Characterization of *Vibrio parahaemolyticus* genes encoding the systems for utilization of enterobactin as a xenosiderophore

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We determined the ability of *Vibrio parahaemolyticus* to utilize enterobactin (Ent) as a xenosiderophore. Homology searches of the *V. parahaemolyticus* genomic sequence revealed the presence of genes that are homologous to the *V. cholerae* ferric Ent utilization genes, which consist of the iron-repressible outer-membrane protein genes *irgA* and *vctA*, and the ATP-binding cassette transport system operon *vctPDGC*. Moreover, the *irgB* and *vctR* genes, which encode transcriptional regulators, were also found immediately upstream of *irgA* and *vctA*, respectively. Growth assays of *V. parahaemolyticus* indicated that both *irgA* and *vctA* mutants grew well in the presence of Ent under iron-limiting conditions, whereas both the *irgA*/*vctA* double mutant and the *vctPDGC* mutant barely grew under the same conditions. In addition, growth assays of three isogenic *tonB* mutants demonstrated that the TonB2 system, and to a lesser extent the TonB1 system, can provide energy for both IrgA and VctA to transport ferric Ent. SDS-PAGE analysis showed that expression of both IrgA and VctA was enhanced by the presence of Ent. Complementation of the *irgB* and *vctR* mutants with their respective genes resulted in the increased expression of IrgA and VctA, respectively. Finally, reverse transcriptase-quantitative PCR revealed that transcription of the Ent utilization system genes is iron-regulated, and that transcription of *irgA* and *vctA* under iron-limiting conditions is further activated by proteins encoded by *irgB* and *vctR*, respectively, together with Ent.

INTRODUCTION

Iron is an essential element for virtually all forms of cellular life, including most bacteria, because the iron ion is a cofactor for several key enzymes required for many metabolic processes, such as redox reactions, nucleic acid synthesis and electron transfer. However, the bioavailability of iron is extremely low, since it exists in insoluble mineral complexes under aerobic, aqueous and neutral pH conditions, or is bound to mammalian high-affinity iron-binding proteins such as transferrin, lactoferrin and ferritin. Therefore, many bacteria have developed high-affinity iron transport systems to acquire iron (Andrews et al., 2003; Miethke & Marahiel, 2007). One of these strategies is based on low-molecular-mass compounds, siderophores, that specifically chelate ferric iron. Indeed, most bacteria can biosynthesize and secrete cognate siderophores in response to iron limitation. In Gram-negative bacteria, iron-bound siderophores, called ferric siderophores, are transported across the outer and inner membranes by specific iron-repressible outer-membrane proteins (IROMPs) and ATP-binding cassette (ABC) transport systems, respectively (Chu et al., 2010). The transport of ferric siderophores via their specific IROMPs is dependent on the energy transferred...
from the inner-membrane proton motive force by the TonB system, which is generally composed of TonB and its accessory proteins ExbB and ExbD (Moec & Coulton, 1998; Noinaj et al., 2010). The expression of many of the genes engaged in iron acquisition is regulated by the ferric uptake regulator (Fur), which is a global negative regulator ubiquitous in Gram-negative bacteria (Bagg & Neilands, 1987; Escolar et al., 1999). When the intracellular iron concentration is increased, the Fur–ferrous iron complex binds to a consensus sequence, termed the Fur box, which overlaps the −10 and −35 elements in Fur target genes, thereby leading to repressed transcription initiation of the respective genes. In contrast to the Fur−Fe²⁺ complex, other transcriptional regulators such as the AraC-type family members (Anderson & Armstrong, 2004; Fetherston et al., 1996; Heinrichs & Poole, 1993; Hollander et al., 2011; Tanabe et al., 2005) and LysR-type family members (Balado et al., 2008; Visca et al., 2002) have been shown to activate transcription initiation of siderophore synthesis genes and siderophore utilization system genes.

