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Short communication

Influence of iron-chelated growth conditions on outer membrane protein production and virulence of *Vibrio tubiashii*

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ABSTRACT

Growth of two *Vibrio tubiashii* strains under iron-chelated conditions resulted in the production of a hydroxymate-like siderophore, and expression of outer membrane proteins with homologies to proteins in *Vibrio cholerae* and *Vibrio vulnificus* which were not seen in cells grown under non-chelated growth conditions. PCR analysis using primers based on *Listonella anguillarum*'s ferric uptake Repressor protein (*fur*) gene detected a 316 bp *fur* gene homolog which also had sequence homology to the *fur* genes of *V. cholerae* and *V. vulnificus*. *V. tubiashii* cultured under iron-chelated growth conditions induced a greater fluid accumulation (FA) response in suckling mice than cells which were cultured under iron non-chelated growth conditions. Our observations that *V. tubiashii* possesses a *fur*-like gene homolog and expresses unique OMPs, a hydroxymate-like siderophore, and produces an increased fluid accumulation response in suckling mice when grown under iron-chelated condition support previous findings that *V. tubiashii* may have the essential components for the survival and establishment of infections and this report represents the first observations of competent iron acquisition system in *V. tubiashii* which is similar to those produced by other marine vibrios, many of which are pathogenic for humans.

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Vibrio tubiashii is a known pathogen that causes bacillary necrosis or vibriosis (Hasegawa et al., 2008), a disease of larval and juvenile mollusks (Tubiash et al., 1965, 1970). In addition to causing fluid accumulation in suckling mice (Kothary et al., 2001), *V. tubiashii* expresses an extracellular cytolysin and a metalloprotease, both of which possess significant N-terminal amino acid sequence homology with similar exoproteins expressed by other *Vibrio* species, including those species pathogenic for humans (Delston et al., 2003; Kothary and Richardson, 1987; Kothary et al., 2001). Furthermore, *V. tubiashii* possesses a *tox*R homolog that has 80% homology to the *tox*R of *Vibrio cholerae* and *Vibrio vulnificus* (Jean-Gilles Beaubrun et al., 2008). Previous findings also suggest that outer membrane proteins (OMPs) expressed by *V. tubiashii* may be involved in pathogenicity (Jean-Gilles Beaubrun et al., 2008). Extracellular virulence factors such as lipases, exopolysaccharides, extracellular metalloprotease and effectors delivered via type III secretion systems (Hasegawa et al., 2008; Park et al., 2004) found in other marine Vibrio species are similar to those present in *V. tubiashii*. Iron utilization is considered an essential component associated with the virulence of bacterial pathogens (Crosa and Walsh, 2002) and iron is an essential requirement for the growth of most microorganisms (Guan et al., 2001). Microorganisms growing in nature and those associated with hosts live under iron-limited conditions and produce OMPs that are not found in organisms grown under iron-rich conditions. Therefore, iron is an important factor for the survival and establishment of infections in susceptible hosts. In this study, the effect of iron-chelated growth conditions on OMP production, iron metabolism and its subsequent effect on virulence in suckling mice were investigated.

1. Effect of iron on OMP expression

Frozen cultures of *V. tubiashii* ATCC strains 19105 and 19109, *V. cholerae* O139 strain 1837, *V. vulnificus* strain 4965-T1 and *Escherichia coli* strain HB101 were routinely cultured and aseptically inoculated onto Trypticase Soy agar medium (Becton Dickinson Microbiology Systems, BBL, Cockeysville, MD) supplemented with 1% NaCl (TSA-S), pH 7.3. The OMPs were isolated according to the procedure of Johnston et al., (Johnston et al., 1976) as described by Jean-Gilles Beaubrun et al. (Jean-Gilles Beaubrun et al., 2008).

