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FvtA Is the Receptor for the Siderophore Vanchrobactin in *Vibrio anguillarum*: Utility as a Route of Entry for Vanchrobactin Analogues▽†

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Some strains of *Vibrio anguillarum*, the causative agent of vibriosis in a variety of marine animals, produce a catechol-type siderophore named vanchrobactin. The biosynthetic pathway and regulation of vanchrobactin are quite well understood. However, aspects concerning its entry into the cell have remained uncharacterized. In the present study we characterized two genes, *fvtA* and *orf13*, encoding potential TonB-dependent ferric-vanchrobactin receptors in serotype O2 *V. anguillarum* strain RV22. We found that an *fvtA* mutant was defective for growth under iron limitation conditions and for utilization of vanchrobactin, suggesting that *fvtA* encodes the vanchrobactin receptor of *V. anguillarum*. Interestingly, an *orf13* mutant was not significantly affected, and results of reverse transcriptase PCR, as well as analysis of outer membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggested that this gene is not expressed. Furthermore, *fatA*, a plasmid gene coding for the anguibactin receptor in plasmid pJM1-harboring strains, is also present in the chromosome of RV22, although it is inactivated by insertion of transposases. In addition, we found that FvtA is the route of entry for vanchrobactin analogues, and there is evidence that it recognizes primarily the catechol-iron center. These analogues are potential candidate vectors for a Trojan horse strategy aimed at generating antimicrobial compounds exploiting the same route of entry for native siderophores. We found that *fvtA* and vanchrobactin biosynthesis genes are ubiquitous in both vanchrobactin- and anguibactin-producing *V. anguillarum* strains, which reinforces the utility of the vanchrobactin route of entry for the design of future strategies for the control of vibriosis.

Vibrio anguillarum strains possess an array of specific virulence factors that enable them to cause a hemorrhagic septicemia called vibriosis in a variety of marine animals (41). There are more than 20 recognized serotypes, but serotypes O1 and O2 are the serotypes predominantly implicated in vibriosis outbreaks (43). Although the virulence mechanisms of *V. anguillarum* are not fully understood, it is known that the ability to scavenge iron through utilization of siderophores contributes significantly to the virulence of this bacterium (11, 20, 45). Currently, two clearly different siderophore-mediated iron uptake systems in *V. anguillarum* are known. One of them is the vanchrobactin-mediated system encoded by a chromosomal gene cluster. The chemical structure of the siderophore in this system and its biosynthesis and regulation pathways were recently established (3, 4, 36). The other system is a plasmid pJM1-encoded system called the anguibactin system that is found only in pJM1-containing serotype O1 strains (10, 40), whose synthesis requires additional chromosomal genes that are also involved in vanchrobactin production (1, 4).

Uptake of ferric iron-siderophore complexes into the cell requires a specific outer membrane (OM) receptor protein connected to a TonB-ExbB-ExbD complex that produces the energy necessary for active transport (6, 12). Two functional *tonB* systems have been identified in *V. anguillarum*, and the *tonB2* system is essential for transport of siderophores and virulence (39). The ferric iron-anguibactin complex is transported via the OM receptor FatA, the periplasmic binding protein FatB, and the inner membrane proteins FatC and FatD (ABC transporter) (40). Although the genetic basis of anguibactin transport has been well characterized, little is known about how *V. anguillarum* vanchrobactin-producing strains transport the ferric iron-vanchrobactin complexes into the cell.

Increasing antibiotic-mediated selective pressure has led to the emergence of multiresistant strains of many bacterial pathogens, and fish pathogens are no exception. To facilitate penetration of antibiotics into bacterial cells, the so-called "Trojan horse strategy" can be employed, where antimicrobial drugs are transported across the bacterial membranes by exploiting the iron uptake pathways (7, 8, 24, 26, 34, 38). The vanchrobactin chemical structure was recently determined, and a series of vanchrobactin analogues which have functionality (an amino group) appropriate for use as antibiotic vectors and keep their siderophore activity have been synthesized and evaluated (36, 37). Similar approaches have been used and have provided promising results with the pyoverdine-mediated iron uptake system (18) and conjugated siderophore–β-lactamase inhibitors (9).

