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Iron-Regulated Outer Membrane Protein OM2 of *Vibrio anguillarum* Is Encoded by Virulence Plasmid pJM1

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***Vibrio anguillarum* 775 harboring the virulence plasmid pJM1 synthesized an outer membrane protein of 86 kilodaltons, OM2, that was inducible under conditions of iron limitation. pJM1 DNA fragments obtained by digestion with restriction endonucleases were cloned into cosmid vectors and transferred into *Escherichia coli*. The OM2 protein was synthesized in *E. coli*, demonstrating that it is actually encoded by the pJM1 plasmid. Mobilization of the recombinant plasmids to *V. anguillarum* was accomplished by using the transfer factor pRK2013. A *V. anguillarum* exconjugant harboring the recombinant derivative pJHC-T7 and synthesizing the OM2 protein took up $^{55}Fe^{3+}$ and grew under iron-limiting conditions, only in presence of the pJM1-mediated siderophore. Exconjugants harboring recombinant plasmids, such as pJHC-T2 which did not encode the OM2 protein, were transport negative. Membrane protein iodination experiments, together with protease treatment of whole cells, indicated that the OM2 protein is exposed to the outside environment of the *V. anguillarum* cells.**

The high virulence of the fish pathogen *Vibrio anguillarum* 775 is conferred by a 65-kilobase pair plasmid designated pJM1 (9, 11). This virulence plasmid specifies a very efficient iron uptake system (5, 6) which allows bacteria to utilize the otherwise unavailable host iron which is bound to high-affinity iron-binding proteins (3, 7, 14, 20, 22, 31). Genetic characterization of the pJM1-mediated iron uptake system was recently achieved by Tn*1* transposition mutagenesis and restriction endonuclease analysis (30) defining not only the location of the iron uptake genes in the pJM1 plasmid but also the existence of two components involved in the iron uptake process in *V. anguillarum*: a diffusible siderophore activity in the supernatant fluid and a nondiffusible receptor activity for iron-siderophore complexes, tentatively identified as the outer membrane protein OM2 (30). This outer membrane protein is induced under conditions of iron limitation only in those strains that possess a pJM1 plasmid (8). These results suggested that the OM2 protein could either be encoded by the pJM1 plasmid or be of a chromosomal origin but induced by a plasmid-regulatory substance. We report in this work that the iron-regulated OM2 protein of *V. anguillarum* is indeed encoded by the pJM1 plasmid and may play an essential role in the pMJ1-mediated iron uptake process.

MATERIALS AND METHODS

Bacterial strains. The characteristics of the strains used in this work are described in Table 1.

Isolation of plasmid DNA. Large-scale purification of plasmid DNA was performed by the method of Birnboim and Doly (1) which was modified as described previously (19). Further purification was achieved by centrifugation in cesium chloride-ethidium bromide density gradients. Plasmid screening in transformation and recombinant DNA experiments was carried out by using a rapid alkaline lysis method (1).

Restriction endonuclease analysis and molecular cloning of pJM1 DNA fragments. Restriction enzymes were used under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of restriction endonuclease-cleaved DNA was performed in horizontal 0.8% agarose slab gels in a Tris-borate buffer system (10). *Xho*I partial digests of pJM1 DNA ligated with the cosmid vector pVK102 (17) (completely digested with *Xho*I) and packaged in vitro were transduced into *Escherichia coli* HB101 cells as described elsewhere (28). Samples of pJM1 DNA were also completely digested with *Sal*I and cloned into the *Sal*I site of the cloning vector pVK100 (17). Ligation reactions were carried out at 15°C for 12 h at an approximate 1:1 ratio of picomoles of vector ends to target DNA by using T4 DNA ligase prepared as described by Tait et al. (27). The transformation of *E. coli* HB101 cells with a ligation mixture was carried out as previously described (10).

Conjugation experiments. Recombinant plasmids containing pJM1 DNA fragments cloned in the cosmid vectors pVK100 or pVK102 (harbored by *E. coli* HB101) were returned to *V. anguillarum* by conjugation with the helper plasmid pRK2013 (13). Next, 5-ml samples of overnight cultures of *E. coli* MM294(pRK2013), *E. coli* HB101 harboring the recombinant DNA clones and the *V. anguillarum* plasmidless strain H775-3 (used as the recipient), were mixed and filtered through 0.22-μm Millipore filters. The filters were incubated at 30°C for 16 h on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% NaCl. After incubation the cells were suspended and plated on Trypticase soy agar supplemented with 1% NaCl and containing either tetracycline (5 μg/ml) for the recombinant plasmids obtained by cloning in the *Xho*I site of pVK102 or kanamycin (1 mg/ml) for those derived from cloning in the *Sal*I site of pVK100. Ampicillin (20 μg/ml) was used to select for the recipient *V. anguillarum* strain. Plates were incubated at 24°C for 48 h.

