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Molecular Cloning and Expression of Genetic Determinants for the Iron Uptake System Mediated by the *Vibrio anguillarum* Plasmid pJM1

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Plasmid pJM1 from an invasive strain of *Vibrio anguillarum* encodes an iron uptake system which mediates the biosynthesis of a siderophore and a membrane receptor for the iron-siderophore complex. This system has been associated with the ability of *V. anguillarum* to cause hemorrhagic septicemic disease in marine fish. Recombinant derivatives containing essential regions of the pJM1-mediated iron uptake system cloned into cosmid vector pVK102 were introduced into low-virulence iron uptake-deficient *V. anguillarum* strains by using a trifactor mating procedure with helper plasmid pRK2013. Three recombinant clones, pJHC-T7, pJHC-T11, and pJHC-T2612, possessed genetic determinants for receptor activity. Production of receptor activity was correlated in all three cases with the presence of OM2, an 86-kilodalton outer membrane protein which was induced under iron-limiting conditions. Two of the clones, pJHC-T7 and pJHC-T2612, also coded for the production of siderophore activity, although at a much lower level than the wild type. Strains harboring either of these two clones were still unable to grow under iron-limiting conditions. This inability was overcome only when other indigenous pJM1 derivatives were present in the cells in addition to the recombinant cosmids. This restoration of high siderophore production and ability to grow under iron-limiting conditions was achieved even when the indigenous plasmids possessed lesions in genes involved in siderophore activity or in both siderophore and receptor production. Thus, another function mediated by plasmid pJM1, possibly a *trans*-acting factor, may play a role in the regulation of siderophore production. Results of experimental infections demonstrated that restoration of the ability to grow under conditions of iron limitation by introduction of a recombinant clone into one of the low-virulence *V. anguillarum* strains was correlated with an increase in bacterial pathogenicity.

One of the most important marine fish pathogens is *Vibrio anguillarum* (5, 15, 23), which causes the disease vibriosis, characterized by a terminal hemorrhagic septicemia (17, 22). The high-virulence phenotype associated with this bacterium is conferred by a 65-kilobase plasmid designated pJM1 (9, 10), which mediates a very efficient iron transport system (6, 7).

Iron is essential for bacterial growth (4, 20, 21, 26, 26a), and this plasmid-mediated system allows bacteria to compete for the host iron which is bound by high-affinity iron-binding proteins, such as transferrin and lactoferrin (4). Thus, cells of *V. anguillarum* harboring plasmid pJM1 can grow in culture medium in which iron is complexed to non-assimilable chelators. An analysis of the outer membrane proteins synthesized under these conditions showed the induction of two novel proteins. One of these, OM2 (86 kilodaltons [kd]), is mediated by plasmid pJM1 and may play a role as a receptor for iron-siderophore complexes (8). Further characterization of the genetics of the pJM1-mediated iron uptake system has recently been achieved by using the mutagenic capability of transposition sequence Tn*1* (11, 12, 25). Restriction endonuclease analysis of iron uptake-deficient and -proficient derivatives generated by Tn*1* transposition onto plasmid pJM1 defined the location of iron uptake genes to a 20 to 25-kilobase region of pJM1 DNA. Iron uptake-deficient mutants were classified as having lesions in genes associated with the activity or biosynthesis of either a plasmid-mediated siderophore or a receptor for binding of iron-siderophore complexes or both.

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A detailed understanding of the molecular mechanisms of pJM1 plasmid-mediated iron transport required molecular cloning of DNA regions carrying genes involved in this process. In other systems, such as those mediated by aerobactin in *Escherichia coli* and by pseudobactin in *Pseudomonas*, cloning of genes involved in the biosynthesis of siderophores has recently been reported (1, 19). We describe in this paper the cloning of important components of the pJM1 system, as well as the expression of these components in *V. anguillarum*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* HB101 *pro leu thi lacY hsr hsm endA recA* (3) was used as a recipient of phage particles in transduction experiments. *E. coli* MM294 *endA hsdR pro* harboring plasmid pRK2013 (16) was used in the conjugation experiments to mobilize derivatives of cosmid pVK102 (18). *V. anguillarum* 775::Tn*1*-5(pJHC-91) is a derivative obtained by Tn*1* insertion in plasmid pJM1 which does not produce siderophores but still synthesizes receptor activity (25). This strain was used as an indicator strain in the bioassays for detection of siderophore production and also in conjugation experiments. The other *V. anguillarum* strains used in this work are described in Table 1.

Isolation of plasmid DNA. Large-scale preparation of plasmid DNA was performed by the method of Birnboim and Doly (2). Further purification was achieved by centrifugation in cesium chloride-ethidium bromide density gradients (25).