In previous studies, a gene cluster (*vab* cluster) encoding the functions involved in the biosynthesis of vanchrobactin and its

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TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Relevant characteristic(s)	Source or reference
Plasmids		
pGEMT-Easy	PCR cloning vector, Amp^r	Promega
pWKS30	Low-copy-number cloning vector, Amp^r	44
pNidKan	*pir*-dependent suicide plasmid, pCVD442 derivative, *sacB*, Kan^r	27
pHRP309	Low-copy-number vector, Gm^r	30
MBcos167	Cosmid containing *orf13*	3
MBcos69	Cosmid containing *vabD*	3
pMB54	*fvtA* gene and promoter cloned in pHRP309, Gm^r	This study
V. anguillarum strains		
RV22	Serotype O2 strain isolated from diseased turbot	20
MB11	RV22 Δ*vabB*	4
MB84	RV22 Δ*fvtA*	This study
MB70	RV22 Δ*orf13*	This study
MB90	RV22 Δ*fvtA* Δ*orf13*	This study
MB102	RV22 Δ*vabB* Δ*fvtA*	This study
MB104	RV22 Δ*vabB* Δ*orf13*	This study
MB107	RV22 Δ*vabB* Δ*fvtA* Δ*orf13*	This study
E. coli S17-1-λ*pir*	Tp^r Sm^r, *recA thi pro hsdR*-M+RP4::2-Tc::Mu::Km Tn*7* λ*pir*	19

regulation was widely characterized (3, 4). The *fvtA* gene, linked to the biosynthetic genes, was initially postulated to encode a vanchrobactin receptor (3), and it is known that *V. anguillarum* senses ferric-vanchrobactin in the extracellular environment, resulting in upregulation of the *fvtA* gene (3). However, the actual role of *fvtA* in the vanchrobactin uptake process has not been demonstrated yet. In this paper we functionally characterize the role of *fvtA* in acquisition of ferric-vanchrobactin and the possible utility of using the vanchrobactin acquisition pathway as a strategy for therapy against vibriosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used, including those derived in this study, are listed in Tables 1 and Table 3. *V. anguillarum* strains were grown at 25°C on tryptic soy agar (Difco) supplemented with 1% NaCl and in tryptic soy broth (Difco) supplemented with 1% NaCl, as well as in M9 minimal medium (25) supplemented with 0.2% Casamino Acids (Difco) (CM9). *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani medium (Pronadisa) supplemented with the appropriate antibiotics. Antibiotics (Sigma-Aldrich) were used at the following final concentrations: ampicillin (sodium salt) and kanamycin, 50 μg ml^{-1}; and gentamicin, 10 μg ml^{-1}. Ethylenediamine-di-(*o*-hidroxyphenyl acetic acid) (EDDA) and 2,2′-dipyridyl were used as iron chelators at appropriate concentrations.

DNA manipulations. Total genomic DNA from *V. anguillarum* was purified with an Easy-DNA kit (Invitrogen). Plasmid DNA purification and extraction of DNA from agarose gels were carried out using kits from Qiagen (Qiagen). DNA probe labeling and Southern blot analyses were performed with the ECL DNA labeling and detection system (Amersham Biosciences). DNA probes for gene screening by Southern blotting were obtained by PCR amplification using suitable primer pairs (see Table S1 in the supplemental material). PCRs were routinely carried out using a T-Gradient thermal cycler (Biometra) with *Taq* polymerase BioTaq (Bioline).

DNA sequencing and bioinformatics tools. DNA sequences were determined by the dideoxy chain termination method for either cosmid, plasmid, or PCR products using a GenomeLab DTCS quick start kit with a CEQ 8000 DNA sequencer (Beckman Coulter). Sequences were examined and assembled using BioEdit, version 7.0.4.1 (16). The European Bioinformatics Institute and the NCBI services were used to consult DNA and protein sequence databases with FASTA3 and BLAST algorithms.

