An iron-regulated gene required for utilization of aerobactin as an exogenous siderophore in *Vibrio parahaemolyticus*

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A previous investigation using the Fur titration assay system showed that *Vibrio parahaemolyticus* possesses a gene encoding a protein homologous to IutA, the outer-membrane receptor for ferric aerobactin in *Escherichia coli*. In this study, a 5.6 kb DNA region from the *V. parahaemolyticus* WP1 genome was cloned and two entire genes, *iutA* and *alcD* homologues, were identified which are absent from *Vibrio cholerae* genomic sequences. The *V. parahaemolyticus* IutA and AlcD proteins share 43% identity with the *E. coli* IutA protein and 24% identity with the *Bordetella bronchiseptica* AlcD protein of unknown function, respectively. Primer extension analysis revealed that the *iutA* gene is transcribed in response to low-iron availability from a putative promoter overlapped with a sequence resembling a consensus *E. coli* Fur-binding sequence. In agreement with the above finding, *V. parahaemolyticus* effectively utilized exogenously supplied aerobactin for growth under iron-limiting conditions. Moreover, insertional inactivation of *iutA* impaired growth in the presence of aerobactin and incapacitated the outer-membrane fraction from iron-deficient cells for binding 55Fe-labelled aerobactin. These results indicate that the *V. parahaemolyticus* *iutA* homologue encodes an outer-membrane protein which functions as the receptor for ferric aerobactin. Southern blot analysis revealed that the *iutA* homologues are widely distributed in clinical and environmental isolates of *V. parahaemolyticus*. However, additional genes required for ferric aerobactin transport across the inner membrane remain to be clarified.

INTRODUCTION

Iron is an essential cofactor of many enzymes with redox activity and micromolar levels of bioavailable iron are required by most bacteria for optimal growth (Guerinot, 1994). To capture iron from extremely insoluble ferric oxyhydroxide polymers in the natural environment or from iron–protein complexes such as transferrin and lactoferrin in the host, many bacteria have evolved to synthesize and secrete low-molecular-mass, highly ferric ion-specific chelators termed siderophores, together with cell-surface receptors specific for the iron–siderophore complexes (Drechsel & Winkelman, 1997). In Gram-negative bacteria, the iron–siderophore complexes are translocated into the cytoplasm by outer-membrane receptors coupled with ATP-binding cassette type transporters (Braun & Hantke, 1991). In addition to cognate siderophore-mediated iron uptake, some bacteria are also capable of taking up iron by means of exogenous (heterologous) siderophores secreted by other bacteria or fungi (Griffiths, 1999). Although the *in vivo* significance of exogenous siderophore utilization has not yet been characterized, this tactic presumably facilitates iron-scavenging under a wide range of environmental and physiological conditions and provides an advantage of competing with other micro-organisms for iron (Poole et al., 1990). In these cases, expression of most proteins involved in iron acquisition is regulated at the transcriptional level by a global iron-binding repressor protein called Fur (for ferric uptake regulation) (Hantke, 1981; Braun & Hantke, 1991).

*Vibrio parahaemolyticus* is an estuarine pathogen known to be a common cause of seafood-borne acute gastroenteritis worldwide. Production of the siderophore vibrioferrin and utilization of haem and haemoglobin as sole sources
of iron have been demonstrated for *V. parahaemolyticus* grown under iron-deficient conditions (Yamamoto et al., 1994b, 1995). The Fur protein of this species has been shown to mediate iron regulation both in production of vibrioferrin and in expression of two iron-repressible outer-membrane proteins (OMPs) of 78 and 83 kDa (Funahashi et al., 2000). Using the Fur titration assay (FURTA) system (Stojiljkovic et al., 1994), we have previously isolated many Fur target gene fragments from *V. parahaemolyticus*, one of which led us to identify the *pvuA* gene encoding the 78 kDa ferric vibrioferrin receptor protein (Funahashi et al., 2002). At the same time, we also obtained a genomic fragment containing an incomplete ORF, whose predicted protein sequence shares significant homology with the IutA protein that serves as the outer-membrane receptor for ferric aerobactin in *Escherichia coli* (Krone et al., 1987). In addition, the N-terminal amino acid sequence (AEQAQQLASQ) determined for the 83 kDa iron-repressible OMP band (containing three kinds of proteins) of *V. parahaemolyticus* WP1 was correlated with the amino acid sequence deduced from the partial ORF. These results suggested that the protein product of this *iutA* homologue seemed a likely candidate for a ferric aerobactin receptor.

