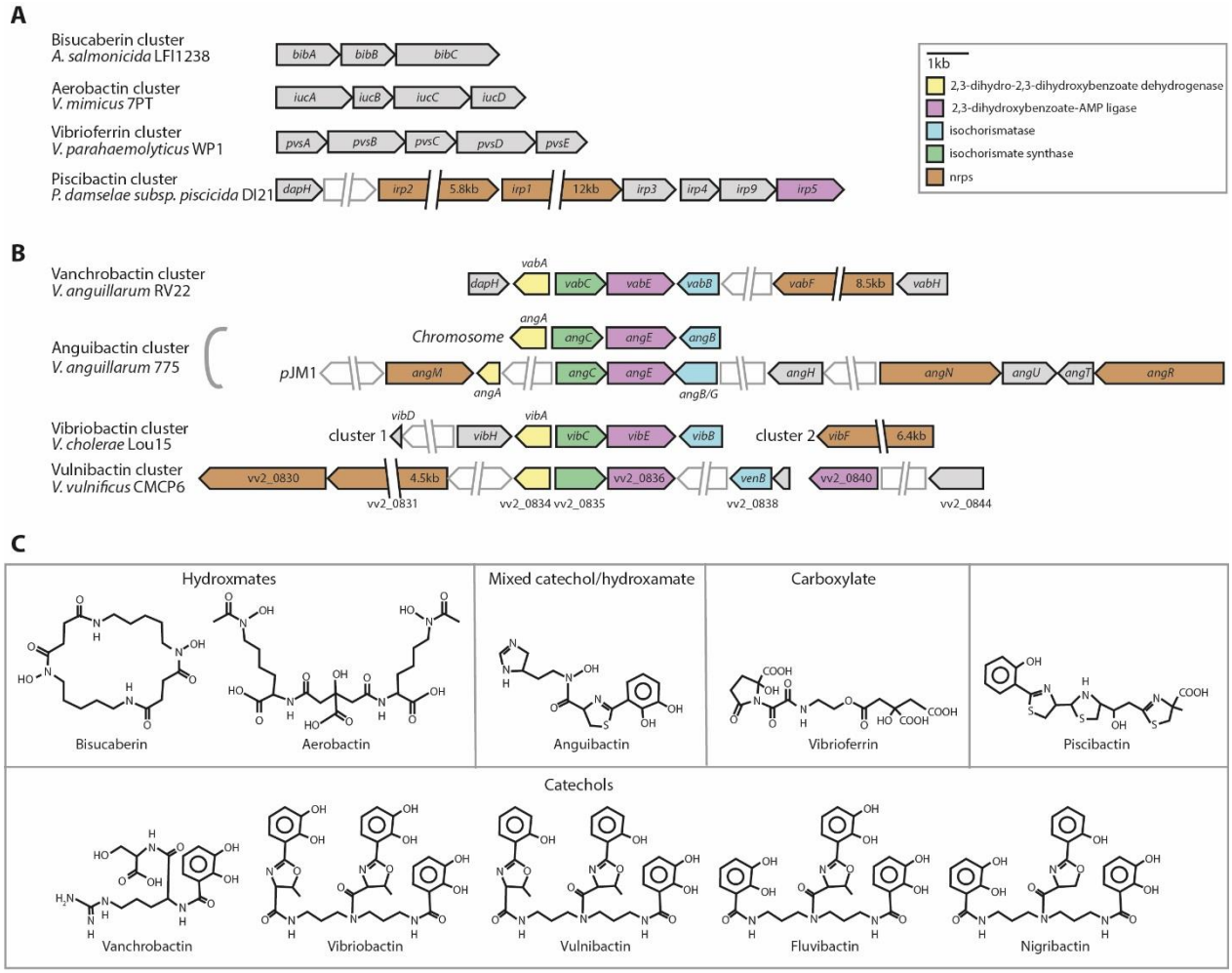

A
Bisucaberin cluster
A. salmonicida LFI1238
bibA
bibB
bibC
Aerobactin cluster
V. mimicus 7PT
iucA
iucB
iucC
iucD
Vibrioferrin cluster
V. parahaemolyticus WP1
pvsA
pvsB
pvsC
pvsD
pvsE
Piscibactin cluster
P. damselae subsp. piscicida DI21
dapH
irp2
5.8kb
irp1
12kb
irp3
irp4
irp9
irp5
1kb
2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
2,3-dihydroxybenzoate-AMP ligase
isochorismatase
isochorismate synthase
nrps
B
Vanchrobactin cluster
V. anguillarum RV22
vabA
dapH
vabC
vabE
vabB
vabF
8.5kb
vabH
Anguibactin cluster
V. anguillarum 775
Chromosome
angA
angC
angE
angB
pJM1
angM
angA
angC
angE
angB/G
angH
angN
angU
angT
angR
Vibriobactin cluster
V. cholerae Lou15
cluster 1
vibD
vibH
vibA
vibC
vibE
vibB
cluster 2
vibF
6.4kb
Vulnibactin cluster
V. vulnificus CMCP6
vv2_0830
4.5kb
vv2_0831
vv2_0834
vv2_0835
vv2_0836
venB
vv2_0838
vv2_0840
vv2_0844
C
Hydroxmates
Bisucaberin
Aerobactin
Mixed catechol/hydroxamate
Anguibactin
Carboxylate
Vibrioferrin
Piscibactin
Catechols
Vanchrobactin
Vibriobactin
Vulnibactin
Fluvibactin
Nigribactin