Molecular cloning of pJM1 DNA. Samples of plasmid pJM1 DNA were treated with restriction endonuclease *Hin*dIII or *Xho*I under conditions (2 min at 37°C for each restriction

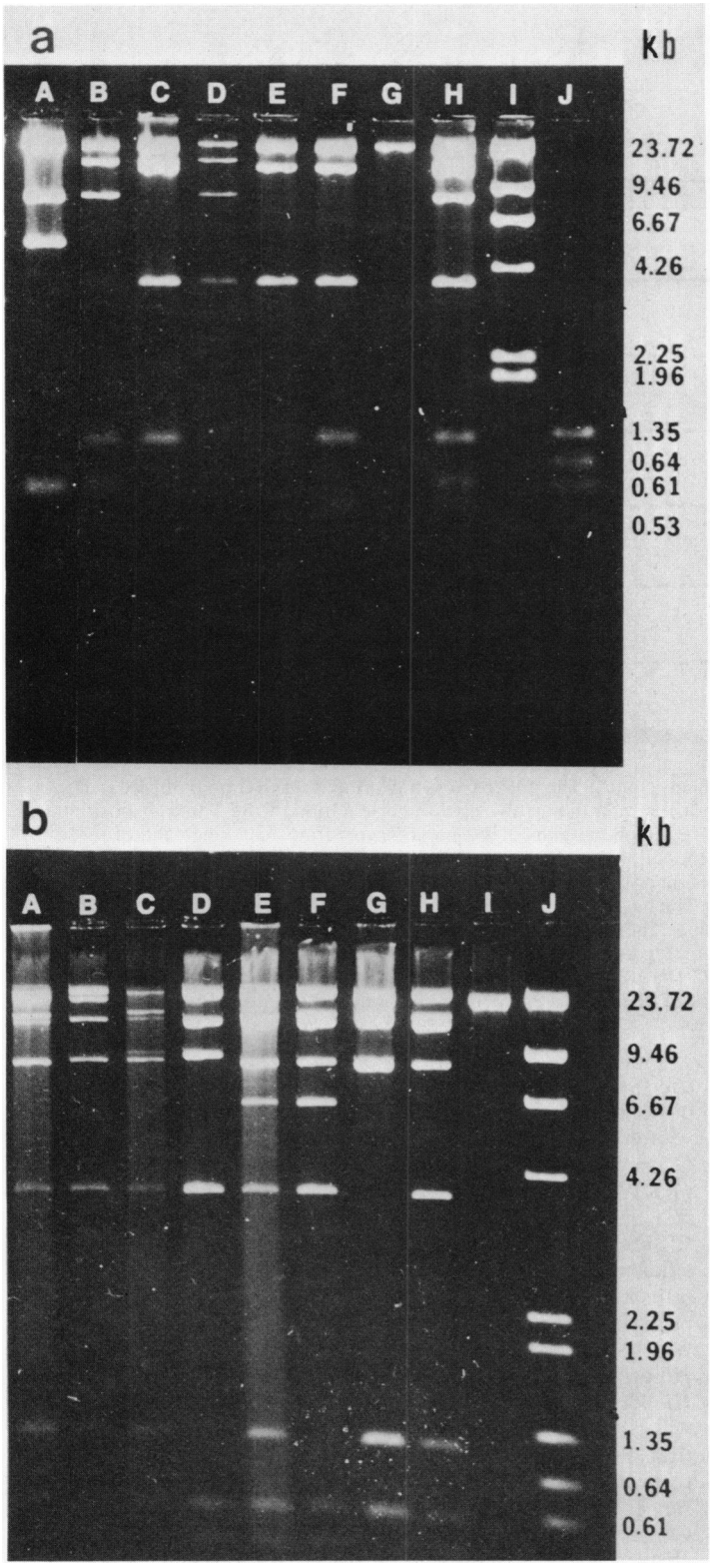

FIG. 1. Agarose gel electrophoresis of *Xho*I-cleaved plasmid DNA. (a) Plasmid DNA obtained from *E. coli*. Lane A, pJHC-T521; lane B, pJHC-T2612; lane C, pJHC-T11; lane D, pJHC-T7; lane E, pJHC-T6; lane F, pJHC-T2; lane G, pVK102; lane H, pJM1; lane I, *Hin*dIII-cleaved lambda DNA; lane J, *Hae*III-cleaved ϕX174 DNA. (b) Plasmid DNAs obtained from *V. anguillarum* strains containing an indigenous plasmid (lanes B, D, and F) and the same strains carrying in addition recombinant plasmid pJHC-T2612 (lanes A, C, and E). The indigenous plasmids used were pJHC-W7 (lanes A and B), pJHC9-8 (lanes C and D), and pJHC-91 (lanes E and F). Lane G, Plasmid DNA from the *V. anguillarum* H775-3 harboring recombinant clone pJHC-T2612; lane H, pJM1; lane I, pVK102; lane J, *Hin*dIII-cleaved lambda DNA plus *Hae*III-cleaved ϕX174 DNA.