Purification of the pJM1-mediated *V. anguillarum* siderophore. The *V. anguillarum* siderophore was partially purified from a culture supernatant of the strain 775(pJM1) by successive adsorption and gel filtration chromatography. Briefly, the culture supernatant of *V. anguillarum* 775 (grown in minimal medium containing the essential amino acids aspartic acid and histidine as well as the non-assimilable iron chelator ethylenediamine-di(*o*-hydroxyphenyl) acetic acid

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TABLE 1. Bacterial strains and properties

Strain	Plasmid	Relevant phenotype[a]	Source or reference
V. anguillarum			
775	pJM1	$Iu^+(S^+R^+)$	11
H775-3		$Iu^-(S^-R^-)$	6
775::Tn*1*-5	pJHC-91	$Iu^-(S^-R^+)$ Ap^r	30
H775-3A	pJHC-T7	$Iu^-(S^-R^+)$ Tc^r	28
H775-3D	pJHC-T2	$Iu^-(S^-R^-)$ Tc^r	28
H775-3A100	pJHC-A100	$Iu^-(S^-R^-)$ Km^r	This work
H775-3A101	pJHC-A101	$Iu^-(S^-R^-)$ Km^r	This work
H775-3102	pVK102	$Iu^-(S^-R^-)$ Km^r Tc^r	28
E. coli			
HB101		F^- *hsdS20 recA*13 *ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	2
HB101-3A	pJHC-T7	Tc^r	28
HB101-3D	pJHC-T2	Tc^r	28
HB101-3A100	pJHC-A100	Km^r	This work
HB101-3A101	pJHC-A101	Km^r	This work
HB101-3102	pVK102	Km^r Tc^r	17
MM294	pRK2013	Km^r	13

[a] Abbreviations: Iu, iron uptake; S, siderophore activity; R, receptor activity; Ap, ampicillin; Tc, tetracycline; and Km, kanamycin.

[EDDA] at a final concentration of 5 μM) was used as the siderophore source. After the centrifugation and removal of the cells, the supernatant fluid was run through an Amberlite XAD-7 column previously equilibrated with M9 salts. The siderophore was eluted with methanol. The active fractions, detected by bioassays (30), were pooled, concentrated with a rotary vacuum evaporator, and dissolved in methanol. This material was further purified on a Bio-Gel P2 gel filtration column in which the impurities came off in the void volume and the pJM1-mediated siderophore came off at ca. 1 column volume later.

Bioassays for detection of receptor activity. Ten microliters of either sterile siderophore or supernatant fluid was applied onto sterile filter disks and placed on M9 minimal medium agar plates (containing 10 μM EDDA) that had been seeded with 0.1 ml of an overnight culture of 10^8 cells per ml of the strain to be tested for receptor activity. The indicator strain 775::Tn*1*-5(pJHC-91), which is deficient in siderophore production but still shows receptor activity, was used as a control.

Measurement of radioactive iron uptake. Bacterial strains were grown for several generations at 24°C in M9 minimal medium containing 10 μM EDDA for iron uptake-proficient bacteria or 2 μM EDDA for iron uptake-deficient bacteria. Exponentially growing cultures were centrifuged, and the cells were washed and suspended in the same volume of similar medium containing the required amino acids aspartic acid and histidine, at 20 μg/ml, instead of Casamino Acids. After further incubation for 2 h to deplete iron and amino acid intracellular pools, the cultures were centrifuged and the cells were washed and resuspended to an absorbance value of 1.00 at 590 nm in either a similar medium lacking essential amino acids but containing 100 μM sodium nitrilotriacetate or cell-free culture supernatant containing siderophore activity from *V. anguillarum* 775 grown in the same medium. In some experiments, 10 mM KCN was added at this stage. The radioactive iron uptake kinetics was performed with 1 μCi/ml of carrier-free $^{55}FeCl_3$ (final iron concentration, 0.2 μM; 4.55×10^8 cpm/μmol) as described previously (8).

Analysis of membrane proteins. Total-cell envelopes as well as outer membranes from *V. anguillarum* were prepared essentially as described previously (8). Pellets from total or outer membranes as well as whole cells were suspended in 50 μl of a buffer containing 62.5 mM Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.001% bromophenol blue, and 5% mercaptoethanol. The suspensions were boiled for 5 min, and samples were applied to a 12.5% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 44:0.8) in 375 mM Tris-hydrochloride (pH 8.8) containing 0.2% SDS and 3% stacking gel in 125 mM Tris-hydrochloride (pH 6.8) containing 0.1% SDS. The electrophoresis buffer was 2.5 mM Tris base–200 mM glycine–0.1% SDS. After electrophoresis (100 V for 16 h), the gels were stained with 0.05% Coomassie blue in 25% isopropanol–10% acetic acid. The gels were destained with 5% acetic acid and photographed, or they were dried and autoradiographed when radioactive proteins were loaded.