Construction of *fvtA* and *orf13* mutants by allelic exchange. In-frame deletions of *fvtA* and *orf13* in *V. anguillarum* RV22 were constructed by using PCR amplification of two fragments of each gene and flanking regions, which, when ligated together, would result in an in-frame (nonpolar) deletion. The oligonucleotides used to amplify the upstream and downstream ends of each gene are shown in Table S1 in the supplemental material. Each deleted allele construction was ligated into the suicide vector pNidKan (27). As a pCVD442 derivative, pNidKan contains R6K *ori*, requiring the *pir* gene product for replication, and the *sacB* gene, conferring sucrose sensitivity. The resulting plasmids were mated from *E. coli* S17-1-λ*pir* into *V. anguillarum* wild-type strain RV22 and into previously constructed mutant strains, and exconjugants with the plasmid (conferring kanamycin resistance) integrated into the chromosome by homologous recombination were selected. A second recombination event involved selecting for sucrose (10%) resistance and further checking for plasmid loss and for allelic exchange. This process led to the generation of *V. anguillarum* single mutant strains MB84 (Δ*fvtA*) and MB70 (Δ*orf13*) and mutants MB102 (Δ*vabB* Δ*fvtA*), MB104 (Δ*vabB* Δ*orf13*), and MB107 (Δ*vabB* Δ*fvtA* Δ*orf13*). Deletion of the parental gene was checked by Southern blot hybridization, and DNA sequencing of the region involved in the deletion was carried out to ensure that all mutations were in frame.

Complementation of *V. anguillarum fvtA* mutants. The *fvtA* gene, along with its promoter sequence, was PCR amplified from the *V. anguillarum* RV22 chromosome using specific primers (see Table S1 in the supplemental material), cloned into the pHRP309 vector (30), and subsequently transformed into *E. coli* strain S17-1-λ*pir*. The resulting plasmid (Table 1) was mated from *E. coli* S17-1-λ*pir* into the *V. anguillarum fvtA* mutant, and transformants were selected on agar medium containing gentamicin (resistance conferred by pHRP309) and ampicillin (to select for *V. anguillarum*).

Growth under iron-limited conditions and test for siderophore production. To test the ability of *V. anguillarum* deletion mutants to grow under iron-limited conditions, the optical densities at 600 nm (OD_{600}) of overnight cultures in Luria-Bertani medium of the parental and mutant strains were adjusted to 0.5, and the cultures were diluted 1:15 in CM9 containing 10 μM EDDA. In the case of the complemented *fvtA* mutant, gentamicin was added to avoid loss of plasmid pMB54. Cultures were incubated at 25°C with shaking at 150 rpm, and growth (OD_{600}) was measured after 12 h of incubation. Siderophore production was measured using the chrome azurol S (CAS) liquid assay (35), which detects the presence of iron-chelating compounds. For siderophore production, strains were grown with 5 μM EDDA instead of 10 μM EDDA to allow cultures to grow enough to make siderophore secretion detectable. A noninoculated sample of CM9 containing EDDA at an appropriate concentration and a sample of the *vabB* mutant (MB11) were used as a spectrophotometric blank and as a negative control for the CAS liquid assay, respectively. Growth curve and CAS assays were carried out in triplicate, and the results shown below are the means of three independent experiments.

OM protein analysis. *V. anguillarum* strains were grown in CM9 supplemented with either 10 μM $Fe_2(SO_4)_3$ or 5 μM EDDA (iron-sufficient or iron-restricted conditions, respectively). Cells were centrifuged, and OM proteins were obtained as previously described (42). The protein concentration was adjusted for all the

samples, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein bands were visualized by staining with Coomassie brilliant blue.

Cross-feeding assays. The biological activities of vanchrobactin and analogues of this compound were determined by using bioassays. Strains MB11 (Δ*vabB*), MB102 (Δ*vabB* Δ*fvtA*), MB104 (Δ*vabB* Δ*orf13*), and MB107 (Δ*vabB* Δ*fvtA* Δ*orf13*), used as indicator strains, were inoculated into CM9 containing the iron chelator 2,2′-dipyridyl at a concentration of 120 μM, a concentration higher than the MIC for the wild-type strain. Paper disks were loaded with 25 μg of each compound and put on the surfaces of the plates. The compounds tested were synthetic vanchrobactin (2,3-dihydroxybenzoic acid [DHBA]–Arg-Ser) and several synthetic derivatives with known siderophore activity, including DHBA-Orn, DHBA-Orn-Ser, DHBA-Ser, DHBA-Ser-Orn, DHBA-Ser-Arg, and DHBA-Arg (37), that could be the vanchrobactin esterase product. The results were considered positive when a compound promoted the growth of indicator strains. A 10 μM $Fe_2(SO_4)_3$ solution was used as a positive growth control.