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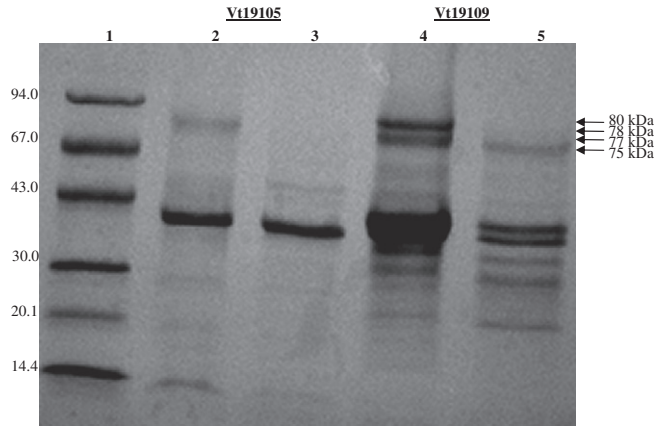

Fig. 1. SDS-PAGE analysis of outer membrane protein expressed by *V. tubiashii* strains 19105 and 19109. Lane 1, Molecular markers and sizes are reported in kDa; Lane 2, *V. tubiashii* 19105 grown on TSA-S supplemented with 125 μM of 2′2′-dipyridyl at 30° C; Lane 3, *V. tubiashii* 19105 grown on TSA-S at 30° C; Lane 4, *V. tubiashii* 19109 grown on TSA-S supplemented with 125 μM of 2′2′-dipyridyl at 30° C; and Lane 5, *V. tubiashii* 19109 grown on TSA-S at 30° C. Note that the 77, 78, and 80 kDa OMPs expressed during growth under iron-chelated growth conditions are identified with arrows.

Based on the minimum inhibitory concentration method (Dai et al., 1992), the iron-chelating agent, 2′, 2-dipyridyl (Sigma–Aldrich Chemical Co., St. Louis, MO, final concentration of 125 μM) was added to TSA-S to achieve optimum concentration for iron-chelated growth conditions for all five organisms. OM complexes and associated OMPs were isolated from *V. tubiashii* cells grown under iron-chelated conditions, and were compared with OM complexes and associated OMPs isolated from cells grown under iron non-chelated conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Payne, 1976) was performed using the Laemmli procedure (Laemmli, 1970) with 8–25% gradient or 7.5% and 12.5% homogenous gels in a PhastSystem (GE Healthcare., Piscataway, NJ). The molecular weights of the denatured and reduced OMPs were estimated by the relative mobility method of Weber et al. (Weber et al., 1972). The purity of the OM complexes were based on methods described by Jean-Gilles Beaubrun et al. (Jean-Gilles Beaubrun et al., 2008), and the protein concentration of each sample was estimated by the method of Bradford (Bradford, 1976). As previously reported by Jean-Gilles Beaubrun et al. (Jean-Gilles Beaubrun et al., 2008), OMP expression was affected by growth conditions. In this study, *V tubiashii* under iron-chelated growth conditions showed that *V. tubiashii* strain 19105 produced a 75 kDa and a 78 kDa OMPs and *V. tubiashii* strain 19109 produced a 77 kDa and an 80 kDa OMPs that were not observed in cells grown under iron non-chelated conditions (Fig. 1). After separation on SDS-PAGE gels, the OMPs were electrophoretically transferred onto ProBlott membranes (Applied Biosystems, Foster City, CA) for N-terminal amino acid (NTAA) sequencing (3 or more repeats for each protein) using a Procise model 491 protein sequencer (Applied Biosystems). BLAST analysis and sequence alignment was carried out using Clustal X analysis. The 77 kDa OMP of *V. tubiashii* 19109 was identified as HutA. This outer membrane protein has been identified in *V. cholerae* and *V. vulnificus* (Henderson and Payne, 1994), and has been previously reported as a hemophore that recognizes free heme or heme bound to host carrier proteins (Crosa and Walsh, 2002). This HutA-like protein had 70% homology to the HutA of *V. cholerae* (Table 1). This finding suggests that the HutA in *V. tubiashii* may be similar to the HutA in *V. cholerae*. The 80 kDa protein was identified as a novel protein.The 78 kDa OMP from *V. tubiashii* 19105 was identified as a *V. cholerae* long chain fatty acid transport protein. In addition to this transport protein, a 75 kDa protein was identified as a metalloprotease-like protein. As previously reported and identified by Jean-Gilles Beaubrun et al., (Jean-Gilles Beaubrun et al., 2008) *V. tubiashii* contains an OmpU-like proteins, an OmpS-like protein, three OMPs (60 kDa, 50 kDa, and 45 kDa) characterized as novel hypothetical proteins expressed by *V. tubiashii* strain 19105 grown in the presence of 3% NaCl and 2% Maltose, and three OMPs, (90 kDa, 70 kDa, and 45 kDa) expressed by *V. tubiashii* strain 19109 grown in the presence of 3% NaCl. These OMPs ranged from 14 kDa to 90 kDa and are inducible under various growth conditions. The most significant OMPs observed in this study were the OMPs produced under iron-chelated conditions, which were the metalloprotease (75 kDa), the transport protein (78 kDa) in *V. tubiashii* 19105, and the HutA-like protein (77 kDa) in *V. tubiashii* 19109. Results of the NTAA sequence and the BLAST analysis of these proteins are listed in Table 1.