Membrane protein iodination. The iodination of membrane proteins was carried out by using a modification of a previously described method (26). *V. anguillarum* 775 was grown in M9 minimal medium containing 10 μM EDDA until it reached a turbidity of 100 Klett units. Cells contained in 5-ml fractions were washed, suspended in 1 ml of M9 salts, and transferred to a Microfuge tube (Beckman Instruments, Inc., Fullerton, Calif.). After the addition of 0.5 mCi of $Na^{125}I$ (56 mCi/ml; ICN, Irvine, Calif.) and six Iodo Beads (Pierce Chemical Co., Rockford, Ill.), the iodination reaction was allowed to proceed for 3 min at room temperature, removing 160-μl samples each 30 s. Free $Na^{125}I$ was removed by extensive washing of the cells with M9 salts, and then total-cell envelopes and outer membranes were prepared as described above.

Preparation of antiserum against outer membrane protein OM2. Antiserum against OM2 was raised in 6-month-old rabbits by injection with crushed polyacrylamide gel fragments containing the OM2 protein band. The material, emulsified in 1 ml of Freund complete adjuvant, was injected at multiple intracutaneous sites on the upper back of the rabbit three times at 15-day intervals. At week 1 after the last injection, the rabbits received a booster dose with the same material emulsified in incomplete Freund adjuvant. Serum was obtained at 10-day intervals after the initial dose, and the immunoglobulin fraction was prepared by $(NH_4)_2SO_4$ precipitation.

Immunoblot analysis. Total cellular proteins and membrane proteins were prepared for SDS-polyacrylamide gel electrophoresis and separated on 12.5% acrylamide gels. After electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose paper (0.45 μm; BA85; Schleicher and Schuell, Inc., Keene, N.H.) essentially as described by Towbin et al. (29). The proteins were electrophoresed onto nitrocellular paper under a constant current (250 mA) at 10°C in a Trans-blot cell (Bio-Rad Laboratories, Richmond, Calif.) with 25 mM Tris base–192 mM glycine–20% methanol. After electrophoresis, the nitrocellulose paper was soaked in Tris-buffered saline (10 mM Tris-hydrochloride [pH 7.4], 0.9% NaCl) containing 3% bovine serum albumin for 30 min at room temperature, followed by incubation with anti-OM2 serum diluted 1:500 in NIBB solution (50 mM Tris-hydrochloride [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100) for 3 h at room temperature. The blots were washed twice in NIBB and incubated in a 1:1,000 dilution of horseradish peroxidase-

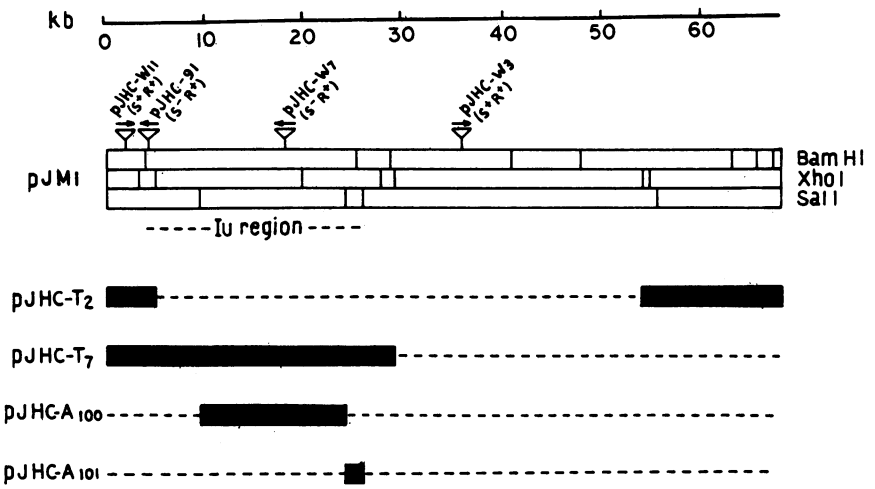

FIG. 1. Genetic and physical map of pJM1 derivatives and recombinant clones. Upper bar, *Bam*HI, *Xho*I and *Sal*I restriction endonuclease cleavage sites obtained from previously published data (28, 30). The restriction endonuclease maps are made colinear to the *Bam*HI map. Therefore, the lines at both ends of the *Bam*HI map show actual restriction sites, and the lines at the ends of the restriction maps for the other enzymes indicate the *Bam*HI cleavage site within the specific restriction endonuclease fragment. ⍱, sites of Tn*1* insertions in several pJM1 derivatives; the arrow over this symbol gives the orientation of Tn*1* with respect to β-lactamase gene transcription. The phenotype of *V. anguillarum* strains carrying each of these derivatives is also noted. Iron uptake (Iu)-proficient derivatives are indicated as follows: S^+R^+, production of siderophore (S) and receptor (R) activities; and S^-R^+, iron uptake-deficient derivatives in which the Tn*1* insertion affected the genes associated with the production or activity of siderophore. The dashed lines correspond to pJM1 DNA regions not included in the cloned DNA. Thicker lines represent the pJM1 DNA cloned in either pVK100 or pVK102.

conjugated protein A (0.5 mg/ml) in NIBB for 3 h at room temperature. The nitrocellulose paper was stained with 0.022% H_2O_2 and 0.075% horseradish peroxidase color development reagent (Bio-Rad) for 5 to 10 min at room temperature.