In this study, to gain more insight into the iron-uptake systems in *V. parahaemolyticus*, we cloned the entire *iutA* gene and characterized it. Primer extension analysis revealed that the *iutA* gene is transcribed from a Fur-repressed promoter upstream of *iutA*. The function of the *iutA* protein product in *V. parahaemolyticus* as the receptor for ferric aerobactin was confirmed by construction of an *iutA*-disrupted mutant followed by phenotypic comparison between the mutant and the parental strain. Another gene encoding a protein homologous to AlcD, predicted as one of the alcaligin biosynthetic enzymes in *Bordetella* species (Pradel et al., 1998), was identified just upstream of *iutA*, although its function is currently unknown.

### METHODS

**Bacterial strains, plasmids and growth conditions.** The strains and plasmids used are described in Table 1. LB broth and agar (2.5 %) plates were used for growth of *E. coli* strains, and plates containing 3 % NaCl were used for growth of *V. parahaemolyticus* strains. When required, appropriate antibiotics were added to the media as follows: ampicillin at 50 μg ml⁻¹ and chloramphenicol at 10 μg ml⁻¹. All strains were routinely grown at 37 °C. Iron-free water was obtained by passage of distilled water through a Milli-Q

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant feature*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>V. parahaemolyticus</strong></td>
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<td>WP1</td>
<td>Clinical isolate</td>
<td>Yamamoto et al. (1994a)</td>
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<tr>
<td>MY-1</td>
<td>Spontaneous vibrioferrin-deficient mutant of a clinical isolate AQ3354</td>
<td>Yamamoto et al. (1994a)</td>
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<td>VPTF3</td>
<td>MY-1, orf2 disrupted; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>VPTF4</td>
<td>MY-1, iutA disrupted; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>E. coli</strong></td>
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<td>H1717</td>
<td>araD139 rpsL150 Δ(argF-lac) relA1 U169 fbb5301 deoC1 ptsF25 rbsR aroB fhuf::ΔlacMu; host strain for FURTA</td>
<td>Stojiljkovic et al. (1994)</td>
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<td>SY327&lt;sup&gt;+&lt;/sup&gt;pi&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Δ(lac pro) argE(Am) rif naldA recA56 Δ(pirR6K); host for π-requiring plasmids</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<td>SM10&lt;sup&gt;+&lt;/sup&gt;pi&lt;sup&gt;r&lt;/sup&gt;</td>
<td>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu ΔpirR6K; Km&lt;sup&gt;+&lt;/sup&gt;; host for π-requiring plasmids; conjugal donor</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<td><strong>Plasmids</strong></td>
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<td>pKTN701</td>
<td>R6K-ori suicide vector for gene replacement; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nishibuchi et al. (1991)</td>
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<td>pVP226</td>
<td>Initially isolated as a FURTA-positive clone; pUC19 containing chromosomal ca 1.3 kb <em>SacA</em>AI fragment from WP1 in the same orientation relative to the <em>lac</em> promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Funahashi et al. (2002)</td>
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<td>pVPA4.1</td>
<td>pUC19 containing a chromosomal 4124 bp <em>HindIII</em> fragment from WP1 in the opposite orientation relative to the <em>lac</em> promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pVPA2.8</td>
<td>pBluescript II KS(+) containing a chromosomal 2940 bp <em>BglII</em> fragment from WP1 in the opposite orientation relative to the <em>lac</em> promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pVPA2.5</td>
<td>pBluescript II KS(+) containing a chromosomal 2482 bp <em>PstI</em>-<em>SacI</em> fragment from WP1 in the same orientation relative to the <em>lac</em> promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pTF3</td>
<td>pKTN701 containing a 634 bp <em>KpnI</em>-<em>EcoRI</em> fragment (internal to <em>orf2</em>) PCR-amplified with primers 1 and 2 using pVPA4.1 as a template; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pTF4</td>
<td>pKTN701 containing a 756 bp <em>KpnI</em>-<em>EcoRI</em> fragment (internal to <em>iutA</em>) PCR-amplified with primers 6 and 7 using pVPA4.1 as a template; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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*Ap<sup>+</sup>, ampicillin resistance; Cm<sup>+</sup>, chloramphenicol resistance; Km<sup>+</sup>, kanamycin resistance.
water filtration unit (Millipore) and all reagent solutions were made with iron-free water. Prior to use, all glassware was washed with 6 M HCl and rinsed several times with iron-free water.