endonuclease) in which the cleavage reaction was not complete, rendering partially digested DNA fragments. Partial digests of pJM1 DNA were ligated with pVK102 DNA which had been completely digested with the appropriate restriction enzyme by using T4 DNA ligase prepared according to the method of Tait et al. (24). Reactions were carried out at 15°C for 12 h at an approximate picomole ratio of ends of vector to target of 1:1. The reaction mixture consisted of 20 mM Tris-hydrochloride (pH 7.4), 10 mM $MgSO_4$, 10 mM dithiothreitol, 0.6 mM ATP, and ligase in a final volume of 20 μl. Successful ligation was tested by agarose gel electrophoresis. Ligated DNA was precipitated with ethanol after phenol extraction. DNA was next packaged in vitro by using a commercially available in vitro packaging system under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md., or Promega Biotech, Madison Wis.). Exponential phase *E. coli* cells from an L-broth culture containing 0.2% maltose and 10 mM $MgSO_4$ grown at 37°C were infected with the phage particles at 30°C in the same medium. After 20 min, 1 volume of L-broth was added, the temperature was raised to 37°C, and incubation was continued for an additional 2 h. The cells were then spread onto L-agar plates containing 20 μg of tetracycline per ml, and the plates were incubated at 37°C for 16 h. Tetracycline-resistant (Tc^r) colonies were then transferred to two sets of plates. One set contained tetracycline (20 μg/ml), and the other contained kanamycin at a concentration of 25 μg/ml. Those colonies that had a Tc^r kanamycin-sensitive (Km^s) phenotype were selected for further analysis.

Conjugation experiments. *E. coli* MM294(pRK2013) and *E. coli* HB101 harboring the recombinant DNA clones were cultured overnight at 37°C in L-broth. The strains of *V. anguillarum* to be used as recipients were grown at 20°C in Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% NaCl. Mixtures containing 5 ml of each of the cultures described above were filtered through 0.2-μm membrane filters (Millipore Corp., Bedford, Mass.). The filters were incubated at 30°C for 16 h on plates containing Trypticase soy agar supplemented with 1% NaCl. After incubation the cells were resuspended, plated onto Trypticase soy agar containing 1% NaCl and either tetracycline (5 μg/ml) or tetracycline (5 μg/ml) and ampicillin (1 mg/ml), depending on whether the *V. anguillarum* recipient harbored a Tn*1* insertion derivative of pJM1, and incubated at 24°C.

Agarose gel electrophoresis of plasmid DNA. Electrophoresis of plasmid DNA was performed as previously described (25).

Bioassays for detection of siderophore activity. The ability of supernatants or total homogenates from *V. anguillarum* strains to crossfeed indicator strain 775::Tn*1*-5(pJHC-91), which is deficient in the production of siderophores but still shows receptor activity, was used as an indication of the level of siderophore activity. The bioassays were carried out as described previously (11, 25).

Analysis of outer membrane proteins. Outer membrane proteins from *V. anguillarum* cells were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Crosa and Hodges (8). Cells were grown in M9 minimal medium containing either 50 μM $FeCl_3$