Papain treatment of whole cells. *V. anguillarum* 775 was grown overnight in M9 minimal medium containing 10 μM EDDA. The cells were washed three times, suspended in M9 salts, and divided into three 10-ml portions. Each portion was incubated at 24°C with 50, 100, and 150 μg of papain, respectively. The cells were harvested and washed with 10 mM Tris-hydrochloride (pH 8.0)–0.3% NaCl containing 1 mM phenylmethylsulfonyl fluoride, and the total membrane and outer membrane fractions were prepared and analyzed electrophoretically in SDS-polyacrylamide gels.

RESULTS

Origin of the OM2 protein. To determine whether the OM2 structural genes were present in pJM1, recombinant clones containing restriction fragments were introduced into *E. coli* HB101 cells. Clones containing the OM2 genes could be detected by immunological methods in *E. coli* extracts. Total-cell lysates and outer membranes from *E. coli* cells harboring the recombinant plasmids were prepared. After SDS-polyacrylamide gel electrophoresis, the total-cell and outer membrane proteins were analyzed by the immunoblot procedure with anti-OM2 antiserum. Recombinant plasmids pJHC-T2 and pJHC-T7 were constructed by cloning *Xho*I partial digests of pJM1 in the cosmid vector pVK102; pJHC-A100 and pJHC-A101 were obtained by cloning *Sal*I total digests of pJM1 in the cosmid vector pVK100. The cloned DNA region in pJHC-T7 and pJHC-A100 encompass the pJM1 iron uptake region defined previously by Tn*1* transposition mutagenesis (30), and the cloned pJM1 DNA in pJHC-T2 and pJHC-A101 lie mostly outside of this region (Fig. 1) and thus serve as negative controls. OM2 was present in the total-cell lysates of *E. coli* cells harboring the clones pJHC-T7 or pJHC-A100 (Fig. 2, lanes E and G). The same immunological profile was obtained when the corresponding outer membrane protein fractions were assayed (data not shown). These results indicate that the structural genes for OM2 must be present in the plasmid pJM1, within the iron uptake region.

Receptor activity of bacteria carrying the OM2 gene clones. Each of the clones described above was transferred to the *V. anguillarum* plasmidless strain H775-3 by using a triparental mating. The ability of the exconjugants to grow under iron-limiting conditions around a paper disk containing either partially purified siderophore or 775 cell-free culture supernatant was used as an indication of receptor activity (Fig. 3). The control strain 775::Tn*1*-5(pJHC-91) (Panel a) and the exconjugant-harboring pJHC-T7 (Panel b) were cross-fed by the *V. anguillarum* siderophore, suggesting that the clone pJHC-T7 possessed the genetic information for receptor activity. *V. anguillarum* cells harboring pJHC-A100 did not grow in the presence of either partially purified siderophore or 775 culture supernatant (Panel c), despite the fact that this recombinant clone expressed an outer membrane protein which is immunologically related to OM2 in *E. coli* HB101 cells (Fig. 2, lane G).

To further characterize the nature of the OM2-like protein encoded by pJHC-A100, we performed electrophoretic analysis and immunoblotting of *V. anguillarum* cells harboring either this plasmid or pJHC-T7 and that had been grown under iron-limiting or iron-rich conditions. As in the case of the wild-type strain 775, cells of the *V. anguillarum* strain H775-3 containing the recombinant plasmid pJHC-T7 expressed, under iron-limiting conditions, an 86-kilodalton outer membrane protein that reacted with anti-OM2 serum (Fig. 4, panels a and b, lane D_2). This protein was absent from the cell preparation obtained from the cultures grown under iron-rich conditions (Panels a and b, lane D_1). Conversely, in the case of *V. anguillarum* exconjugants harboring pJHC-A100, the synthesis of the OM2-like protein occurs under both iron-rich as well as iron-limiting conditions

