Identification and Characterization of pvuA, a Gene Encoding the Ferric Vibrioferrin Receptor Protein in Vibrio parahaemolyticus

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Received 16 August 2001/Accepted 14 November 2001

Iron is an essential element for almost all living organisms by virtue of its two valences, which act as cofactors in various oxidative-reductive enzymatic reactions. However, in an aerobic environment at neutral pH, iron exists as insoluble iron complexes that are largely unavailable to bacteria. In the mammalian host most of the iron is intracellular in the form of heme and the small amount of extracellular iron is sequestered in high-affinity iron-binding proteins such as transferrin in serum and lymph and lactoferrin in mucosal secretions. To overcome such iron-restricted conditions, most potential pathogens can express high-affinity transport systems to efficiently obtain iron from one or more of these host iron sources. One mechanism is the direct assimilation of heme and iron bound to transferrin or lactoferrin through specific bacterial receptors. Another mechanism involves the production and excretion of soluble siderophores that can chelate free ferric iron or bind to transferrin and lactoferrin. Siderophores complexed with ferric ions are subsequently conveyed into the bacterial cell by ligand-specific cell surface receptors and ABC transporters. In addition, to exploit the availability of diverse iron sources that may be present in their surroundings, some bacteria express additional iron transport systems including the receptors that enable them to utilize exogenous or heterologous siderophores produced by other microbial species. Thus, the ability to utilize the host iron sources has been frequently associated with bacterial pathogenesis. Moreover, there is increasing evidence that the restricted availability of iron in the host constitutes a major signal which coordinately regulates the expression of a number of virulence and metabolic genes.

In Escherichia coli, the iron transport systems characterized to date are all regulated at the transcriptional level by iron-binding protein Fur (ferrous iron uptake regulation), which requires ferrous iron as a cofactor and which acts as a repressor when environmental iron levels are low. Fur homologs with similar functions have been identified in many other bacterial species. The genus Vibrio includes 46 species, and 11 of these are human pathogens or have been isolated from human clinical specimens. Vibrio parahaemolyticus, one of the pathogenic vibrios, is a gram-negative, halophilic bacterium that naturally inhabits marine and estuarine environments and that causes three major syndromes of clinical illness: gastroenteritis, wound infections, and septicemia.
TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>V. parahaemolyticus</strong></td>
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<tr>
<td>WP1</td>
<td>Clinical isolate</td>
<td>70</td>
</tr>
<tr>
<td>AQ3354</td>
<td>Clinical isolate</td>
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<tr>
<td>MY-1</td>
<td>Spontaneous vibrioferrin-deficient AQ3354 mutant</td>
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<tr>
<td>VPTF1</td>
<td>AQ3354, psud disrupted</td>
<td>This study</td>
</tr>
<tr>
<td>VPTF2</td>
<td>AQ3354, psud disrupted</td>
<td>This study</td>
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<td>E. coli H1717</td>
<td>aprD139 psuL150 Δ(argF-lac) relA1 U169 fblB5301 deoC1 ptsF25 rbsR araB fhaF::λ p lacMu; host strain for FURTA</td>
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</tr>
<tr>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
<td>endA1 hisdR77 (r&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;−&lt;/sup&gt; supE44 thi-1 recA1 gva96 relA1 Δ(argF-lacZYA)169 deoR::(868d lac Δ(lacZ)M15)) general cloning host</td>
<td>Promega</td>
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<td>JM109</td>
<td>recA1 endA1 g496 thi hisdR77 supE44 relA1 Δ(lac-proAB)F&lt;sup&gt;+&lt;/sup&gt; (traD36 proAB&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;+&lt;/sup&gt; λ μXZΔM15); general cloning host</td>
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<td>SY327 λ pir</td>
<td>Δ(lac pro) argEAm recA56 gva4 rpoB λ pir; host for λ requiring plasmids</td>
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<tr>
<td>SM10 λ pir</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu, λ pir; Km&lt;sup&gt;+&lt;/sup&gt;; host for λ requiring plasmids; conjugal donor</td>
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<td><strong>Plasmids</strong></td>
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<td>pUC19</td>
<td>High-copy-number cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pBluescript II KS(+)</td>
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<td>pMW118</td>
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<td>Nippon Gene</td>
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<tr>
<td>pKTN701</td>
<td>R6K ori suicide vector for gene replacement; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pRK415</td>
<td>Broad-host-range plasmid (~10.5 kb); Te&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pVP3151</td>
<td>Initially isolated FURTA-positive clone; pUC19 containing chromosomal 3,151-bp PstI fragment from AQ3354 in the orientation opposite to the lac promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pVPD1</td>
<td>pUC19 containing the EcoRI (in pUC19)-EcoRI fragment from pVP3151; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pVPD2</td>
<td>pUC19 containing the KpnI-EcoRI fragment internal to psud from pVP3151; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pVPV2995</td>
<td>pBluescript II KS(+) containing the chromosomal 2,995-bp EcoRI-SacI fragment from AQ3354 in the same orientation relative to the lac promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pVPV2995-1</td>
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<td>pVPV4780</td>
<td>pMW118 containing 39-bp EcoRI-PstI (from pUC19) and 4,780-bp PstI-SacI fragments; ligated 1.8-kb EcoRI (in pUC19)-EcoRI fragment from pVPD1 into the EcoRI site of pVPV2995-1 in the same orientation relative to the lac promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pVPV4780R</td>
<td>pMW118 containing 4,813-bp SacI-SacI fragment of pVPV4780 in the opposite orientation relative to the lac promoter; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pTF1</td>
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<td>This study</td>
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<td>pTF2</td>
<td>pKTN701 containing 877-bp EcoRI-KpnI fragment PCR amplified with primers 5 and 6 using pVPV2995 as a template; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRKVP4.8</td>
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<td>pRKVP3</td>
<td>pRK415 containing 2,995-bp EcoRI-SacI fragment of pVPV2995 in the opposite orientation relative to the lac promoter; Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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* Ap<sup>+</sup>, ampicillin resistant; Cm<sup>+</sup>, chloramphenicol resistant; Km<sup>+</sup>, kanamycin resistant; Te<sup>+</sup>, tetracycline resistant.

regulator (35) have been demonstrated in this species. Besides these pathogenic factors, adherence to and proliferation within the host intestine are the prerequisites for pathogenesis of *V. parahaemolyticus*. It seems likely that the ability to acquire iron for proliferation in the host is another important virulence factor in this species.

