Iron assimilation and siderophore production by *Vibrio ordalii* strains isolated from diseased Atlantic salmon *Salmo salar* in Chile

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**ABSTRACT:** *Vibrio ordalii* is the causative agent of vibriosis in several cultured salmonid species worldwide. Despite its impact on aquaculture, relatively little information is available about its virulence factors. The present study demonstrates for the first time that *V. ordalii* possesses different systems of iron acquisition, one involving siderophore synthesis and another one that uses direct binding of heme to use iron. Using 6 strains of *V. ordalii* from Atlantic salmon *Salmo salar* and the *V. ordalii* type strain, we could demonstrate that all strains could grow in presence of the chelating agent 2,2'-dipyridyl and produced siderophores in solid and liquid media. Cross-feeding assays among *V. ordalii* strains evidenced variability in the siderophores produced. Bioassays and PCR data suggest that *V. ordalii* could produce a siderophore with a structure similar to piscibactin, although the production of a second siderophore in certain strains cannot be discarded. Furthermore, all strains were able to use hemin and hemoglobin as the only iron sources, although the cell yield was higher when using hemoglobin. A hemin-binding assay indicated the presence of constitutive heme-binding molecules at the cell surface of *V. ordalii*. Virulence tests using rainbow trout as a model of infection revealed a clear relationship between iron-uptake ability and pathogenicity in *V. ordalii*.

**KEY WORDS:** *Vibrio ordalii* · Siderophore · Iron sources · Virulence mechanisms · Atlantic salmon

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**INTRODUCTION**

*Vibrio ordalii*, formerly *Vibrio anguillarum* biovar II (Schiewe et al. 1981), is an important causative agent of vibriosis in several salmonid species cultured in the USA, Japan, Australia, New Zealand and Chile (Harrell et al. 1976, Ransom et al. 1984, Toranzo et al. 1997, Colquhoun et al. 2004), although it has also been reported as a pathogen for other fish species such as gilthead sea bream *Sparus aurata* in Turkey (Akayli et al. 2010). This pathogen is a Gram-negative, motile, rod-shaped bacterium that is fermentative, catalase and oxidase positive, and sensitive to the vibriostatic agent O/129 (Farmer et al. 2005). *V. ordalii* causes hemorrhagic septicemia on the base of the fins, exophthalmos, and injury, especially on the area surrounding the anus, including the anal pore (Ransom et al. 1984).
Currently, the pathogenic mechanisms of *V. ordalii* are not yet fully understood. We have previously shown that the pathogenicity is not correlated to the hemagglutination capacity of Atlantic salmon *Salmo salar* red blood cells or biofilm characteristics, but it could be associated with its hydrophobic properties (Ruiz et al. 2015). *V. ordalii* can also survive in *S. salar* mucus, which likely facilitates *in vivo* host colonization and helps to neutralize or evade the host immune system, including bactericidal activities (Ruiz et al. 2015).

A critical virulence factor for most Gram-negative bacteria is the ability to obtain iron (Wolf & Crosa 1986). In hosts, iron is a cofactor involved in general metabolism, DNA replication, and the electron transport chain (Ratledge & Dover 2000), and it is mostly bound to proteins, such as hemoglobin, transferrin, lactoferrin, or ferritin (Wooldridge & Williams 1993, Ratledge & Dover 2000). Therefore, within a host, free iron is often highly limited. Thus, most pathogenic bacteria possess 2 main iron-uptake mechanisms: (1) a direct utilization of iron contained in host proteins, such as transferrin or hemoglobin; and (2) the production of intermediaries, such as hemophores and siderophores, to scavenge iron from heme- or iron-containing compounds (Wooldridge & Williams 1993, Ratledge & Dover 2000, Debarbieux & Wandersman 2004, Raymond & Dertz 2004). In *V. anguillarum*, it has been demonstrated that both types of mechanisms are present. *V. anguillarum* can efficiently use heme and hemoglobin as iron sources (Mazoy & Lemos 1991, Mourño et al. 2004) and also produces sidero- and scavengers that can efficiently use heme and hemoglobin as iron sources (Mazoy & Lemos 1991, Mourño et al. 2004) and also produces sidero- from heme and hemoglobin as iron sources (Mazoy & Lemos 1991, Mourño et al. 2004) and also produces sidero-