Figure 2

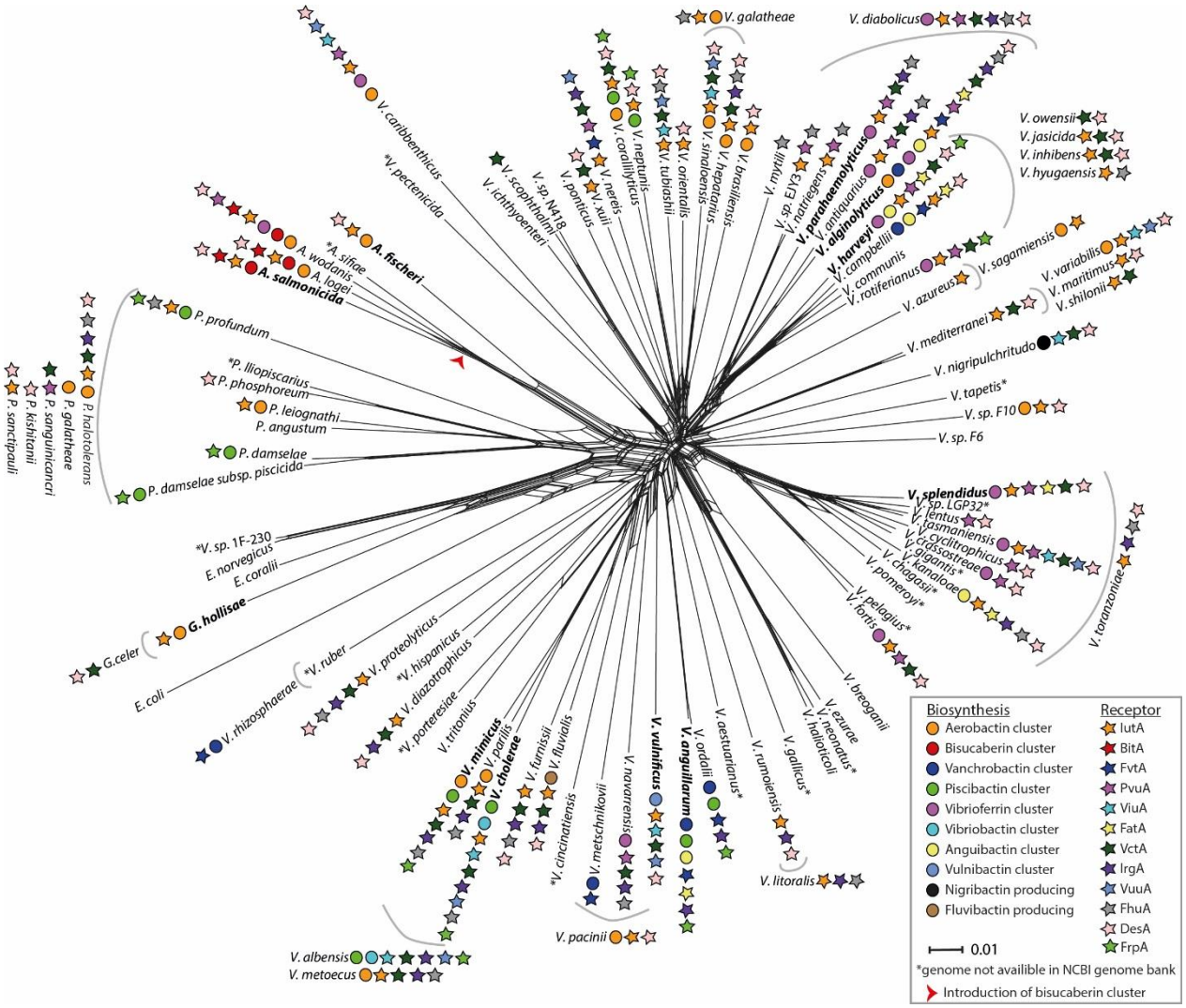

V. galatheae
V. diabolicus
V. owensii
V. jasicida
V. inhibens
V. hyugaensis
V. caribbenthicus
*V. pectenicida
V. scophthalmi
V. ichthyoenteri
V. sp. N418
V. ponticus
V. xuii
V. nereis
V. coralliilyticus
V. neptunis
V. tubiashii
V. orientalis
V. sinaloensis
V. hepatarius
V. bivalvicida
V. mytili
V. sp. EJY3
V. natriegens
V. parahaemolyticus
V. antiquarius
V. alginolyticus
V. harveyi
V. campbellii
V. communis
V. rotiferianus
V. azureus
V. sagamiensis
V. variabilis
V. maritimus
V. shilonii
V. mediterranei
V. nigripulchritudo
V. tapetis*
V. sp. F10
V. sp. F6
*A. sifiae
A. wodanis
A. logei
A. fischeri
A. salmonicida
P. profundum
*P. liliopiscarius
P. phosphoreum
P. leiognathi
P. angustum
P. damselae
P. damselae subsp. piscicida
P. halotolerans
P. galatheae
P. sanguinicancri
P. kishitanii
P. sanctipauli
*V. sp. 1F-230
E. norvegicus
E. coralii
G. hollisae
G. celer
E. coli
V. splendidus
V. sp. LGP32*
V. lentus
V. tasmaniensis
V. cyclitrophicus
V. crassostreae
V. gigantis*
V. kanaloae
V. chagasi*
V. pomeroyi*
V. pelagius*
V. fortis
V. toranzoniae
V. breoganii
V. ezurae
V. neonatus*
V. halioticoli
V. gallicus*
V. rumoiensis*
V. litoralis
V. aestuarianus*
V. ordalii
V. anguillarum
V. vulnificus
V. navarrensis
V. metschnikovii
V. pacinii
*V. cincinatiensis
V. furnissii
V. fluvialis
V. cholerae
V. mimicus
V. parilis
V. tritonius
*V. porteresiae
*V. diazotrophicus
*V. hispanicus
V. proteolyticus
*V. ruber
V. rhizosphaerae
V. albensis
V. metoecus
Biosynthesis
Aerobactin cluster
Bisucaberin cluster
Vanchrobactin cluster
Piscibactin cluster
Vibrioferrin cluster
Vibriobactin cluster
Anguibactin cluster
Vulnibactin cluster
Nigribactin producing
Fluvibactin producing
Receptor
IutA
BitA
FvtA
PvuA
ViuA
FatA
VctA
IrgA
VuuA
FhuA
DesA
FrpA
0.01
*genome not availible in NCBI genome bank
Introduction of bisucaberin cluster

Figure 3

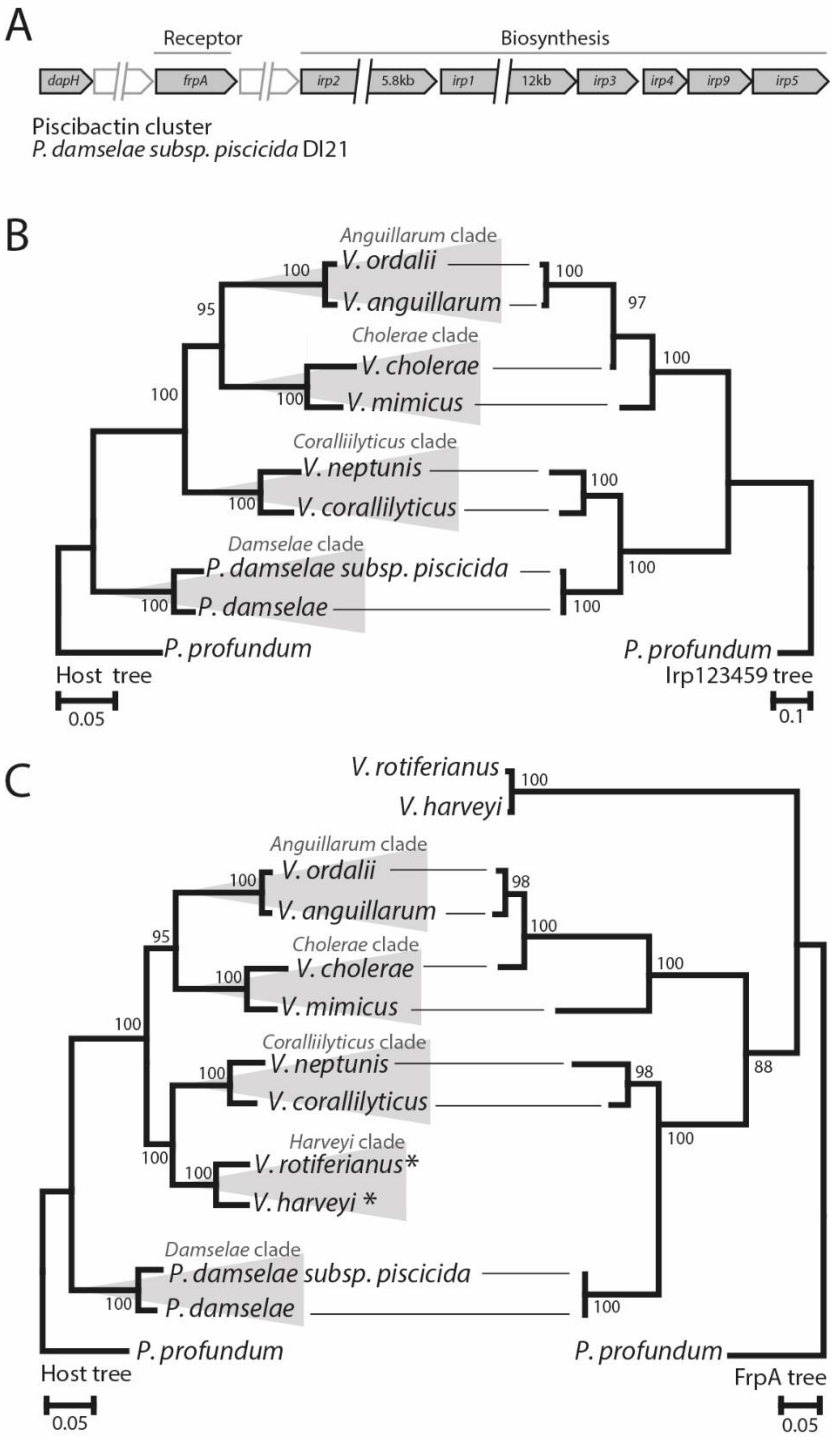
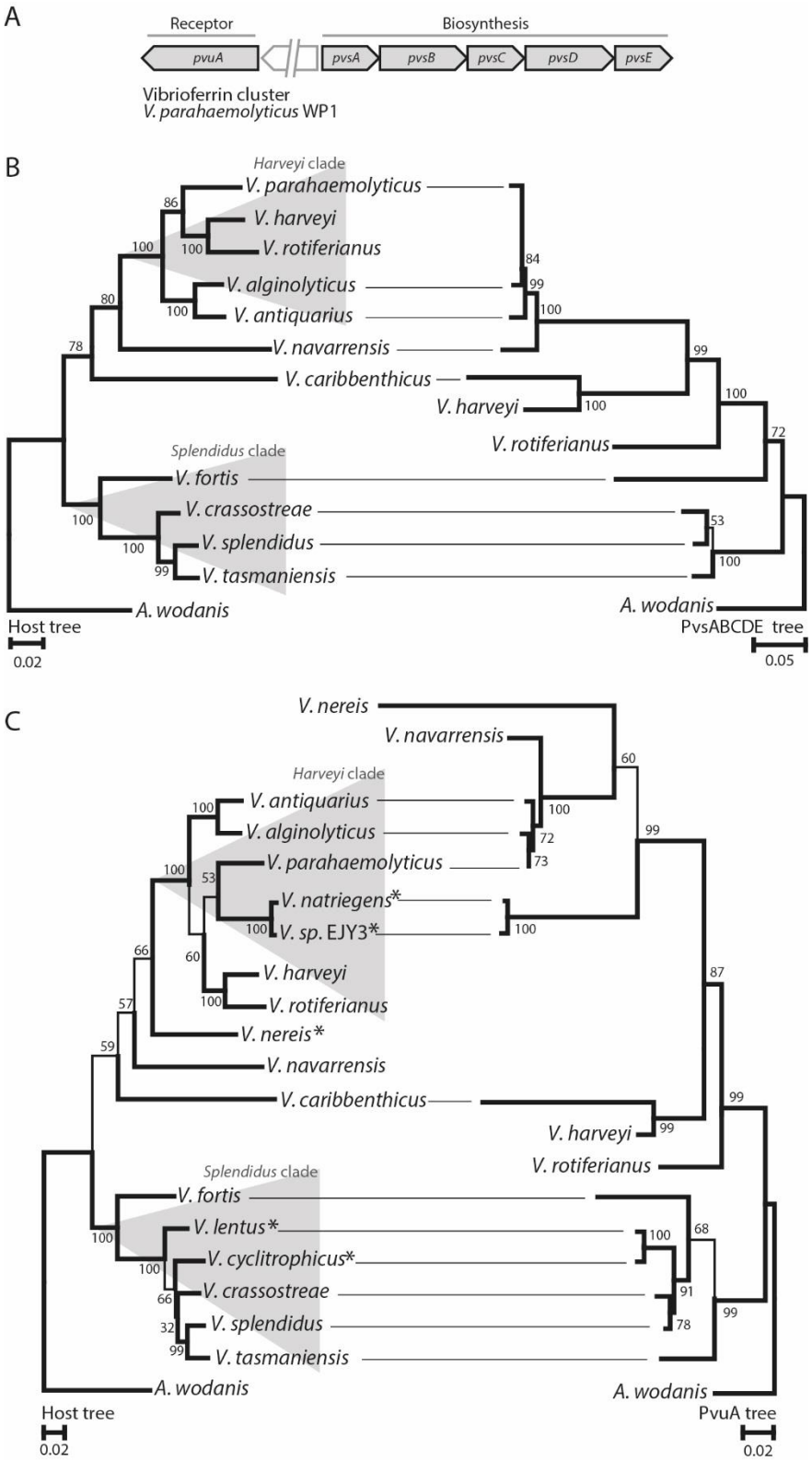