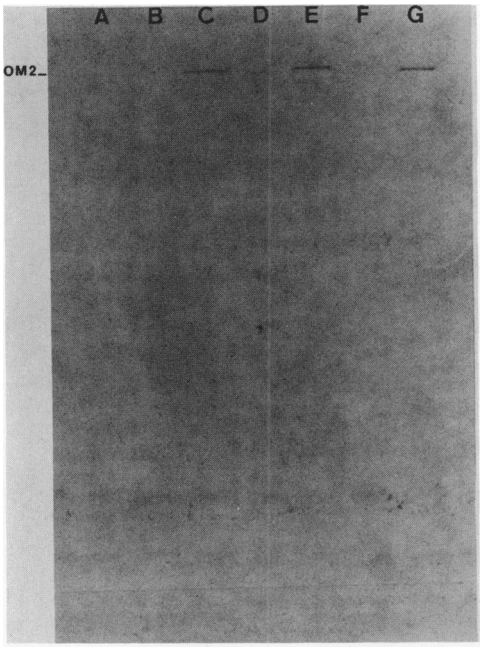

FIG. 2. Immunoblot analysis of total proteins of *E. coli* HB101 cells harboring recombinant plasmids. Lanes: (A) plasmidless HB101; (B) HB101(pVK102); (C) control *V. anguillarum* 775(pJM1) outer membrane proteins; (D) HB101(pJHC-T2); (E) HB101(pJHC-T7); (F) HB101(pJHC-A101); and (G) HB101(pJHC-A100). The proteins were electrophoresed in 12.5% polyacrylamide gels and transferred to nitrocellulose filters as described in the text. Western blots were successively reacted with anti-OM2 serum and protein A peroxidase and stained with H_2O_2 and horseradish peroxidase color development reagent.

(Panels a and b, lanes E_1 and E_2). Therefore, its expression is no longer under the control of the iron status of the cells.

$^{55}Fe^{3+}$ uptake by *V. anguillarum*. The role of the outer membrane protein OM2 in the pJM1-mediated iron uptake process was assessed by experiments in which the uptake of radioactive iron was measured with nongrowing cells either in the presence or absence of pJM1 plasmid-mediated siderophore activity (Fig. 5). Only cells of the wild-type 775 strain harboring pJM1 were capable of taking up radioactive iron without the added siderophore. As expected, no uptake was detected with the plasmidless strain H775-3. The strain 775::Tn*1*-5(pJHC-91), a Tn*1* derivative that is iron uptake deficient but still produces OM2, could take up radioactive iron only in the presence of the pJM1-produced *V. anguillarum* siderophore (Fig. 5b). The recombinant clone containing pJHC-T7 also took up iron only in the presence of the pJM1-mediated siderophore (Fig. 5c), whereas *V. anguillarum* cells harboring pJHC-T2 or pJHC-A100 did not take up iron either in the absence or presence of the pJM1 siderophore (Fig. 5d). In all cases in which uptake occurred, the iron accumulation was inhibited by KCN (data only shown for strain 775), indicating an energy dependence of this process and suggesting uptake into the cytosol rather than just binding at the cellular membrane (4).

Surface exposure of outer membrane proteins. Whole cells of *V. anguillarum* were labeled with $Na^{125}I$, and the cell envelope fractions were extracted with Sarkosyl to separate the cytoplasmic and outer membrane proteins. Electrophoretic analysis of the ^{125}I-labeled membrane proteins produced under iron limitation showed that the OM2 protein and other membrane proteins were labeled with ^{125}I (Fig. 6a,

