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# Outer Membrane Proteins Induced Under Conditions of Iron Limitation in the Marine Fish Pathogen *Vibrio anguillarum* 775

JORGE H. CROSA†* AND LINDA L. HODGES

*Department of Microbiology and Immunology, School of Medicine, University of Washington, Seattle, Washington 98195*

Cells of the marine fish pathogen *Vibrio anguillarum* 775 harboring a plasmid associated with virulence can grow unaffected in the presence of iron-binding compounds such as transferrin. In contrast, the growth of isogenic plasmidless derivatives is inhibited by the presence of iron chelators. Radioactive iron ($^{55}Fe^{3+}$) uptake experiments indicate that this plasmid-linked ability of *V. anguillarum* cells to grow under conditions of iron limitation is indeed due to a more rapid and efficient iron uptake mediated by the virulence plasmid. In addition, *V. anguillarum* cells growing under iron limitation show at least two novel outer membrane proteins. One of them, a 86,000-dalton protein we called OM2, is inducible only in those cells in which the virulence plasmid is present.

Vibriosis is one of the most devastating diseases affecting salmonids and many other species of fish throughout the world (2, 9, 17, 18). Several biotypes of the causative agent, *Vibrio anguillarum*, can be isolated from epizootics (11, 19), although our past work has been concentrated on the so-called biotype I.

We have reported that many of the virulent strains of *V. anguillarum* biotype I isolated in the Pacific Northwest harbored a specific plasmid class, pJM1, which was absent from low-virulence strains (7). Recently, the transposon A sequence Tn*1* containing the ampicillin resistance determinants was transposed to this plasmid (5). Curing experiments in which plasmid loss was assessed by analysis of the segregation of the ampicillin resistance phenotype showed the association of virulence with the specific *V. anguillarum* plasmid class (5). We also reported that the pJM1 plasmid in *V. anguillarum* mediates a novel virulence mechanism—that of an efficient iron-sequestering system (4). On losing the plasmid, *V. anguillarum* loses its virulence and also its ability to grow under conditions of iron limitation like those imposed by the iron-binding proteins transferrin and lactoferrin in the host fish system. This ability to scavenge bound iron allows the invading bacteria to proliferate in body fluids and tissues (4).

We have now initiated the characterization of the plasmid-mediated iron uptake system in *V. anguillarum*. The results of those preliminary studies indicate that concomitant with an efficient uptake of radioactive iron by plasmid-containing strains, new outer membrane proteins are induced under conditions of iron limitation. At least one of these proteins is produced only when the virulence plasmid is present.

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† Present address: Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, OR 97201.

## MATERIALS AND METHODS

**Bacteria.** The characteristics and sources of the strains of *V. anguillarum* used in this project are described in Table 1. Cured derivatives of plasmid-carrying strains were obtained as described previously (5).

**Measurement of radioactive iron uptake.** Bacterial strains were grown for several generations at 22°C in a low-iron minimal medium (14) (iron content, ~2 μM) supplemented with 0.5% (wt/vol) glucose and the required amino acids: aspartic acid and histidine at 20 μg/ml. Exponentially growing cultures were centrifuged, and cells were washed and resuspended to a density of $0.4 \times 10^8$ cells per ml in similar medium except that the iron content was now less than 0.5 μM. After further incubation for 2 h at 22°C to deplete iron intracellular pools, cultures were centrifuged and cells were washed and resuspended to a density of $4 \times 10^8$ cells per ml in similar medium lacking essential amino acids but containing 100 μM sodium nitrilotriacetate. In some experiments 2 mM KCN was included in this stage. Carrier-free $^{55}FeCl_3$ (1 μCi/ml) was added to shaking cell suspensions, and 1-ml samples were removed at intervals and filtered through membrane filters (0.45-μm pore size; Millipore Corp.). Filters were washed with 100 mM sodium citrate, dried, and counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co.) using a toluene-based scin-

TABLE 1. *Correlation between iron uptake, presence of plasmid, and virulence of strains of V. anguillarum*

| *V. anguillarum* strain | Presence of the virulence plasmid[a] | Uptake of $^{55}Fe$[b] (cpm/min) | $LD_{50}$[c] |
|---|---|---|---|
| 775(pJM1) | + | 11,500 | $1.0 \times 10^3$ |
| 133S(pJM1) | + | 12,000 | $1.3 \times 10^3$ |
| LS174(pJM1) | + | 15,000 | $5.0 \times 10^2$ |
| LS174-WTI | − | 700 | $2.0 \times 10^7$ |
| H775-3 | − | 550 | $3 \times 10^7$ |
| H775-8 | − | 450 | $2.5 \times 10^6$ |
| E775-100 | − | 600 | $2 \times 10^6$ |

[a] Plasmid DNA was determined by an agarose electrophoretic method as previously described (5).