A
Receptor
Biosynthesis
dapH
frpA
irp2
5.8kb
irp1
12kb
irp3
irp4
irp9
irp5
Piscibactin cluster
P. damselae subsp. piscicida DI21
B
Anguillarum clade
V. ordalii
V. anguillarum
Cholerae clade
V. cholerae
V. mimicus
Coralliilyticus clade
V. neptunis
V. corallilyticus
Damselae clade
P. damselae subsp. piscicida
P. damselae
P. profundum
Host tree
0.05
P. profundum
Irp123459 tree
0.1
C
V. rotiferianus
V. harveyi
Anguillarum clade
V. ordalii
V. anguillarum
Cholerae clade
V. cholerae
V. mimicus
Coralliilyticus clade
V. neptunis
V. corallilyticus
Harveyi clade
V. rotiferianus*
V. harveyi *
Damselae clade
P. damselae subsp. piscicida
P. damselae
P. profundum
Host tree
0.05
P. profundum
FrpA tree
0.05

Figure 4


A
Receptor
Biosynthesis
pvuA
pvsA
pvsB
pvsC
pvsD
pvsE
Vibrioferrin cluster
V. parahaemolyticus WP1
B
Harveyi clade
V. parahaemolyticus
V. harveyi
V. rotiferianus
V. alginolyticus
V. antiquarius
V. navarrensis
V. caribbenthicus
V. harveyi
V. rotiferianus
Splendidus clade
V. fortis
V. crassostreae
V. splendidus
V. tasmaniensis
A. wodanis
Host tree
0.02
A. wodanis
PvsABCDE tree
0.05
C
V. nereis
V. navarrensis
Harveyi clade
V. antiquarius
V. alginolyticus
V. parahaemolyticus
V. natriegens*
V. sp. EJY3*
V. harveyi
V. rotiferianus
V. nereis*
V. navarrensis
V. caribbenthicus
V. harveyi
V. rotiferianus
Splendidus clade
V. fortis
V. lentus*
V. cyclitrophicus*
V. crassostreae
V. splendidus
V. tasmaniensis
A. wodanis
Host tree
0.02
A. wodanis
PvuA tree
0.02

Supplementary file S1

Aerobactin biosynthesis cluster

Organism	IucA	Cov/Ident/E	IucB	Cov/Ident/E	IucC	Cov/Ident/E	IucD	Cov/Ident/E
Aliivibrio fischeri	WP_063668674.1	96/61/0	WP_063649197.1	100/63/8e-140	WP_063646176.1	99/68/0	WP_063656611.1	97/71/0
Aliivibrio logei	WP_017023525.1	96/61/0	WP_017023526.1	100/60/3e-137	WP_017023527.1	99/69/0	WP_017023528.1	98/72/0
Aliivibrio salmonicida	pseudo		WP_012551639.1	100/60/3e-135	pseudo		WP_012551640.1	98/72/0
Aliivibrio wodanis	WP_061029794.1	96/61/0	WP_061029792.1	99/61/5e-137	WP_061003472.1	99/71/0	WP_061003473.1	98/72/0
Grimontia hollisae	WP_005505275.1	94/53/0	WP_005505267.1	93/51/2e-101	WP_005505265.1	98/60/0	WP_040529008.1	97/61/0
Photobacterium halotolerans	WP_046220649.1	98/62/0	WP_046220648.1	94/65/2e-136	WP_036752138.1	99/71/0	WP_036752136.1	98/71/0
Photobacterium leiognathi	WP_053988888.1	100/75/0	WP_053988887.1	100/68/4e-161	WP_053988886.1	99/79/0	WP_053988885.1	99/81/0
Photobacterium sp. SKA34	WP_006645231.1	95/52/0	WP_050764797.1	89/55/3e-107	EAR55206.1	83/60/0	WP_006645235.1	97/61/0
Vibrio alginolyticus	WP_053311479.1	96/58/0	WP_053311480.1	100/62/3e-137	WP_053311481.1	99/67/0	WP_053311482.1	99/71/0
Vibrio brasiliensis	WP_006880411.1	100/75/0	WP_006880410.1	100/69/5e-164	WP_006880409.1	99/80/0	WP_006880408.1	99/81/0
Vibrio caribbeanicus	WP_052131268.1	97/67/0	WP_038134820.1	100/67/3e-155	WP_038134818.1	98/75/0	WP_038134815.1	100/76/0
Vibrio coralliilyticus	WP_019276335.1	100/64/0	WP_045986472.1	100/63/2e-144	WP_019276337.1	98/71/0	WP_040122006.1	100/74/0
Vibrio galatheae	WP_045956523.1	99/73/0	WP_045956524.1	100/67/3e-154	WP_045956525.1	99/77/0	WP_045956526.1	99/81/0
Vibrio genomosp. F10	WP_017041124.1	96/61/0	WP_017041125.1	100/60/8e-137	WP_017033971.1	99/67/0	WP_017033970.1	95/71/0
Vibrio hepatarius	WP_053407993.1	100/81/0	WP_053407868.1	100/82/0	WP_053407869.1	99/84/0	WP_053407870.1	100/83/0
Vibrio metoecus	WP_055051039.1	100/99/0	WP_055051038.1	100/99/0	WP_055032970.1	100/98/0	WP_055051036.1	100/99/0
Vibrio mimicus	WP_000554936.1		WP_000033134.1		WP_000372426.1		WP_000401386.1	
Vibrio pacinii	WP_038173493.1	100/81/0	WP_038173492.1	100/83/0	WP_038173490.1	99/82/0	WP_038173488.1	100/84/0
Vibrio parilis	WP_000554940.1	100/96/0	WP_000033136.1	100/95/0	WP_000436133.1	100/97/0	WP_000401383.1	100/97/0
Vibrio sagamiensis	WP_039980470.1	99/70/0	WP_039980472.1	100/62/2e-137	WP_039980473.1	99/74/0	WP_039980474.1	98/82/0
Vibrio sinaloensis	WP_052319139.1	97/67/0	WP_039623864.1	100/67/3e-157	WP_039477140.1	98/74/0	WP_039477142.1	100/76/0
Vibrio sp. 16	WP_043886533.1	97/67/0	WP_043886505.1	100/67/4e-155	WP_005469810.1	98/74/0	WP_043886506.1	100/76/0
Vibrio sp. AND4	WP_009841203.1	99/74/0	WP_009841202.1	100/66/4e-155	WP_009841201.1	99/78/0	WP_009841200.1	99/78/0
Vibrio sp. B183	WP_051912559.1	99/64/0	WP_038160278.1	100/63/1e-143	WP_038160275.1	98/70/0	WP_038160274.1	99/74/0
Vibrio variabilis	WP_052132383.1	97/67/0	WP_038212532.1	100/67/3e-158	WP_038212530.1	98/74/0	WP_043886506.1	100/75/0