RNA purification and RT-PCR. *V. anguillarum* cultures (5 ml) were grown until exponential phase in low-iron CM9 containing 5 μM EDDA, and total RNA was isolated with the RNA isolation reagent RNAwiz (Ambion) by following the manufacturer's recommendations. Reverse transcriptase (RT) PCRs were performed with 0.5 to 3 μg of RNA pretreated with RQ1 RNase-free DNase (Promega) by using the Moloney murine leukemia virus RT (Invitrogen). Negative controls for PCR were performed with total RNA without Moloney murine leukemia virus RT to confirm the lack of genomic DNA contamination in each reaction mixture. A primer pair flanking a 639-bp internal fragment of the *fvtA* gene was used to PCR amplify the cDNA from *fvtA* transcripts (see Table S1 in the supplemental material).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been deposited in the EMBL database under accession number AM168450.

RESULTS AND DISCUSSION

The vanchrobactin gene cluster includes two genes encoding predicted TonB-dependent ferric iron-siderophore receptors. The vanchrobactin biosynthesis gene cluster (*vab* cluster) includes a gene designated *fvtA* encoding a protein with high levels of similarity to TonB-dependent siderophore receptors. Expression of *fvtA* is known to be iron regulated via the Fur repressor, and *fvtA* is cotranscribed with *vabD*, a gene encoding a phosphopantetheinyl transferase essential for vanchrobactin biosynthesis. In addition, expression of *fvtA* was found to be vanchrobactin dependent (3). These findings, together with homology data, suggested that FvtA could be the vanchrobactin receptor, although no direct experimental data confirmed this hypothesis.

Using *V. anguillarum* RV22 cosmid clones identified in a previous study (3), we extended DNA sequencing on both sides of the *vab* cluster and identified a previously undescribed gene designated *orf13* downstream of *vabR* and transcribed from the same strand (Fig. 1). VabR is a predicted LysR family regulator that activates expression of *vabG*, a gene encoding a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase involved in vanchrobactin biosynthesis (3). Downstream of *orf13* we found a putative lipase gene. On the other side of the *vab* cluster, sequencing downstream of *vabD* yielded a gene encoding a putative NADH-quinone reductase (Fig. 1). We believe that the *vab* cluster sequence might now be complete, since the proteins encoded by the two new genes at the borders of this cluster (lipase and NADH-quinone reductase) do not show any known relationship with siderophore synthesis or transport proteins, as deduced from protein database homology searches. Interestingly, the predicted protein encoded by *orf13* showed homology with, among other proteins, FepA, the OM receptor for ferrienterochelin and colicins of *Vibrio*

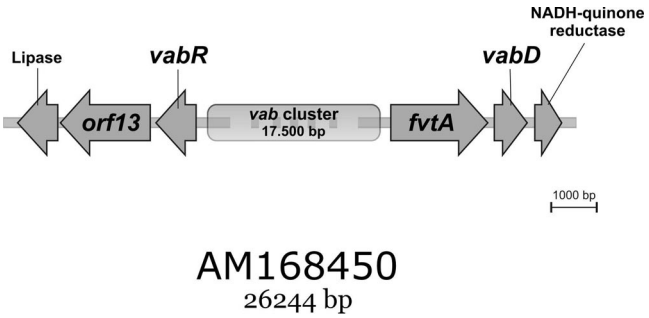

FIG. 1. Physical map of the *V. anguillarum* RV22 *vab* cluster, showing newly sequenced genes at the borders of the previously sequenced *vab* genes (4). Flanking genes encoding a predicted lipase and an NADH-quinone reductase are thought to be not part of the *vab* cluster.

alginolyticus (accession no. ZP_01260504; 59% identity and 77% similarity), and IrgA, the enterobactin receptor of *Vibrio cholerae* (accession no. ZP_01950374; 44% identity and 60% similarity) (14).