2. Expression of siderophore

Since iron availability is limited in most aerobic environments, many microorganisms have developed efficient means to obtain iron under these conditions by producing iron-chelating compounds called siderophores (Wyckoff et al., 1997, 2007). Siderophore production in *V. tubiashii* was investigated in cells grown under iron-chelated and iron non-chelated conditions using the universal siderophore detection procedure described by Schwyn and Neilands (Schwyn and Neilands, 1987). This assay is based on competition for iron between the ferric complex of the indicator dye, chrome Azurol S or CAS, and a siderophore produced by the microorganism. Orange halos around the colonies on the blue CAS agar are indicative of siderophore production and occurs in the medium around colonies only when the Fe^{+3} is removed from the dye complex. CAS plates were inoculated with 18–24 h cultures of organisms grown under iron-chelated and iron non-chelated conditions, which were incubated at 30 °C and examined over a 5 day period. *V. tubiashii* strain 19105 grown on CAS plates under iron non-chelated conditions did not produce the characteristic orange halo, while cells grown under iron-chelated growth conditions (in

Table 1
N-Terminal amino acid sequences of OMPs expressed by *V. tubiashii* strains 19105 and 19109[a].

V. tubiashii	Growth condition	OMP (kDa)	N-Terminal AA Sequence	% ID[a]	% (+)[b]	Related Protein[c]
ATCC19105	Iron-chelated	75	AQIGTGPD	60	76	Metalloprotease of Vh
		78	AGFQLNAQSATELDRYXADAAV	70	80	Vc fatty acid transport protein
ATCC19109	Iron-chelated	30	AVNVNTNVAAMTAQRYKNVAA	80	90	Flagellin subunit A FLA Vv
		77	DDYASFDEVVVSAT	70	75	Hut A Heme transport protein
		80	EDTNAFQPDAVVXA	None	None	*None
		25	AEVFFTDDDAVNFRGQGIRRSV	None	None	*None

The abbreviated terms: Vh, *Vibrio harveyii*; Vc, *Vibrio cholerae*; Vv, *Vibrio vulnificus*; FLA, flagellin.
[a] %ID, percentage of the sequenced amino acid residues that were identified in the database.
[b] %+, percentage of the sequenced amino acid residues that were identical or similar to other *Vibrio* sp.
[c] "None" indicates that the protein is novel.

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presence of the 2′, 2′-dipyridyl) did produce the characteristic orange halo (2 mm), which confirms that the expressed siderophore is inducible and expressed only under conditions of iron-chelated concentrations (Supplemental Figure 1a). Conversely, the results of the CAS assay for *V. tubiashii* strain 19109 (Supplemental Figure 1b) showed that the siderophore was constitutively expressed in iron-chelated and iron non-chelated conditions. However, a larger halo (1 mm) was produced by growth of this strain under iron-chelated conditions than what was seen with growth under iron non-chelated conditions (0.5 mm); suggesting that siderophore expression was up regulated under iron-chelated growth conditions. *V. vulnificus* strain 4965-T1 was used as control in this study and the results show it did express a siderophore under the iron-chelated condition (Supplemental Figure 1c). The results were similar to that of *V. tubiashii* strain 19105. It is known that *V. vulnificus* expresses both the hydroxamate and catechol types of siderophores (Simpson and Oliver, 1983). The presence of a *hut*A gene and the expression of siderophores in both strains of *V. tubiashii* is significant, suggesting that *V. tubiashii* contains essential components of an iron acquisition system.

