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Identification of the Vibriobactin Receptor of *Vibrio cholerae*

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Vibrio cholerae **produces the novel phenolate siderophore vibriobactin and several outer membrane proteins in response to iron starvation. To determine whether any of these iron-regulated outer membrane proteins serves as the receptor for vibriobactin, the classical *V. cholerae* strain 0395 was mutagenized by using Tn*phoA*, and iron-regulated fusions were analyzed for vibriobactin transport. One mutant, MBG14, was unable to bind or utilize exogenous vibriobactin and did not grow in low-iron medium. However, synthesis of the siderophore and transport of other iron complexes, including ferrichrome, hemin, and ferric citrate, were unaffected in MBG14. Analysis of membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated the loss from the mutant of a 74-kDa iron-regulated outer membrane protein present in the parental strain when grown in iron-limiting conditions. This protein partitioned into the detergent phase during Triton X-114 extraction, suggesting that it is a hydrophobic membrane protein. DNA sequences encoding the gene into which Tn*phoA* had inserted, designated *viuA* (vibriobactin uptake), restored the wild-type phenotype to the mutant; the complemented mutant expressed the 74-kDa outer membrane protein under iron-limiting conditions and possessed normal vibriobactin binding and uptake. These data indicate that the 74-kDa outer membrane protein of *V. cholerae* serves as the vibriobactin receptor.**

Vibrio cholerae, the causative agent of cholera, requires iron for growth and survival. However, this element is not freely available either in the environment or within a human host. To acquire sufficient iron, *V. cholerae* depends on one or more high-affinity transport systems which function in iron uptake in vivo and in vitro. In iron-limiting environments, the organism secretes vibriobactin, a high-affinity, iron-binding catechol which can solubilize iron and transport it into the cell (10, 16). Synthesis of vibriobactin is tightly regulated by iron, and the compound is not detected in supernatants of iron-sufficient cultures (20). Additionally, *V. cholerae* can utilize ferric citrate (21) and possesses a heme-iron transport system which allows the organism to utilize iron contained in heme or hemoglobin (24). The heme transport system may be important during colonization and infection of the host.

Iron restriction also serves as a regulatory signal for other genes in *V. cholerae*. The production of a cytotoxic hemolysin and synthesis of several outer membrane proteins (9, 19, 20, 24) are induced in low-iron cultures. The hemolysin may aid in iron acquisition in vivo by liberating host intracellular iron compounds, such as heme and hemoglobin, which can be used by the bacterium. Synthesis of the hemolysin, like that of vibriobactin, is repressed by iron, and both systems are regulated by a Fur-like protein (24). The *Vibrio* Fur system is functionally analogous to the *Escherichia coli* Fur (4) system, and a recombinant plasmid containing the *E. coli fur* sequences complements a *V. cholerae* mutation which resulted in a loss of the ability to repress synthesis of the hemolysin and vibriobactin in the presence of iron (24).

The iron-regulated outer membrane proteins include a group of five proteins ranging in size from 62 to 77 kDa and a 220-kDa protein (20). These proteins were detected in in vitro-grown cells of both classical and El Tor biotypes. Additionally, Sciortino and Finkelstein (19) have shown that iron-regulated outer membrane proteins ranging in size from 16 to 68 kDa are expressed in vivo, and Amaro et al. (1) demonstrated the presence of outer membrane proteins ranging in size from 70 to 78 kDa in iron-starved O1 and non-O1 *V. cholerae*. The functions of these iron-regulated outer membrane proteins are unknown, although at least one is required for virulence. Goldberg et al. (9) have demonstrated that the loss of the 77-kDa protein results in reduced virulence in mice. Although this protein is iron regulated, it does not appear to be involved in iron transport. The mutant shows normal transport of vibriobactin, heme, ferrichrome, and ferric citrate, and growth was comparable to that of the wild type in low-iron media (8). It is likely that one or more of the other proteins functions as a receptor(s) for these iron complexes. This study was undertaken to identify the iron-regulated outer membrane protein which is the receptor for ferric vibriobactin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The classical *V. cholerae* strain 0395 and the Tn*phoA* insertion mutant strains derived from it have been described previously (9). *V. cholerae* Lou1510 (10) is a vibriobactin transport-deficient El Tor strain used for the production of vibriobactin. Plasmid pJRB15 contains 0395 sequences encoding *viuA* cloned into pUC18 (6). All strains were maintained at −80°C in L broth plus 20% glycerol.