The halophilic Gram-negative bacterium Vibrio parahaemolyticus is a causative agent of watery diarrhoea that possesses multiple iron acquisition systems. We previously demonstrated that V. parahaemolyticus produces the cognate siderophore vibrioferrin belonging to the hydroxycarboxylate-type siderophore family (Yamamoto et al., 1994), and transports ferric vibrioferrin into cells via the outer-membrane receptors PvuA1 and PvuA2, and the inner-membrane ABC transport system PvuBCDE (Tanabe et al., 2003, 2011). Three sets of TonB systems, termed TonB1, TonB2 and TonB3, are present in this bacterium (Kuehl & Crosa, 2010), and the energy required for PvuA1 and PvuA2 to transport ferric vibrioferrin is provided by the TonB2 system, and both the TonB1 and TonB2 systems, respectively (Tanabe et al., 2011). In addition, V. parahaemolyticus can utilize hydroxamate-type xenosiderophores such as desferri-ferriochrome and aerobactin (Funashashi et al., 2003, 2009). Previous studies have demonstrated that several Vibrio species, including Vibrio cholerae and Vibrio anguillarum, can use catecholate-type siderophores, such as their cognate siderophores (Actis et al., 1986; Balado et al., 2009; Griffiths et al., 1984) and a xenosiderophore enterobactin (Ent) mainly produced by members of the family Enterobacteriaceae (Carson et al., 1999; Mey et al., 2002; Naka & Crosa, 2012; Stork et al., 2004; Wyckoff et al., 2007). However, to our knowledge, the ability of V. parahaemolyticus to utilize catecholate-type xenosiderophores has not yet been reported.

In this study, we show that V. parahaemolyticus can utilize Ent as an iron source when grown in iron-limiting medium, through two newly identified receptors, namely, IrgA and VctA, and an ABC transport system encoded by vctPDGC. In addition, we show that both IrgA and VctA from V. parahaemolyticus can gain energy from the TonB1 and TonB2 systems. Finally, we demonstrate that under iron-limiting conditions in the presence of Ent, the irgB and vctR genes encoding the transcriptional regulators, which are homologous to V. cholerae IrgB (Goldberg et al., 1991) and VCA0231 (Mey et al., 2002), are responsible for the activation of irgA and vctA expression.

METHODS

Bacterial strains, plasmids, growth conditions and primers. The bacterial strains and plasmids used in this study are listed in Tables 1 and S1 (available with the online version of this paper), respectively. The V. parahaemolyticus strains were routinely cultivated in Luria–Bertani (LB) medium (pH 7.0) containing 3 % NaCl (+ Fe medium). The Escherichia coli β2155 strain, which is a dianimopinic acid auxotroph, was grown in LB medium containing 0.5 % NaCl and 0.5 mM dianimopinic acid. To impose iron limitation on the V. parahaemolyticus strains, they were cultured in −Fe medium that was composed of LB medium with 25 μM ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDA; Sigma-Aldrich). When required, the antibiotics chloramphenicol (10 μg ml⁻¹) and tetracycline (15 μg ml⁻¹) were added to the media. The PCR primers used in this study are listed in Table S2.