Under iron-limited conditions, *V. parahaemolyticus* produces native siderophore vibrioferrin to facilitate iron acquisition (72) and also utilizes heme as a sole source of iron (70). *V. parahaemolyticus* expresses two iron-repressible outer membrane proteins of 78 and 83 kDa, which were identified as the receptors for ferric vibrioferrin (68) and heme and hemoglobin (70), respectively. In addition, the fur gene of this bacterium was cloned (69) and shown to mediate iron regulation both in the production of vibrioferrin and in the expression of the outer membrane proteins (16). However, little is known about iron assimilation systems in this bacterium at the gene level.

To gain insight into the Fur-regulated genes in *V. parahaemolyticus*, we used the Fur titration assay (FURTA) system, originally established for *E. coli* (60), to isolate Fur target genes from *V. parahaemolyticus*. As a result, we identified an operon consisting of psud* (V. parahaemolyticus* siderophore utilization), encoding a new TonB-dependent receptor for an unidentified ligand, probably an exogenous siderophore, and psuA (V. parahaemolyticus* vibrioferrin utilization), encoding the ferric vibrioferrin receptor. The function of the PvuA protein in vibrioferrin-mediated iron transport was confirmed by insertion mutation studies coupled with genetic complementation studies.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the *V. parahaemolyticus* strains were cultured with shaking in Luria-Bertani (LB) broth (1% tryptone [Difco], 0.5% yeast extract [Difco], 3% NaCl, pH 7.5), which was determined to contain ferric ion at a sufficient concentration (approximately 8 μM). The *E. coli* DH5α and JM109 strains (74), used for propagation of various plasmids, and the *E. coli* λpir strains (40), used for construction of the *V. parahaemolyticus* AQ3354 mutants by homologous recombination, were grown in LB broth or on LB agar containing 0.5% NaCl. When required, appropriate antibiotics were added to the media as follows: ampicillin at 50 μg/ml, chloram-
chromosomal Fur-repressible E. coli potential difference of 2.5 kV, a resistance of 200 kΩ.

General DNA manipulations. Chromosomal DNAs of V. parahaemolyticus WP1 and other Vibrio species were extracted from overnight cultures with a Wizard genomic DNA purification kit (Promega) according to the manufacturer’s protocol. Cloning, restriction endonuclease digestion, and DNA ligation were carried out according to standard protocols (56). Electroporation was performed in a Gene Pulser apparatus (Bio-Rad) at a capacitance of 25 μF, a potential difference of 2.5 kV, a resistance of 200 Ω, and an electrode distance of 2 mm. Restriction enzymes and a DNA ligation kit (version 2) were purchased from Takara Biomedicals (Kyoto, Japan).

Nucleotide sequence determination. Nucleotide sequencing was carried out by a Hitachi (Tokyo, Japan) DNA sequencing kit (SQ5505E) with the Thermo Sequenase premix or core cycle sequencing kit and appropriate primers, which were labeled with a 5'-oligonucleotide Texas red labeling kit (Amersham Pharmacia Biotech). Sequence analysis was conducted with the Genetyx-Mac, version 9.0, software package (GENETYX Software Development Co., Tokyo, Japan). The BLASTP and FASTA programs (1, 50) of the Institute for Chemical Research, Kyoto University, were used to determine homologies of the deduced amino acid sequences to other proteins.

Oligonucleotide primers. Primers 1 and 2 (nucleotide positions 2140 to 2159 and 2807 to 2826) and primers 3 and 4 (nucleotide positions 3634 to 3653 and 4259 to 4278) were used for preparation of digoxigenin (DIG)-labeled hybridization probes A and B, respectively, under the PCR conditions recommended in the PCR DIG probe synthesis kit (Roche Diagnostics). The EcoRI-KpnI fragment internal to pvuA, which was ligated into pKTN701 to construct pvuA-disruptant VPTF2, was prepared with primers 5 (AAGCTTGAATTCGTACACCCG CC; nucleotide positions 3046 to 3065) and 6 (ATACGCCTTACCATGGC TTAC; nucleotide positions 3912 to 3931) (the nucleotides changed to generate the respective restriction enzyme sites are underlined) under the following PCR conditions. KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used, and after initial denaturation of 94°C for 2 min, a cycle of 94°C for 15 s, 55°C for 30 s, 72°C for 2 min, a cycle of 94°C for 15 s, 55°C for 30 s, 68°C for 2 min, a cycle of 94°C for 15 s, 55°C for 30 s, 68°C for 2 min, 6°C for 1 min was repeated 30 times.