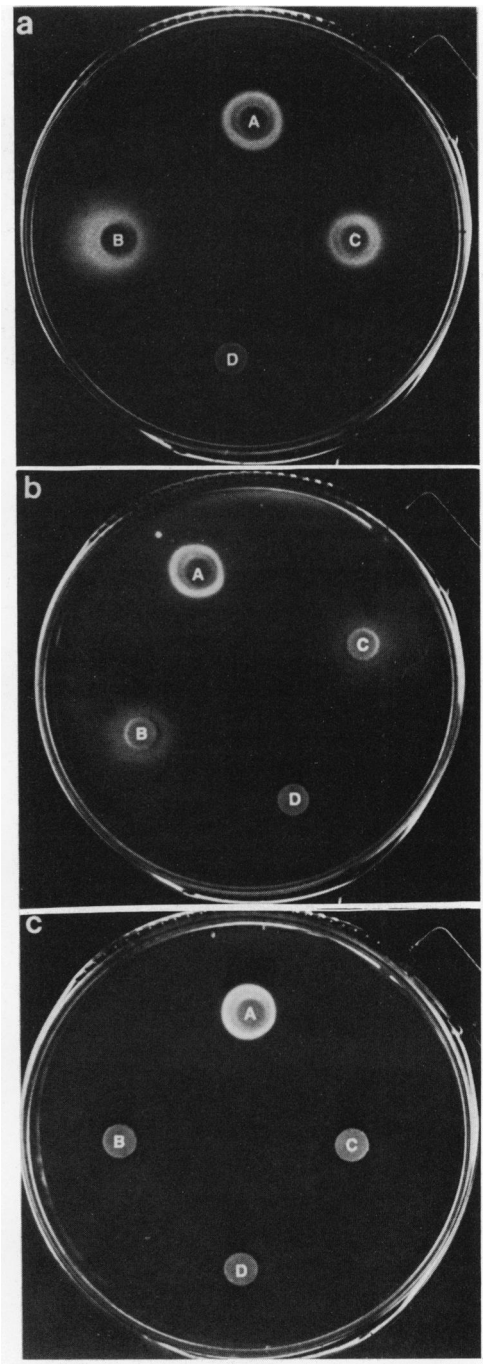

FIG. 3. Bioassay of receptor activity of *V. anguillarum* exconjugants harboring recombinant plasmids. *V. anguillarum* cells to be used as lawns in bioassays were grown overnight in M9 minimal medium containing 2 μM EDDA and seeded onto 0.7% agarose plates containing 10 μM EDDA in M9 minimal medium. Panel a, 775::Tn*1*-5(pJHC-91); panel b, H775-3A(pJHC-T7); and panel c, H775-3A100(pJHC-A100). Disks containing 10 μl of either siderophore preparations or $FeCl_3$ were placed on top of the seeded plates, and incubation was carried out overnight at 24°C. Disk A, 10 mM $FeCl_3$; disk B, partially purified *V. anguillarum* siderophore; disk C, cell-free 775(pJM1) culture supernatant; and disk D, control M9 minimal medium. Receptor activity of the lawn strain is evidenced by the growth halo around the disks containing either partially purified siderophore or 775(pJM1) culture supernatant. Controls not shown are *V. anguillarum* cells carrying pJHC-T2, pJHC-A101, or the cosmid vector pVK102 which grew only around the disk containing $FeCl_3$.

lanes B and C). It was recently reported for *Haemophilus influenzae* that iodination procedures lead to nonspecific labeling of cytoplasmic membranes (18). Since this finding could invalidate our approach, we included different times of iodination and comparison between the patterns corresponding to total-cell lysates, total-cell envelopes, and outer membranes to determine the specificity of the method of labeling only surface-exposed proteins. The autoradiographic profile of the three cellular fractions obtained after 3 min of iodination was the same (Fig. 6a, lane A to C). In addition, much shorter reaction times rendered a very similar pattern of iodinated proteins for both total envelopes and outer membranes, and the OM2 protein was one of the most prominently labeled products, together with the major outer membrane protein (Fig. 6b). The fact that similar profiles were obtained at shorter times of iodination for both total-cell envelopes and outer membranes indicated that none other than surface-exposed proteins were labeled. To confirm these results, whole cells were treated with different amounts of papain which, like other macromolecules, is presumed not to penetrate the outer membrane when stabilized by divalent cations such as Mg^{2+} (12, 25). The results indicated that OM2 as well as most of the outer membrane proteins were sensitive to papain digestion in the presence of Mg^{2+} (Fig. 7). The combined results of this section indicate that OM2 is a cell surface protein that is exposed to the environment.

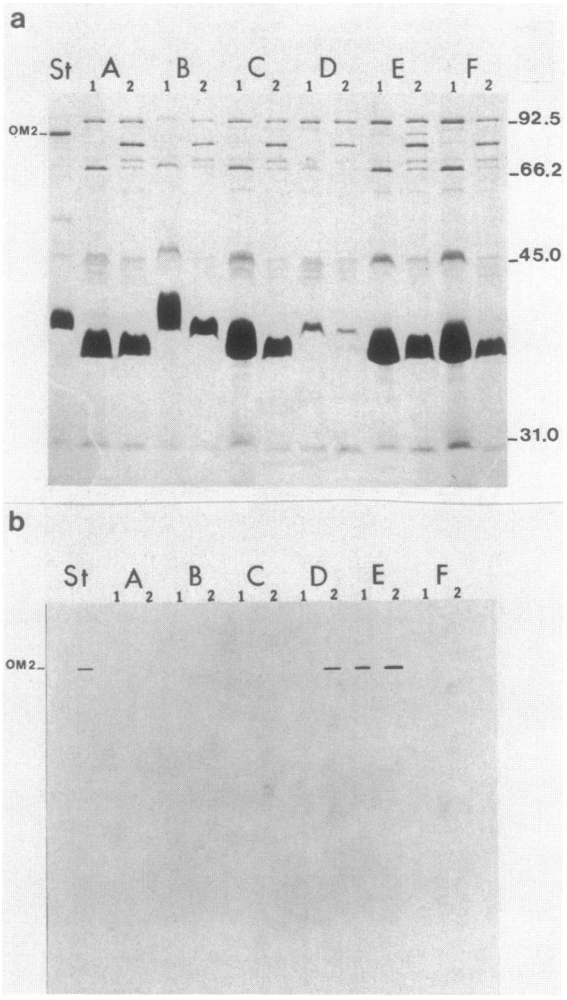

FIG. 4. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of outer membrane proteins from *V. anguillarum* exconjugants harboring recombinant plasmids. Panel a, Proteins stained with Coomassie blue. Lanes: St, outer membrane proteins from 775(pJM1) grown under iron-limiting conditions; A, plasmidless H775-3; B, H775-3102(pVK102); C, H775-3D(pJHC-T2); D, H775-3A(pJHC-T7); E, H775-3A100(pJHC-A100); and F, H775-A101(pJHC-A101). Outer membranes were obtained from cells grown under either iron-sufficient (20 μM $FeCl_3$; subscript 1) or iron-limiting conditions (2 μM EDDA; subscript 2). The numbers on the right of the gel show the molecular weight standards in kilodaltons. Panel b, Immunoblot of an SDS-polyacrylamide gel similar to that shown in panel a.