**DNA manipulations.** Isolation of DNA, recovery of DNA fragments from agarose gels, cleavage with restriction endonucleases, ligation, transformation and agarose gel electrophoresis were done as outlined by Sambrook et al. (1989), or as recommended by the commercial suppliers of the materials. A model 785 vacuum blotter (Bio-Rad) was used for transfer of DNA in agarose gels onto nylon membranes. Electroporation was performed in a Gene Pulser apparatus (Bio-Rad) at a capacitance of 25 μF, a resistance of 200 Ω and an electrode distance of 2 mm. Unless otherwise noted, PCR was performed under the following conditions: after initial denaturation at 94 °C for 2 min, a cycle of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 1 min was repeated 30 times.

**Southern and colony hybridization.** Hybridization followed by immunological detection of DNA was performed according to the digoxigenin (DIG) system user’s guide for filter hybridization (Roche Diagnostics). Hybridization with appropriate DIG-labelled probes was carried out overnight at 68 °C. Hybridization probes A and B (see Fig. 1c) were prepared using primers 1 and 2 (positions 2884–2908 and 3412–3436) and primers 3 and 4 (positions 4003–4022 and 4457–4476), respectively, with the PCR DIG probe synthesis kit (Roche Diagnostics) under the recommended PCR conditions.

**Cloning of the iutA gene and its flanking regions.** Plasmid pVP226, bearing a partial iutA gene on a 1-3 kb SatI3AI fragment, was initially isolated by the FURTA system from a V. parahaemolyticus WP1 genomic library as described previously (Funahashi et al., 2002). For cloning of the entire iutA gene and its flanking regions, V. parahaemolyticus WP1 genomic DNA was first digested with various restriction enzymes and the DNA fragments were examined by Southern blotting with DIG-labelled probe A that had been designed with the same probe to isolate plasmids pVPA4.1 and pVPA2.8 (see Fig. 1). In a similar way, plasmid pVPA2.5 containing a genomic Pst−Sac fragment was isolated by hybridization with DIG-labelled probe B.

**Nucleotide sequencing and homology search.** Nucleotide sequences were determined by a Hitachi DNA sequencer (SQ5500E) with the Thermo Sequenase premixed cycle sequencing kit and appropriate primers previously labelled with the 5’-oligonucleotide Texas red labelling kit (Amersham Pharmacia Biotech). Sequence analysis and alignment were performed with the Genetyx-Mac, version 10.1, software package (Genetyx Software Development). The BLASTP program (Altschul et al., 1997) of the Institute for Chemical Research, Kyoto University, Japan, was used for a homology search of the deduced amino acid sequences.

**Primer extension.** Iron-deficient and iron-sufficient cells of V. parahaemolyticus WP1 were prepared as follows. The culture was grown in LB broth to an OD660 of 0.15 and was split into two aliquots; one was left untreated (iron-sufficient cells) and the other was supplemented with 2,2’-dipyridyl at a final concentration of 200 μM to achieve iron depletion (iron-deficient cells). Then, both aliquots were further incubated until an OD660 of 0.5 was reached. Total RNA was prepared from each cell sample using ISOGEN (Nippon Gene), according to the manufacturer’s instructions. The primer 5’-CGTTTGGAGACGCCAGTTGC-3’, complementary to positions 3097–3116 of the iutA sequence, was 5’-labelled with Texas red as described above. Approximately 20 fmol of the labelled primer was annealed to 30 μg total RNA at 50 °C and extended at 50 °C for 60 min using avian myeloblastosis virus reverse transcriptase XL (Takara Biomedicals), according to the manufacturer’s protocol. The extension product was sized on a 6 % (w/v) denaturing polyacrylamide gel by using a Hitachi DNA sequencer (SQ5500E) alongside the DNA sequence ladder of the control region synthesized with the same labelled primer to map the start site of the transcript.