FURTA. The FURTA was essentially performed as described by Stoijilkovic et al. (60). V. parahaemolyticus WP1 chromosomal DNA fragments (2 to 5 kb) completely digested with PstI were cloned into the PstI site of pUC19. The resulting recombinant plasmids were introduced into E. coli H1717 carrying the chromosomal Fur-repressible fmuA: lacZ fusion, and ampicillin-resistant transformants were screened for the Lac õ phenotype on MacConkey lactose agar plates (Difco) supplemented with 20 μM ferrous ammonium sulfate after 15 h of growth at 37°C. Following several rounds of screening, we isolated more than 20 FURTA-positive clones with inserts of different sizes (data not shown).

N-terminal amino acid sequence determination. Sarkosyl (Sigma)-insoluble outer membrane proteins were prepared from V. parahaemolyticus WP1 cells grown in LB broth supplemented (iron-limited) or not (iron-sufficient) with ethylenediaminetetraacetic acid (EDTA; Sigma) at 25 μM, and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 10% separating gel (34). The separated proteins were electrophoretically transferred to a pretwisted polyvinylidene difluoride (PVDF) membrane (ProBlott; Applied Biosystems) using a Trans-Blot semidyem sceptrophoretic transfer cell (Bio-Rad) essentially as described by Tow- bin et al. (63) and stained with Coomassie brilliant blue. The membrane was rinsed several times with distilled water and air-dried, and the iron-repressible protein bands of 78 and 83 kDa were excised from the membrane with a razor blade. The N-terminal amino acid sequence was determined by automated Edman degradation with a model 491 protein sequencer (Applied Biosystems) equipped with an online model 120A PTH-amino acid analyzer. The amino acid sequences were compared with those deduced from the nucleotide sequences of the FURTA-positive clones.

Southern blot analysis and colony hybridization. These procedures were performed according to the DIG system user’s guide for filter hybridization (Roche Diagnostics). Digested chromosomal DNA was resolved in 1% agarose gel, transferred onto a positively charged nylon membrane (Roche Diagnostics) with a model 785 vacuum blotter (Bio-Rad), and fixed to the membrane by baking it for 30 min at 120°C. Colonies on a nylon membrane for colony and plaque hybridization (Roche Diagnostics) were denatured and neutralized, and the transferred DNA was fixed to the membrane by baking it for 30 min at 80°C. Hybridization with an appropriate DIG-labeled probe was carried out overnight at 68°C, and, after treatment of the membrane with alkaline phosphatase-labeled anti-DIG Fab fragments, the hybridized DNA was detected by a CSPD reagent for Southern blot analysis and by colorimetric detection reagents nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate) for colony hybridization, according to the DIG system user’s guide for filter hybridization (Roche Diagnostics). The DIG system user’s guide for filter hybridization (Roche Diagnostics). The DIG system user’s guide for filter hybridization (Roche Diagnostics). The DIG system user’s guide for filter hybridization (Roche Diagnostics).

Cloning of the pvuA gene. V. parahaemolyticus WP1 chromosomal DNA was first digested by the combination of EcoRI with various restriction enzymes, and the DNA fragments were examined by Southern blotting with DIG-labeled probe A (see Fig. 1C). Then, approximately 3-kb band fragments in the EcoRI-SauI digest which hybridized with probe A as a single band were ligated into the same restriction sites of phleomycin H Ks+(-). Colonies on LB agar plates were screened by colony blot hybridization with the same probe. The nucleotide sequence of the insert of positive plasmid pVPV2995 was determined.

Western blot analysis. Expression of the pvuA protein was examined by Western blot analysis using rabbit antiserum against the 78-kDa ferric vibrio-ferrin receptor of V. parahaemolyticus WP1 (68). The Sarkosyl-insoluble outer membrane fractions were prepared from washed stationary-phase cells broken by sonication, as previously described (68). Protein concentrations were determined by the Lowry method. After SDS-PAGE, protein bands were transferred onto a PVDF membrane (63) and the membrane was treated with blocking solution (8% skim milk, 20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 0.02% NaN₃) for 1 h. The membrane was then incubated with antiserum diluted 1:500 with gentle agitation and was reacted for 1 h with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibodies (Roche Diagnostics) diluted 1:2,000. After thorough washing, the membrane was dipped in a BCP-nitroblue tetrazolium substrate solution (Bio-Rad).

RNA isolation and analysis. V. parahaemolyticus WP1 was grown in LB broth in the presence (iron-limited cells) and absence (iron-deficient cells) of 25 μM EDDA to an A₅₆₀ of 0.5. Total RNA from each cell sample was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions, and the amount of total RNA was quantified by measuring A₂₆₀.

(i) Primer extension. Oligonucleotide primers 7 (nucleotide positions 414 to 433) and 8 (nucleotide positions 2610 to 2629), complementary to the 5'-end regions of pvuA and pvuA', respectively, were 5'-labeled with Texas red as a donor and transferred to a Hitachi DNA sequencer (SQ5500E) along the DNA sequence ladder of each control region synthesized with the same labeled primer.

(ii) Northern blot analysis. Total RNA (approximately 10 μg) from each cell sample was separated electrophoretically on a 1% agarose–2.2 M formaldehyde gel. The gel was rinsed in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, and the RNA was transferred to a positively charged nylon membrane by a model 785 vacuum blotter. The SaI-EcoRI and PstI-ClaI fragments from pVP3151 and pVPV2995, respectively, were labeled with alkaline phosphatase according to the manufacturer’s protocol (AlkPhos Direct; Amersham Pharmacia Biotech) to yield AlkPhos-labeled probes A and B, respectively (see Fig. 1C). Alkaline phosphatase activity was visualized fluoroscopically using Chemi-Lumi II (Roche Diagnostics) according to the manufacturer’s instructions. The extension products were sized in a 6% denaturing polyacrylamide gel by a Hitachi DNA sequencer (SQ5500E) alongside the DNA sequence ladder of each control region synthesized with the same labeled primer.