Vanchrobactin biosynthesis cluster

Organism	DahP	Cov/Ident/E	VabA	Cov/Ident/E	VabB	Cov/Ident/E	VabC	Cov/Ident/E
Vibrio alginolyticus	WP_047101776.1	97/80/6e-161	WP_047101854.1	100/54/2e-97	WP_053306972.1	83/65/4e-144	WP_047101778.1	99/69/0
Vibrio anguillarum	WP_011154675.1		WP_064624836.1		WP_064624831.1		WP_043004165.1	
Vibrio campbellii	WP_005425950.1	93/53/5e-93	WP_045397519.1	96/48/7e-73	WP_045378486.1	82/64/2e-136	WP_051117323.1	98/49/6e-130
Vibrio metschnikovii	WP_004396943.1	94/70/3e-136	WP_004396942.1	100/66/4e-124	WP_004396939.1	83/67/1e-139	WP_004396941.1	100/76/0
Vibrio ordalii	WP_017044377.1	93/69/4e-135	WP_017050150.1	100/99/0	WP_017050153.1	100/99/0	WP_010318455.1	100/99/0
Vibrio rhizosphaerae	WP_038184547.1	93/52/7e-93	WP_038181208.1	98/68/2e-121	WP_038181214.1	85/63/2e-134	WP_038181209.1	100/77/0
Vibrio sp. MEBiC08052	WP_059122076.1	93/52/7e-93	WP_059122676.1	98/66/2e-118	WP_059120568.1	84/64/9e-139	WP_059120570.1	100/78/0

Organism	VabE	Cov/Ident/E	VabF	Cov/Ident/E	VabH	Cov/Ident/E
Vibrio alginolyticus	WP_017821446.1	99/69/0	WP_054579172.1	99/63/0	WP_054579171.1	99/59/2e-180
Vibrio anguillarum	WP_019281788.1		WP_019281791.1		WP_019281793.1	
Vibrio campbellii	WP_045370524.1	98/56/0	WP_010648829.1	99/62/0	WP_050545982.1	99/59/0
Vibrio metschnikovii	WP_004396940.1	99/68/0	WP_004396933.1	99/60/0	WP_040905265.1	99/61/0
Vibrio ordalii	WP_017045629.1	100/99/0	WP_017044383.1	99/97/0	WP_017044384.1	100/98/0
Vibrio rhizosphaerae	WP_038181212.1	99/68/0	WP_038181227.1	99/61/0	WP_038181233.1	98/56(1e-173
Vibrio sp. MEBiC08052	WP_059120569.1	99/69/0	WP_059120563.1	99/61/0	WP_059120561.1	98/60/0

Anguibactin biosynthesis cluster

Organism	AngC	Cov/Ident/E	AngB	Cov/Ident/E	AngA	Cov/Ident/E	AngE	Cov/Ident/E
Vibrio alginolyticus	WP_047101778.1	99/69/0	WP_053306972.1	83/65/4e-144	WP_047101854.1	100/54/2e-97	WP_017821446.1	99/69/0
Vibrio anguillarum	WP_043004165.1		WP_013857270.1		WP_013857267.1		WP_013857269.1	
Vibrio campbellii	WP_051117323.1	98/49/6e-130	WP_045378486.1	82/64/2e-136	WP_045397519.1	96/48/7e-73	WP_045370524.1	98/56/0
Vibrio harveyi	WP_017188367.1	98/49/2e-132	WP_050913426.1	82/64/8e-127	WP_050902411.1	96/48/2e-73	WP_061065702.1	98/58/0
Vibrio kanaloae	WP_017055790.1	99/70/0	WP_017055788.1	82/64/3e-118	WP_032547875.1	99/56/2e-101	WP_032547877.1	98/63/0

Organism	AngB/G	Cov/Ident/E	AngM	Cov/Ident/E	AngT	Cov/Ident/E	AngH	Cov/Ident/E
Vibrio alginolyticus	WP_047101780.1	100/80/3e-173	WP_047101757.1	100/64/0	WP_047101764.1	99/70/2e-130	WP_047101770.1	96/86/0
Vibrio anguillarum	WP_011154672.1		WP_011154633.1		WP_011154640.1		WP_011154645.1	
Vibrio campbellii	WP_045378486.1	100/79/1e-168	WP_045418437.1	100/63/0	WP_050918968.1	98/63/2e-119	WP_047479187.1	96/81/0
Vibrio harveyi	WP_050913426.1	100/80/9e-160	WP_050913415.1	99/63/0	WP_050913420.1	98/63/5e-118	WP_050913423.1	96/81/0
Vibrio kanaloae	WP_017055788.1	100/95/0	WP_017055763.1	100/90/0	WP_017055797.1	99/91/1e-172	WP_017055794.1	96/96/0

Organism	AngR	Cov/Ident/E	AngN	Cov/Ident/E	AngU	Cov/Ident/E
Vibrio alginolyticus	WP_047101763.1	99/67/0	WP_047101768.1	99/71/0	WP_047101766.1	99/84/0
Vibrio anguillarum	WP_011154639.1		WP_011154642.1		WP_011154641.1	
Vibrio campbellii	WP_050910520.1	99/64/0	WP_005533427.1	99/69/0	WP_005533426.1	99/79/0
Vibrio harveyi	WP_050913419.1	99/63/0	WP_050913422.1	99/69/0	WP_050913421.1	99/79/0
Vibrio kanaloae	AKN37366.1	99/90/0	WP_017055795.1	100/91/0	WP_017055796.1	99/95/0

Vibriobactin biosynthesis cluster

Organism	VibA	Cov/Ident/E	VibB	Cov/Ident/E	VibC	Cov/Ident/E	VibD	Cov/Ident/E
Vibrio albensis	EEO04018.1	100/99/0	WP_000997090.1	100/99/0	WP_032468518.1	99/98/0	WP_001907346.1	100/96/1e-169
Vibrio cholerae	WP_000654285.1		WP_000997093.1		WP_000245175.1		WP_000874996.1	