**Growth and binding assays.** In these assays for elucidation of aerobactin utilization, a spontaneously arising, vibrioferrin-deficient mutant, MY-1 (Yamamoto et al., 1994a) derived from V. parahaemolyticus AQ3354, was used to avoid the effect of the endogenous siderophore, vibrioferrin, on growth under iron-limiting conditions.
Stationary-phase cells of MY-1 were diluted to an OD_{600} of 0·005 with fresh LB broth (3 % NaCl) containing 20 μM ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDA) with or without 10 μM aerobactin. Cultures were shaken (125 r.p.m.) at 37 °C and the OD_{600} was determined at regular intervals. Aerobactin was prepared as described previously (Okujo & Yamamoto, 1994).

V. parahaemolyticus MY-1 was cultured under the same conditions as WP1 for primer extension analysis, and OMP-enriched fractions from the iron-deficient and iron-sufficient cell samples were prepared by sonication followed by high-speed centrifugation and Sarkosyl extraction as described previously (Yamamoto et al., 1995). Aerobactin was incubated with radioactive $^{55}$FeCl$_3$ as described for desferrioxamine B (Asso et al., 2002) to prepare a 5 μM $^{55}$Fe-labelled aerobactin solution (680 c.p.m. pmol$^{-1}$). An equal volume of this solution was mixed with 0·3 ml 100 mM Tris/HCl buffer, pH 7·4, containing 100 μg of the OMP. The mixture was shaken at 37 °C for 10 min, 0·5 ml of which was filtered onto a 0·45 μm pore-size nitrocellulose filter. The filter was washed twice with 5 ml of the Tris/HCl buffer and the radioactivity on the filter was measured. Digestion of the OMP fraction with proteinase K (19 units mg$^{-1}$) (Wako) was carried out by incubating a mixture consisting of 500 μg of the enzyme and 500 μg of the OMP in a total volume of 1 ml at 37 °C for 1 h. The mixture was centrifuged for 20 min at 20 000 g and then the precipitate was used for SDS-PAGE (Laemmli, 1970) or for further incubation with $^{55}$Fe-labelled aerobactin as described above.

Construction of iutA- and orf2-disruptants. A mutant with disruption in iutA was prepared by inserting a suicide vector into the chromosome of V. parahaemolyticus MY-1, using the strategy originally described by Miller & Mekalanos (1988). MY-1 has the same gene arrangement with respect to orf2-alcD-iutA as WP1. Briefly, the KpnI–EcoRI fragment internal to iutA was prepared by PCR amplification with a set of primers (5’-ATATTCCGGTACCC- GTTTGG-3'; positions 3171–3190 and 5’-CCGAAATCCGTGGTGC- GTGCC-3'; positions 3917–3936) (the bases changed for introduction of the respective restriction enzyme sites are underlined) followed by digestion with the respective restriction enzymes. This fragment was subsequently inserted into a suicide vector pKT701 with a chloramphenicol resistance cassette (Nishibuchi et al., 1991) to generate pTF4, which was propagated in E. coli SY327/pir. The extracted plasmid was transformed into E. coli SM10/pir as a donor and transferred to MY-1 by membrane-filter mating conjugation. Some transconjugants on LB plates containing ampicillin and chloramphenicol were confirmed by Southern blot analysis with DIG-labelled probe B (data not shown). One of the disruptants was designated VPTF4. In a similar fashion, chromosomal orf2 was disrupted by plasmid pTF3 to generate VPTF3. For construction of pTF3, the KpnI–EcoRI fragment internal to orf2 was PCR-amplified with a set of primers (5’-TCCGTACCATCTACAAATGC-3'; positions 759–779 and 5’-AACGGGATTCTGTCCTGACGG-3'; positions 1379–1398); the bases changed for introduction of the appropriate restriction enzyme sites are underlined.