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

A total of 6 *Vibrio ordalii* strains isolated from Atlantic salmon from enzootics on farms in southern Chile during 2003 to 2011 were studied. The Atlantic salmon isolates were obtained from internal organs (kidney or spleen) of diseased cage-cultured fish. The strains chosen for this work were selected on the basis of their cell-surface characteristics (Ruiz et al. 2015) because a first requisite for successful colonization is the ability to adhere to the host, and hydrophobicity (water repelling) of bacterial cells has been associated with bacterial pathogenicity (González-Contreras et al. 2011). Strains Vo-LM-03 and Vo-LM-05 (Group 1) were classified as strongly hydrophobic (hydrophobicity >29.5%), strains Vo-LM-01, Vo-LM-13, and Vo-LM-18 (Group 2) were classified as relatively hydrophobic (hydrophobicity ranging from 19.6 to 29.5%), and strain Vo-LM-06 (Group 3) was classified as non-hydrophobic (hydrophobicity ranging from 9.6 to 19.5%). *V. ordalii* type strain ATCC 33509 and *V. anguillarum* ATCC 43307 (obtained from the American Type Culture Collection) were also used for comparative purposes. Identification of each strain as *V. ordalii* was achieved by standard phenotypic characterization procedures (Silva-Rubio et al. 2008, Poblete-Morales et al. 2013) and confirmed by the PCR-based analysis described by Avendaño-Herrera et al. (2014). *V. ordalii* strains were routinely cultivated on trypticase soy agar or broth supplemented with 1% (w/v) NaCl (TSA-1 and TSB-1, respectively) under aerobic conditions at 18°C for 48 to 72 h. Stock cultures were maintained frozen at −80°C in Cryobrilles tubes (AES Laboratory) or in TSB-1 containing 15% (v/v) glycerol.

**Ability to grow under iron-limiting conditions**

The ability of the *V. ordalii* strains to grow under iron-restricted conditions was determined by their capacity to grow in TSB-1 supplemented with the non-assimilable iron chelator 2,2′-dipyridyl (Sigma-Aldrich). The minimal inhibitory concentration (MIC) of 2,2′-dipyridyl was determined by culturing each *V. ordalii* strain in TSB-1 containing increasing concentrations of 2,2′-dipyridyl (from 0 to 400 μM) and was defined as the lowest concentration at which no bacterial growth was observed. Growth was determined using optical density at 620 nm (OD620) in a Multi-
skan Ex (Thermo Scientific) spectrophotometer. All experiments were carried out in triplicate using different batches of media.

**Utilization of different iron sources**

To test the use of different sources of iron by *V. ordalii*, TSB-1 medium with 2,2′-dipyridyl (at inhibitory concentrations for each strain) was supplemented with various iron sources: hemin (bovine), hemoglobin (bovine), ferric ammonium citrate (FAC), and ferric chloride (Sigma), and growth in each condition was determined by OD₆₂₀. Hemin (Sigma) stock solution at 5 mM was prepared in 10 mM NaOH, and hemoglobin (Becton Dickinson) was dissolved in distilled water at 1 mM. FAC was dissolved in phosphate buffered saline (PBS) and ferric chloride in distilled water, both at 10 mM. All stock solutions were sterilized by filtration through 0.22 µm pore size membranes (Millipore) and maintained at 4°C until use. Final concentrations of the iron sources ranged from 0.1 to 20 µM. The growth was assessed in triplicate in 96-well plates (NUNC Surface) containing 100 µl of TSB-1, supplemented with 2,2′-dipyridyl and the iron source to be tested, seeded with a 1:100 (v/v) inoculum from TSB-1. The OD₆₂₀ was measured after 48 h of incubation at 18°C in a shaker at 100 rpm.

**Hemin-binding assays**

Once the ability to use hemin as iron source was identified, the existence of possible outer membrane receptors for heme compounds in *V. ordalii* was determined using the Congo red and hemin-binding assays in liquid media, according to the procedures described by Kay et al. (1985) with some modifications. Congo red dye-binding ability is an indicator of heme-binding capacity because Congo red structurally resembles heme (Surgalla & Beesley 1969). Briefly, *V. ordalii* cells grown in (1) TSB-1 with 15 µM FeCl₃, (2) TSB-1 with 20 µM hemoglobin, and (3) TSB-1 supplemented with half of the MIC of 2,2′-dipyridyl and the iron source to be tested, seeded with a 1:100 (v/v) inoculum from TSB-1. The OD₂₀ was measured after 48 h of incubation at 18°C in a shaker at 100 rpm.