Media and reagents. Strains were grown on L agar or in L broth at 37°C. Kanamycin (50 μg/ml) was added to cultures of strains containing Tn*phoA* insertions, and carbenicillin (250 μg/ml) was added to cultures of cells containing pJRB15. The iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) (Sigma Chemical Co.), deferrated by the method of Rogers (18), was added to L broth or

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L agar to induce iron limitation. Tris-buffered minimal medium (T medium) (22) without added iron was used as described previously (20) to assay for siderophore production, to determine growth in low-iron medium, and in vibriobactin binding assays. Hemin was obtained from Sigma Chemical Co., and ferrichrome was a gift from Paul Szaniszlo, University of Texas, Austin.

Growth stimulation assay. Strains were grown overnight at 37°C in L broth and washed once in saline. L agar containing EDDA (75 μg/ml) was inoculated with 10^4 bacteria per ml and poured into plates. Solutions of iron complexes or fully grown bacterial cultures were spotted (10-μl drops) onto the surface of the hardened agar and allowed to dry. The plates were incubated for 18 to 24 h and then observed for zones of growth around each compound. Iron compounds or siderophores tested were $FeSO_4$ (10 mM), hemin (8 mM), vibriobactin (2 mM), and ferrichrome (1 μM).

Analysis of membrane proteins. *V. cholerae* cells were fractionated to prepare outer membranes as previously described (20). Overnight cultures were diluted 1:100 into L broth and grown to stationary phase. To induce iron starvation, EDDA (100 μg/ml) was added when cells reached an A_{650} of 0.5. The procedure of Inouye and Guthrie (12) was used to prepare total cell envelopes. Outer membranes were isolated by the method of Filip et al. (7) by using 1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) for 20 min to solubilize the inner membrane. Outer membrane fractions were solubilized in Laemmli solubilization buffer (13) and heated at 100°C for 3 min, and the polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Alternatively, Triton X-114 (5, 17) was used to extract surface proteins from cells grown in either high- or low-iron media as follows. Cultures were grown to stationary phase as described above, 1.5-ml aliquots were centrifuged, and the pellets were resuspended in 100 μl of ice-cold 100 mM Tris buffer, pH 7.5. One hundred microliters of ice-cold 2% Triton X-114 was added, and the mixture was vortexed gently and kept on ice for 20 min. The mixture was vortexed gently two additional times during incubation and then centrifuged at 4°C for 10 min. The supernatant was transferred to a new tube, placed in a 37°C water bath for 10 min, and then centrifuged at room temperature for 5 min. To separate the lower detergent-enriched (hydrophobic) phase and the upper detergent-depleted (hydrophilic) phase, each phase was transferred to a new tube and washed as follows. Seven microliters of 20% Triton X-114 (room temperature) was added to the hydrophilic phase and mixed. Fifty microliters of 100 mM Tris (room temperature) was added to the hydrophobic phase and mixed. Each tube was reincubated at 37°C for 10 min and then centrifuged for 5 min at room temperature. The Tris wash of the hydrophobic phase was discarded, and the detergent wash of the hydrophilic phase was discarded. The procedure was repeated once more for a total of two washes. Proteins were precipitated by adding 4 volumes of acetone and incubating at −20°C for 2 to 4 h. Samples were centrifuged at 4°C for 10 min to pellet proteins. The proteins were resuspended in 50 to 100 μl of sterile deionized water. An equal volume of 2× Laemmli solubilization buffer (13) was added, the mixture was boiled for 5 min, and samples were analyzed by SDS-PAGE.