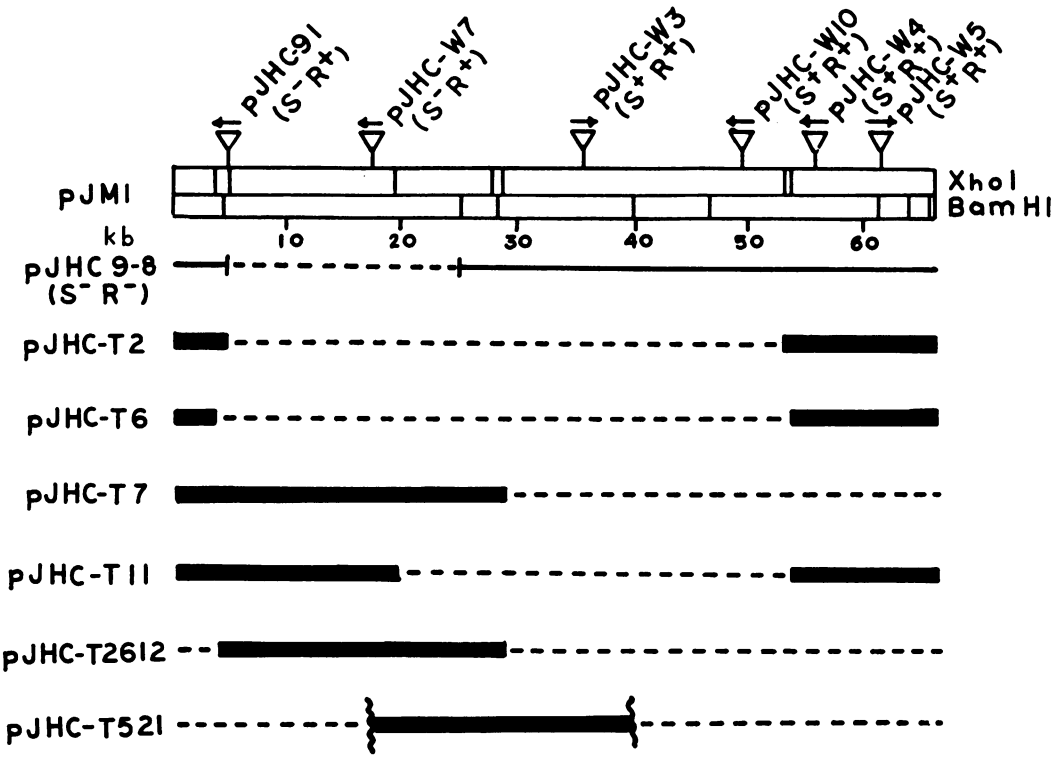

FIG. 2. Genetic and physical maps of pJM1 derivatives and recombinant clones. The upper bar is a *Xho*I and *Bam*HI map of pJM1. The open triangles indicate the sites of Tn*1* insertions in several pJM1 derivatives, and the arrows give the orientation of the Tn*1* transposition sequence with respect to β-lactamase gene transcription. Also shown are the phenotypes of *V. anguillarum* strains carrying each of these derivatives. Iron uptake-proficient derivatives are indicated as S^+R^+ (production of siderophore [S] and receptor [R]); iron uptake-deficient derivatives are classified depending on whether the Tn*1* insertion or deletion affected the genes associated with the production or activity or either the siderophore (S^-R^+) or both siderophore and receptor (S^-R^-). The dashed lines correspond to pJM1 DNA regions that were either deleted (e.g., in pJHC9-8) or were not included in the cloned DNA. The thick lines represent the pJM1 DNA cloned in pVK102. Clones pJHC-T2, pJHC-T6, pJHC-T7, pJHC-T11, and pJHC-T2612 were obtained by cloning *Xho*I partial digests of pJM1 DNA into pVK102. Recombinant clone pJHC-T521 was the result of cloning *Hin*dIII partial digests of pJM1 DNA in the same vector. The interrupted thick line on the diagram for this clone indicates that the cloned region extends a short stretch beyond either end as mapped by positioning the *Hin*dIII fragments on the *Xho*I map. The numbers below the upper bar indicate kilobase pairs (kb).

(iron sufficiency), 10 μM ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA) (for iron uptake-proficient strains), or 2 μM EDDA (for iron uptake-deficient strains). In the case of iron uptake-deficient strains still expressing receptor activity, we also used a concentration of 10 μM EDDA, but in the presence of 50% sterile supernatant from wild-type strain 775(pJM1).

Infectivity assays. The virulence assays were performed on juvenile rainbow trout (*Salmo gairdneri*) weighing about 5 g. For each bacterial dilution tested four fish were anesthetized with tricaine methane sulfonate (100 ppm [1 mg/liter]) and inoculated subcutaneously at the posterior base of the dorsal fin with 0.05-ml dilutions of a bacterial suspension from a 12-h M9 minimal medium culture. Fish were kept at 15°C for 7 days, and mortalities were examined by bacteriological culture techniques. Kidney material was streaked, and bacteria were reisolated and tested for purity. Mortalities were considered to be due to *V. anguillarum* only when the bacterium was reisolated in a pure culture. Mean lethal dose values (number of microorganisms that killed 50% of the animals inoculated) were determined as described previously (9).

RESULTS

Cloning of plasmid pJM1 DNA fragments in *E. coli* and transfer to *V. anguillarum*. Since plasmid pJM1 DNA can be transformed into *E. coli* but there is no detectable expression of the iron transport system, we decided to use as a cloning vector a plasmid that could be mobilized to *V. anguillarum*. Consequently, we used the vector pVK102, a derivative of pRK290 (13), which carries the lambda cos site and genes for resistance to the antibiotics tetracycline and kanamycin (18). We recently determined that this plasmid could be mobilized efficiently from *E. coli* to *V. anguillarum* by using the transfer factor pRK2013 described by Ditta et al. (14). To perform the cloning experiments, pJM1 DNA fragments, which were obtained by partial digestion with either *Xho*I or *Hin*dIII restriction endonuclease, were ligated in the *Xho*I or *Hin*dIII sites of cosmid vector pVK102. The ligated DNA was packaged in vitro, and the phage particles were used to transduce *E. coli* HB101. Digestion of pJM1 DNA with restriction endonuclease *Xho*I yielded eight fragments which ranged in size from 24.4 to 0.55 kilobases (Fig. 1a, lane H). Consequently, plasmid DNA from the Tc^r Km^s clones was cleaved with restriction endonuclease *Xho*I to determine the *Xho*I fragments of pJM1 DNA that had been cloned. Several clones carrying pJM1 DNA fragments encompassing regions associated with iron uptake, as mapped by Tn*1* insertions (25), were selected for further studies. The *Xho*I restriction endonuclease cleavage patterns and a physical map of these clones are shown in Fig. 1a and 2, respectively. Each of these clones was then transferred to *V. anguillarum* by using