**Production of siderophores**

Siderophore activity was tested using the Chrome Azurol S (CAS) assay of Schwyn & Neilands (1987) in solid and liquid media. This test detects the presence of compounds with iron-chelating activity using a ferri–chromogen complex that changes color when it loses Fe³⁺ ions. Siderophores, which have more affinity for iron than the chromogen (CAS), can capture iron from the ferri–chromogen complex, resulting in a color change of the dye from blue to yellow-orange. The assays were performed by spotting 10 µl of a bacterial suspension in PBS obtained from a 3 d TSA-1 culture onto CAS agar plates. The siderophore levels produced by the strains on CAS agar were expressed as the ratio of the orange halo diameter to the growth diameter after 48 to 72 h of incubation. Plates were also spotted with *V. anguillarum* ATCC 43307 as a positive control. For siderophore detection in liquid media, supernatants from a culture in TSB-1 containing 2,2′-dipyridyl were mixed with a CAS supernatant solution, and the absorbance (A₆₃₀) of the mixture was measured.

The presence of catecholate-type and hydroxamate-type siderophores was detected in cell-free culture supernatants obtained from *V. ordalii* strains grown under iron-limiting conditions (TSA-1 plus 2,2′-dipyridyl) using the colorimetric assays described by Arnow (1937) and Csáky (1948), modified by Andrus et al. (1983), respectively. Deferoxamine mesylate (a trihydroxamate siderophore; Sigma) and 2,3-dihydroxybenzoic acid (Sigma) were used as positive controls. The experiments were performed twice.

**Cross-feeding assays**

To test the ability of each *V. ordalii* strain to induce the growth of the other strains subjected to iron starvation, cross-feeding assays were used (Avendaño-Herrera et al. 2005). Briefly, *V. ordalii* strains were grown in iron-limiting conditions (TSB-1 supplemented with half of the MIC of 2,2′-dipyridyl) and
centrifuged at 12,000 × g for 5 min. Cell-free supernatants from the strains to be tested were pipetted onto sterile filter paper disks and, after being dried, were placed on the agar surface of TSA-1 plates seeded with the strain to be used as an indicator and containing an inhibitory concentration of 2,2′-dipyridyl for that strain. The detection of a growth halo around the disks indicated the production by the tested strain of a diffusible siderophore that can be utilized by the indicator strain.

Purified siderophores vanchrobactin (from *V. anguillarum*) and piscibactin (from *Photobacterium damselae* subsp. *piscicida*), obtained as previously described (Soengas et al. 2006, Souto et al. 2012), were also used to test the ability of *V. ordalii* strains to utilize them as iron sources. Different concentrations of each siderophore were added to liquid cultures of each strain in TSB-1 with an inhibitory concentration of 2,2′-dipyridyl for each particular strain. Growth was measured by determining the OD600.

### DNA isolation and PCR assays

To test the possible presence in *V. ordalii* strains of genes known in *V. anguillarum* to be involved in iron assimilation, PCR assays were performed to test the presence of genes *vabF* (siderophore vanchorbactin biosynthesis), *irp1* (piscibactin biosynthesis), *huvA* and *huvS* (encoding heme receptors), and *tonB1* and *tonB2* (encoding TonB systems). Genomic DNA from each *V. ordalii* strain was extracted using the Genome® DNA Kit (Q-Biogene) following the manufacturer's instructions. Primers employed for detection of each gene are shown in Table 1. PCR reactions were performed following standard protocols as described in the sources shown in Table 1.

### Table 1. Genes and primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Nucleotide sequence 5’–3’</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>irp1</em></td>
<td><em>irp1_ang_1</em></td>
<td>TGA TGC ATT AGC CCA TCA GG</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td><em>irp1_ang_2</em></td>
<td>AAA GCA AGG GTC GAG AGT GT</td>
<td></td>
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<tr>
<td><em>vabF</em></td>
<td><em>pMB5_R</em></td>
<td>AAT CAC CGC CAC TCC AGG A</td>
<td>Balado et al. (2008)</td>
</tr>
<tr>
<td></td>
<td><em>pMB5_-R</em></td>
<td>TAG ATG GCA ACG TGG TGC A</td>
<td></td>
</tr>
<tr>
<td><em>huvA</em></td>
<td><em>P3-1</em></td>
<td>GGA ATG TGG TCC CAG CAC TA</td>
<td>Mouriño et al. (2005)</td>
</tr>
<tr>
<td></td>
<td><em>HuvA-Short 3′</em></td>
<td>CAT GGA ACA ACA AAG CCA GC</td>
<td></td>
</tr>
<tr>
<td><em>huvS</em></td>
<td><em>huvS3</em></td>
<td>CGA AGA CCA GCG GGT AAT AT</td>
<td>Mouriño et al. (2005)</td>
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<tr>
<td></td>
<td><em>huvS6-rev</em></td>
<td>GCT CTC GCA GAA GAA GTT TC</td>
<td></td>
</tr>
<tr>
<td><em>tonB1</em></td>
<td><em>cDNA-tonB1</em></td>
<td>AAA CCC CAC CTC CAG AAA AG</td>
<td>Stork et al. (2004)</td>
</tr>
<tr>
<td></td>
<td><em>cDNA-tonB2</em></td>
<td>TGG GCG AGT AAC AAA AGA CG</td>
<td></td>
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<tr>
<td><em>tonB2</em></td>
<td><em>TonB2-1</em></td>
<td>TTA CTG CTT GCT TTG CCA A</td>
<td>Stork et al. (2004)</td>
</tr>
<tr>
<td></td>
<td><em>TonB2-2</em></td>
<td>TTT GTC CCG GCT CTT AAT A</td>
<td></td>
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</tbody>
</table>