SDS-PAGE of outer membranes. Membrane polypeptides were separated by the SDS-PAGE system of Ames (2) as described previously (20). Stacking and separating gels consisted of 4% (wt/vol) and 12% (wt/vol) acrylamide, respectively. Gels were stained with Coomassie brilliant blue R (Sigma), destained, and dried. Protein standards (molecular weights) were phosphorylase *b* (94,000), albumin (67,000), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lactalbumin (14,400) (Pharmacia Fine Chemicals, Piscataway, N.J.).

Isolation of vibriobactin. *V. cholerae* Lou1510 was grown in low-iron T medium at 37°C for 24 h, and vibriobactin was extracted from the culture supernatant according to the procedure of Griffiths et al. (10). Purified vibriobactin was dissolved in methanol and stored at −20°C. Activity of purified vibriobactin was verified by measuring its ability to stimulate growth of iron-starved *V. cholerae* 0395 in a bioassay.

^{55}Fe-vibriobactin binding to outer membrane proteins. Binding of ferric vibriobactin to purified outer membrane proteins was performed by a modification of the procedure of Ichihara and Mizushima (11). Cells were grown overnight in L broth with or without EDDA, and outer membranes were isolated by the Sarkosyl procedure as described above. Outer membranes were resuspended in distilled H_2O and stored at −20°C. Protein concentrations were determined by using the Bio-Rad microprotein assay, and 100 μg of protein was used for each binding assay.

Purified vibriobactin was labeled with $^{55}FeCl_3$ as follows. Ten microliters each of 100 μM nitrilotriacetate, 2 mM vibriobactin, and 1 mM $^{55}FeCl_3$ was mixed in 5 ml of T medium (22). The mixture was incubated at 37°C for 45 min, filtered through a Millipore filter, and stored at −20°C in a polypropylene tube. The specific activity of the labeled vibriobactin was 560 cpm/pmol. For each assay, 0.3 ml of ^{55}Fe-vibriobactin was warmed to 37°C, added to the outer membrane sample suspended in an equal volume of T medium, and incubated for 5 min at 37°C. The mixtures were filtered through a Millipore filter (0.45 μm pore size; presoaked in 10 mM EDTA, pH 7.0) and washed with T medium. The filters were air dried and counted in scintillation fluid.

RESULTS

Characterization of Tn*phoA* fusions in iron-regulated genes of *V. cholerae*. Classical *V. cholerae* strain 0395 was mutagenized with the transposon vector Tn*phoA* (14), and phosphatase expression was monitored to detect fusion of the *phoA* sequences to genes encoding membrane or secreted proteins. The construction and isolation of these Tn*phoA* insertion mutant strains have been described previously (9).

Insertion mutants containing iron-regulated gene fusions were obtained and analyzed for vibriobactin transport by using a growth stimulation assay. One strain, MBG14, failed to utilize exogenous vibriobactin for growth in iron-restricted medium, and its growth was not stimulated by the vibriobactin-producing parental strain (Table 1). This mutant produced vibriobactin as determined by the Arnow (3) assay for catechols (23) and was able to stimulate growth of the parental strain 0395 (Table 1), indicating that the Tn*phoA* fusion had not interrupted any vibriobactin synthesis genes. The mutant was assayed for utilization of other iron compounds. It was found to utilize exogenous iron, hemin, and ferrichrome for growth in the growth stimulation assay (Table 1) and could grow with ferric citrate as the sole iron source in T medium (23). The ability to grow with all the iron sources except vibriobactin indicated that the mutation was specific to vibriobactin transport and was not a more general transport defect such as a *tonB*-like mutation. This mutation was designated *viuA*, for vibriobactin uptake.

TABLE 1. Growth stimulation of *V. cholerae* strains by producer strains and various iron sources

Producer strain or iron compound	Zone of growth of indicator strain[a]	
	0395	MBG14
0395	26	0
MBG14	20	0
Vibriobactin	30	0
Ferrichrome	34	30
Hemin	33	32
Fe	16	15

[a] Diameter (in millimeters) of zone of growth around the producer strain or iron source.