Construction of mutant strains. To investigate their specific functions, we attempted to inactivate the pvuA and pvuA' genes of V. parahaemolyticus by homologous recombination as described below. Unfortunately, no mutants in these genes were obtained from strain WP1. Since a similar event has been reported for the same strain by Nishibuchi et al. (46), we decided to use another strain, AQ3354, which appeared to have an arrangement of pvuA and pvuA' identical to that of strain WP1. The KpnI-EcoRI fragment derived from pVP3151 was subcloned into R6K ori suicide vector pKTN701 (46) digested with the same restriction enzymes to yield pFT1. For the construction of pFT2, PCR was done with oligonucleotide primers 5 and 6 using pVPV2995 as the template to introduce EcoRI and KpnI linkers, and then an EcoRI-KpnI-digested fragment of this PCR product was inserted into pKTN701. The obtained plasmids were transformed into E. coli SM10 λ pir as a donor and transferred to V. parahaemolyticus AQ3354 by membrane-filter mating conjugation followed by incubation for 3 h at 37°C of a nitrocellulose membrane laid over LB agar plates with 1.5% NaCl. Transconjugants were selected by overnight incubation at 37°C on thiosulfate-citrate-bile-sucrose agar (Difco) (to select against the E. coli donor) containing chloramphenicol at 10 μg/ml. Some of the chloramphenicol-resistant transconjugants were isolated, and single colonies from each were independently plated onto LB agar plates. The mutants thus obtained were confirmed by Southern blot analysis with DIG-labeled probe A (data not shown).

For complementation studies, the SacI-EcoRI fragment of pVP3V4780-
ing an intact copy of the V. parahaemolyticus WP1 psuA-pvuA locus was subcloned into the SacI site of broad-host-range plasmid pRK415 (28), and resulting replicative plasmid pRKVP4.8 was conjugated into pvuA mutant VPTF2, with E. coli SM10 pir used as a donor. One of the chloramphenicol- and tetracycline-resistant colonies was selected, and correct transfer of the plasmid was confirmed by restriction enzyme analysis. Plasmid pRKVP3, pRK415 bearing the intact copy of the pvuA gene, was prepared and introduced into VPTF1 and -2 in the same manner.

Growth assay. Overnight cultures of V. parahaemolyticus AQ3354 and its mutant strains in LB medium were subcultured into the same medium containing 25 mM EDDA at an initial cell density corresponding to an \( A_{660} \) of 0.05. Vibrio-ferrin (72) or ferrichrome (Sigma) was added to the medium at a final concentration of 20 mM. Cultures were shaken (125 rpm) at 37°C, and growth was evaluated by determining the \( A_{660} \) of the culture. Three independent experiments were conducted on each strain.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in EMBL, GenBank, and DDBJ databases under accession no. AB048250.

RESULTS

Identification of the V. parahaemolyticus ferric vibrioferrin receptor gene. Application of the FURTA system to V. parahaemolyticus WP1 allowed us to isolate many different positive clones which contained potential Fur-regulated gene fragments. This was consistent with our hypothesis that the FURTA system might be applicable to V. parahaemolyticus because the Fur protein of this bacterium showed the high degree of homology (81% identity) with that of E. coli (16). Nucleotide sequences of the 5' and 3' regions in the insert of plasmid pVP3151 suggested the presence of full-length and partial open reading frames (ORFs) in the same direction (Fig. 1). Interestingly, as described below, the amino acid sequences deduced from these ORFs were each compatible with one of the extracted amino acid sequences in the 83-kDa band and with the N-terminal amino acid sequence of the 78-kDa ferric vibrioferrin receptor. The putative Fur box which overlaps the 10 region of the predicted promoter of the upstream gene and which shares 11 of 19 nucleotides (GTAAATAATATTGTTCTT; the matched nucleotides are underlined) with the E. coli consensus Fur box (12) was found. In agreement with this, subclone pVPD1, containing the EcoRI (in pUC19)-EcoRI fragment of pVP3151 conferred a Lac\(^{-}\) phenotype to H1717 in the FURTA, whereas subclone pVPD2, with a deletion of the PstI-KpnI portion containing the putative Fur box sequence from pVP3151, did not (Fig. 1).

N-terminal amino acid sequences of iron-repressible outer membrane proteins from V. parahaemolyticus. Separating the Sarkosyl-insoluble outer membrane proteins from V. parahaemolyticus WP1 grown in iron-limited medium revealed two major protein bands of 78 and 83 kDa, which were normally suppressed by growth in iron-replete medium (Fig. 2A). These iron-repressible proteins were blotted from a preparative gel onto a PVDF membrane, and the area of the membrane corresponding to each band was cut out. The amino acid sequences determined by Edman degradation are shown in Fig.
2B. The N-terminal amino acid sequence of the 78-kDa protein, which had been identified as the ferric vibrioferrin receptor protein (68), matched the amino acid sequence deduced from pvuA nucleotide positions 2588 to 2617 (Fig. 3B). Unexpectedly, sequence determination of the 83-kDa protein band, in which the heme and hemoglobin receptor had been identified (70), revealed the presence of three kinds of proteins, three amino acid residues being detected in most of the 10 cycles (Fig. 2B). However, the amino acid sequence (SEET NSTPSA) extracted from the amino acid residues detected in every cycle was correlated with that deduced from psuA nucleotide positions 393 to 422 (Fig. 3A). In addition, the other extracted amino acid sequence (AEQAQQLASQ) corresponded with the deduced N-terminal amino acid sequence encoded by an incomplete ORF detected in another V. parahaemolyticus FURTA-positive clone, whose deduced amino acid sequence had 27% identity (in a 73-amino-acid overlap) with the outer membrane receptor IutA in the E. coli ferric aerobactin transport system (T. Funahashi and S. Yamamoto, unpublished data). Therefore, the remaining amino acid sequence (EQHSTFNEVV) is suggestive of the 83-kDa heme and hemoglobin receptor (70) because it is similar to the N-terminal amino acid sequences of V. cholerae HtuA (DDYASFDEVV) (21) and V. vulnificus HupA (QDAGLFDEVV) (36), identified as heme receptors; the amino acid residues identical to those of the TonB box sequences proposed for HtuA and HupA are underlined.