The extended *vab* cluster, as described here, includes two genes which encode potential OM receptors for vanchrobactin. Alignment of these two protein sequences with the sequences of described TonB-dependent receptors (data not shown) showed that there was conservation of some residues near the N termini of both FvtA (DETVVVVGE) and ORF13 (METLVVTAS) (residues conserved in other TonB-dependent ferric siderophore receptors are underlined), which might be involved in direct interaction with the TonB protein (the so-called TonB box) (15, 22, 29). In addition, various OM proteins possess a highly conserved C-terminal sequence, which was proposed to form an amphipathic β-sheet important for correct assembly of the protein in the OM (13). This sequence motif also is present in ORF13 and FvtA and in other OM TonB-dependent siderophore and heme receptors of other *Vibrio* species.

FvtA is directly involved in ferric-vanchrobactin uptake. Since *fvtA* and *orf13* are part of the *vab* cluster and are closely linked to other genes whose role in vanchrobactin biosynthesis has already been demonstrated, these two genes are candidates for the genes that encode the ferric-vanchrobactin OM receptor. In order to assess this possibility, in-frame deletion mutants were constructed by allelic exchange, and their ability to grow under iron limitation conditions was evaluated. As a control, we also included in these experiments the vanchrobactin biosynthesis mutant MB11 (Δ*vabB*) (3). Under iron-sufficient conditions (CM9 plus 10 μM ferric sulfate), no significant differences in growth rates between the mutants and parental strain RV22 were observed (Fig. 2). However, when the same strains were cultured under iron-restricted conditions (CM9 with 10 μM EDDA), the growth of the Δ*fvtA* mutant (MB84) was significantly impaired (Fig. 2), resulting in growth levels similar to those of the vanchrobactin-deficient mutant, whereas the growth of the Δ*orf13* mutant (MB70) was not affected. As expected, a Δ*fvtA* Δ*orf13* double mutant (MB90) had a phenotype similar to that of the Δ*fvtA* single mutant (Fig. 2). Interestingly, analysis of the culture supernatants by the CAS assay showed an increase in the siderophore concentration in

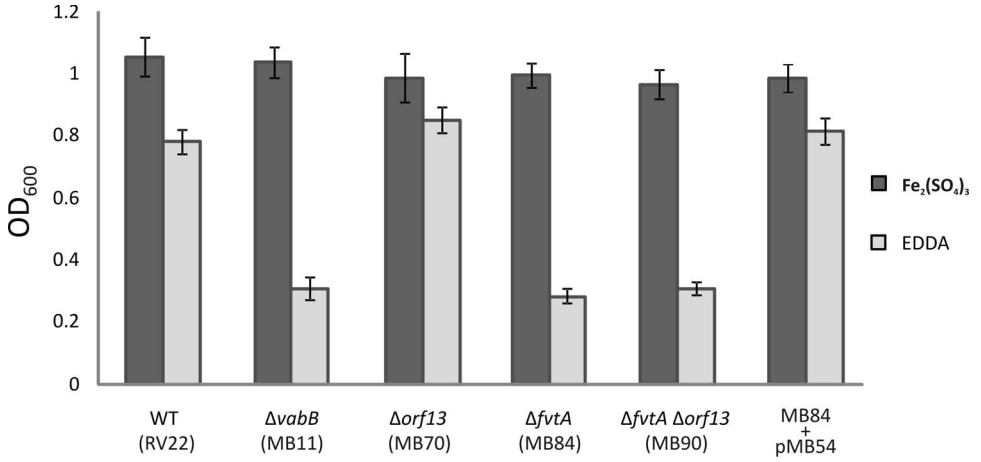

FIG. 2. Growth (OD_{600}) after 12 h of incubation of *V. anguillarum* RV22, Δ*vabB* (MB11), Δ*orf13* (MB70), Δ*fvtA* (MB84), and Δ*fvtA* Δ*orf13* (MB90) mutants, and the MB84 mutant complemented with plasmid pMB54 in CM9 supplemented with $Fe_2(SO_4)_3$ (10 μM) or the iron chelator EDDA (10 μM). WT, wild type.