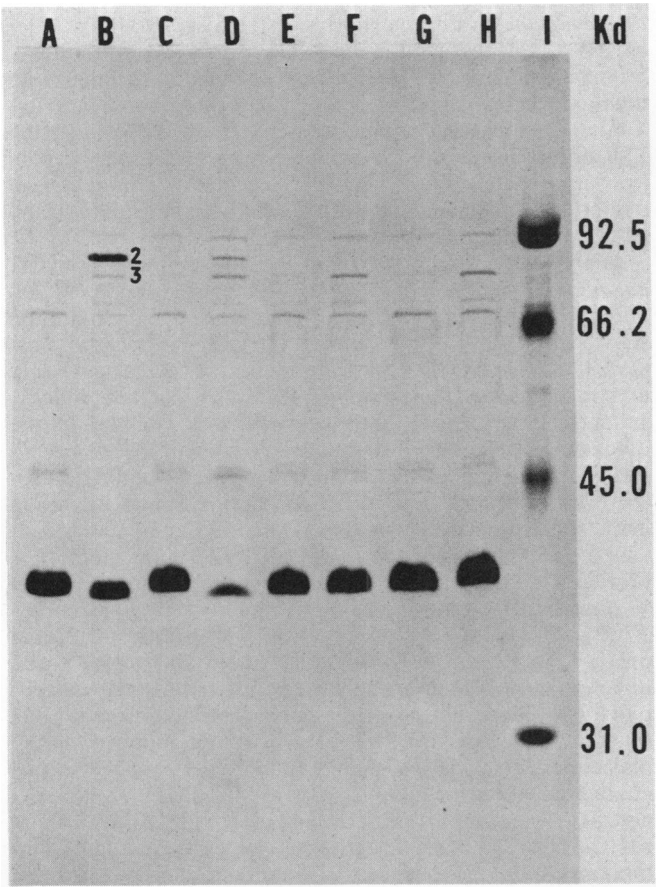

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of outer membrane proteins of *V. anguillarum* harboring recombinant cosmid pJHC-T7. *V. anguillarum* cells were cultured under either iron-proficient conditions (in the presence of 50 μM $FeCl_3$) or conditions of iron limitation, which were achieved by adding 10 μM EDDA for wild-type *V. anguillarum* 775(pJM1), 10 μM EDDA supplemented with 50% sterile supernatant from *V. anguillarum* 775(pJM1) for *V. anguillarum* H775-3A(pJHC-T7), or 2 μM EDDA for *V. anguillarum* H775-3 and H775-3 containing pVK102. Lanes A, C, E, and G, Outer membrane proteins obtained from cultures growing under iron-proficient conditions; lanes B, D, F, and H, outer membrane proteins obtained from cultures grown under iron limitation conditions. Lanes A and B, 775(pJM1); lanes C and D, H775-3A(pJHC-T7); lanes E and F, H775-3(pVK102); lanes G and H, plasmidless strain H775-3; lane I, molecular weight standards. Electrophoresis was performed in a 12.5% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 44:0.8) as described previously (8).

a triparental mating. An *E. coli* strain harboring the pJM1 DNA clones was mixed with *E. coli* (pRK2013) and the appropriate *V. anguillarum* recipients as described above. The *V. anguillarum* strains used as recipients were plasmidless strain H775-3, strain 775::Tn*1*-5 carrying Tn*1* insertion derivative pJHC-91, strain 775::Tn*1*-6 carrying deletion plasmid pJHC9-8, or strain 775::Tn*1*-7 carrying insertion plasmid pJHC-W7. The locations of the insertions and deletions on pJM1 DNA are shown in Fig. 2. To determine that the recombinant clones had the same topological configuration after transfer to *V. anguillarum*, plasmid DNAs from the exconjugant clones were analyzed by cleavage with restriction endonuclease *Xho*I. For the sake of simplicity, Fig. 1b only shows the results for exconjugants obtained by transfer of recombinant cosmid pJHC-T2612 to each of the *V. anguillarum* recipients. It is evident that the recombinant cosmid in *V. anguillarum* H775-3 had the same *Xho*I banding pattern as the cosmid isolated from *E. coli*. This recombinant cosmid and all of the others examined were stably maintained in *V. anguillarum* strain H775-3. In the case of recipient *V. anguillarum* strains 775::Tn*1*-5, 775::Tn*1*-6, and 775::Tn*1*-7, the banding patterns resulted from the addition of the recombinant cosmid bands and the bands obtained from cleavage of the indigenous pJM1 derivatives. Moreover, in all of these latter cases, the presence of the two independent plasmids was confirmed by agarose gel electrophoresis, indicating that they were not recombined as a cointegrate. Similar results were also found with the other recombinant clones (pJHC-T2, pJHC-T6, pJHC-T7, and pJHC-T11) independently of whether they originated in either *E. coli* or *V. anguillarum*. The plasmids carried by each exconjugant clone are listed in Table 1.

Siderophore and receptor activity of *V. anguillarum* cells harboring the recombinant cosmids. *V. anguillarum* 775::Tn*1*-5 carries plasmid pJHC-91, which confers to the cells the capacity to produce the postulated receptor for complexes of iron and siderophore (25) (Table 1). Since this strain does not produce the pJM1-mediated siderophore due to a Tn*1* insertion in the pJM1 iron uptake region, which generated pJHC-91, it was used as an indicator in bioassays to detect the production of the diffusible siderophore by the *V. anguillarum* exconjugants carrying the recombinant cosmids. The results of this type of bioassay are shown in Table 1. Exconjugants harboring either cosmid pJHC-T7 or pJHC-T2612, in addition to an indigenous pJM1 derivative, such as pJHC-91 or the deleted plasmid pJHC9-8, expressed siderophore activity (Table 1). Similar results were obtained when the indigenous *V. anguillarum* plasmid was pJHC-W7, which, like pJHC-91, has an insertion in genes determining siderophore activity and possesses an intact receptor gene. Conversely, Table 1 also shows that supernatants from exconjugants harboring recombinant cosmid pJHC-T11 in addition to either pJHC-91 or pJHC-W7 were unable to crossfeed the indicator strain. Plasmidless strains of *V. anguillarum* or strains harboring either one of the indigenous plasmids but not carrying a recombinant cosmid did not show any siderophore activity under the conditions of the bioassay (Table 1). Exconjugants harboring either pJHC-T7 or pJHC-T2612 in the absence of an indigenous plasmid produced a consistently lower amount of siderophore (barely detectable but reproducible in the plate bioassay), whereas supernatants from exconjugants harboring only either pJHC-T2, pJHC-T6, pJHC-T11, pJHC-T521, or cloning vector pVK102 did not show any siderophore activity (Table 1). To determine whether the lower siderophore activity of supernatants from exconjugants harboring either pJHC-T7 or pJHC-T2612 alone was due to a less efficient export mechanism (that is, whether the siderophore was being synthesized but not transported outside the bacterium), cells of the exconjugants harboring either pJHC-T7 or pJHC-T2612 were sonically treated, and the homogenates were tested before and after filtration. The results (data not shown) indicated that homogenates from *V. anguillarum* harboring either pJHC-T7 or pJHC-T2612 also had very low siderophore activity, about the same as the activities found in the respective supernatants. Since similar low levels were obtained with the homogenate before and after filtration, these results suggest that the siderophore neither is in the cell cytoplasm nor is bound to the cell membrane. Thus, a deficiency in siderophore export did not appear to be the reason for our findings. Homogenates of the plasmidless *V.*