DNA techniques. All plasmids were extracted using a High Pure Plasmid Isolation kit (Roche). Standard DNA manipulations were performed according to the procedures of Sambrook et al. (1989). Oligonucleotide primers employed in this study (Table S2) were designed on the basis of the V. parahaemolyticus RIMD2210633 genome sequence from the Genome Information Research Center (GIRC) at Osaka University (http://genome.gen-info.osaka-u.ac.jp/bacteria/vparahaemo/) (Makino et al., 2003). A homology search was performed using the BLAST program of the GIRC or the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/). Conserved domain searches were performed using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Construction of V. parahaemolyticus deletion mutants. Deletion mutants were created by allelic exchange using the R6K-ori suicide vector pXAC623, according to the procedure described by Kuroda et al. (2005). DNA fragments with deletions in the V. parahaemolyticus irgA, vctA, irgB, vctR and vctPDGC genes were prepared by PCR-driven overlap extension (Heckman & Pease, 2007) as previously described (Tanabe et al., 2011), and then ligated into appropriately digested pXAC623. Each resulting plasmid was transformed into E. coli β2155 (Demarre et al., 2005), and mobilized into an appropriate V. parahaemolyticus strain by filter mating. Chloramphenicol-resistant merodiploids were spread on VDS-broth agar plates (1 % polypeptone, 0.5 % yeast extract, 30 mM NaCl, 55 mM KCl, 10 % sucrose, 2.5 % agar) at 25 °C for 30 h. Sucrose-resistant and chloramphenicol-sensitive colonies were selected, and the deleted DNA regions were confirmed by PCR using their chromosomal DNAs (Fig. S1). To complement the deletion mutants of irgA, vctA, irgB, vctR and vctPDGC, PCR amplicons containing the respective genes were ligated into appropriately digested pRK415 (Keen et al., 1988), and the complementing plasmids thus obtained were transformed into the respective V. parahaemolyticus mutant strains.

Growth assay. Stationary phase V. parahaemolyticus cells were diluted with the −Fe medium to OD₅₀₀ 0.005. When required, the −Fe medium was supplemented with Ent at a final concentration of 5 μM (−Fe + Ent medium). The cultures were shaken at 70 r.p.m. at 37 °C, and the OD₅₀₀ was measured every 3 h with a biophotometer (TVS062CA, Advantec). The siderophore Ent was purchased from EMC Microcollections GmbH and Sigma-Aldrich.
Preparation of outer-membrane protein (OMP)-rich fractions.
Stationary phase V. parahaemolyticus cells were inoculated at a final OD₆₀₀ of 0.005 into the +Fe and −Fe + Ent media, or OD₆₀₀ 0.01 into the −Fe medium, and the cultures were shaken at 37 °C for 12 h. The OMP-rich extracts were prepared and analysed by SDS-PAGE, as previously described (Yamamoto et al., 1995). The IROMPs were electroblotted onto a wet PVDF membrane, and their N-terminal amino acid sequences were determined using the Edman degradation method with a Procise 491 HT protein sequencer (Applied Biosystems).

RNA analysis. V. parahaemolyticus cells were inoculated as described for the OMP-rich fractions, and the cultures were then shaken at 37 °C until they reached OD₆₀₀ 0.3–0.5. The total RNA was isolated from each cell sample by using an RNeasy Protect Bacteria Mini kit (Qiagen). Primer extension of irgA and vctA was performed with the primers irgA-PE and vctA-PE, respectively, which had been 5'-labelled with Texas red. The labelled primers were annealed with 5 μg total RNA and extended with avian myeloblastosis virus reverse transcriptase XL (Takara) for 1 h at 50 °C, and each extension product was separated, in addition to the DNA sequence ladder of the control region synthesized with the same primer, on a 6% polyacrylamide gel by using an SQ5500 DNA sequencer (Hitachi). For reverse transcriptase-quantitative PCR (RT-qPCR), the total RNA was treated with RNase-free DNase I (Ambion), and 0.5 μg DNase I-treated total RNA was then used to generate the cDNA product by using ReverTra Ace reverse transcriptase (Toyobo) and a random hexamer primer (Takara). The PCRs were performed with Thunderbird SYBR qPCR Mix (Toyobo) in a Chromo4 Real-Time PCR detection system (Bio-Rad). The values were quantified using the comparative threshold cycle method, and the samples were normalized to 16S rRNA.