Most siderophores isolated so far fall into two main groups; those based on catechol and those derived from hydroxamic acid and are called hydroxamate-like siderophores. Both of these types of siderophores are identified using specific colorimetric assays described by Arnow (Arnow, 1937) and Csàky (Csáky, 1948). Using these colorimetric assays, hydroxamate activity was detected in the supernatant of iron-chelated cultures of both strains of *V. tubiashii*, whereas under the same conditions, no catechol activity was observed. This indicates that *V. tubiashii* expresses and secretes a hydroxamate-like siderophore.

3. Detection of the *fur* gene in *V. tubiashii*

Molecular analyses were conducted to support the hypothesis that an iron transport system was present in *V. tubiashii*. Previous investigators have shown that iron uptake is regulated by several genes, one of which is *fur*, which encodes for the ferric uptake regulatory protein (Wyckoff et al., 1997, 2007). When there is little to no free iron in the environment, bacteria rely on high-affinity surface receptor proteins that bind Fe^{3+}, such as siderophores, and than subsequently facilitate their translocation into the cell for respiratory and metabolic functions (Wyckoff et al., 1997, 2007). Fur is a regulatory protein that controls the transcription of iron uptake genes in many bacteria (Wyckoff et al., 1997). Under iron non-limited conditions, the Fe^{3+}- Fur complexes bind to the Fur box (Fur binding sequence) and represses transcription of the iron uptake genes. Under iron-limited conditions, Fur does not bind to the Fur box and transcription of the iron uptake genes is allowed to occur. The presence of *fur* and siderophore production is significant in demonstrating that *V. tubiashii* has the ability to bind free iron under iron-chelated conditions. To screen for *fur* in *V. tubiashii*, primers designed by Colquhoun and Sorum (Colquhoun and Sørum, 2002) that are based on the *fur* genes of *Listonella anguillarum* and *Vibrio salmonicida* were used in this investigation. The primers sets are as follows: *fur L. anguillarum* 5′-GGAAAGTR-TATGTCAGAYATT-3′ and 5′-GAAAACTCAATNACTTCACC-3′and *fur V. salmonicida* 5′-CCTCCATATTGGGTACGATTCG-3′ and 5′-GGAAGC-TGGCTATCAATTTG-3′.

L. anguillarum and *V. salmonicida* primers were obtained from Invitrogen Life Technologies, Chicago IL, and these primer sequences flank a 316 bp and 985 bp fragments of these *fur* genes, respectively. The controls used in this investigation were *Vibrio wodanis* and *L. anguillarum* instead of *V. salmonicida*. PCR analysis was carried out using a master mix containing 5 μl of 10X Tris-Borate-EDTA buffer (Qiagen, Inc., Valencia, CA), 2 μl of 25 mM MgCl2 (Qiagen, Inc.), 2 μl of a 10 mM concentration of the deoxynucleoside triphosphates or 4 μl of a 2 mM concentration of the deoxynucleoside triphosphates (Invitrogen Corporation), and 0.5 μl of Hot Star Taq (Qiagen, Inc.). PCR amplification of each isolate was conducted using 5 μl of the DNA template, prepared according to the procedure described by Kothary et al. (Kothary et al., 2001), and 45 μl of a master mix containing primers, for a total volume of 50 μl. The amplification cycle was as follows: the total reaction occurred in 35 PCR cycles, after a period of 15 min at 95 °C to activate Hot-start Taq, each cycle included 1 min denaturing step at 95 °C, 2 min at 50 °C, and 3 min at 72 °C. This was followed by a final extension step of 7 min. A theoretical-sized 316 bp amplicon was obtained in a PCR reaction using the primers based on *fur* of *L. anguillarum* using DNA templates from *V. tubiashii* strains 19105 and 19109 (Supplemental Figure 2). The sequence of the 316 bp amplicon has 85% nucleic acid sequence homology to *fur* of *V. cholerae*, *L. anguillarum*, and *V. vulnificus* (Supplemental Figure 3).