Growth of 0395 and MBG14. To determine the effect of the mutation on growth, strains 0395 and MBG14 were grown in L broth with or without EDDA. Both parental 0395 and mutant MBG14 grew equally well under iron-replete conditions (23). A striking difference was observed, however, when the two strains were grown under iron restriction. When grown in L broth plus EDDA, MBG14 grew poorly, whereas parental 0395 in the presence of EDDA grew to a density comparable to that in L broth (23).

^{55}Fe binding to *V. cholerae* outer membrane proteins. If the insertion in MBG14 interrupted the gene for the vibriobactin receptor, the mutant should lack the ability to bind vibriobactin to the outer membrane. Outer membranes were isolated from the wild-type and mutant strains and assayed for the ability to bind ^{55}Fe-labeled vibriobactin (Table 2). Binding of vibriobactin to the parental strain was increased when the cells were starved for iron, indicating that iron limitation derepressed expression of the receptor. Levels of siderophore binding to the outer membrane were similar to those observed for the binding of enterobactin to the outer membrane of *E. coli* (15). In contrast, there was no increased binding when the mutant was starved for iron. Thus, the defect in the mutant is in the ability to bind ferric vibriobactin to the outer membrane.

A plasmid containing the wild-type *viuA* gene, pJRB15 (6), was introduced into the mutant to determine whether it would restore vibriobactin binding (Table 2). The presence of *viuA* on a high-copy-number vector resulted in high levels of vibriobactin binding to the outer membrane. Binding was reduced to background levels when the recombinant strain was grown in the presence of excess iron, indicating that the cloned gene was regulated in the same manner as the chromosomal locus.

Characterization of envelope proteins produced by MBG14. In response to iron starvation, *V. cholerae* produces increased amounts of several outer membrane proteins, one of which could serve as the receptor for vibriobactin. Therefore, the outer membrane protein profiles of 0395 and MBG14 were compared to determine whether a loss of vibriobactin transport was associated with a loss of a specific outer membrane protein. Previous studies have shown that the Sarkosyl procedure can be used to identify outer membrane proteins of *V. cholerae* (20). Therefore, cells were grown in L broth or L broth plus EDDA, and the outer membrane proteins obtained from Sarkosyl-solubilized total envelopes were analyzed by SDS-PAGE. Unlike the parental strain 0395, MBG14 lacks the 74-kDa iron-repressible outer membrane protein (Fig. 1). A 62-kDa protein was also absent in some membrane preparations from the mutant strain. However, expression of this protein appears to be influenced by the growth phase of the mutant, and it was present in some preparations (Fig. 2), whereas the 74-kDa protein was uniformly absent.

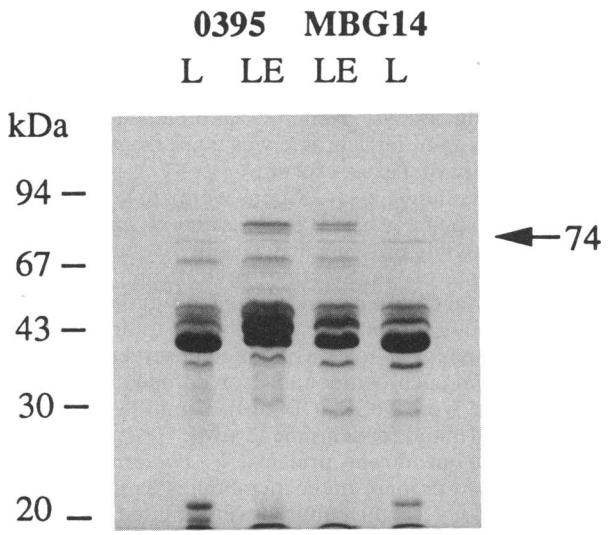

FIG. 1. Outer membrane proteins of *V. cholerae* 0395 and MBG14 grown in high- and low-iron media. Lanes (from left to right): L, 0395 grown in high-iron L broth; LE, 0395 grown in low-iron L broth plus EDDA; LE, MBG14 grown in low-iron L broth plus EDDA; L, MBG14 grown in high-iron L broth. Arrow indicates the 74-kDa protein which is absent from MBG14. Sizes and positions of protein standards are shown at the left.