**RESULTS AND DISCUSSION**

**Utilization of Ent by V. parahaemolyticus**
To examine whether V. parahaemolyticus is capable of utilizing Ent, growth assays were performed in the −Fe media in the absence and presence of Ent. The VPD5 strain, which is defective in the vibrioferrin biosynthetic gene pvsB, was used to eliminate background growth resulting from vibrioferrin-mediated iron uptake in the −Fe medium. As shown in Fig. 1, the VPD5 strain was unable to grow in the −Fe medium; however, growth of VPD5 was restored to a level similar to that observed in the +Fe medium by the addition of Ent (5 μM). This demonstrates that V. parahaemolyticus is capable of utilizing Ent as a source of iron for growth.
Identification of genes involved in the utilization of ferric Ent in *V. parahaemolyticus*

We performed a BLAST search of the *V. parahaemolyticus* genome with the amino acid sequences of *V. cholerae* ferric Ent receptors IrgA and VctA (Mey et al., 2002). We identified potential IrgA (VP2602; 652 aa) and VctA (VPA0664; 668 aa) orthologues (Fig. 2a) that are 67 and 65% identical to IrgA and VctA in *V. cholerae*, respectively. Both IrgA and VctA in *V. parahaemolyticus* contain putative TonB box sequences (IrgA, 33ETVVVTA39; VctA, 36EEVGVVG42) close to their respective N termini (Fig. 2b) (Peacock et al., 2005) and C-terminal Phe residues characteristic of TonB-dependent siderophore receptors (Struyve et al., 1991). The amino acid composition of these TonB boxes supports the notion that the TonB boxes of many TonB-dependent receptors contain primarily hydrophobic and acidic residues (Kuehl & Crosa, 2010). In addition, Fig. 2(b) demonstrates that the promoters of *V. parahaemolyticus* irgA and vctA contain potential Fur boxes sharing 17 of 19 and 14 of 19 base matches, respectively, with the *E. coli* consensus Fur box (Braun & Hantke, 1991).

To study the function of *V. parahaemolyticus* IrgA and VctA proteins, isogenic deletion mutants, namely VPD51, VPD52 and VPD54, were generated in the VPD5 background and analysed using growth assays (Fig. 3). The VPD51 and VPD52 strains, which possess the *irgA* and *vctA* genes, respectively, grew in the −Fe + Ent medium to an extent similar to that of the parental VPD5 strain; in comparison, VPD54, which is defective in both the *irgA* and *vctA* genes, showed no Ent-mediated growth. However, complementation of VPD54 with either *irgA* or *vctA* harboured in the broad-host-range plasmid pRK415 allowed the strain to regain growth to a level comparable with that of VPD5 in the −Fe + Ent medium. These results indicate that the *V. parahaemolyticus* *irgA* and *vctA* genes encode TonB-dependent IROMPs involved in the uptake of ferric Ent.

A search of the region upstream of the *V. parahaemolyticus* *vctA* gene revealed the existence of an operon in the same gene order as that of *V. cholerae* that encodes the putative ABC transporter components VPA0657–0660 (Fig. 2a). In addition, the protein products share 56, 74, 70 and 69% identity with *V. cholerae* VctPDGC, respectively, responsible for ferric catecholate siderophore transport (Mey et al., 2002). To determine whether *V. parahaemolyticus* VctPDGC also functions as the ABC transporter system for ferric Ent, a growth assay was performed with the vctPDGC deletion mutant VPD95 constructed from VPD5. VPD95 showed no growth in the −Fe + Ent medium, whereas the complemented strain VPD95/pRK415-vctPDGC regained the ability to grow in this medium (Fig. 3). Therefore we concluded that the ABC transport system encoded by vctPDGC in *V. parahaemolyticus* also functions in ferric Ent transport.