Further characterization of the *fur* gene was conducted in this study, by using internal primers based on the partial sequence to screen for the entire *fur* gene. The conceptual partial translation product of this DNA sequence was used to identify closely related *Vibrio* proteins using NCBI Blast (http://www.ncbi.nlm.nih.gov/blast). The primers for the cloning of the complete coding region of *fur* in *V. tubiashii* 19105 were designed based on multiple-alignment of homologous coding sequences of the Fur protein in *Vibrio coralliitycus*, *Vibrio furnicii*, and *V. vulnificus* (Johnson et al., 2008). Primers were as follows vt-furxF 5′ CCGATTGGCGTTCTTGTTCCACCAGCG and vt-furxR 5′ GGCTTACGT-GATACTTGGGCTAA and the internal primers Vtfur1F 5′-TGGTCTAAAAGTTACCCTTCCGAGACTGand Vtfur1R 5′- TAACTTCAC-CACAGTCCAGACACACAAG. The primers were designed from the genomic regions flanking the *fur* gene and were used to sequence upstream, downstream of the complete *fur* gene sequences of *V. tubiashii* 19105. The Accession numbers listed in Genbank are as follows: *V. tubiashii* strains 19105, Accession # GQ465203, and 19109 Accession # GQ465202.

V. tubiashii entire *fur* coding sequence was amplified using the primers Vt-cl1F 5′- GGCTAGTCGAATTCTCAGACAATAACCAAGCT-CTTAAG and *Vt*-clR 5′- ATTGCCTGCTCGAGTTTCTTCTTGTGAGCGTC-TGGGTTATC and cloned into pET30(+) T7 promoter-based expression vector following the manufacturer's protocol (Novagen, San Diego, CA). Enzymes T4 DNA ligase, EcoR1 and Xho1 (New England Biolabs, MA) were used for cloning. The recombinant plasmid was transformed into *E. coli* DH5α competent cells (Invitrogen, CA) according to the manufacturer's protocol. Kanamycin resistant transformants were screened using internal primers and two clones were selected for sequencing using vector-based T7 promoter and terminator primers (Macrogen Inc., Gaithersburg, MD). The sequences were compared with the complete sequence obtained from *V. tubiashii* strain 19105 genomic DNA as described earlier and found to match 100%.

4. Virulence of *V. tubiashii* in suckling mice

The suckling mouse model as described by Kothary et al. (Kothary et al., 2001) was used to determine the effect of iron limitation on the ability of *V. tubiashii* strains 19105 and 19109 to cause fluid accumulation, comparatively with that for *V. cholerae* O139 strain 1837, *V. vulnificus*, and *E. coli*. Pregnant ICR mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and studies were carried out in accordance with Institutional Animal Care and Use Committee-approved protocol number 413. Prior to the experiment, food was withdrawn from suckling mice weighing 2.5 to 4.0 g (2–4 day old) for 6 h and were orally fed with 50 μl of 10^8 CFU/ml of *V. tubiashii* strains 19105 and 19109, *E. coli* HB101, *V. cholerae* O139 strain 1837 cells suspended in casamino acids

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Table 2
Fluid accumulation induced by *V. tubiashii*, *V. cholerae*, and *E. coli*, grown under iron non-chelated and iron-chelated conditions[a], and the presence of antisera prepared against OMPs obtained from *V. tubiashii* 19109.