the Δ*fvtA* mutant compared with the parental strain and the Δ*orf13* mutant; at an OD_{600} of approximately 0.6 in CM9 plus 5 μM EDDA, the parental and Δ*orf13* strains showed CAS values (A_{630}) of ca. −0.2, while the Δ*fvtA* mutant showed CAS assay values (A_{630}) of −0.3 or less (lower values indicate higher siderophore concentrations [35]). Under these conditions, the Δ*vabB* mutant showed mean CAS assay values (A_{630}) of −0.015. These results demonstrate that the impaired-growth phenotype of the Δ*fvtA* mutant is not due to the lack of vanchrobactin production. The increase in siderophore concentration could be explained by the extracellular accumulation of vanchrobactin, which is not transported back into the cell. When the Δ*fvtA* mutant was complemented with a plasmid harboring an intact *fvtA* gene (pMB54), the growth and CAS assay values were restored to wild-type levels (Fig. 2). All these results clearly suggest that FvtA plays a crucial role in the transport of ferric-vanchrobactin.

The OM protein profiles under iron-sufficient and iron-deficient conditions were analyzed for *V. anguillarum* RV22, for three mutants, and for the complemented Δ*fvtA* mutant (Fig. 3). The molecular masses of most of the TonB-dependent OM proteins involved in iron acquisition in gram-negative bacteria fall in the range from 70 to 80 kDa. Several protein bands at molecular masses in this range could be visualized for the parental strain under iron limitation conditions but not under iron-sufficient conditions. However, one of these iron-regulated protein bands (Fig. 3, RV22 Fe− lane) was absent in the lane containing the Δ*fvtA* mutant, and it presumably corresponded to the FvtA protein (the predicted molecular mass of FvtA, based on its amino acid sequence, is 78 kDa). This band was also absent in the lane containing the double mutant but not in the lane containing the Δ*orf13* mutant, while it was present in the lane containing the complemented Δ*fvtA* mutant (Fig. 3). Interestingly, the Δ*orf13* mutant showed a pattern identical to that of the parental strain, and similarly, the pattern of the Δ*fvtA* Δ*orf13* double mutant was the same as that of the Δ*fvtA* mutant. Together, these results suggest that *orf13* is not expressed (the predicted molecular mass of ORF13, based on its amino acid sequence, is 71 kDa). This hypothesis was reinforced by the negative results obtained in repeated attempts to detect the presence of *orf13* transcripts by RT-PCR (data not shown). The other iron-regulated protein bands

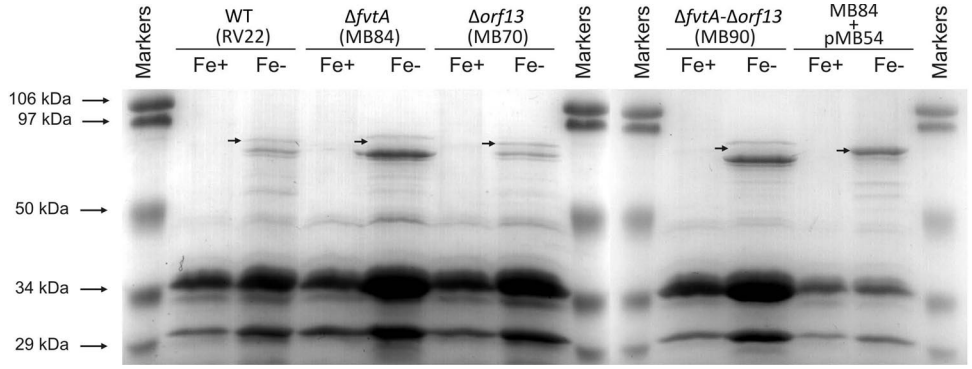

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of OM proteins obtained from cultures of *V. anguillarum* RV22 (wild type [WT]), Δ*fvtA* (MB84), Δ*orf13* (MB70), and Δ*fvtA* Δ*orf13* (MB90) mutants, and the MB84 mutant complemented with plasmid pMB54 under iron-sufficient (Fe+) and iron-deficient (Fe−) conditions. The arrows indicate the locations of the bands corresponding to the iron-regulated putative FvtA protein. The numbers on the left indicate the molecular masses of the protein markers.