Cloning of the full-length pvuA gene. V. parahaemolyticus WP1 genomic DNA samples digested with various combinations of restriction enzymes were separated by agarose gel electrophoresis followed by hybridization with DIG-labeled probes.
probe A (Fig. 1). The gel-extracted 3-kb EcoRI-SacI fragments that would be sufficient in length to encode psuA were ligated into the same sites of pBluescript II KS(+) (3). Positive clones were screened by colony blot hybridization with the same probe, and recombinant plasmid pVPV2995, which produced a Lac" phenotype when introduced into E. coli H1717, was selected (Fig. 1).

Nucleotide sequences of the psuA and pvuA genes and homology search of the deduced amino acid sequences. The combined nucleotide sequences of pVP3151 and pVPV2995 yielded two complete ORFs in the same orientation and 122 bp apart. The G+C content of the sequenced DNA was 49%, which is similar to the V. parahaemolyticus overall G+C content of 46 to 47% (3). The nucleotide sequences, including the promoter region of psuA, the intergenic region between psuA and pvuA, and the end of pvuA including the putative transcriptional termination signal, are presented in Fig. 3. Possible Shine-Dalgarno sequences (GGAA for psuA and AAGGAG for pvuA) are located just upstream of the starting methionine codons. In contrast to what was found for psuA, the obvious promoter sequences similar to both components of the E. coli σ70-like promoter consensus were not detectable in the upstream region of pvuA. An inverted repeat suggestive of a potential rho-independent transcriptional terminator begins 28 bp beyond the termination codon of pvuA. Interestingly, an inverted repeat and two pairs of direct repeats are also present in the intergenic region between psuA and pvuA, but their roles are unclear because, as described below, primer extension and Northern blot analyses indicated that pvuA and psuA were cotranscribed under iron-limited conditions.

Comparison of the N-terminal amino acid sequences determined for PsaA and PvuA with the amino acid sequences deduced from the nucleotide sequences of psuA and pvuA disclosed additional amino acid residues at the amino termini, indicating that the psuA and pvuA genes encode 25- and 37-amino-acid signal peptides, respectively, which are cleaved during translocation across the membrane. Indeed, each of the putative signal peptides has a typical signal sequence and a potential peptidase cleavage site (66). The entire protein encoded by psuA consists of 678 residues, and the mature protein has a calculated molecular mass of 72,364 Da. This is much less than the 83-kDa molecular mass of PsaA estimated from the electrophoretic mobility by SDS-PAGE. The difference may be due to aberrant migration of PsaA on SDS-PAGE gel as frequently reported for outer membrane proteins, but the possibility that this protein is posttranslationally modified prior to incorporation into the outer membrane cannot be ruled out. The second gene, pvuA, encodes a protein of 712 residues, and the molecular mass of the mature protein is 75,080 Da, which is almost compatible with the 78-kDa estimate based on SDS-PAGE. Furthermore, the predicted isoelectric points of the mature PsaA and PvuA, 4.50 and 4.71, respectively, are similar to the acidic isoelectric points of TonB-dependent outer membrane proteins of E. coli (43).

The BLASTP or FASTA algorithm for protein homology was used to compare PsuA and PvuA with other proteins. The deduced PsaA protein sequence had homology with those of a variety of the known TonB-dependent siderophore receptors from many bacteria and was the most similar to that of the putative ferrichrome iron receptor of Synechocystis species (P3 accession no. S74457; 27% identity, 41% similarity). However, PsaA exhibited no homology to the recently characterized V. cholerae, needed for ferrichrome iron utilization (55). On the other hand, PvuA had homology with RumA (31% identity, 48% similarity), the ferric rhizoferrin receptor of Morganella morganii (33), and FecA (31% identity, 47% similarity), the receptor of the E. coli ferric citrate transport system (53), but no PvuA homolog was found in the genomic sequence of V. cholerae. This is reminiscent of some structural analogy because rhizoferrin consists of two citrate moieties linked to putrescine (13). However, the mature PvuA lacked the homologous counterpart of the N-terminal extension of FecA, which is required to mediate transcription induction by the cognate ferric citrate (30). The predicted TonB box amino acid sequences which may be involved in direct interaction with the TonB protein are detected near the N-termini of PsaA (ETIQV) and PvuA (ETVVY) (Fig. 3A and B); the amino acid residues identical to those highly conserved in many TonB-dependent ferric siderophore receptors are underlined (4, 29, 33, 43). In addition, various outer membrane proteins possess the highly conserved C-terminal sequences which were proposed to form an amphipathic α-sheet important for the correct assembly of the protein into the outer membrane (4, 29, 61). This peculiar sequence motif also exists in PsaA and PvuA and in TonB-dependent outer membranes of other Vibrio species listed in Fig. 4.