Organism	VibE	Cov/Ident/E	VibF	Cov/Ident/E	VibH	Cov/Ident/E
Vibrio albensis	WP_000205529.1	100/98/0	WP_000523401.1	100/98/0	EEO04019.1	88/98/0
Vibrio cholerae	WP_000205544.1		WP_000523394.1		WP_001880577.1	

Bisucaberin biosynthesis cluster

Organism	BibA	Cov/Ident/E	BibB	Cov/Ident/E	BibC	Cov/Ident/E
Aliivibrio logei	WP_017021624.1	100/99/0	WP_017021623.1	99/99/0	WP_017021622.1	100/99/0
Aliivibrio salmonicida LFI1238	WP_012549025.1		WP_012549026.1		WP_012549027.1	
Aliivibrio wodanis	WP_060991935.1	100/87/0	WP_060991934.1	99/90/0	WP_060991933.1	100/89/0

Vibrioferrin biosynthesis cluster

Organism	PvsA	Cov/Ident/E	PvsB	Cov/Ident/E	PvsC	Cov/Ident/E	PvsD	Cov/Ident/E
Aliivibrio wodanis	WP_045102246.1	100/74/0	WP_045102247.1	99/73/0	WP_045102248.1	100/74/0	WP_045102249.1	99/77/0
Vibrio alginolyticus	WP_054575389.1	100/97/0	WP_054575388.1	100/98/0	WP_054576553.1	100/99/0	WP_031780477.1	100/99/0
Vibrio antiquarius	WP_006740841.1	100/97/0	WP_006740842.1	100/96/0	WP_006740843.1	100/98/0	WP_006740844.1	100/99/0
Vibrio caribbeanicus	WP_009603302.1	100/72/0	WP_009603303.1	99/74/0	WP_009603304.1	100/70/0	WP_009603305.1	99/73/0
Vibrio crassostreae	WP_057623651.1	100/74/0	WP_057623654.1	99/75/0	WP_017064382.1	100/74/0	WP_048659876.1	100/77/0
Vibrio diabolicus	WP_048625457.1	100/97/0	WP_048625458.1	100/98/0	WP_048625459.1	100/98/0	WP_048625460.1	100/98/0
Vibrio fortis	WP_032550983.1	100/74/0	WP_032550984.1	100/74/0	WP_032550985.1	100/75/0	WP_032550986.1	99/74/0
Vibrio harveyi	WP_029789871.1	100/75/0	WP_049537122.1	99/76/0	WP_050933242.1	100/73/0	WP_050908359.1	100/76/0
Vibrio navarrensis	WP_039433588.1	99/92/0	WP_039439003.1	100/94/0	WP_039433594.1	100/96/0	WP_039439008.1	100/98/0
Vibrio parahaemolyticus	WP_015313675.1		WP_015313676.1		WP_015313677.1		WP_015313678.1	
Vibrio rotiferianus	WP_010453821.1	100/77/0	WP_038887496.1	99/78/0	WP_010453819.1	100/78/0	WP_045390572.1	100/79/0
Vibrio sp. HENC-01	WP_009696138.1	100/74/0	WP_009696137.1	99/76/0	WP_050554537.1	100/73/0	WP_033007960.1	100/76/0
Vibrio sp. J2-12	WP_050644303.1	100/74/0	WP_050644304.1	99/74/0	WP_050644305.1	100/74/0	WP_050644306.1	99/76/0
Vibrio sp. J2-15	WP_050632945.1	100/74/0	WP_050632944.1	99/75/0	WP_050632943.1	100/74/0	WP_050632942.1	99/76/0
Vibrio sp. J2-17	WP_050652641.1	100/74/0	WP_050652640.1	99/75/0	WP_050652639.1	100/74/0	WP_050652638.1	99/76/0
Vibrio sp. J2-29	WP_048614849.1	100/74/0	WP_048614847.1	99/75/0	WP_048614845.1	100/74/0	WP_048614843.1	100/76/0
Vibrio sp. J2-3	WP_050620543.1	100/74/0	WP_050620544.1	99/75/0	WP_050620545.1	100/74/0	WP_050620546.1	99/76/0
Vibrio sp. J2-31	WP_048606054.1	100/74/0	WP_050650869.1	99/74/0	WP_048606050.1	100/74/0	WP_050650871.1	100/76/0
Vibrio sp. OY15	WP_033906632.1	100/96/0	WP_033906631.1	100/94/0	WP_033906630.1	100/98/0	WP_033906629.1	100/98/0
Vibrio splendidus	WP_017089235.1	100/75/0	WP_017092038.1	99/75/0	WP_061038080.1	100/74/0	WP_061022001.1	100/77/0
Vibrio tasmaniensis	WP_012600978.1	100/74/0	WP_012600979.1	99/75/0	WP_012600980.1	100/74/0	WP_012600981.1	100/77/0

Organism	PvsE	Cov/Ident/E
Aliivibrio wodanis	WP_045102250.1	100/83/0
Vibrio alginolyticus	WP_053350159.1	100/98/0
Vibrio antiquarius	WP_006740845.1	100/98/0
Vibrio caribbeanicus	WP_009603306.1	100/83/0
Vibrio crassostreae	WP_059017340.1	100/84/0
Vibrio diabolicus	WP_048625461.1	100/97/0
Vibrio fortis	WP_032550987.1	100/78/0
Vibrio harveyi	WP_050922940.1	100/83/0
Vibrio navarrensis	WP_039439012.1	100/95/0
Vibrio parahaemolyticus	WP_015313679.1	
Vibrio rotiferianus	WP_045390569.1	100/83/0
Vibrio sp. HENC-01	WP_009696133.1	100/84/0
Vibrio sp. J2-12	WP_050644307.1	100/84/0
Vibrio sp. J2-15		
Vibrio sp. J2-17	WP_050652637.1	100/84/0
Vibrio sp. J2-29	WP_048614841.1	100/84/0
Vibrio sp. J2-3	WP_050620547.1	100/82/0
Vibrio sp. J2-31	WP_050650872.1	100/84/0
Vibrio sp. OY15	WP_033906628.1	100/97/0
Vibrio splendidus	WP_060980475.1	100/84/0
Vibrio tasmaniensis	WP_012600982.1	100/83/0

Piscibactin biosynthesis cluster

Organism	Irp1	Cov/Ident/E	Irp2	Cov/Ident/E	Irp3	Cov/Ident/E	Irp4	Cov/Ident/E
Photobacterium damselae	WP_044179415.1	100/99/0	WP_044179418.1	100/99/0	WP_044179412.1	100/100/0	WP_044179406.1	100/100/0
Photobacterium damselae subsp. piscicida	AKQ52532.1		AKQ52531.1		AKQ52533.1		AKQ52534.1	
Photobacterium profundum	CAG20078.1	83/52/0	WP_011218392.1	99/55/0	WP_011218389.1	96/59/2e-140	WP_011218388.1	91/59/5e-114
Vibrio albensis	WP_027694620.1	99/52/0	WP_027694619.1	99/57/0	WP_000842271.1	98/61/2e-144	WP_001077536.1	92/56/2e-98
Vibrio anguillarum	WP_019281879.1	99/52/0	WP_019281878.1	99/57/0	WP_019281880.1	98/61/4e-144	WP_019281881.1	95/54/1e-98
Vibrio cholerae	WP_032479734.1	100/54/0	WP_057558264.1	100/57/0	WP_000842272.1	98/62/9e-145	WP_001077534.1	97/54/2e-99
Vibrio coralliilyticus	WP_006961718.1	100/73/0	WP_006961717.1	100/75/0	WP_019276303.1	100/80/0	WP_045986445.1	100/79/2e-166
Vibrio mimicus	WP_022578908.1	100/54/0	WP_061051252.1	99/58/0	WP_001065237.1	96/61/2e-145	WP_005526076.1	90/60/2e-108
Vibrio neptunius	WP_045975018.1	100/71/0	WP_045975017.1	100/75/0	WP_045975019.1	100/74/0	WP_045975020.1	99/75/2e-156
Vibrio ordalii	WP_017046027.1	99/52/0	WP_017046028.1	99/57/0	WP_010317038.1	98/62/1e-145	WP_017050345.1	97/54/1e-99