Organism	Growth condition[b]	FA Ratio + SD	*p* value[c] among strains	*p* value[d] overall comparison
V.tubiashii ATCC19105	iron-chelated	80 ± 13.00	=0.214	<0.001
	iron non-chelated	75 ± 14.00		<0.001
V.tubiashii ATCC19109	iron-chelated[f]	90 ± 8.00	=0.002	<0.001
	iron non-chelated[f]	74 ± 12.00		<0.001
	Iron-chelated[g] antisera[f]	74 ± 3.00		<0.001
V. cholerae O139	iron-chelated	76 ± 9.00	=0.051	<0.001
	iron non-chelated	65 ± 5.00		<0.001
E. coli HB101	iron-chelated	68 ± 6.00	=0.898	<0.001
	iron non-chelated	68 ± 5.00		<0.001
CYE	Control	60 ± 6.00	N/A[e]	<0.001

[a] 50 μL of 10^8 CFU/mL cell suspension was fed to each mouse.
[b] Iron-chelated; TSA-S supplemented with 125 μM of 2′2′-dipyridyl; iron non-chelated, TSA-S.
[c] These *p* values represent the comparison between iron-chelated versus iron non-chelated growth conditions.
[d] *p* values of <0.001 showed a significant differences as compared to CYE.
[e] N/A not applicable.
[f] Represent the value that showed significant differences in FA between iron-chelated versus iron non-chelated growth conditions.
[g] Iron-chelated with antisera was fed to each mouse to induce fluid accumulation.

yeast extract broth (CYE) and CYE (a negative control). A time course study was conducted and it was determined that the optimal dose and time of incubation to be used in comparative studies were 50 μl of a 10^8 CFU/ml and 3 h (time course data not shown). The fluid accumulation ratio (FA ratio) was expressed as 1000 times the ratio of the weight of the stomach plus intestine to the remaining body weight. The FA ratios were analyzed by using paired Student's *t* test. The results show that *V. tubiashii* strains 19105 and 19109 caused significantly more fluid accumulation in the suckling mice as compared to the negative control CYE. *V. tubiashii* strain 19109 grown under iron-chelated conditions (90 ± 8) caused significantly more fluid accumulation than cells which were grown under iron non-chelated (74 ± 12) conditions (Table 2). Conversely, the fluid accumulation caused by the *V. tubiashii* strain 19105 grown under iron-chelated conditions as compared to iron non-chelated condition was not statistically significant (Table 2). These results suggest that the presence or absence of iron may influence the amount intestinal fluid accumulated in suckling mice. The results also showed that *V. tubiashii* strain 19109 caused significantly more fluid accumulation than *V. tubiashii* strain 19105, *E. coli* HB101, and *V. cholerae* O139 strain 1837 after a 3 h incubation period (Table 2). In this infection model,