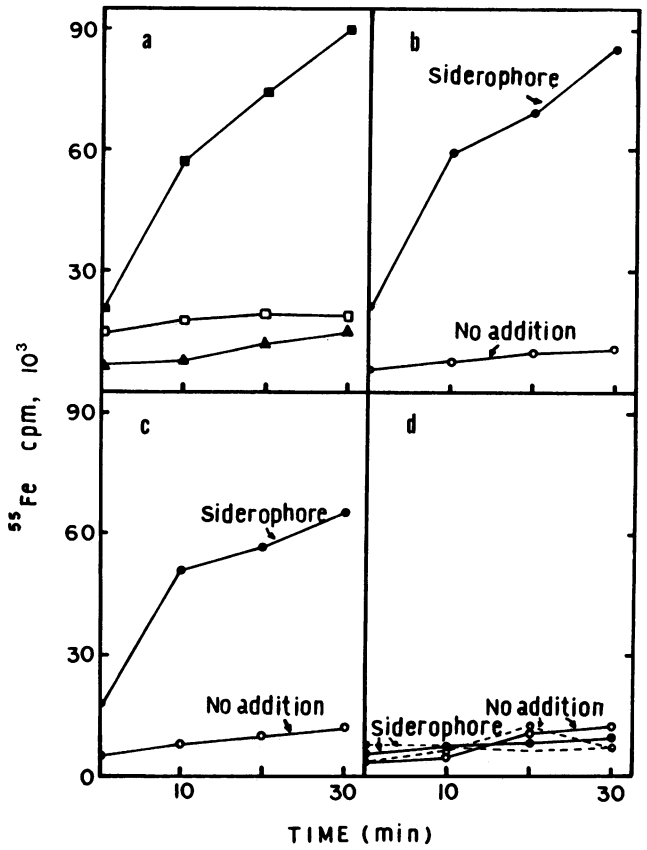

FIG. 5. ^{55}Fe uptake by nongrowing cells of various *V. anguillarum* strains under iron-limiting conditions. (a) Strains 775(pJM1) (■), H775-3 (▲), or 775(pJM1) in the presence of 10 mM KCN (□); (b) strain 775::Tn*1*-5(pJHC-91); (c) strain H775-3A(pJHC-T7); (d) strains H775-3D(pJHC-T2) (——) and H775-3A100(pJHC-A100) (– – –). The iron uptake experiments in panels b, c, and d were carried out either in the presence (●) or absence (○) of cell-free supernatant from *V. anguillarum* 775(pJM1).

DISCUSSION

Recently, two pJM1 plasmid-mediated components involved in the iron uptake process in *V. anguillarum* were defined: a diffusible substance which functions as a siderophore and a nondiffusible receptor for iron-siderophore complexes which is associated with the iron-regulated outer membrane protein OM2 (30). Those results suggested that the OM2 protein was either encoded by the pJM1 plasmid or was of a chromosomal origin but induced by a plasmid-regulatory substance.

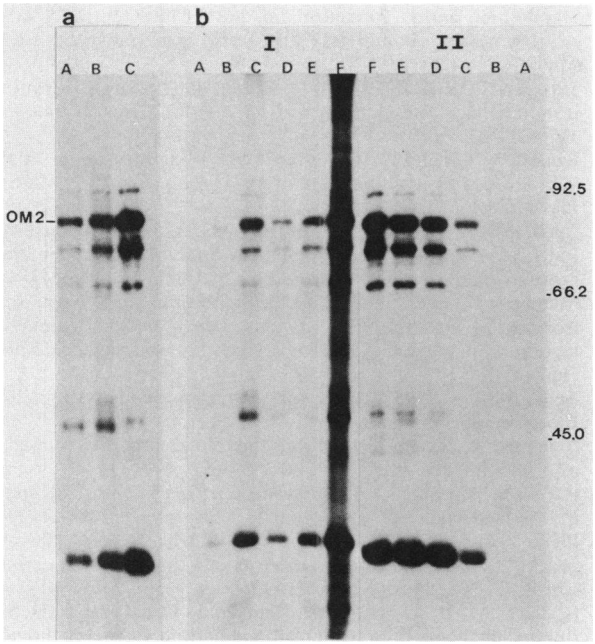

FIG. 6. SDS-polyacrylamide gel electrophoresis of proteins labeled with $Na^{125}I$. Panel a, Autoradiogram of proteins from strain 775 iodinated for 180 s; (A) total-cell lysate; (B) total-cell envelopes; and (C) outer membranes. Panel b, Autoradiogram of total membrane (I) and outer membrane (II) proteins from strain 775 iodinated for the following times: 30 s (A); 60 s (B); 90 s (C); 120 s (D); 150 s (E); and 180 s (F). The numbers on the right of the gel show the molecular weight standard in kilodaltons.