Effect of iron availability on the lethality of *V. ordalii* strains for fish

The influence of iron levels on the pathogenicity of *V. ordalii* strains from different virulence categories (Ruiz et al. 2015) was tested by comparing the virulence of strain LM-Vo-18 and the type strain ATCC 33509T grown under iron-limitation (TSB-1 with half of the MIC of 2,2′-dipyridyl) and under an excess of iron (TSB-1).

Groups of 10 fishes (rainbow trout weighing 28 to 30 g) were inoculated by intraperitoneal injection with the *V. ordalii* strains grown in both conditions with or without the iron-chelating agent. All fish were maintained in tanks at 16 ± 1°C with aeration during the course of the experiments (21 d), with 50% of the tank water refreshed daily. Dead fish were removed from each tank daily, and the cause of death was confirmed by streaking kidney, liver, and spleen samples directly onto TSA-1 plates that were incubated at 18°C for 1 wk. Pure or mixed cultures from these plates, as well as fish tissues (kidney, liver, and spleen) samples, were tested by PCR using a specific protocol (Avendaño-Herrera et al. 2014) to confirm the presence of *V. ordalii*. In addition, the identity of each isolate was confirmed by standard phenotyping procedures (Silva-Rubio et al. 2008, Poblete-Morales et al. 2013).

### RESULTS AND DISCUSSION

#### Growth under iron-limiting conditions and utilization of different iron sources

The ability to grow under iron-limiting conditions was tested in all strains examined. The results show that all *Vibrio ordalii* strains, including the type strain ATCC 33509T, were able to grow under severe iron-limiting growth conditions in TSB-1 medium supplemented with the iron-chelating agent 2,2′-dipyridyl. The MICs of 2,2′-dipyridyl ranged between 90 and
180 µM (Table 2), which suggests that V. ordalii possess at least 1 high-affinity iron uptake system. Based on these results, concentrations of 45 to 90 µM were used as the iron-restrictive conditions for subsequent experiments.

To identify the iron sources that could be used by V. ordalii, we first tested the ability of strains to use heme and hemoglobin, 2 iron sources present in tissues of all animal hosts. Under high iron-restricted conditions in TSB-1 medium, all V. ordalii strains could utilize hemoglobin as the only iron source with high efficiency: cell yield was 3-fold higher (increase in the OD620 values from 0.5 to 1.6) in relation to control grown in plain TSB-1 (Fig. 1A). Growth values were dependent on the hemoglobin concentration, with the highest values obtained when using 20 µM hemoglobin (Fig. 1A). There were no differences among the 3 groups of strains (based on their hydrophobicity) analyzed, suggesting that the mechanisms of iron uptake are independent of this surface characteristic (Fig. 1A).

All strains could also use hemin as the only iron source, although the utilization was much less efficient, since the cell yield reached was 4-fold lower than that obtained when using hemoglobin and lower than the yield obtained in TSB-1 (Fig. 1B). This is accordance with the results obtained in V. anguillarum in which all strains could utilize hemin and hemoglobin as sole iron sources (Mazoy & Lemos 1991, Mouriño et al. 2005).

To test the genetic relationship among the heme uptake systems between V. anguillarum and V. ordalii, we checked by PCR the presence in V. ordalii strains of the genes that encode the outer membrane heme receptors in V. anguillarum: huvA and huvS. As shown in Table 3, all V. ordalii strains tested, including the type strain ATCC 33509T, were positive for the presence of huvS, an alternative receptor gene to huvA that is present in many strains of V. anguillarum (Mouriño et al. 2005). When searching genomes of other V. ordalii strains deposited in GenBank, we found that some strains harbor huvA gene while others harbor huvS, a situation similar to what has been previously reported for V. anguillarum (Mouriño et al. 2005).