Organism	Irp5	Cov/Ident/E	Irp9	Cov/Ident/E
Photobacterium damselae	WP_044179402.1	100/100/0	WP_044179404.1	100/100/0
Photobacterium damselae subsp. piscicida	AKQ52536.1		AKQ52535.1	
Photobacterium profundum	WP_011218386.1	96/60/0	WP_011218387.1	95/64/0
Vibrio albensis	EEO01925.1	94/63/0	WP_001273873.1	96/61/0
Vibrio anguillarum	WP_019281883.1	96/63/0	WP_019281882.1	98/61/0
Vibrio cholerae	WP_042990239.1	96/63/0	WP_001273875.1	98/61/0
Vibrio coralliilyticus	WP_040122045.1	100/77/0	WP_040122044.1	99/79/0
Vibrio mimicus	WP_001000061.1	97/65/0	WP_022578911.1	95/63/0
Vibrio neptunius	WP_045975022.1	98/76/0	WP_045975021.1	100/77/0
Vibrio ordalii	WP_010317045.1	96/64/0	WP_017044479.1	97/61/0

Vulnbactin biosynthesis cluster

Organism	VV2_0830	Cov/Ident/E	VV2_0831	Cov/Ident/E	VV2_0834	Cov/Ident/E	VV2_0835	Cov/Ident/E
Vibrio vulnificus	WP_011081748.1		AAO07755.1		WP_011081751.1		WP_011081752.1	
Organism	**VV2_0836**	**Cov/Ident/E**	**VV2_0838**	**Cov/Ident/E**	**VV2_0839**	**Cov/Ident/E**	**VV2_0840**	**Cov/Ident/E**
Vibrio vulnificus	WP_011081753.1		WP_011081755.1		WP_011081756.1		WP_011081757.1	

Organism	VV2_0844	Cov/Ident/E
Vibrio vulnificus	AAO07767.2	

Supplementary file S2

Organism	IutA	Cov/Ident/E
Aliivibrio fischeri	WP_063646174.1	98/74/0
Aliivibrio logei	WP_017023529.1	100/73/0
Aliivibrio salmonicida	WP_012551641.1	100/73/0
Aliivibrio wodanis	WP_060992051.1	98/74/0
Grimontia hollisae	WP_005505256.1	99/56/0
Photobacterium halotolerans	WP_027252270.1	95/73/0
Photobacterium leiognathi	WP_053988884.1	98/81/0
Photobacterium profundum	WP_036802510.1	100/56/0
Photobacterium sanctipauli	WP_036821338.1	91/60/0
Photobacterium sp. SKA34	WP_006645229.1	97/54/0
Vibrio alginolyticus	WP_053311483.1	98/69/0
Vibrio antiquarius	WP_012842231.1	98/68/0
Vibrio azureus	WP_021709526.1	94/56/0
Vibrio brasiliensis	WP_006880407.1	98/57/0
Vibrio campbellii	WP_005533358.1	100/61/0
Vibrio caribbeanicus	WP_038134813.1	100/72/0
Vibrio cholerae	KFE32191.1	98/97/0
Vibrio coralliilyticus	WP_040122005.1	100/72/0
Vibrio diabolicus	WP_048625083.1	98/68/0
Vibrio diazotrophicus	WP_042480676.1	100/80/0
Vibrio fluvialis	WP_044365758.1	95/84/0
Vibrio fortis	WP_032550606.1	100/81/0
Vibrio furnissii	WP_047458045.1	95/84/0
Vibrio galatheae	WP_045956527.1	100/80/0
Vibrio genomosp. F10	WP_017036918.1	100/61/0
Vibrio harveyi	WP_038898973.1	95/68/0
Vibrio hepatarius	WP_053407871.1	100/59/0
Vibrio hyugaensis	WP_045401698.1	98/66/0
Vibrio inhibens	WP_063344320.1	95/67/0
Vibrio jasicida	WP_038865785.1	98/66/0
Vibrio kanaloae	WP_017058734.1	98/84/0
Vibrio litoralis	WP_027696548.1	100/57/0
Vibrio maritimus	GAL38192.1	97/79/0
Vibrio mediterranei	WP_062456094.1	98/59/0
Vibrio metoecus	WP_055064999.1	100/99/0
Vibrio mimicus	WP_000843157.1	
Vibrio natriegens	WP_020336334.1	98/80/0
Vibrio neptunius	WP_045977518.1	100/81/0
Vibrio nereis	WP_053394631.1	98/59/0
Vibrio orientalis	WP_004417036.1	95/83/0
Vibrio pacinii	WP_038173486.1	100/85/0
Vibrio parahaemolyticus	WP_031853589.1	94/70/0
Vibrio parilis	WP_000644798.1	100/98/0
Vibrio proteolyticus	WP_021706161.1	98/70/0
Vibrio rotiferianus	WP_045388840.1	94/68/0
Vibrio rumoiensis	WP_026025691.1	100/57/0
Vibrio sagamiensis	WP_039980475.1	94/56/0
Vibrio shilonii	WP_006070381.1	98/59/0
Vibrio sinaloensis	WP_038187421.1	100/72/0
Vibrio sp. 16	WP_005469571.1	100/72/0
Vibrio sp. 3062	WP_063603061.1	98/58/0
Vibrio sp. 712i1	WP_017634648.1	98/67/0
Vibrio sp. AND4	WP_009841199.1	98/57/0
Vibrio sp. B183	WP_038160271.1	100/62/0
Vibrio sp. ECSMB14106	WP_046225040.1	98/84/0
Vibrio sp. EJY3	WP_014231930.1	98/80/0
Vibrio sp. ER1A	WP_038227377.1	98/58/0
Vibrio sp. HENC-01	EKM20725.1	98/66/0
Vibrio sp. HI00D65	WP_063522285.1	100/58/0
Vibrio sp. JCM 18905	GAJ76753.1	98/68/0
Vibrio sp. MED222	WP_009847194.1	100/81/0
Vibrio splendidus	WP_004736323.1	100/82/0
Vibrio tasmaniensis	WP_017104136.1	100/80/0
Vibrio toranzoniae	WP_060469444.1	98/84/0
Vibrio tubiashii	WP_004743922.1	100/83/0
Vibrio variabilis	WP_038212523.1	100/72/0
Vibrio vulnificus	WP_011152720.1	100/82/0
Vibrio xuii	WP_053440998.1	98/82/0

Organism	BitA	Cov/Ident/E
Aliivibrio logei	WP_017021621.1	100/99/0
Aliivibrio salmonicida	WP_012549028.1	
Aliivibrio wodanis	WP_060991932.1	100/85/0

Organism	ViuA	Cov/Ident/E
Vibrio albensis	WP_000279436.1	100/99/0
Vibrio caribbeanicus	WP_038136065.1	93/57/0
Vibrio cholerae	WP_000279435.1	
Vibrio nigripulchritudo	WP_004405074.1	100/60/0
Vibrio sinaloensis	WP_039625029.1	93/57/0
Vibrio sp. 16	WP_043886606.1	93/57/0
Vibrio sp. MED222	WP_009846388.1	100/57/0
Vibrio tasmaniensis	WP_017104270.1	100/57/0
Vibrio tubiashii	WP_038201292.1	100/55/0
Vibrio variabilis	WP_038217008.1	93/57/0
Vibrio vulnificus	WP_045590134.1	100/76/0

Organism	VuuA	Cov/Ident/E
Vibrio albensis	WP_000279436.1	100/75/0
Vibrio caribbeanicus	WP_038136065.1	92/58/0
Vibrio cholerae	WP_046126980.1	100/75/0
Vibrio nigripulchritudo	WP_004405074.1	100/62/0
Vibrio sinaloensis	WP_038188975.1	92/59/0
Vibrio sp. 16	WP_043886606.1	92/58/0
Vibrio sp. MED222	WP_009846388.1	100/57/0
Vibrio tasmaniensis	WP_017104270.1	100/57/0
Vibrio tubiashii	WP_038201292.1	99/59/0
Vibrio variabilis	WP_038217008.1	92/58/0
Vibrio vulnificus	WP_015728225.1	