RESULTS AND DISCUSSION

Nucleotide sequence and iron-regulated transcription of the iutA gene

Appropriate DNA segments from the V. parahaemolyticus WP1 genome were cloned by gene walking with DIG-labelled probes A and B to complete the iutA sequence. A restriction map of the 5·6 kb HindIII–Sacl region cloned, and the relevant plasmid inserts and the probes constructed are shown in Fig. 1. In contrast to pVP226, pVPA2.5 did not confer a Lac$^{+}$ phenotype to E. coli H1717, suggesting that a potential Fur box maps between the ClaI and PstI sites. Finally, the combined nucleotide sequences of the plasmid inserts disclosed four entire and one partial ORF, among which orf3 and iutA are transcribed in the same direction. The G+C contents of the sequenced region and the iutA gene were 45·4 and 46·7 mol%, respectively, these values being similar to the overall G+C content of V. parahaemolyticus (46–47 mol%) (Baumann et al., 1984). The intergenic region between orf3 and iutA is shown in Fig. 2(a). A search for hexameric sequences with significant homology to the consensus −10 and −35 sequences with appropriate spacing (17 ± 1 bp) (Hawley & McClure, 1983) yielded a putative promoter site. In accordance with the results obtained by FURTA, the predicted Fur box sequence matching the E. coli Fur box consensus (Calderwood & Mekalanos, 1984) was found at 15 of 19 positions, which overlaps the −10 region of the predicted promoter of the iutA gene. The proposed translational start site is preceded by a Shine–Dalgarno-like sequence (Shine & Dalgarno, 1974). Just downstream of the iutA stop codon lies an inverted repeat followed by a thymidine-rich sequence, which possibly functions as a ρ-independent transcriptional terminator (positions 5211–5233).

To clarify whether transcription of iutA is regulated by iron and to identify the transcriptional start site of this gene, primer extension analysis was carried out for total RNA preparations from iron-deficient and iron-sufficient cells of V. parahaemolyticus WP1. As shown in Fig. 2(b), primer extension revealed a transcript with a 5′ terminus mapping to position 2978 only for total RNA from iron-deficient cells. This 5′ transcript terminus maps just downstream of the putative promoter sequence. As judged by the result of primer extension, iutA transcription was repressed in cells cultured in LB broth without 2,2′-dipyridyl (iron-deficient conditions). This fact was consistent with the presence of the potential Fur-binding sequence upstream of the iutA gene.

Homology of predicted protein sequences

A search of the protein database revealed significant homology of the deduced iutA product with several proteins in the family of TonB-dependent receptors. It shows the highest amino acid identity to Vibrio orientalis IutA (55 %) (Murakami et al., 2000), followed by E. coli IutA (43 %) (Krone et al., 1987). Alignment of the V. parahaemolyticus, V. orientalis and E. coli IutA proteins is shown in Fig. 3. The amino acid sequence derived from the nucleotide sequence of iutA contains a typical leader peptide of 25 residues (von Heijne, 1983). The TonB box sequence close to the N terminus was found in the V. parahaemolyticus IutA, with a similarity to sequences conserved in TonB-dependent receptors (Braun & Hantke, 1991). A C-terminal phenylalanine residue and an arginine residue at position −11 relative to the C terminus are widely conserved among OMPs (Struyvë et al., 1991; Bäumler & Hantke, 1992).
Interestingly, in the case of *V. parahaemolyticus* IutA, the C-terminal residue is hydrophobic tyrosine. The *V. parahaemolyticus* IutA protein consists of 725 residues and the mature protein has a calculated molecular mass of 76,654 Da, which is somewhat less than the 83 kDa estimate from the electrophoretic mobility in SDS-PAGE. The difference may be due to aberrant migration on SDS-PAGE as frequently reported for OMPs.