[b] Values were obtained from kinetic experiments similar to that in Fig. 1 by determining the ratio of $^{55}Fe$ counts per minute/time at the 20-min time point.

[c] $LD_{50}$ values were determined as described in the text.

tillation cocktail containing Omnifluor (New England Nuclear Corp.) at 4 g/liter.

**Infectivity studies.** Virulence tests were carried out on juvenile coho salmon (*Oncorhynchus kisutch*) weighing about 15 g, as described in a previous publication (7). Virulence was quantitated as the number of microorganisms that will kill 50% of the animals inoculated ($LD_{50}$ [50% lethal dose]) as determined by the method of Reed and Muench (16).

**Analysis of membrane proteins.** Five-milliliter cultures of bacteria were grown overnight at 22°C in M9 minimal medium containing various concentrations of $FeCl_3$. Cells were harvested by centrifugation, resuspended in 1.5 ml of 10 mM tris (hydroxymethyl)aminomethane (Tris)-hydrochloride–0.3% NaCl (pH 8.0) and sonically treated. After a 60-s centrifugation in a microcentrifuge (Eppendorf) the supernatant was decanted into another tube, and the cell envelopes were centrifuged for 60 min at 20,000 × *g* in a Beckman J2-21 centrifuge. To prepare outer membranes, we treated the cell envelopes with 1.5% Sarkosyl (wt/vol) in distilled water at room temperature for 20 min to dissolve the inner membrane. Outer membrane material was collected by centrifugation (20,000 × *g*, 60 min). This treatment was repeated at least twice. Pellets from total cell envelopes or outer membranes were dissolved in 50 μl of a buffer consisting of 62.5 mM Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.001% bromophenol blue, and 5% beta-mercaptoethanol. Suspensions were boiled for 5 min, and portions were applied to a 12.5% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 44:0.8) in 0.375 M Tris-hydrochloride (pH 8.8) containing 0.2% SDS with a 3% stacking gel in 0.125 M Tris-hydrochloride (pH 6.8) containing 0.1% SDS. The electrophoresis buffer was 2.5 mM Tris base, 200 mM glycine, and 0.1% SDS. After electrophoresis (100 V, 16 h), gels were stained with 0.05% Coomassie blue in 25% isopropanol–10% acetic acid. Gels were destained with 5% acetic acid and photographed.

**Determination of plasmid copy numbers.** Two-milliliter bacterial cultures were grown in minimal medium (containing glucose [0.5%] and essential amino acids at 20 μg/ml) to which transferrin (3 μM) or $FeCl_3$ was added at various concentrations. Labeling of the DNA was accomplished by adding [$^3H$]thymidine (50 Ci/mmol) at 10 μCi/ml in the presence of 250 μg of deoxyadenosine per ml. Cells were lysed as described previously (6). Radioactive DNA (about 500,000 cpm) was centrifuged in 5 ml of cesium chloride-ethidium bromide gradients in a 50 rotor of a Beckman L3-50 ultracentrifuge at 40,000 rpm for 40 h at 15°C. Gradients were fractionated directly onto filters (Whatman 3MM). Radioactivity was measured in a Tri-Carb counter (Packard) using a scintillation cocktail containing Omnifluor (New England Nuclear Corp.).

## RESULTS

**Radioactive iron ($^{55}Fe$) uptake by cells of *V. anguillarum*.** To determine whether the ability of plasmid-carrying high-virulence *V. anguillarum* strains to grow in iron-limited conditions is due to an efficient iron uptake system, we directly measured the uptake of radioactive iron by nongrowing cells. The plasmid-carrying *V. anguillarum* 775(pJM1) and the plasmidless derivative H775-3 were grown in a minimal medium containing about 2 μM $FeCl_3$ (minimal iron concentration at which the H775-3 strain can grow) for several generations. Exponentially growing cultures were centrifuged, cells were washed with low-iron medium (iron content less than 0.5 μM) and resuspended to $0.4 \times 10^8$ cells per ml in similar medium containing less than 0.5 μM iron. After incubation in this medium to deplete intracellular iron pools, cultures were centrifuged, and cells were washed and resuspended to a density of $4 \times 10^8$ cells per ml in similar medium lacking essential amino acids but containing nitrilotriacetate and then exposed to $^{55}Fe$ as described in Materials and Methods. Figure 1 and Table 1 show that under the same uptake conditions nongrowing cells of the plasmid-carrying, high-virulence *V. anguillarum* 775(pJM1), 133S(pJM1), or LS174(pJM1) take up radioactive iron more rapidly than the isogenic, plasmidless, low-virulence derivative H775-3. A similar low uptake was obtained with other plasmidless low-virulence derivatives (Table 1).