Organism	PvuA	Cov/Ident/E
Aliivibrio wodanis	WP_045102244.1	100/82/0
Photobacterium sanguinicancri	WP_062689355.1	97/69/0
Vibrio alginolyticus	WP_046875681.1	100/99/0
Vibrio antiquarius	WP_006740839.1	100/99/0
Vibrio caribbeanicus	WP_009603300.1	100/79/0
Vibrio crassostreae	WP_017064386.1	100/83/0
Vibrio cyclitrophicus	WP_016795660.1	100/82/0
Vibrio diabolicus	WP_048625455.1	100/91/0
Vibrio fortis	WP_032550981.1	100/81/0
Vibrio harveyi	WP_050908356.1	100/85/0
Vibrio lentus	WP_050613138.1	100/82/0
Vibrio natriegens	WP_049873777.1	98/85/0
Vibrio navarrensis	WP_039433582.1	100/97/0
Vibrio nereis	WP_053395920.1	100/82/0
Vibrio parahaemolyticus	WP_057620147.1	
Vibrio rotiferianus	WP_045390585.1	100/86/0
Vibrio sp. EJY3	WP_049794697.1	98/85/0
Vibrio sp. HENC-01	WP_009696140.1	100/85/0
Vibrio sp. J2-12	WP_050644301.1	100/83/0
Vibrio sp. J2-15	WP_050632946.1	100/83/0
Vibrio sp. J2-17	WP_050652643.1	100/83/0
Vibrio sp. J2-29	WP_048614853.1	100/83/0
Vibrio sp. J2-3	WP_050620541.1	100/83/0
Vibrio sp. J2-31	WP_050650866.1	100/83/0
Vibrio sp. MED222	WP_009844801.1	100/82/0
Vibrio sp. OY15	KFJ86288.1	100/98/0
Vibrio splendidus	WP_061016597.1	100/82/0
Vibrio tasmaniensis	WP_017103812.1	100/82/0

Organism	FvtA	Cov/Ident/E
Vibrio anguillarum	WP_019281795.1	
Vibrio ordalii	WP_017050158.1	100/99/0
Vibrio campbellii	WP_029388829.1	100/75/0
Vibrio alginolyticus	WP_054579170.1	100/76/0
Vibrio sp. MEBiC08052	WP_059120560.1	100/73/0
Vibrio rhizosphaerae	WP_038181236.1	100/73/0
Vibrio metschnikovii	WP_004396931.1	100/72/0
Vibrio nereis	WP_053394472.1	100/72/0

Organism	FatA	Cov/Ident/E
Vibrio alginolyticus	WP_005375288.1	100/80/0
Vibrio anguillarum	WP_011154638.1	
Vibrio campbellii	WP_050910521.1	99/78/0
Vibrio harveyi	WP_050913418.1	99/78/0
Vibrio kanaloae	WP_017055759.1	100/95/0
Vibrio sp. AND4	WP_009841728.1	99/69/0
Vibrio sp. OY15	WP_033906769.1	100/80/0
Vibrio splendidus	WP_017094255.1	100/99/0

Organism	FrpA	Cov/Ident/E
Photobacterium damselae	WP_044179775.1	94/100/0
Photobacterium damselae subsp. piscicida	AKQ52529.1	
Photobacterium profundum	WP_011218394.1	100/66/0
Salinivibrio sp. DV	WP_069588086.1	94/94/0
Vibrio albensis	WP_032468567.1	95/65/0
Vibrio anguillarum	WP_064626367.1	94/66/0
Vibrio atlanticus	WP_065679247.1	100/56/0
Vibrio cholerae	WP_069648789.1	95/65/0
Vibrio coralliilyticus	WP_045986450.1	100/84/0
Vibrio harveyi	WP_050922393.1	100/57/0
Vibrio mimicus	WP_061051254.1	100/65/0
Vibrio neptunius	WP_045975015.1	100/80/0
Vibrio ordalii	WP_017044474.1	94/64/0
Vibrio rotiferianus	WP_045392643.1	100/57/0
Vibrio sp. HENC-01	WP_009695982.1	100/57/0

Organism	IrgA	Cov/Ident/E
Photobacterium ganghwense	WP_047884933.1	100/63/0
Photobacterium halotolerans	WP_036756649.1	98/63/0
Salinivibrio socompensis	WP_025674231.1	97/58/0
Salinivibrio sp. KP-1	WP_046075193.1	97/60/0
Vibrio albensis	WP_000088795.1	100/98/0
Vibrio alginolyticus	WP_053311394.1	100/97/0
Vibrio anguillarum	WP_013857808.1	100/68/0
Vibrio antiquarius	WP_006743085.1	100/68/0
Vibrio cholerae	WP_000086048.1	
Vibrio diabolicus	CDT94561.1	100/68/0
Vibrio diazotrophicus	WP_042483111.1	96/59/0
Vibrio fluvialis	WP_020332665.1	100/60/0
Vibrio furnissii	WP_047459440.1	100/60/0
Vibrio hepatarius	WP_053408789.1	100/68/0
Vibrio kanaloae	WP_050546269.1	96/70/0
Vibrio litoralis	WP_027696627.1	97/62/0
Vibrio metoecus	WP_055065300.1	100/99/0
Vibrio mimicus	WP_000086042.1	100/94/0
Vibrio navarrensis	WP_039439461.1	100/67/0
Vibrio nereis	WP_053394255.1	98/66/0
Vibrio ordalii	WP_017045434.1	100/68/0
Vibrio parahaemolyticus	WP_025502123.1	100/67/0
Vibrio parilis	WP_000086453.1	100/96/0
Vibrio proteolyticus	WP_040903254.1	97/69/0
Vibrio rumoiensis	WP_017025449.1	100/60/0
Vibrio sp. 2538-88	WP_061897465.1	97/68/0
Vibrio sp. 712i1	WP_017633560.1	100/69/0
Vibrio sp. ECSMB14106	WP_046223384.1	96/70/0
Vibrio sp. JCM 18904	GAJ73542.1	100/68/0
Vibrio sp. JCM 18905	GAJ77987.1	100/68/0
Vibrio sp. OY15	WP_033907452.1	100/68/0
Vibrio sp. S512-13	KJQ87368.1	100/68/0
Vibrio sp. ZOR0018	WP_047688185.1	100/68/0
Vibrio toranzoniae	WP_060468643.1	96/70/0