**Involvement of *irgB* and *vctR*, together with *Ent*, in *Ent* utilization**

We noted the two *V. parahaemolyticus* genes VP2603 and VPA0663, which are divergently located immediately upstream of *irgA* and *vctA*, respectively. *V. parahaemolyticus* VP2603 shares 53% identity with a LysR-type transcriptional regulator, IrgB (VC0474) (Goldberg et al., 1991), and VPA0663 has 56% identity with an AraC-type transcriptional regulator, VCA0231, of unknown function (Mey et al., 2002), in *V. cholerae*. Hereafter, VPA0663 was termed *vctR*. In *V. cholerae*, IrgB is well documented as a transcriptional regulator of *irgA* (Goldberg et al., 1991; Watnick et al., 1998). However, it is unknown whether in *V. cholerae*, *Ent* is also involved in the transcriptional activation of *irgA* by IrgB. The conserved domain sequences for DNA binding and co-inducer binding reported for LysR-type and AraC-type family regulator members (Gallegos et al., 1997; Maddocks & Oyston, 2008) were also found in the *V. parahaemolyticus* proteins IrgB and VctR, respectively (data not shown). Furthermore, the predicted regulatory binding site consisting of a palindromic T-N_{11}-A sequence characteristic of LysR-type regulators (Maddocks & Oyston, 2008) was found upstream of the *V. parahaemolyticus* *irgA* gene (Fig. 2b).

The organization of the *irgBA* and *vctPDGC-vctR-vctA* Ent utilization system genes in *V. parahaemolyticus* is similar to that of *V. cholerae*. However, the *vctC-vctR* intergenic regions are not the same length in *V. parahaemolyticus* (641 bp) and *V. cholerae* (45 bp). In *V. parahaemolyticus*, two other annotated ORFs, namely, VPA0661 and VPA0662, intervene in this region (Fig. 2a). VPA0662 shares homology with MerR-type transcriptional regulators (Brown et al., 2003) that function in response to the presence of heavy metals, whereas VPA0661 shows no convincing homology to other bacterial proteins. The functions of VPA0661 and VPA0662 are currently unknown.
To evaluate the necessity of IrgB and VctR for Ent utilization in V. parahaemolyticus, we generated the irgB mutant VPD93 and vctR mutant VPD94 from VPD51 and VPD52, respectively. The Ent-mediated growth promotion that was observed in VPD51 and VPD52 was almost completely eliminated in VPD93 and VPD94, independently of the presence of irgA and vctA (Table 2). In contrast, the complementing strains VPD93/pRK415-irgB and VPD94/pRK415-vctR grew in the Fe²⁺ Ent medium to almost the same levels as VPD51 and VPD52 (Table 2). These results suggest that both irgB and vctR encode the transcriptional factors that promote expression of the corresponding IROMPs IrgA and VctA for ferric Ent.

**Necessity of irgB and vctR together with Ent for IrgA and VctA production**

To identify the IrgA and VctA proteins, Sarkosyl-insoluble OMPs prepared from VPD5 cells grown in the Fe²⁺ medium were separated by SDS-PAGE, and their N-terminal amino acid sequences were determined. Two bands, with N-terminal amino acid sequences of NDSVSKMETV and KMETVVVTAS, were derived from IrgA (Fig. 4, lane 2), whereas the band corresponding to VctA was not observed even when VPD8, which is defective in both pvuA1 and pvuA2 (ferric vibrioferrin receptor genes) (Tanabe et al., 2011), was used to avoid overlapping with other possible IROMPs (Fig. 4, lane 3). Interestingly, when VPD8 was cultured in the Fe²⁺ + Ent medium, a fairly strong band with an N-terminal amino acid sequence of QESDSHFEEV appeared (Fig. 4, lane 4), and was determined to be VctA based on the amino acid sequence deduced from the predicted gene. Unexpectedly, the expression of the two protein bands due to IrgA was only slightly promoted in the Fe²⁺ + Ent medium when these bands were compared between lanes 3 and 4 in Fig. 4. However, the VctA band was clearly detected in the presence of Ent (Fig. 4, lane 4) but not in the absence of Ent (Fig. 4, lane 3). These data suggest that Ent produces VctA more efficiently than IrgA.
We also examined the effects of irgB and vctR on IrgA and VctA production. SDS-PAGE analysis of IROMPs prepared from the irgB mutant VPD99 and vctR mutant VPD98 grown in the −Fe + Ent medium exhibited slightly decreased production of IrgA and markedly diminished production of VctA, respectively (Fig. 4, lanes 5 and 7), compared with their parental VPD8 (Fig. 4, lane 4). However, the complementing strains VPD99/pRK415-irgB and VPD98/pRK415-vctR possessed the ability to produce IrgA and VctA, respectively (Fig. 4, lanes 6 and 8). These data suggest that the products of irgB and vctR serve as transcriptional activators with the help of Ent for their corresponding target genes irgA and vctA. In addition, the expression levels of IrgA and VctA were unaffected by the presence of vctR and irgB (Fig. 4, lanes 5–8), indicating that the Ent-mediated actions of IrgB and VctR are specific to irgA and vctA.