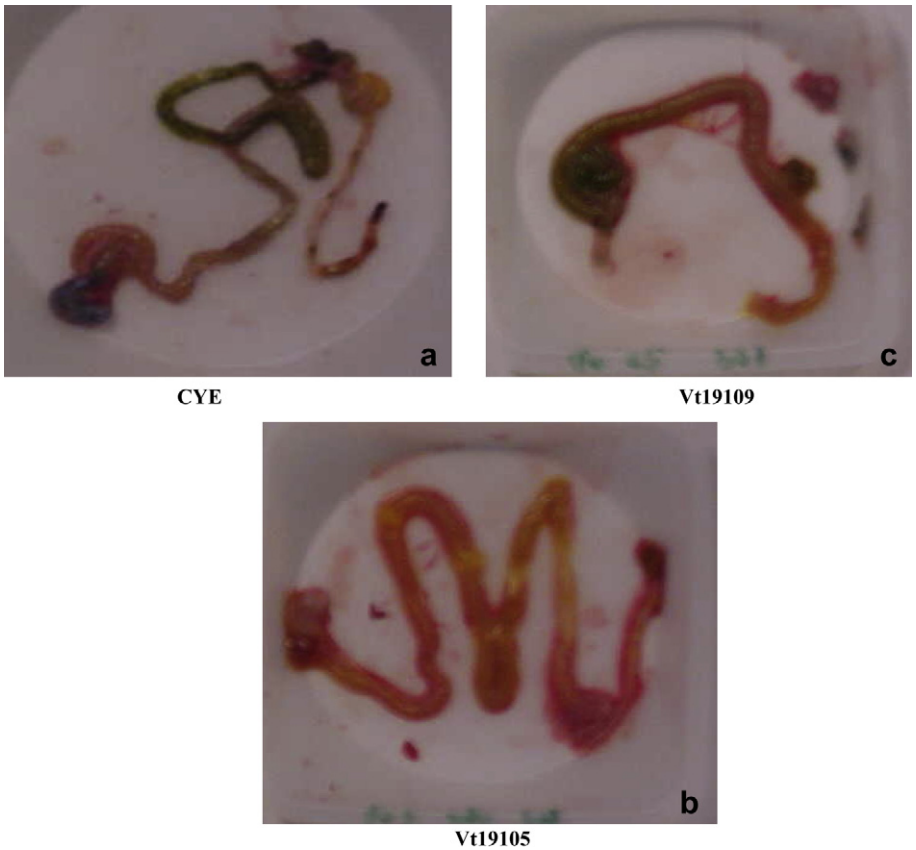

Fig. 2. Stomach and large intestine of suckling mice challenged orally with 50 μL of 10^8 CFU/ml cell suspension of *V. tubiashii* strains 19105 and 19109 cells grown on TSA-S supplemented with 125 μM of 2′2′-dipyridyl (iron-chelated). a. CYE; b. *V. tubiashii* strain 19105; c. *V. tubiashii* strain 19109.

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V. tubiashii strain 19105 appears to be less virulent than *V. tubiashii* strain 19109. Furthermore, fluid accumulation could be abated when suckling mice were challenged with *V. tubiashii* strain 19109 and antiserum (Jean-Gilles Beaubrun et al., 2008) prepared against the OMP of the same strain as the results suggest in Table 2. These findings suggest that OMPs may also influence fluid accumulation. These findings are supported by previous reports of bacteria relying on high-affinity surface receptor proteins, such as iron-loaded siderophores, to facilitate the translocation of Fe^{3+} into the periplasmic space (Henderson and Payne, 1994; Wyckoff et al., 1997).

Fig. 2 represents a typical observation of the stomach, large and small intestine of the suckling mice 3 h post challenge with *V. tubiashii* strains 19105 and 19109, compared to a mouse challenged with CYE. As can be seen, more fluid accumulation occurred in the intestines of the mice that were challenged with the *V. tubiashii* strains than in the mouse that was challenged with CYE. These results suggest that iron-chelated growth did induce the fluid accumulation in the suckling mice, which also suggest that induction, and or up regulation of an iron acquisition system in *V. tubiashii* may influence virulence in suckling mice.

In summary, the expression of unique OMPs produced only under iron-chelated conditions, the expression of siderophores, the identification of a *fur* homolog in *V. tubiashii* and the influence of iron in fluid accumulation in suckling mice suggest the presence of an competent iron acquisition system in *V. tubiashii* similar to that of *V. cholerae* and *V. vulnificus* (Biosca et al., 1996). Based on these findings, it can be concluded that under iron-chelated conditions the expression of siderophores in *V. tubiashii* is inducible and/or up regulated (in a strain-dependent fashion) and that it enables the binding of iron from its environment and that growth of the cells under iron-limited conditions influence the level of diarrheal response in the suckling mouse model caused by these organisms. An organism needs to have the capability to sequester and metabolize iron in order to cause infection (Biosca et al., 1996; Faraldo-Gómez and Sansom, 2003). Lee et al., stated that the ability to acquire iron is essential to maintain growth as well as virulence in *Vibrio* spp. (Lee et al., 2007). Hasegawa et al., (Hasegawa et al., 2008) has reported that the metalloprotease described by Kothary et al. (Delston et al., 2003) is a critical factor in the virulence of *V. tubiashii* in molluscan hosts. Since not much is known about *V. tubiashii*, and with the increase of unknown *Vibrio* spp. causing diseases in the U.S., this investigation has yielded new information on the OMP profiles of *V. tubiashii* and possibly virulent components possessed by *V. tubiashii* and other human pathogenic marine *Vibrio* species.

Furthermore, we have provided evidence for the variable expression of OMPs and a hydroxymate-like iron acquisition system in *V. tubiashii* that are regulated by the presence of iron. Although *V. tubiashii* has emerged as a pathogen capable of infecting fish and mollusks, understanding how this organism is able to overcome species barriers and adapt to new hosts is crucial in producing disease-free seafoods. These finding may be essential to increase food safety.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fm.2011.04.003.

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