We also demonstrated that V. ordalii strains could also grow using FAC or ferric chloride (Fig. 1C,D) as iron sources when these compounds were added to TSB-1 supplemented with inhibitory concentrations of 2,2’-dipyridyl. When using ferric chloride, all V. ordalii strains exhibited identical growth than when they were grown in plain TSB-1 (Fig. 1D).

### Table 2. Growth of Vibrio ordalii strains under iron-limiting conditions; siderophore production, cross-feeding assays, and use of pure siderophores.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µM)</th>
<th>Chemical test</th>
<th>CAS agar</th>
<th>CAS in liquid</th>
<th>Use of siderophores</th>
<th>Use of siderophores</th>
<th>Cross-feeding assay</th>
<th>Cross-feeding assay</th>
<th>Use of siderophores</th>
<th>Use of siderophores</th>
<th>Use of siderophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vo-LM-01</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Vo-LM-03</td>
<td>150</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Vo-LM-05</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
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<tr>
<td>Vo-LM-06</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Vo-LM-13</td>
<td>120</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
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<td>(+)</td>
</tr>
<tr>
<td>ATCC 33509T</td>
<td>90</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>
To determine whether the *V. ordalii* cells display iron-regulated heme-binding activity, tested strains were cultured under iron excess (TSB-1 plus ferric chloride or hemoglobin) and under iron-restricted conditions (TSB-1 plus 2,2'-dipyridyl), and their hemin and Congo red-binding abilities were measured (Fig. 2). None of the strains showed an increase in Congo red absorption when cells were grown in TSB-1 with 2,2'-dipyridyl (Fig. 2A). However, when the strains
were grown in TSB-1 with hemoglobin, they rapidly bound most of the Congo red during the first 15 min after adding the dye (Fig. 2B), with no additional binding observed after that time. The same effect was noted when we measured the hemin-binding ability (Fig. 2E). These results suggest that binding could be due to cell surface components that are induced in presence of hemoglobin or heme groups. Furthermore, when *V. ordalii* cells were grown in TSB-1 with 2,2'-dipyridyl or FeCl₃, a gradual increase of hemin-binding was observed, reaching the highest value after 240 min of exposure to hemin (Fig. 2D,F). This observation suggests that binding is not iron-regulated and could be non-specific. It has been demonstrated that in *V. anguillarum*, hemin binding is mediated by cell surface proteins and that heme-binding ability is independent of the iron levels in the medium, suggesting that constitutive outer membrane proteins play a role in this function (Mazoy & Lemos 1996, Mouriño et al. 2005). This could also be the case for *V. ordalii*.

In *V. anguillarum*, it has also been demonstrated that the presence of a heme uptake system is not a marker of virulence because all strains regardless of their virulence potential have the same ability to use heme (Mouriño et al. 2005). However, this does not rule out a possible role for heme utilization during the infective process of *V. ordalii*. The ability of a bacterial pathogen to acquire iron from heme compounds present in host tissues could constitute an advantage for colonization and invasion, as has been demonstrated in other pathogens (Lemos & Osorio 2007, Contreras et al. 2014).
Production of siderophores

The ability to grow under severely iron-restricted conditions (see ‘Heme-binding activity’) suggests that *V. ordalii* may possess high-affinity iron uptake mechanisms. In siderophore production CAS test, all *V. ordalii* strains and the type strain ATCC 33509\(^T\) gave positive reactions on CAS agar, showing an orange halo on the blue agar plates, clearly indicating the production of siderophores. The *V. ordalii* isolates showed halo/growth diameter ratios ranging from 1.73 to 2.97 (Table 2). As expected, a positive reaction was also found for *V. anguillarum* ATCC 43307\(^T\) used as a control. No differences among the groups of strains (based on their hydrophobicity) were observed, suggesting that the mechanisms of iron uptake are independent of this surface characteristic. Furthermore, the cross-feeding assays among the *V. ordalii* strains showed that all them were able to be cross-fed by the type strain ATCC 33509\(^T\) and by strain Vo-LM-01. However, the remaining 5 strains were unable to promote the growth of any other strain or just gave a small weak growth halo (Table 2). This result points to the existence of an intraspecific variability in *V. ordalii* regarding siderophore production.