Nucleotide sequence analysis of the region upstream of *iutA* revealed two additional genes, *orf2* and *orf3*. The predicted product of *orf2* has 47% identity to *E. coli* DcuB, one of the anaerobic C₄-dicarboxylate membrane transporters (Six et al., 1994). The *orf2* product also shows a high degree of identity (90%) to the putative DcuB of *V. cholerae* (VCA0205, chromosome 2). The *orf3* gene probably encodes a protein with 44 amino acid residues which shows weak homology (24% identity in 224 residues) to the putative AlcD protein of *B. bronchiseptica* (Pradel et al., 1998). AlcD, although showing no homology with proteins in the database, has been proposed to be one of the biosynthetic enzymes for the siderophore alcaligin, owing to its location within the *alc* operon involved in alcaligin production. However, the *V. parahaemolyticus* AlcD homologue seems unlikely to play a role in aerobactin-mediated iron uptake and its function is currently unknown.

Other features of the sequence determined include the *orf1* gene which encodes the C-terminal portion of an apparent homologue of *V. cholerae* VC1811 (71% identity in 113 residues, chromosome 1). Downstream of *iutA* lies *orf4* whose predicted product amino acid sequence is completely consistent with that of the *V. parahaemolyticus* hypothetical protein (AY026362) and also homologous to *V. cholerae* VC1810 (60% identity, chromosome 1). However, the functions of the *orf1* and *orf4* products are currently unknown in both *V. parahaemolyticus* and in *V. cholerae*.

**Utilization of aerobactin by *V. parahaemolyticus* and characterization of its *iutA*-disrupted mutant**

Fig. 4 shows growth curves of strain MY-1 and its *iutA* disruptant under iron-limiting conditions with or without added aerobactin. Iron restriction imposed by the addition of 20 μM EDDA to the LB broth resulted in poor growth of MY-1. However, supplementation of this iron-restricted LB broth with aerobactin at 10 μM restored the growth of MY-1 to a level comparable to that of the same strain grown in iron-replete LB broth, indicating that aerobactin is capable of effectively providing iron to *V. parahaemolyticus*. This is in accordance with the detection of the *iutA* homologue in *V. parahaemolyticus*. The *iutA*-disrupted mutant, VPTF4, however, grew poorly even in the medium supplemented with aerobactin (Fig. 4), confirming that aerobactin-dependent iron uptake for growth is mediated by the IutA protein.

The OMP profiles of MY-1 are shown in Fig. 5(a), both the 78 and 83 kDa protein bands being detected only in cells grown in an iron-limiting medium. However, lack of the IutA protein in the *iutA* disruptant was not clearly demonstrated by SDS-PAGE owing to coexistence of other OMPs with similar mobility (Fig. 5a, OMP fraction 4). Then, to provide further evidence that the receptor protein for ferric aerobactin is indeed expressed under iron-limiting conditions, the OMP fractions from both
iron-limiting and iron-sufficient cells were assayed to compare their capacity to bind $^{55}$Fe-labelled aerobactin. The outer-membrane fraction (containing these iron-repressible OMPs) prepared from cells grown under iron-limiting conditions bound significantly large amounts of the radioactive $^{55}$Fe-labelled aerobactin complex compared with that prepared from cells grown under iron-sufficient conditions (Fig. 5b). No radioactivity was detected when radioactive mineral iron ($^{55}$FeCl$_3$) was incubated with the OMP fraction from iron-limiting cells. In addition, preincubation with proteinase K of the outer-membrane fraction prepared from cells grown under iron-limiting conditions led to a loss in its ability to bind ferric aerobactin. These results indicated that a proteinaceous component, most presumably IutA, which possesses the capability of binding ferric aerobactin, is expressed as its receptor in response to iron starvation stress. Finally, the $iutA$ disruption incapacitated the outer-membrane fraction from the $iutA$-disrupted mutant in its ability to bind ferric aerobactin (Fig. 5b), demonstrating that the outer-membrane receptor protein relevant to utilization of aerobactin is in fact encoded by the $iutA$ gene. Unfortunately,
our initial attempt failed to provide definite evidence that ferric aerobactin directly binds to the IutA protein.

On the other hand, its location immediately upstream of the \( alcD \) homologue as well as the hydrophobicity of the predicted gene product suggested the possibility that the product of \( orf2 \) might function as one of the components for transportation of ferric aerobactin across the inner membrane into the cytosol. To explore this possibility, \( orf2 \) was also disrupted by insertion mutation. Growth of the resultant disruptant VPTF3 in the presence of aerobactin under iron-limiting conditions, however, was comparable with that of the parental strain MY-1 (Fig. 4), indicating that the predicted \( orf2 \) product is not involved in ferric aerobactin transport.