In this report, we demonstrate the plasmid-borne nature of the OM2 protein gene by immunological experiments in conjunction with molecular cloning. It was possible to detect the presence of OM2 in the lysates as well as in the outer membranes of *E. coli* cells harboring the recombinant plasmids pJHC-T7 or pJHC-A100 both containing pJM1 restriction fragments mapping within the iron uptake region as defined previously by Tn*1* transposition mutagenesis (30). The expression of OM2 in an unrelated genetic environment (24) rules out the contribution of the *V. anguillarum* chromosomal DNA to the synthesis of this outer membrane protein.

It is of interest that *V. anguillarum* strains harboring the Tn*1* derivatives pJHC-91 and pJHC-W7 do not produce the pJM1-mediated siderophore although they synthesize OM2 and possess receptor activity (30). Since the Tn*1* insertions which generated pJHC-91 and pJHC-W7 occur at both ends of the iron uptake region which contain the OM2 gene (see pJHC-A100 in Fig. 1), siderophore biosynthetic genes must be located in noncontiguous regions separated by a DNA sequence which includes the OM2 protein gene.

The conjugation experiments combined with bioassays showed that receptor activity and iron-regulated OM2 synthesis in *V. anguillarum* is recovered upon the introduction of pJHC-T7. This is not the case with pJHC-A100 which codes for a protein that is immunologically and electrophoretically indistinguishable from OM2 and which is no longer under iron regulation. Thus, the plasmid pJHC-A100, when introduced into *V. anguillarum*, does not confer a receptor function as determined by bioassays, nor does it enhance the uptake of $^{55}Fe^{3+}$. One possible explanation for our results is that pJHC-A100 contains a protein which is a fusion product between the OM2 protein and a polypeptide initiated adjacent to the vector cloning site. This OM2-like fused polypeptide from pJHC-A100 shows a molecular weight essentially identical to the OM2 protein of pJM1 but lacks regulatory regions as well as transport functions. Another possible explanation is that, in addition to the OM2 protein, another product encoded in the pJM1 DNA included in pJHC-T7 but absent from pJHC-A100 is necessary for successful iron transport. The requirement of at least two genes for a functional iron transport, such as *fepA* and *fepB* for ferric enterobactin uptake in *E. coli* (23, 33), is not unusual and has been described for several other iron uptake systems (7, 16, 32). Furthermore, the essential functions provided by the *tonB* and other genes in high-affinity iron transport (15, 21) may have a counterpart in *V. anguillarum*. Experiments to assess the contribution of other pJM1 regions in addition to that carrying the OM2 gene are being carried out.

To investigate the possible function of OM2 in the pJM1-mediated iron transport process, we performed radioactive iron uptake experiments with strains harboring either the recombinant plasmid pJHC-T7 or the Tn*1* insertion plasmid pJHC-91, both with intact OM2 protein genes. These strains were able to take up radioactive iron only in the presence of the pJM1-mediated siderophore. *V. anguillarum* cells harboring the recombinant plasmid pJHC-T2 which did not possess the OM2 genes were unable to take up radioactive

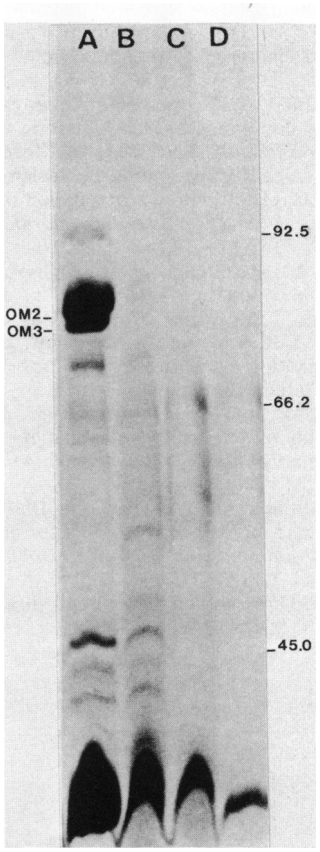

FIG. 7. SDS-polyacrylamide gel electrophoresis of outer membrane proteins obtained from papain-treated, iron-limited whole cells of *V. anguillarum* 775(pJM1). (A) Outer membrane proteins from untreated cells. Outer membrane proteins from treated cells contained the following amounts of papain: (B) 50 μg; (C) 100 μg; and (D) 150 μg. The numbers on the right of the gel show the molecular weight standards in kilodaltons.

iron, even in the presence of the *V. anguillarum* siderophore. We also showed in this report that this outer membrane protein is indeed exposed to the outside environment of the *V. anguillarum* cells, a location that is compatible with its possible function as a receptor for iron-siderophore complexes.

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