Organism	VctA	Cov/Ident/E
Grimontia celer	WP_062665201.1	99/62/0
Grimontia sp. AD028	WP_046303643.1	99/62/0
Photobacterium halotolerans	WP_036756816.1	100/54/0
Photobacterium jeanii	OAN11739.1	99/55/0
Photobacterium sanguinicancri	WP_062691924.1	99/55/0
Vibrio albensis	EEO01325.1	100/97/0
Vibrio alginolyticus	WP_046875988.1	99/65/0
Vibrio antiquarius	WP_006742779.1	99/65/0
Vibrio cholerae	WP_000350325.1	
Vibrio coralliilyticus	WP_043010499.1	99/67/0
Vibrio diabolicus	WP_048624949.1	99/65/0
Vibrio diazotrophicus	WP_042479836.1	99/74/0
Vibrio fluvialis	WP_044362327.1	100/71/0
Vibrio fortis	WP_032552715.1	99/67/0
Vibrio furnissii	WP_047460619.1	100/70/0
Vibrio harveyi	WP_017190702.1	99/65/0
Vibrio hepatarius	WP_053407879.1	99/64/0
Vibrio inhibens	WP_063345324.1	99/64/0
Vibrio jasicida	WP_038880693.1	99/65/0
Vibrio mediterranei	WP_062457880.1	99/60/0
Vibrio metoecus	WP_055050054.1	100/92/0
Vibrio mimicus	WP_000350338.1	100/91/0
Vibrio navarrensis	WP_039427354.1	99/61/0
Vibrio nereis	WP_053394603.1	99/66/0
Vibrio nigripulchritudo	WP_022561450.1	99/50/0
Vibrio owensii	WP_045412290.1	99/65/0
Vibrio parahaemolyticus	WP_024701351.1	99/66/0
Vibrio parilis	WP_000350334.1	100/90/0
Vibrio proteolyticus	WP_021704796.1	99/67/0
Vibrio rotiferianus	WP_045389041.1	99/65/0
Vibrio scophthalmi	WP_040757791.1	99/58/0
Vibrio shilonii	WP_006070015.1	99/61/0
Vibrio sinaloensis	WP_008072564.1	99/64/0
Vibrio sp. 1048-83	WP_061900259.1	99/61/0
Vibrio sp. 16	WP_043886787.1	99/63/0
Vibrio sp. 2423-01	WP_061894514.1	99/60/0
Vibrio sp. 2538-88	WP_061898659.1	99/61/0
Vibrio sp. 712i1	WP_017635378.1	99/65/0
Vibrio sp. ER1A	WP_038229421.1	99/61/0
Vibrio sp. HENC-01	WP_009695870.1	99/65/0
Vibrio sp. HENC-02	KM28232.1	97/65/0
Vibrio sp. HENC-03	WP_009704150.1	99/65/0
Vibrio sp. JCM 18904	GAJ70526.1	99/65/0
Vibrio sp. JCM 19053	GAK19199.1	98/65/0
Vibrio sp. MED222	WP_009845519.1	99/65/0
Vibrio sp. N418	WP_038218985.1	100/58/0
Vibrio sp. OY15	KFJ87325.1	99/65/0
Vibrio sp. S234-5	WP_045569166.1	99/61/0
Vibrio splendidus	WP_004737311.1	99/66/0
Vibrio tasmaniensis	WP_017101366.1	99/65/0
Vibrio tubiashii	WP_004742845.1	99/67/0
Vibrio vulnificus	WP_060533657.1	99/61/0
Vibrio xuii	WP_053439033.1	99/65/0

Organism	DesA	Cov/Ident/E
Aliivibrio fischeri	WP_011263609.1	99/59/0
Aliivibrio logei	WP_017021400.1	99/60/0
Aliivibrio salmonicida	WP_012552174.1	99/60/0
Aliivibrio wodanis	WP_061029250.1	99/61/0
Photobacterium halotolerans	WP_052729927.1	99/52/0
Photobacterium kishitanii	WP_045044119.1	94/59/0
Photobacterium phosphoreum	WP_045030314.1	100/56/0
Photobacterium sanctipauli	WP_036832716.1	97/53/0
Photobacterium swingsii	WP_048897482.1	98/53/0
Vibrio alginolyticus	WP_047009840.1	98/50/0
Vibrio brasiliensis	EGA65619.1	88/58/0
Vibrio campbellii	WP_005533381.1	99/60/0
Vibrio caribbeanicus	WP_038136114.1	99/60/0
Vibrio coralliilyticus	WP_006960102.1	98/59/0
Vibrio crassostreae	WP_048668812.1	99/61/0
Vibrio cyclitrophicus	WP_010430390.1	99/60/0
Vibrio diabolicus	WP_048626473.1	98/50/0
Vibrio diazotrophicus	WP_042481746.1	98/72/0
Vibrio fluvialis	WP_032081496.1	100/96/0
Vibrio fortis	WP_032552731.1	100/53/0
Vibrio furnissii	WP_004725209.1	
Vibrio genomosp. F10	WP_017033505.1	99/60/0
Vibrio harveyi	WP_061035573.1	99/60/0
Vibrio hepatarius	WP_053409345.1	99/59/0
Vibrio inhibens	WP_063344221.1	99/60/0
Vibrio jasicida	WP_038865297.1	99/61/0
Vibrio kanaloae	WP_017058127.1	99/60/0
Vibrio litoralis	WP_027697455.1	97/60/0
Vibrio maritimus	WP_042496488.1	99/51/0
Vibrio mediterranei	WP_062457030.1	99/50/0
Vibrio neptunius	WP_045974315.1	98/59/0
Vibrio nigripulchritudo	WP_022610261.1	99/53/0
Vibrio orientalis	WP_004412948.1	98/65/0
Vibrio owensii	WP_045483410.1	99/60/0
Vibrio pacinii	WP_038172738.1	99/58/0
Vibrio proteolyticus	WP_021706544.1	100/66/0
Vibrio rumoiensis	WP_039836685.1	98/59/0
Vibrio sinaloensis	WP_039482114.1	99/61/0
Vibrio sp. 16	EED28639.1	99/60/0
Vibrio sp. 3062	WP_063606139.1	99/50/0
Vibrio sp. 712i1	WP_017634564.1	98/50/0
Vibrio sp. AND4	WP_009841883.1	99/59/0
Vibrio sp. B183	WP_038157617.1	98/60/0
Vibrio sp. CAIM 1540	WP_047043906.1	98/63/0
Vibrio sp. ECSMB14106	WP_046224905.1	99/60/0
Vibrio sp. ER1A	WP_038225588.1	99/50/0
Vibrio sp. HI00D65	WP_063525104.1	99/53/0
Vibrio sp. J2-15	WP_050633003.1	99/61/0
Vibrio sp. J2-26	WP_050711850.1	99/61/0
Vibrio sp. J2-3	WP_050620734.1	99/61/0
Vibrio sp. J2-31	WP_048606204.1	99/60/0
Vibrio sp. J2-6	WP_050645620.1	99/52/0
Vibrio sp. JCM 19052	GAK23108.1	99/60/0
Vibrio sp. MED222	WP_009845539.1	99/52/0
Vibrio splendidus	WP_019822843.1	99/61/0
Vibrio tasmaniensis	WP_017109449.1	99/61/0
Vibrio toranzoniae	WP_060469588.1	99/60/0
Vibrio tubiashii	WP_004745724.1	98/60/0
Vibrio variabilis	GAL24804.1	97/52/0
Vibrio vulnificus	WP_061057750.1	98/59/0
Vibrio xuii	WP_053439432.1	98/65/0

Organism	FhuA	Cov/Ident/E
Photobacterium ganghwense	WP_047886182.1	99/60/0
Photobacterium halotolerans	WP_046220079.1	99/51/0
Photobacterium profundum	WP_011218298.1	100/64/0
Vibrio alginolyticus	WP_053350062.1	98/65/0
Vibrio antiquarius	WP_012842430.1	98/65/0
Vibrio cholerae	WP_053043596.1	
Vibrio diabolicus	WP_048625353.1	98/64/0
Vibrio furnissii	WP_055466431.1	100/73/0
Vibrio galatheae	WP_045954466.1	99/57/0
Vibrio hepatarius	WP_053407570.1	99/60/0
Vibrio hyugaensis	WP_045462290.1	95/66/0
Vibrio kanaloae	WP_017058066.1	100/63/0
Vibrio litoralis	WP_038150238.1	95/50/0
Vibrio metoecus	WP_055051882.1	100/98/0
Vibrio mimicus	WP_000763684.1	100/97/0
Vibrio mytili	WP_041154089.1	98/63/0
Vibrio natriegens	WP_020335445.1	98/63/0
Vibrio navarrensis	WP_039435429.1	98/63/0
Vibrio parahaemolyticus	WP_025553671.1	98/65/0
Vibrio parilis	WP_001204838.1	100/98/0
Vibrio proteolyticus	WP_021706425.1	100/64/0
Vibrio sp. 2423-01	WP_061895609.1	98/64/0
Vibrio sp. 2538-88	WP_061897033.1	98/64/0
Vibrio sp. ECSMB14106	WP_046225153.1	100/64/0
Vibrio sp. EJY3	WP_014234892.1	98/63/0
Vibrio sp. JCM 18904	GAJ71497.1	96/66/0
Vibrio sp. OY15	KFJ86518.1	98/65/0
Vibrio toranzoniae	WP_060469744.1	100/64/0
Vibrio tubiashii	WP_038204349.1	100/57/0

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