**Distribution of the \( iutA \) gene in \( V.\ parahaemolyticus \)**

Southern blot hybridization was performed with DIG-labelled probe B on chromosomal DNA samples from some clinical and environmental isolates of \( V.\ parahaemolyticus \) (Fig. 6). Prior to electrophoresis, all chromosomal DNA samples were completely digested with \( BglII \). In WP1, the probe hybridized to an approximately 2-9 kb \( BglII \) fragment, the size predicted from the DNA sequence analysis. In UST-4-1, the size of the \( BglII \) fragment was slightly different from WP1, but in the other strains the probe hybridized to fragments of the same size. In accordance with these results, growth promotion by aerobactin was observed for each of these strains under iron-limiting conditions, indicating that the \( iutA \) homologues are widely distributed in \( V.\ parahaemolyticus \) to assimilate iron as a ferric aerobactin complex. However, a homologous counterpart of the \( V.\ parahaemolyticus\ iutA \) gene was not found in the \( V.\ cholerae \) genome sequences.

Aerobactin was first isolated from cultures of \( Aerobacter\ aerogenes \) (Gibson & Magrath, 1969). The aerobactin...
iron-uptake system, consisting of a cluster of four genes, iucABCD, the aerobactin biosynthesis genes and iutA, the receptor gene for ferric aerobactin, are found not only on plasmids pColV and F1me in some E. coli and Salmonella strains, respectively, but also in the chromosomes of other E. coli and Shigella strains causing infections in humans (de Lorenzo & Marinez, 1988; Payne, 1988; Crosa, 1999). In the case of Shigella species, the aerobactin systems have recently been found to be carried on pathogenicity islands (Moss et al., 1999; Vokes et al., 1999; Purdy & Payne, 2001). Interestingly, some Vibrio species, including pathogenic Vibrio hollisae and Vibrio mimicus have also been reported to produce aerobactin (Haygood et al., 1993; Okujo & Yamamoto, 1994; Murakami et al., 1995). It is reasonable to assume that these aerobactin-producing species may have aerobactin-mediated iron-uptake systems similar to those of the above species in the Enterobacteriaceae. However, until now only the iutA homologue has been cloned in V. orientalis, but without functional definition of the protein product (Murakami et al., 2000). Moreover, some bacterial pathogens such as Corynebacterium diphtheriae (Russell & Holmes, 1985), Neisseria gonorrhoeae (West & Sparling, 1987), Pseudomonas aeruginosa (Meyer, 1992), Pseudomonas putida (Loper & Henkels, 1999) and Staphylococcus aureus (Sebulsky et al., 2000) have also been reported to be capable of utilizing exogenous aerobactin to promote their growth under iron-limiting conditions, but none of the genes responsible for the ferric aerobactin receptor has been clarified yet.

Our data demonstrate that V. parahaemolyticus possesses the iutA homologue encoding the ferric aerobactin receptor which is responsible for utilization of aerobactin as an exogenous siderophore in iron-restricted environments. This is the first report of the ferric aerobactin receptor gene being carried on the chromosome of a bacterium which does not produce aerobactin. However, it is currently unclear whether the aerobactin-dependent iron acquisition system confers on V. parahaemolyticus a greater ability to survive in different niches outside or inside the host or to establish an infection. Besides the siderophore-specific outer-membrane receptors, cytoplasmic membrane-associated components are also necessary for ferric siderophore transport and the corresponding genes are generally clustered (Braun et al., 1998). However, the present data indicate that the genes responsible are not located in the close vicinity of iutA on the V. parahaemolyticus chromosome. Further studies will be required to identify and characterize the genes encoding the components responsible for the inner-membrane transport of ferric aerobactin into the cytoplasm.

ACKNOWLEDGEMENTS

We are indebted to I. Stojiljkovic for providing the host strain E. coli H1717 in the FURTA system. We also thank H. Yamada for determining the N-terminal amino acid sequence. This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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