Localization of the transcriptional start site by primer extension. To identify the transcriptional start sites of psuA and pvuA and to clarify whether transcription of these genes is regulated by iron, primer extension analysis with total RNA from strain WP1 cells was performed with oligonucleotide primers 7 and 8, complementary to the DNA sequences located 97 and 134 bp downstream of the methionine start codons of psuA and pvuA, respectively. As shown in Fig. 5A,
primer extension analysis with primer 7 for total RNA from the
iron-limited cells identified a potential transcriptional start site
34 bp upstream of the translational start site (Fig. 3A). However,
the same analysis with primer 8 was unable to identify any
transcriptional start site within the junction between the two
genes, even when total RNAs from both iron-limited and iron-
sufficient cells were used (data not shown). Therefore, we
assumed that the \textit{pvuA} gene might be cotranscribed with the
\textit{psuA} gene under iron-limited conditions.

\textbf{Identification of the transcript by Northern blot analysis.}

To clarify the aforementioned assumption, Northern blot anal-
ysis was performed for the same total RNA preparations as in
primer extension analysis. The blots were probed with AlkPhos-labeled probe A, internal to \textit{psuA} (a), and AlkPhos-labeled probe B, internal to \textit{pvuA} (b). Positions of RNA stan-
dards (in kilobases) are shown on the right.

\textbf{Expression of \textit{pvuA} in an \textit{E. coli} background.} Contrary to
our finding that the \textit{psuA} gene has no promoter, Western
blotting of the outer membrane preparation from \textit{E. coli}
JM109 carrying pVPV2995 [pBluescript II KS(+) containing the
chromosomal \textit{Eco}RI-SacI fragment in the DNA sequence ladder of the noncoding strand] revealed the production of a
small amount of PsuA irrespective of iron status in the growth
medium (data not shown). However, \textit{E. coli} JM109 carrying
pBluescript II SK(+) containing the \textit{Eco}RI-SacI fragment of
pVPV2995 in the opposite orientation relative to the \textit{lac}
operator produced no PsuA, indicating that \textit{pvuA} has no pro-
moter functional in an \textit{E. coli} background and therefore that
the production of PsuA by \textit{E. coli} JM109 carrying pVPV2995,
the derivative of pBluescript II KS(+), may be due to leaky
activity of the \textit{lac} promoter. Taking these results into consid-
eration, we attempted to reconstruct the entire \textit{psuA} and \textit{pvuA}
genesis including the promoter region in a single high-copy-
number plasmid to examine the iron-regulated production of
the PvuA protein by Western blotting. However, such a plas-
mid was not obtained, suggesting that some overproduction of
PsuA and/or PvuA may be toxic to \textit{E. coli}. Thus, pVPV4780R
was constructed by means of low-copy-number plasmid
pMW118, into which the \textit{Sac}I-SacI fragment from pVPV4780
was inserted in the orientation opposite to that of the \textit{lac}
promoter to transcribe \textit{psuA} and \textit{pvuA} from the promoter
upstream of \textit{psuA}. \textit{E. coli} JM109 carrying either pMW118 itself
or pVPV4780R was then grown under iron-limited-condi-
tions, and the outer membrane preparations were analyzed by
SDS-PAGE and Western blotting. Although the outer mem-
brane proteins expressed by the host strain itself hindered
observing any apparent difference in the SDS-PAGE band
profile between these two outer membrane preparations (Fig.
6A, lanes 2 and 3), a band reactive with the antisera against
the \textit{V. parahaemolyticus} \textit{Eco}RI fragment at 78-kDa outer membrane protein
was seen in \textit{E. coli} JM109 carrying pVPV4780R (Fig. 6B, lane 3). In contrast, \textit{E. coli} JM109 carrying pMW118 displayed no corresponding band (Fig. 6B, lane 2). These results proved that in an \textit{E. coli} background also the potential Fur box located
upstream of \textit{psuA} can mediate iron regulation, leading to the
transcription of these two genes as a bicistronic message.

\textbf{Characterization of the \textit{psuA} and \textit{pvuA} mutants.} Gene-disrup-
ted mutants VPTF1 and VPTF2 were created with suicide
plasmids pTF1 and pTF2, respectively. Table 2 shows the abil-
ities of the wild-type and the mutant strains to utilize vibrioferrin
or ferrichrome for growth. Wild-type strain AQ3354 grew
well even without supplementation of vibrioferrin under the
iron-restricted conditions imposed by EDDA, whereas mutant
strains VPTF1 and VPTF2 as well as MY-1, a spontaneous
vibrioferrin-deficient mutant of wild-type AQ3354 (71), failed
to grow under the same conditions. The result for MY-1 indi-
cates that vibrioferrin can capture iron chelated to EDDA
and deliver it to the cytoplasm. Failure of VPTF1 to grow under
the same conditions as those for VPTF2 suggested a polar effect
of the \textit{psuA} disruption on the transcription of downstream gene
\textit{pvuA}. However, \textit{psuA} mutant VPTF1 was still able to utilize
ferrichrome iron, at least suggesting that the \textit{psuA} gene is not
responsible for the utilization of ferrichrome iron.

To confirm by Western blotting that disruptants VPTF1 and
VPTF2 indeed lack the 78-kDa protein, their outer membrane
proteins were prepared from a culture treated as follows. The
culture was grown in LB broth to an \textit{A}_{660} of 0.3 and was split
into aliquots; one aliquot was left untreated, and the other was
supplemented with EDDA at a final concentration of 100 \mu M
before adding a sufficient amount of vibrioferrin to achieve
iron depletion. Then, both aliquots were further incubated until an \textit{A}_{660} of 0.5 was reached. SDS-PAGE anal-
alysis of the outer membrane proteins from these disruptants
revealed loss of the 78-kDa protein band, in contrast to appar-
tent induction of the 83-kDa protein in response to suddenly
imposed iron restriction (Fig. 6A, lanes 2 and 5). Consistent
with this result, no band corresponding to the 78-kDa protein
was detected by Western blotting (Fig. 6B, lanes 4 and 5).