Envelope proteins were also extracted with the detergent Triton X-114 under conditions in which cell lysis did not occur (Fig. 2). Surface proteins are extracted by this procedure, and the extracted proteins then partition into either a hydrophilic, detergent-depleted phase or a hydrophobic, detergent-enriched phase. The major outer membrane protein and at least four of the iron-regulated outer membrane proteins (77, 76, 74, and 62 kDa) partitioned into the detergent-enriched phase, indicating that they are hydrophobic in nature. Again, it was observed that the 74-kDa iron-repressible protein was missing in mutant MBG14. Other *V. cholerae* 0395 mutants containing Tn*phoA* insertions which did not disrupt vibriobactin transport were similarly analyzed, and all produced a 74-kDa hydrophobic membrane protein (23). Only MBG14 lacked the 74-kDa protein.

When mutant MBG14 was supplied with the wild-type *viuA* encoded by pJRB15, the 74-kDa protein was restored (Fig. 3). Levels of the protein in the outer membrane were somewhat lower than might have been expected from a high-copy-number plasmid, and increased amounts of a

TABLE 2. Binding of ferric vibriobactin to outer membranes of *V. cholerae*

Strain	Growth medium	^{55}Fe-vibriobactin bound[a]
0395	+Fe	3,668
0395	−Fe	6,646
MBG14	+Fe	2,741
MBG14	−Fe	2,875
MBG14/pJRB15	+Fe	2,058
MBG14/pJRB15	−Fe	29,502

[a] Counts per minute per 100 μg of total membrane protein.

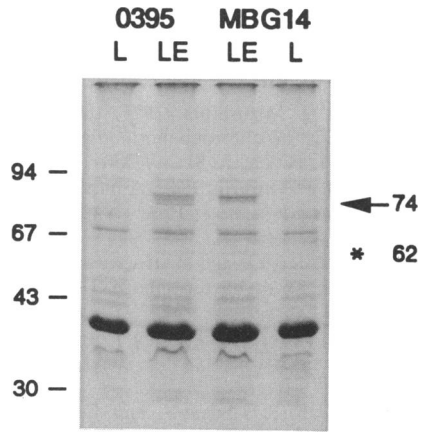

FIG. 2. Hydrophobic membrane proteins of *V. cholerae* 0395 and MBG14 grown in high- and low-iron media. Lanes (from left to right): L, 0395 grown in high-iron L broth; LE, 0395 grown in low-iron L broth plus EDDA; LE, MBG14 grown in low-iron L broth plus EDDA; L, MBG14 grown in high-iron L broth. Hydrophobic membrane proteins were isolated by using the detergent Triton X-114. Arrow points to the 74-kDa protein, which is missing in MBG14. Asterisk indicates the position of the 62-kDa protein present in both 0395 and MBG14 grown under low-iron conditions. Sizes and positions of protein standards are shown at the left.

70-kDa protein are seen. It is not known how overexpression of *viuA* affects the levels of other membrane proteins or whether the 70-kDa protein is a degraded or processed form of the 74-kDa species. It is unlikely, however, that the 70-kDa protein is a separate protein encoded by pJRB15 since the sequencing data indicate that only a single open reading frame is contained on pJRB15 (6). The expression of the protein from the cloned sequences, like vibriobactin binding, was repressed by iron (23), indicating that the promoter and essential regulatory sequences are also contained on the cloned DNA fragment.