anguillarum cells were devoid of siderophore activity, whereas homogenates obtained from cells harboring wild-type plasmid pJM1 exhibited, as expected, high siderophore activity. These results indicate that the sonication and filtering procedures did not affect the stability of the siderophore, and thus the amount of siderophore present in the cells carrying recombinant cosmid pJHC-T7 or pJHC-T2612 must have been actually very low. Thus, these results suggest that an additional factor present in those *V. anguillarum* strains harboring pJHC-91, pJHC-W7, or pJHC9-8 was necessary for the production of a normal level of siderophore activity. To investigate whether any of the recombinant cosmids carried information for the pJM1-mediated receptor activity, we performed bioassays by using as indicators each of the *V. anguillarum* exconjugants harboring the recombinant cosmids. The results of this experiment are presented in Table 1. *V. anguillarum* strains carrying either pJHC-T7, pJHC-T11, or pJHC-T2612 alone could be specifically crossfed with supernatants from the wild-type strain harboring pJM1. Similarly, receptor activity was also found when pJHC-T11 was introduced into a *V. anguillarum* recipient already harboring plasmid pJHC9-8, which has a deletion affecting the genes for both siderophore and receptor activity. Of course, transfer of pJHC-T11 into *V. anguillarum* strains harboring plasmids such as pJHC-91 or pJHC-W7, which are only affected in DNA regions concerned with siderophore production, also resulted in derivatives with receptor activity. In the case of pJHC-T7 and pJHC-T2612, introduction of these cosmids into *V. anguillarum* strains harboring pJHC-91, pJHC-W7, or pJHC9-8 resulted in derivatives that showed confluent growth when they were used as indicator strains in the bioassay; therefore, these derivatives must possess an iron uptake-proficient phenotype (see below). Neither cloning vector pVK102 nor recombinant cosmids pJHC-T2, pJHC-T6, and pJHC-T521, when introduced into *V. anguillarum*, were able to mediate receptor activity.

Ability of *V. anguillarum* strains carrying recombinant cosmids to grow under iron-limiting conditions. The results described above suggest that recombinant cosmids pJHC-T7 and pJHC-T2612 apparently carry genetic determinants for expression of both siderophore and receptor activity, whereas recombinant cosmid pJHC-T11 only contains genes involved in receptor activity.

Since possession of siderophore and receptor activity should be correlated with iron uptake proficiency, whereas lack of either one of these two properties should be reflected in an iron uptake-deficient phenotype, we tested the iron uptake properties of the cloned derivatives by determining the MIC of the iron-chelating agent EDDA for the growth of these strains. Table 1 shows that iron uptake-deficient strains of *V. anguillarum* already harboring either pJHC-91 or pJHC-W7 or the deletion plasmid pJHC9-8 increased their MICs for EDDA from 2 to 40 μM upon introduction of either pJHC-T7 or pJHC-T2612, thus becoming iron uptake proficient, since 40 μM is the MIC of EDDA for wild-type strain 775 harboring pJM1. Surprisingly, introduction of these same recombinant plasmids into plasmidless *V. anguillarum* strain H775-3 resulted in no modification of the MIC of EDDA, and thus such strains remained iron uptake deficient. This result was somewhat correlated with the poor siderophore activity encountered with these derivatives. Since these clones showed an active receptor, the reduced amount of siderophore activity could have been the reason behind their inability to grow under conditions of iron limitation. Thus, an additional factor present in those *V. anguillarum* strains harboring pJHC-91, pJHC-W7, or pJHC9-8 is necessary for the expression of an iron uptake-proficient phenotype, which must then be associated with the production of an efficient amount of siderophore activity. Table 1 also shows that neither pJHC-T2, pJHC-T6, pJHC-T11, nor pJHC-T521 nor cloning vector pVK102 affected the iron uptake-deficient phenotype of the *V. anguillarum* strains tested, independently of whether they carried any additional indigenous plasmids.