For complementation analysis, \textit{psuA} mutant VPTF2 was
transformed with either pRK415 or pRKVP4.8, a pRK415
derivative bearing an intact copy of the \textit{psuA} and \textit{pvuA} genes.
Growth of transformant VPTF2 (pRKVP4.8) regardless of the
supplementation with vibrioferrin was restored to about 60%
of the level of wild-type AQ3354, whereas that of the mock transformant was not restored at all (Table 2). Incomplete restoration observed for VPTF2(pRKVP4.8) may be due to the maintenance of a relatively large plasmid (ca. 15 kb) with concomitant expression of the two antibiotic resistance genes. Consistent with the results of growth complementation assay, both SDS-PAGE and Western blotting of the outer membrane preparation from VPTF2(pRKVP4.8) cells grown under iron-limited conditions disclosed the expression of the 78-kDa protein (Fig. 6, lane 6). The mock transformant induced only the 83-kDa protein (Fig. 6, lane 7). Moreover, growth of VPTF1 and VPTF2 was not complemented with pRKVP3, a pRK415 derivative bearing an intact copy of the pvuA gene (data not shown), indicating that the pvuA gene has no promoter. This constitutes better proof that the insertion mutation in the pvuA gene was polar to pvuA expression. These results clearly demonstrated that the pvuA gene in fact encodes the 78-kDa receptor protein necessary for ferric vibrioferrin utilization.

**Distribution of pvuA in other Vibrio species.** Southern blot analysis with DIG-labeled probe B (Fig. 1C) prepared with primers 3 and 4 internal to pvuA was performed to detect homologous pvuA genes in other pathogenic Vibrio species. Genomic DNAs from Vibrio species were completely digested with SacI, and hybridization was carried out under a stringent conditions (at 68°C). All of the seven other *V. parahaemolyticus* clinical and environmental isolates and the four *Vibrio alginolyticus* environmental isolates tested exhibited a single hybridization band of ca. 5.2 kb, similar to the WP1 strain. However, the American Type Culture Collection type strains of *V. cholerae*, *Vibrio mimicus*, *Vibrio vulnificus*, *Vibrio furnissii*, *Vibrio fluvialis*, and *Vibrio hollisae* gave no signal (data not shown), demonstrating that the pvuA gene occurs exclusively in *V. parahaemolyticus* and *V. alginolyticus*. The detection of pvuA in *V. alginolyticus* is reinforced by the fact that the strains of this species produced vibrioferrin and expressed the iron-repressible outer membrane protein, which strongly cross-reacted with the antiserum against the 78-kDa *V. parahaemolyticus* outer membrane protein (68).

**DISCUSSION**

Siderophores detected to date in pathogenic vibrios have displayed considerable structural diversity based on their main chelating groups, but *Vibrio* species generally produce a single cognate siderophore. *V. cholerae* (19), *V. fluvialis* (73), and *V. vulnificus* (47) produce catecholate-type siderophores, all of which characteristically contain a norspermidine (one of the unusual polyamines) moiety as a framework. We also found

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**TABLE 2. Utilization of vibrioferrin and ferrichrome by *V. parahaemolyticus* AQ3354 and its mutant strains**

<table>
<thead>
<tr>
<th>LB broth supplement</th>
<th>Growth of indicator strain:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AQ3354</td>
</tr>
<tr>
<td>EDDA (25 μM)</td>
<td>++</td>
</tr>
<tr>
<td>EDDA (25 μM) + 20 μM vibrioferrin</td>
<td>++</td>
</tr>
<tr>
<td>EDDA (25 μM) + 20 μM ferrichrome</td>
<td>++</td>
</tr>
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<sup>a</sup> $A_{660}$ of the culture after incubation for 25 h at 37°C was measured except for the AQ3354 culture, whose $A_{660}$ was measured after incubation for 17 h. ++, $A_{660} = 0.77$ to 0.94; +, $A_{660} = 0.55$ to 0.59; −, no growth; ND, not determined. The data from three independent experiments are shown.

<sup>b</sup> MY-1 is a spontaneous vibrioferrin-deficient mutant derived from wild-type AQ3354 (71).
that *V. furnissii* produces the same catecholate as *V. fluvialis*. Anguibactin, a member of a unique structural class of plasmid-mediated siderophores isolated from *Vibrio anguillarum*, possesses both catecholate and hydroxamate functional groups (25). Interestingly, *V. mimicus* and *V. hollisae* produce hydroxamate-type siderophore aerobactin (48), which is mainly found in the species *Enterobacteriaceae* (49). In contrast, *V. parahaemolyticus*, as well as *V. alginolyticus*, produces structurally novel siderophore vibrioferrin (68, 72), which is classified as a citrate-based polyhydroxyxcarboxylate along with staphyloferrins A and B in staphylococcal species (14, 32) and rhizoferrin in fungus *Rhizopus microsporus* and other Zygomycetes (13). Transport components for this type of siderophores in the producer strains have been poorly characterized at the gene level, although genes *rumAB*, essential for uptake of ferric rhizoferrin as an exogenous siderophore, have been characterized in *M. morganii* (33).