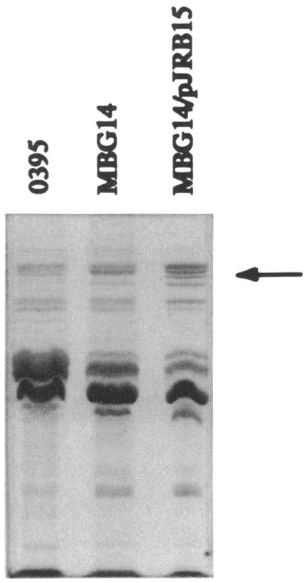

FIG. 3. Outer membrane proteins of *V. cholerae* 0395, MBG14, and MBG14/pJRB15 grown in low-iron L broth plus EDDA. Arrow points to the 74-kDa protein, which is restored by plasmid pJRB15.

The ability to bind and transport vibriobactin, therefore, correlated with the expression of the iron-regulated 74-kDa protein alone. Since MBG14 fails to bind or utilize exogenous vibriobactin and does not grow in low-iron media, these data indicate that the 74-kDa protein is required for vibriobactin transport in *V. cholerae* and acts as the receptor for ferric vibriobactin complexes.

DISCUSSION

Microorganisms have the ability to alter their metabolism rapidly in response to environmental changes. In response to iron deprivation, *V. cholerae* derepresses synthesis of a number of proteins, some of which are involved in iron acquisition. These include proteins involved in siderophore-mediated iron transport and heme-iron uptake as well as a number of outer membrane proteins.

The outer membrane proteins produced in response to iron deprivation presumably include surface receptors required for transport of iron complexes, but specific functions have not been assigned previously to any of these iron-regulated membrane proteins. The 77-kDa outer membrane protein, IrgA, is required for virulence, but its mechanism of action in vivo is unknown (8, 9). Although this protein is iron regulated, it does not appear to be involved in iron transport; MBG40, a Tn*phoA* insertion mutant lacking the 77-kDa protein, has no defect in growth in low-iron media, and transport of vibriobactin, heme, and ferric citrate in MBG40 is normal (8).

Analysis of additional Tn*phoA* mutants of *V. cholerae* 0395 indicated that one, MBG14, contained an iron-regulated gene fusion which eliminated the ability of this strain to grow in low-iron media. This mutant synthesized vibriobactin but failed to utilize exogenously supplied vibriobactin in a growth stimulation assay, indicating that the defect was in transport of the siderophore. Although MBG14 could not use vibriobactin, it could transport and utilize ferrichrome, heme, and ferric citrate for growth. Thus, the mutation in this strain was specific for vibriobactin and not in a general transport function, like *tonB*.

The failure to utilize vibriobactin was associated with the inability of the mutant to bind the siderophore to the outer membrane. Outer membranes isolated from the mutant failed to bind ^{55}Fe-vibriobactin, while those from the parent strain bound the siderophore. The introduction of a plasmid containing the wild-type *viuA* gene restored the ability of the mutant to bind the ferric siderophore. Subjecting wild-type cells to iron starvation increased binding, as would be predicted for an iron-regulated receptor.

Analysis of proteins of the mutant revealed the absence of a 74-kDa, iron-regulated outer membrane protein present in wild-type *V. cholerae*. Complementation of the mutant with *viuA* in *trans* restored synthesis of the 74-kDa protein and restored the ability to bind ferric vibriobactin. Previous studies have demonstrated the presence of an iron-regulated protein of 73 to 74 kDa in clinical isolates of both classical and El Tor biotypes (20), environmental El Tor strains (20), and non-O1 *V. cholerae* (1), all of which synthesize and use vibriobactin (1, 20, 24). Amaro et al. (1) noted that the 74-kDa protein was consistently derepressed by iron starvation in all the non-O1 strains tested. In the classical strain used in this study, the 74-kDa protein appears to be located on the cell surface; it is found in the outer membrane fraction, and it can be extracted into the detergent Triton

X-114 in the absence of cell lysis. The protein is hydrophobic in nature, as it partitions into the detergent phase during Triton X-114 extraction. Because the loss of expression of the 74-kDa protein correlated with the failure to utilize vibriobactin and the inability to bind vibriobactin to the outer membrane, we propose that this iron-regulated outer membrane protein of *V. cholerae* is the vibriobactin receptor.

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