Table 2. Effect of irgB and vctR on Ent utilization

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<tr>
<th>Strain*</th>
<th>Description†</th>
<th>Ent-mediated growth promotion‡</th>
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<tr>
<td></td>
<td>irgA</td>
<td>vctA</td>
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<tr>
<td>VPD51 (parental strain)</td>
<td>+</td>
<td>−</td>
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<tr>
<td>VPD93</td>
<td>+</td>
<td>−</td>
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<tr>
<td>VPD93/pRK415-irgB</td>
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<tr>
<td>VPD52</td>
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<tr>
<td>VPD94</td>
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<tr>
<td>VPD94/pRK415-vctR</td>
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*Deletion mutants were constructed from VPD5, which is unable to produce vibrioferrin.
†The corresponding genes are present (+) and deleted (−).
‡Cells were inoculated into the −Fe + Ent medium at a final OD₆₀₀ of 0.005, and the OD₆₀₀ was measured after incubation for 18 h. Growth curves of the parental strains VPD51 and VPD52 are shown in Fig. 3. +, Growth approximately equal to that of the parental strain; −, Ent-mediated growth promotion mostly disappeared, similar to VPD54 (Fig. 3).

Fig. 3. Involvement of the V. parahaemolyticus irgA, vctA and vctPDCG genes in Ent utilization. The growth assay was performed as described in Fig. 1. All strains used in this experiment lack the pvsB gene, and therefore are unable to produce the cognate siderophore vibrioferrin. Data represent the mean ± SD from three separate experiments.

TonB specificity of the two ferric Ent receptors IrgA and VctA

V. parahaemolyticus possesses both the genes encoding the TonB1 and TonB2 systems in the small chromosome and the TonB3 system in the large chromosome. This is distinct from the gene distribution in V. cholerae, in which the genes encoding the TonB1 system are present in the small chromosome and those encoding the TonB2 system are localized to the large chromosome, and no TonB3 system is present (Kuehl & Crosa, 2010). To clarify which TonB proteins are involved in the uptake of ferric Ent through IrgA and VctA, we constructed a set of isogenic deletion mutants of tonB1, tonB2 and tonB3 from VPD51 and VPD52, which originate from VPD5. Their growth was then measured in the presence of Ent under iron-limiting conditions. The growth assays of the tonB mutants that
possess irgA, but not vctA (Fig. 5a), and the tonB mutants that possess vctA, but not irgA (Fig. 5b), were analysed. The VPD79 and VPD83 strains, which possess irgA and tonB2, but not tonB1, as well as their parental strain, VPD51, grew in the −Fe + Ent medium; however, although the VPD80 and VPD84 strains, which contain the irgA and tonB1 genes, but not tonB2, also grew in the same medium, their growth curves showed a delayed initial rise compared with that of VPD51. No growth was observed in the VPD82 and VPD85 strains, which possess irgA, but not tonB1 and tonB2. Similarly, the growth curves of the tonB mutants generated from VPD52 resembled those of the tonB mutants generated from VPD51. Taken together, these findings indicate that, although both TonB1 and TonB2 can supply the energy necessary for IrgA and VctA to transport ferric Ent, TonB2 is functionally more active for both receptors than TonB1. This is distinct from the V. cholerae IrgA and VctA ferric Ent receptors, which are energized only by the TonB2 system (Seliger et al., 2001). The results also indicate that TonB3 is not associated with the transport of ferric Ent by IrgA and VctA.