Analysis of outer membrane proteins synthesized by *V. anguillarum* strains harboring recombinant cosmids. *V. anguillarum* strains growing under conditions of iron limitation showed induction of two novel outer membrane proteins, OM2 (86 kd) and OM3 (79 kd). Induction of OM2 occurs only in strains carrying plasmid pJM1 (8). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the outer membrane proteins synthesized by various *V. anguillarum* strains indicated that an 86-kd outer membrane protein was indeed induced under conditions of iron limitation in the strain carrying plasmid pJM1 (Fig. 3, lanes A and B). It was of interest that induction of an 86-kd outer membrane protein under conditions of iron limitation also occurred in *V. anguillarum* strains carrying recombinant cosmids pJHC-T7 (Fig. 3, lanes C and D, and Table 1), pJHC-T2612, and pJHC-T11 (Table 1). This 86-kd protein was found to be immunologically identical to OM2 by immunological western blots with antiserum prepared against purified protein OM2 (data not shown). Protein OM2 was not induced in the plasmidless strain (Fig. 3, lanes E and F) or in those strains containing either the cloning vector pVK102 (Fig. 3, lanes G and H) or recombinant cosmid pJHC-T2, pJHC-T6, or pJHC-T521 (Table 1). Of course, in all cases, under iron-limiting conditions there was also induction of 79-kd protein OM3, which was the only protein induced either in the isogenic plasmidless derivative (Fig. 3, lanes G and H) or in the strain harboring cloning vector pVK102 (Fig. 3, lanes E and F). This was also the case for strains harboring pJHC-T2, pJHC-T6, or pJHC-T521.

Virulence of *V. anguillarum* strains harboring recombinant clones. Strains of *V. anguillarum* harboring recombinant clones were used in experimental infections. Table 2 shows that the virulence of strain 775::Tn*1*-5 was greatly increased when it harbored, in addition to indigenous plasmid pJHC-91, either pJHC-T7 or pJHC-T2612 recombinant plasmid. The mean lethal dose values for these strains were of the same order as the value obtained with wild-type strain 775 carrying pJM1, in contrast to the low-virulence phenotype of the original recipient strain carrying only plasmid pJHC-91 or the controls (plasmidless strain H775-3 and strain H775-3A harboring only pJHC-T7). Conversely, when the plasmid carried by strain 775::Tn*1*-5 was pJHC-T11 in addition to pJHC-91, the virulence phenotype was not affected. Since, unlike pJHC-T11, both pJHC-T7 and pJHC-T2612 could confer an iron uptake-proficient phenotype to strain 775::Tn*1*-5(pJHC-91) (Table 1), our results show a correlation between iron uptake proficiency and the virulence phenotype conferred by these recombinant plasmids.

DISCUSSION

The presence of plasmid-mediated iron uptake systems has been associated with pathogenicity in *V. anguillarum* and also in invasive strains of *E. coli* (6, 7, 7a, 27, 28). In the case of *V. anguillarum*, plasmid pJM1 has been demonstrated to contain the genes for the iron uptake system which mediates the high-virulence phenotype of certain strains of this bacterium. To elucidate in detail the molecular mecha-

TABLE 1. Properties of *V. anguillarum* strains

Strain	Plasmid(s)	Siderophore activity[a]	Receptor activity[b]	Presence of OM2[c]	MIC for EDDA (μM)[d]
775	pJM1	+	C	+	40
H775-3	None	−	−	−	2
775::Tn*1*-5	pJHC-91	−	+	+	2
775::Tn*1*-6	pJHC9-8	−	−	−	2
775::Tn*1*-7	pJHC-W7	−	+	+	2
775::Tn*1*-5A	pJHC-91, pJHC-T7	+	C	+	40
775::Tn*1*-6A	pJHC9-8, pJHC-T7	+	C	+	40
775::Tn*1*-7A	pJHC-W7, pJHC-T7	+	C	+	40
H775-3A	pJHC-T7	Low	+	+	2
775::Tn*1*-5B	pJHC-91, pJHC-T2612	+	C	+	40
775::Tn*1*-6B	pJHC9-8, pJHC-T2612	+	C	+	40
775::Tn*1*-7B	pJHC-W7, pJHC-T2612	+	C	+	40
H775-3B	pJHC-T2612	Low	+	+	2
775::Tn*1*-5C	pJHC-91, pJHC-T11	−	+	+	2
775::Tn*1*-7C	pJHC-W7, pJHC-T11	−	+	+	2
H775-3C	pJHC-T11	−	+	+	2
H775-3D	pJHC-T2	−	−	−	2
H775-3E	pJHC-T6	−	−	−	2
H775-3F	pJHC-T521	−	−	−	2
H775-3102	pVK102	−	−	−	2

[a] Siderophore activity was determined by a crossfeeding bioassay as described previously (11, 25), using supernatants from each strain and indicator strain 775::Tn*1*(pJHC-91) as the lawn strain.

[b] Receptor activity was determined by using the crossfeeding bioassay used for siderophore activity, although in this case each of the strains tested was used as the lawn strain to be checked for crossfeeding with supernatant from wild-type strain 775(pJM1). C, Confluent growth due to the iron uptake-proficient phenotype.

[c] Induction of the protein OM2 under iron-limiting conditions was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological western blots with antiserum against purified protein OM2 from strain 775(pJM1).