In this study, by a combination of the FURTA system to isolate the Fur box-containing gene fragments with information on the N-terminal amino acid sequences of the iron-repressible outer membrane proteins, we identified *V. parahaemolyticus* *psuA* and *pvuA* genes, which encode the TonB-dependent outer membrane receptors for a putative ferric siderophore and ferric vibrioferrin, respectively. The same genetic approach using the FURTA system led to the recent identification of Fur-repressed genes in bacteria other than *E. coli*, for example, in *Bordetella*, *Salmonella*, and *Vibrio* spp. (5, 7, 17, 31, 51, 52, 64). A sequence homologous to the *E. coli* Fur box consensus was also detected upstream of *psuA*. Primer extension analysis of mRNA from *V. parahaemolyticus* grown under iron-limited or iron-sufficient conditions defined a transcriptional start site adjacent to the proposed Fur box and demonstrated iron regulation of these genes at the transcriptional level. The iron regulation of these genes through the Fur box was consistent with the FURTA-positive phenotype of the isolated clone and with the constitutive expression of the iron-repressible outer membrane proteins, including the 78-kDa protein in the manganese-resistant mutants (*fur* mutants) (16). Examination of the size of the *psuA-pvuA* transcript by Northern blot analysis indicated that these genes are cotranscribed as a single unit from a Fur-repressed promoter and that the inverted repeat located just downstream of the second gene, *pvuA*, functions as a transcriptional termination signal. Such an operon comprising the two different siderophore receptor genes whose transcription is controlled by a common Fur box is unique, since many of the siderophore receptor genes characterized to date are always arranged in an operon with the relevant genes encoding the other iron transport components and the siderophore biosynthesis enzymes (6). However, genetic organization around the *psuA* and *pvuA* genes appeared to be conserved among the *V. parahaemolyticus* strains tested. In all of the seven other strains tested, the probe was hybridized with the SacI fragments of a similar size, suggesting that this operon may play an important role in iron assimilation of this species.

On the other hand, there is a stem-loop structure located 68 nucleotides downstream of the *psuA* translational stop codon, which seems to fit the criteria for a potential rho-independent transcriptional termination site (54). However, either this transcriptional termination site may be inefficient or there may be an antitermination mechanism operating only under iron-limited conditions, since Northern blot analysis showed that the *psuA* and *pvuA* genes are organized in an operon structure. Interestingly, two pairs of seven-nucleotide direct repeat sequences, 5'-TTTTGCC-3' and 5'-ATGTTTT-3', are identifiable in the same intergenic region. They seemed to be typical of sites of the binding of an antitermination factor to the mRNA under transcription, and binding may prevent the formation of the termination structure (42, 58). However, since *E. coli* JM109 carrying pVPVS4785R also expressed the PvuA protein under iron-limited conditions, it seems unlikely that the antitermination mechanism, if one exists, is specific to *V. parahaemolyticus*. Further studies are needed to clarify possible functions of these characteristic sequences.

Because of the similarity of PvuA to the putative ferrichrome iron receptor of *Synechocystis* species and because of the ability of *V. parahaemolyticus* to utilize ferrichrome as an exogenous siderophore, we hypothesized that *psuA* was the ferrichrome iron receptor gene. The disruption of this gene, however, did not affect ferrichrome utilization. Since the PsvA protein is in fact expressed by *V. parahaemolyticus*, it is not unexpected that this protein may be a receptor for a ferric siderophore of unknown origin. Therefore, the present study suggests that *V. parahaemolyticus* has two other siderophore-mediated iron acquisition systems, which may be associated with ferrichrome and aerobactin. Recent studies have demonstrated that, besides their cognate siderophores, many bacteria can utilize a wide variety of different exogenous siderophores (6, 8, 11, 33). This strategy may enhance the organism’s ability to acquire iron under a range of environmental conditions and reflects the importance of iron competition in the natural habitat of the bacteria. An operon required for utilization of ferrichrome as an exogenous siderophore has recently been identified in *V. cholerae* (55). Studies in our laboratory to isolate and characterize the genes involved in the ferrichrome- and aerobactin-mediated iron acquisition systems in *V. parahaemolyticus* are under way.

Presumptive proof for the function of *pvuA* in ferric vibrioferrin utilization was obtained by phenotypic analysis of *V. parahaemolyticus* *pvuA* insertion mutants VPTF1 and VPTF2. These mutants were incapable of utilizing ferric vibrioferrin in the growth assay and of expressing the 78-kDa iron-repressible outer membrane protein. Moreover, the *psuA* insertion mutation resulted in a polar effect on *pvuA* expression, confirming that these two genes are organized in an operon structure. Introduction of pRKVP4.8, containing the wild-type *psuA-pvuA* locus, into mutant VPTF2 restored its abilities to utilize vibrioferrin and to express the 78-kDa protein for growth under iron-limiting conditions.

Genetic and biochemical evidence presented in this study corroborates our previous identification of the 78-kDa outer membrane protein as the receptor in the vibrioferrin-mediated iron uptake system of *V. parahaemolyticus*. At the same time, FURTA methodology originally established for *E. coli* has proven to be an effective approach to isolate the iron-regulated genes in *V. parahaemolyticus*. DNA probes internal to the isolated Fur box-containing gene fragments will be very useful in exploring the related neighboring genes by gene walking. Since siderophore biosynthesis genes as well as ferric siderophore transport genes are frequently organized in an operon struc-
ture or are closely related, further investigation into the regions upstream and downstream of the psaA-psaA operon may disclose the genes necessary for vibrioferrin biosynthesis and uptake of ferric vibrioferrin into the cytoplasm.

ACKNOWLEDGMENTS

We are indebted to I. Stojiljkovic and S. H. Choi for providing E. coli H7177 and pRK415, respectively. We thank H. Yamada for determining the N-terminal amino acid sequences and T. Kuroda for providing H1717 and pRK415, respectively. We thank H. Yamada for determination of ferric vibrioferrin into the cytoplasm.

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