Evaluation of the transcriptional levels of the Ent utilization system genes by RT-qPCR

RT-qPCR analysis was performed to evaluate the irgA and vctA transcription levels. The transcription levels of irgA and vctA in VPD5 were higher in the −Fe medium than in the +Fe medium, and a further dramatic induction of transcription of both genes was caused by the addition of Ent to the −Fe medium (Fig. 6a). These results were consistent with those of the primer extension analysis carried out for the irgA and vctA transcripts expressed in the presence of Ent (Fig. 5c). In contrast to the parental VPD5, the transcription levels of irgA and vctA were drastically diminished in the irgB mutant VPD100 and vctR mutant VPD101, respectively, independently of the presence of Ent in the −Fe medium (Fig. 6a). These data indicate that irgA and vctA transcription in the irgB and vctR mutants was unresponsive to the Ent inducer. The presence of a potential Fur box in the promoter region of the vctR gene indicated that the operon is, in fact, regulated by iron. The results showed that the transcription of vctP and vctC was completely derepressed in the −Fe medium, which suggests that the operon is, in fact, regulated by iron. The effect of Ent on the transcription of these genes was not significant, regardless of the presence of vctR (Fig. 5c). In addition, RT-qPCR analysis indicated that the irgB and vctR genes are also regulated by iron (Fig. 6c), implying that the Fur boxes predicted for irgA and vctA (Fig. 2) also function for irgB and vctR, respectively.

To effectively acquire iron in iron-restricted environments, some bacteria have evolved positive regulatory mechanisms, in which a siderophore itself serves as an extracellular stimulus or inducer, termed a ferrimone (Brickman & Armstrong, 2009), to evoke optimum expression of the corresponding outer membrane receptor gene (Crosa, 1997; Vasil & Ochsner, 1999; Visca et al., 2002). The catecholate-type siderophore Ent is utilized as the iron source by several Gram-negative bacteria, including V. cholerae (Mey et al., 2002), V. anguillarum (Balado et al., 2009; Naka & Crosa, 2012), Pseudomonas aeruginosa (Dean & Poole, 1993; Gysels et al., 2005), Neisseria gonorrhoeae (Carson et al., 1999), Bordetella species (Beall & Sanden, 1995) and Campylobacter species (Palyada et al., 2004; Xu et al., 2010). In this study, we also showed that V. parahaemolyticus
parahaemolyticus, which possesses the ferric Ent receptors IrgA and VctA, which are iron-repressible and Ent-inducible, can utilize Ent. The induction by Ent of its receptor genes has been reported in other bacteria; for example, pfeA in P. aeruginosa (Dean et al., 1996) and bfeA in Bordetella species (Anderson & Armstrong, 2004). In these regulatory systems, extracellular Ent may act as a signalling molecule, possibly in its iron-loaded state. Therefore, this type of transcriptional regulation is practical in terms of cellular physiology, since responding to or sensing extracellular Ent benefits bacterial cells by permitting economical regulation of the levels of expression of the corresponding receptor protein. However, it remains unknown whether V. parahaemolyticus can utilize Ent in its surroundings. Alternatively, as proposed by Brickman & Armstrong (2009) for the Bordetella BfeA ferric Ent receptor, the authentic ligand for IrgA and VctA may also be another molecule, which is structurally similar to Ent, produced by other organisms.