[d] MICs of EDDA were determined on plates containing M9 minimal medium on 0.7% agarose with increasing concentrations of EDDA.

nisms of the *V. anguillarum* iron uptake process mediated by pJM1 and, concomitantly, to understand how this mechanism affects the pathogenicity of the bacterium, we molecularly cloned the essential components of this system. Since a functional pJM1 iron uptake system is not expressed in *E. coli* (J. H. Crosa and L. L. Hodges, unpublished data), we had to use a cloning vector that permitted us to return the cloned genetic information to the *V. anguillarum* cytoplasm. By using cosmid vector pVK102 (18), a derivative of wide-host-range plasmid pRK290 constructed by Ditta et al. (13), we were able to mobilize back to *V. anguillarum* clones carrying regions of pJM1 DNA concerned with iron uptake. We describe above three clones, pJHC-T7, pJHC-T11, and pJHC-T2612, which were able to mediate receptor activity upon transfer to *V. anguillarum*. Figure 2 shows that these three clones have in common a stretch of pJM1 DNA about 15.5 kilobases long that includes two *Xho*I fragments (about 14.2 and 1.35 kilobases). Thus, this region must carry all of the genetic information for receptor biosynthesis or activity. In addition, an 86-kd protein immunologically identical to OM2, an outer membrane protein presumed to be the putative receptor (8, 25), is also induced under conditions of iron limitation in *V. anguillarum* cells harboring any of the aforementioned clones. Thus, it is possible that if OM2 is the actual receptor, the cloned region may also carry the necessary information for the regulation of its transcription, since its activity in the cloned derivatives is still regulated by the iron status of the cell.

It was of interest that transfer of either pJHC-T7 or pJHC-T2612 to plasmidless *V. anguillarum* strain H775-3 was correlated not only with receptor activity but also with the production of low, but detectable, siderophore activity. These clones did not overcome the iron uptake-deficient phenotype of the recipient strain. However, transfer of these same clones to strains such as 775::Tn*1*-5, 775::Tn*1*-6, and 775::Tn*1*-7, which carry plasmids pJHC-91, pJHC9-8, and pJHC-W7, respectively, resulted in higher siderophore activity and, concomitantly, an iron uptake-proficient phenotype. Since the recombinant cosmids are stably maintained in strain H775-3 and the only difference between this strain and the other recipients is the presence of a plasmid pJM1 derivative in the latter strains, any explanation of our results should involve the existence of additional genetic information for siderophore activity that is present in the pJM1 derivatives but absent from the clones. One explanation for these results is the existence of a *trans*-acting factor that regulates the transcription or translation or both of the siderophore genes. This genetic information must be encoded on a plasmid pJM1 DNA region different from the regions cloned. An alternative explanation for our findings is that the clones are deficient in genetic information for export of the siderophore outside the bacterial cell. This possibility was tested in this work by investigating the siderophore activity present in cell sonicates, which should be a measure of the actual amount of siderophore that is present inside the cells. Our results showed that the strain which contained wild-type plasmid pJM1 and thus was iron uptake proficient showed a high level of internal siderophore activity, whereas strain H775-3 harboring either pJHC-T7 or pJHC-T2612, which had supernatants with low siderophore activity, also had correspondingly low levels of activity inside the cells. Thus, we believe that deficient export is not an explanation for our findings. Of course, other models could explain our data. For instance, the clones may lack one or more of the genes encoding enzymes of the siderophore biosynthetic pathway, resulting in a "defective" siderophore which cannot form stable complexes with iron, or the complexes, once formed, cannot interact properly with the bacterial cell receptor complex. Our results with *V. anguillarum* strains carrying plasmid pJHC9-8, which has an extensive deletion of the pJM1 iron uptake region, suggest that this is not the case. The pJHC-T7 and pJHC-T2612 recombinant clones, when introduced into this strain, yield iron uptake-proficient derivatives despite the lack of most of the pJM1 iron uptake

TABLE 2. Virulence of *V. anguillarum* strains harboring recombinant plasmids

Strain	Plasmid(s)	Virulence[a]
775	pJM1	0.2×10
H775-3	None	1.0×10^6
775::Tn*1*-5	pJHC-91	1.0×10^6
775::Tn*1*-5A	pJHC-91 and pJHC-T7	0.9×10^2
775::Tn*1*-5B	pJHC-91 and pJHC-T2612	0.3×10^2
775::Tn*1*-5C	pJHC-91 and pJHC-T11	1.2×10^6
H775-3A	pJHC-T7	3.0×10^6

[a] The values are the mean lethal dose values (number of microorganisms that killed 50% of the animals inoculated). The experimental infections and mean lethal dose determinations were performed as described in the text.

region on the indigenous plasmid, as determined by Tn*1* transposition analysis (25). Determination of whether the *trans*-acting factor is either a regulatory substance or actually a biosynthetic enzyme that is necessary for the generation of a complete siderophore molecule requires knowledge of the structure of the pJM1-mediated siderophore. Experiments to purify this siderophore and determine its molecular nature are in progress. Another important feature of the pJM1 system shown in this work is that restoration of the ability to grow under conditions of iron limitation by introduction of a recombinant clone into one of the low-virulence *V. anguillarum* strains was correlated with an increase in bacterial pathogenicity, demonstrating the intimate relationship between these two properties.

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