Although all *V. ordalii* strains seem to produce siderophores, the chemical assays performed with cell-free supernatants showed that they do not contain typical hydroxamate or catecholate-type compounds, suggesting that *V. ordalii* could produce a ‘mixed type’ siderophore (Hu et al. 1986). However, according to in *silico* searches and to the genomic data reported by Naka et al. (2011), *V. ordalii* ATCC 33509\(^T\) contains the vab cluster genes, which encode the synthesis of vanchrobactin, a typical catechol siderophore identified in *V. anguillarum* (Balado et al. 2006). In addition, the type strain harbors a gene cluster that is homologous to the piscibactin (a mixed type siderophore) biosynthesis genes described in *Photobacteria damselae* subsp. *piscicida* (Osorio et al. 2006, Souto et al. 2012), genes that seem well conserved in several *Vibrio* species (M. Balado & M. L. Lemos pers. obs.). Thus, we performed a PCR assay to test for the presence of both gene clusters in the 6 strains of *V. ordalii* tested. Interestingly, all strains were positive for the 2 markers used, vabF and irp1 (Table 3). The PCR result does not mean that all necessary genes for synthesis and transport of vanchrobactin and piscibactin are present in *V. ordalii* or that all them are actually expressed. In fact, an in *silico* analysis suggests that the vanchrobactin transport system in strain ATCC 33509\(^T\), although having the outer membrane receptor gene fvtA, seems to lack the ABC transporters fvtB-fvtE, necessary for ferric vanchrobactin internalization, as well as the fetAB genes, necessary for enterobactin (a xenosiderophore) transport (Naka et al. 2011).

To test if vanchrobactin and/or piscibactin could be actually used by *V. ordalii* as iron sources, we performed a bioassay with pure siderophores. The results (Table 2) indicate that none of the strains could utilize vanchrobactin, but all of them could use piscibactin as a siderophore. This observation is in agreement with the absence of fvtB–fvtE ABC transporter for vanchrobactin in the genome of *V. ordalii*. We also checked by PCR the presence of TonB sytems (which transduce energy for siderophore transport) in *V. ordalii* strains. All of them were positive for the presence of tonb1 and tonb2 genes (Table 3), which are necessary for siderophore transport across the outer membrane in many Gram-negative bacteria, although in *V. anguillarum*, only tonB2 is essential for siderophore transport and virulence (Stork et al. 2004).

These results altogether suggest that *V. ordalii* likely produces a siderophore with a structure similar to piscibactin, which is in agreement with the chemical tests results described above. However, the production of a second siderophore in certain strains cannot be ruled out. Further studies are currently underway to elucidate the precise chemical structure of the siderophores produced by *V. ordalii*.

Effect of iron availability on the lethality of *V. ordalii* strains for fish

To identify the effect of iron on the virulence of *V. ordalii*, the virulence of strains LM-Vo-18 and ATCC 33509\(^T\) grown under iron limitation (TSB-1 with 2,2′-dipyridyl) and under an excess of iron (TSB-1) was tested in rainbow trout. The results show that both strains increased in virulence degree when cultured under iron-restriction. Strain LM-Vo-18 grown under iron restriction (TSB-1 with 2,2′-dipyridyl) was able to kill 65% of the challenged fish, while this strain when cultured in TSB-1 killed 55% of fishes. When fish were intraperitoneally injected with the type strain ATCC 33509\(^T\), the iron effect was more profound: the mortality increased from 15% (strain grown in TSB-1) to 35% (strain grown under iron-restriction). In all cases, *V. ordalii* was recovered and identified from all dead fish, which showed typical symptoms of a *V. ordalii* infection. These results suggest that the virulent strain Vo-LM-18 could constitutively express most of its virulence factors, while the
non-virulent isolate ATCC 33509T under iron-limiting conditions can induce expression of genes involved not only in the synthesis of siderophores but also in other iron-regulated factors. Similar results have been described in *P. damselae* subsp. *piscicida*, which has been classically considered a non-proteolytic bacterium in normal culture conditions (Magariños et al. 1992), but low-iron environments within fish trigger the expression of these enzymatic activities (Magariños et al. 1994).

In summary, the investigation reported here clearly demonstrated for the first time that *V. ordalii* possesses different systems of iron acquisition, one involving the synthesis of siderophores and another that allows the utilization of heme groups as an iron source by direct binding. In addition, our results establish a clear relationship between iron uptake ability and the pathogenicity of *V. ordalii*.

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**LITERATURE CITED**