We showed that the V. parahaemolyticus TonB1 and TonB2 systems provide energy for IrgA and VctA for the transport of ferric Ent. This distinguishes V. parahaemolyticus from V. cholerae and V. anguillarum, in which only the TonB2 system can energize ferric Ent transport through the IrgA and VctA receptors (Mey & Payne, 2003; Stork et al., 2004). Mey & Payne (2003) proposed that a large hydrophobic amino acid residue at the TonB box 2 position allows the ferric siderophore receptors to receive energy from the TonB1 system, albeit somewhat less effectively than from TonB2. Interestingly, the TonB box 2 residues in V. cholerae IrgA and VctA, which depend on the TonB2 system, are the acidic residues Asp and Glu, respectively. Comparatively, V. parahaemolyticus IrgA and VctA, which depend on both the TonB1 and TonB2 systems, possess large hydrophobic Met and Phe residues, respectively, at this position (Fig. 2b). Similarly, in V. parahaemolyticus, the Met residue is found at this position of the cognate ferric vibrioferrin receptor PvuA2, which depends on both

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**Fig. 5.** TonB specificity of IrgA and VctA in V. parahaemolyticus. Growth assays of IrgA (a) and VctA (b) were performed as described in Fig. 1, except that the incubation time was increased to 24 h. A series of deletion mutants were constructed from VPDS5, which is a mutant unable to produce vibrioferrin. The Ent-mediated growth in the strains tested was categorized: + +, growth highly similar to the parental strains VPDS1 and VPDS2; +, growth comparable with the parental strains, but requiring a prolonged lag time; –, no Ent-mediated growth. Data plotted are the mean ± SD from three separate experiments. The growth curves of VPDS7, VPDS8, VPDS9, VPDS10, VPDS86, VPDS88 and VPDS92 are omitted to avoid complicating the figure, and are described as 'growth curve not shown'.

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<tr>
<th>Strains</th>
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<td>VPDS1</td>
<td>+ – + + +</td>
<td>+ + (growth curve not shown)</td>
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<td>VPDS7</td>
<td>+ – + + –</td>
<td>+ – (growth curve not shown)</td>
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<tr>
<td>VPDS8</td>
<td>+ – + – +</td>
<td>– (growth curve not shown)</td>
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<tr>
<td>VPDS9</td>
<td>+ – + – +</td>
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Ent. The additional iron assimilation system mediated by Ent in *V. parahaemolyticus* may reflect the importance of iron competition among resident microbes in its natural habitat. This iron assimilation system may aid in the survival and proliferation of the bacterium, and therefore increase its chances of gaining access to mammalian hosts. It will be of interest to investigate the molecular mechanisms by which Ent interacts with IrgB and VctR to activate the *irgA* and *vctA* receptor genes, respectively.

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**REFERENCES**


In conclusion, we have demonstrated that *V. parahaemolyticus* can utilize Ent, and that the ferric Ent receptors IrgA and VctA are induced by iron-limiting conditions through the action of IrgB and VctR, respectively, with the help of the TonB1 and TonB2 systems; however, another ferric vibrio ferrin receptor, PvuA1, that depends only on the TonB2 system possesses the hydrophilic Thr residue at this position (Tanabe et al., 2011). These data also imply that the hydrophilic or acidic amino acid residues at the TonB box –1 position may determine the TonB2 specificity of ferric siderophore receptors, at least in *Vibrio* species.

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**Fig. 6.** RT-qPCR validation of the transcriptional activation of *V. parahaemolyticus* *irgA* and *vctA* by the presence of Ent under iron-limiting conditions. The transcriptional levels of *irgA* and *vctA* (a), an ABC transporter system operon (b), and *irgB* and *vctR* (c) were evaluated by RT-qPCR. Total RNAs were extracted from the VPD5, VPD100 and VPD101 strains grown in +Fe, –Fe and –Fe + Ent media. Error bars, SD; asterisks indicate a P value of <0.05.


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