Additional information regarding the events leading to iron accumulation was obtained by utilizing the respiratory inhibitor KCN. An energy-dependent process like transport of iron inside the cell is inhibited by KCN, whereas binding of iron to the bacterial membrane which is energy-independent is not (3). Figure 1 shows that iron accumulation by the plasmid-carrying strain of *V. anguillarum* 775(pJM1) is greatly inhibited by 2 mM KCN, suggesting that the process must be uptake rather than simply binding to the bacterial membranes, although the

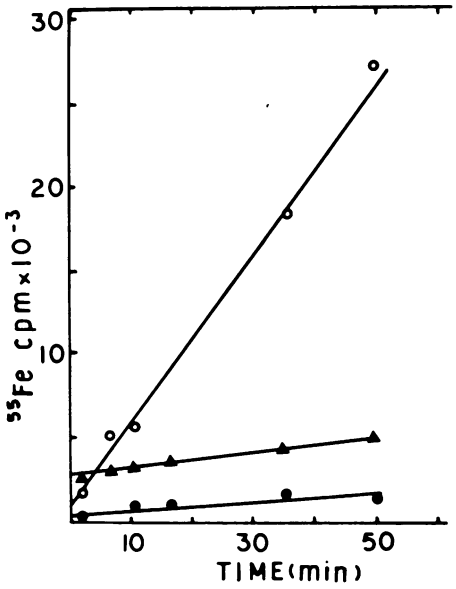


FIG. 1. *Kinetics of radioactive iron uptake by non-growing cells of V. anguillarum under conditions of iron limitation. Plasmid-carrying 775(pJM1) and the plasmidless isogenic derivative H775-3 were grown to 0.4 × 10$^8$ cell per ml density in a low-iron medium, and the uptake of radioactive iron ($^{55}FeCl_3$) in the presence of the chelator nitrilotriacetate was determined as described in the text. Symbols: ○, Plasmid-carrying 775(pJM1); ▲, plasmid-carrying 775(pJM1) in medium containing 2 mM KCN; ●, plasmidless H775-3.*

presence of the small amount of energy-independent accumulation of iron (in the presence of KCN) could be due to some kind of association step which also appears to be plasmid mediated.

These results demonstrate that presence of the virulence plasmid in *V. anguillarum* indeed involves a more rapid and efficient iron uptake. This observation explains the ability of plasmid-carrying *V. anguillarum* to grow in the presence of iron chelators.

**Induction of *V. anguillarum* outer membrane proteins in conditions of iron limitation.** To further investigate the presence of a plasmid-mediated iron uptake system in *V. anguillarum*, we determined whether any specific cell envelope protein was induced under conditions of iron limitation, as is the case for the iron uptake systems previously described for enteric bacteria (1, 15).

*V. anguillarum* 775(pJM1) and its plasmidless, low-virulence derivative H775-3 were grown in minimal medium to which iron as $FeCl_3$ was added to various concentrations. In some cases 3 μM transferrin was added to the growth medium. Total cell envelopes, as well as outer membranes, were prepared from cells grown under these various conditions and were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 2, lanes A to H show total cell envelopes for both strains at different $FeCl_3$ concentrations, whereas lanes I and J are outer membrane proteins obtained from cells grown at 2 μM $FeCl_3$. There are five major outer membrane proteins (OM1–5) present in the plasmid-carrying strains grown at 2 μM $FeCl_3$. One of these, OM2, is missing from the plasmidless derivative. Analysis of total cell envelopes of the plasmid-containing strain shows clearly that there is a threshold of $FeCl_3$ concentration (below 4 μM) at which both OM2 and OM3 are inducible, whereas these proteins are not detectable at higher iron concentrations in plasmid-carrying strains (lanes A to F). Lane A shows that both OM2 and OM3, as expected, are present in the plasmid-carrying strain grown in the presence of 3 μM transferrin. In the case of the plasmidless strain, the OM3 protein is the only protein induced at 2 μM $FeCl_3$ (lowest iron concentration to allow growth of this strain) (cf. lanes G, J, and H). Thus, iron limitation conditions which allow a very rapid uptake of radioactive iron by plasmid-carrying *V. anguillarum* strains also induce the synthesis

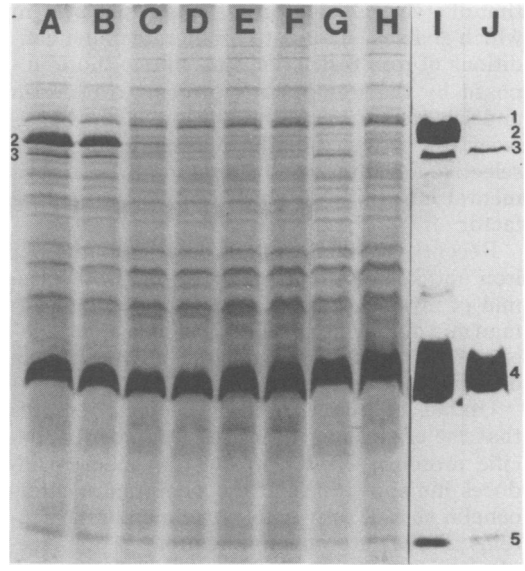


FIG. 2. *Range of $FeCl_3$ concentrations at which OM2 and OM3 proteins are induced. (A to F) Total cell envelopes of plasmid-carrying V. anguillarum 775(pJM1). (A) Cells grown in 3 μM transferrin. (B to F) Cells grown in $FeCl_3$. (B) 2 μM; (C) 4 μM; (D) 8 μM; (E) 12 μM; (F) 24 μM $FeCl_3$. Total cell envelopes of plasmidless derivative H775-3: (G) 2 μM $FeCl_3$; (H) 8 μM $FeCl_3$. Outer membranes obtained from cells grown at 2 μM $FeCl_3$: (I) plasmid-carrying V. anguillarum 775(pJM1); (J) plasmidless H775-3.*

of two specific outer membrane proteins OM2 and OM3 of 86,000 and 79,000 daltons, respectively (as measured with protein molecular weight standards [not shown]). The OM2 protein is associated with the presence of the plasmid pJM1.

**Plasmid copy number and iron concentration.** An elevated synthesis of the OM2 protein under iron limitation conditions could be a reflection of an increased number of copies of the virulence plasmid under these same conditions. Our data show this is not the case. Plasmid copy numbers determined in the presence of transferrin (3 μM, copy number 2) and at various iron concentrations (0.05 μM, copy number 2; 2 μM, copy number 2.1; 4 μM, copy number 2.5; 8 μM, copy number 1.8; 12 μM, copy number 2.1) show that there are no significant changes of this parameter, although at this same range of concentrations there are dramatic changes in the induction of the OM2 protein (Fig. 2).

## DISCUSSION

Investigations of the correlation between virulence of strains of the marine fish pathogen *V. anguillarum* and presence of a specific plasmid class led to the identification of a novel virulence mechanism associated with this plasmid class—that of a very efficient iron-sequestering system which enables bacteria to proliferate under conditions of iron restriction that mimic those imposed by the iron-binding proteins transferrin and lactoferrin in the host-fish system. Moreover, the presence of the plasmid conferred a selective advantage to bacteria in mixed experimental infections in which iron was a limiting factor (4).

Recently, Williams (20) described another iron uptake system mediated by the ColV plasmid of invasive *Escherichia coli* strains of human and animal origin. This system is independent of the enterochelin system of iron uptake described for enteric bacteria (1, 15).

Uptake of iron by enteric bacteria requires that the organism synthesizes a number of specific proteins. Growth in low-iron medium induces the appearance of the siderophore enterochelin as well as specific outer membrane proteins. Three outer membrane proteins of molecular weights 82,000, 79,000, and 77,000 in *Salmonella typhimurum* (1) and molecular weights 83,000 81,000, and 74,000 in *E. coli* (15) are expressed as major proteins under conditions of iron limitation. The binding of ferric enterochelin by outer membrane preparations is increased by iron limitation (12, 13). This evidence supported by genetic results (8, 10, 13, 15) thus suggests that these outer membrane proteins form a receptor for ferric enterochelin necessary for its transport into the cell.

In the case we have examined, the presence of the plasmid pJM1 in *V. anguillarum* greatly stimulates the incorporation of radioactive iron into nongrowing cells, indicating the presence of a very efficient plasmid-mediated iron uptake system.

The preliminary evidence presented in this paper suggests that plasmid-mediated iron accumulation by *V. anguillarum* cells has a major component that is energy dependent. Presence of a small energy-independent component could be due to some degree of plasmid-mediated iron association with the bacterial membrane presumably via complexes of iron with *V. anguillarum* siderophores. A detailed analysis of these processes is currently under way.

Analysis of the outer membrane proteins synthesized by the plasmid-containing *V. anguillarum* 775(pJM1) showed that at least two proteins are induced under conditions of iron limitation: OM2, an 86,000-dalton protein associated with the presence of the pJM1 plasmid, and OM3, a 79,000-dalton protein. We did not detect changes in the copy number of the virulence plasmid as a function of the iron concentration in the growth medium. Although OM2 is present only in strains containing the pJM1 plasmid, it remains to be seen whether this protein is actually coded for by the plasmid and induced in response to a decrease in the iron concentration or is a chromosomal product that is regulated by a plasmid-specific substance. It is of interest that both OM2 and OM3 proteins are induced in cells of *V. anguillarum* 775(pJM1) that are obtained from experimentally infected fish (manuscript in preparation). The possible role of these two proteins in the iron metabolism and virulence of *V. anguillarum* is presently under investigation.

### ACKNOWLEDGMENTS

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