TABLE 2. Results of bioassays using vanchrobactin analogues and purified siderophores

Compound	Promotion of the growth of *V. anguillarum* indicator strains[a]				
	MB11 (Δ*vabB*)	MB102 (Δ*vabB* Δ*fvtA*)	MB104 (Δ*vabB* Δ*orf13*)	MB107 (Δ*vabB* Δ*fvtA* Δ*orf13*)	MB102 (pMB54)
Vanchrobactin (DHBA-Arg-Ser)	+	−	+	−	+
DHBA-Orn	+	−	+	−	+
DHBA-Orn-Ser	+	−	+	−	+
DHBA-Ser	+	−	+	−	+
DHBA-Ser-Arg	+	−	+	−	+
DHBA-Arg	+	−	+	−	+
Amonabactin	−	−	−	−	−
Anguibactin	−	−	−	−	−
Enterobactin	+	(+)	+	(+)	+
Ferrichrome[b]	+	+	+	+	+
Aerobactin[b]	−	−	−	−	−

[a] The abilities of the different compounds to promote the growth of each indicator strain are indicated as follows: +, positive; −, negative; (+), weakly positive.

[b] Hidroxamate-type siderophore.

whose sizes are close to that of the FvtA band can be attributed to other iron-regulated OM iron transporters that have molecular masses in the range from 70 to 80 kDa, like the heme receptor HuvA (ca. 79 kDa) (23), and molecular masses like those of uncharacterized receptors for exogenous siderophores like ferrichrome (see below).

Synthetic vanchrobactin and vanchrobactin analogues use FvtA as the sole route of entry. As mentioned above, the route of entry of Fe-siderophore complexes into bacterial cells can be exploited by using a Trojan horse strategy for introduction of modified molecules with antimicrobial activity (24, 26). In a previous study, a series of vanchrobactin analogues were found to promote growth of *V. anguillarum* (RV22 and 775 strains) under iron-deficient conditions, indicating that they are indeed internalized and utilized as siderophores (37). However, the route of entry for these analogues into the *V. anguillarum* cell remained unknown. In addition, we ignored which part of the siderophore mediated the specific recognition by its cognate receptor, information that would be of great interest for selecting which region of the siderophore can be modified without altering its recognition. Bacteria often possess multiple OM receptors, each of which provides the bacterium with specificity for different siderophores, and some receptors have been found to efficiently transport derivatives of their cognate siderophores (12, 17, 32). We therefore wanted to assess the role of FvtA and ORF13 in the uptake of vanchrobactin analogues, using agar plate bioassays. For this purpose, a *vabB* mutant strain (MB11) (mutation of *vabB* abolishes vanchrobactin production) (4) was used as the parental strain to construct Δ*fvtA* and Δ*orf13* mutants to specifically assay the transport of synthetic vanchrobactin and the most relevant vanchrobactin analogues. Using a Δ*vabB* strain, we made sure that the growth halo was directly related to the ability to use the compound on the paper disk rather than to the utilization of endogenous vanchrobactin. The double mutants MB102 (Δ*vabB* Δ*fvtA*) and MB104 (Δ*vabB* Δ*orf13*), as well as the triple mutant MB107 (Δ*vabB* Δ*fvtA* Δ*orf13*), were used as indicator strains (Table 2), and the *vabB* single mutant was used as a positive control. Although *orf13* seems not to be expressed, we cannot rule out the possibility that expression of this gene was induced under other conditions (e.g., when a cognate ligand was present). Therefore, *orf13* mutant strains were included in the bioassay experiments as well. The compounds tested are shown in Table 2.

As expected, synthetic vanchrobactin and its analogues promoted growth of MB11 (Δ*vabB*) and MB104 (Δ*vabB* Δ*orf13*) (Table 2). However, MB102 (Δ*vabB* Δ*fvtA*) and MB107 (Δ*vabB* Δ*fvtA* Δ*orf13*) were unable to use any of these compounds, which indicates that FvtA is the transporter for vanchrobactin, as well as for the analogues tested. When MB102 (Δ*vabB* Δ*fvtA*) was complemented with plasmid pMB54 (harboring the *fvtA* gene and its promoter), transport of vanchrobactin and its analogues was restored (Table 2). An interesting finding is that all of the analogues tested share the same moiety, the DHBA molecule. This suggests that the specificity of the *V. anguillarum* vanchrobactin receptor FvtA could be mediated by the iron-catecholate center, whereas the D-

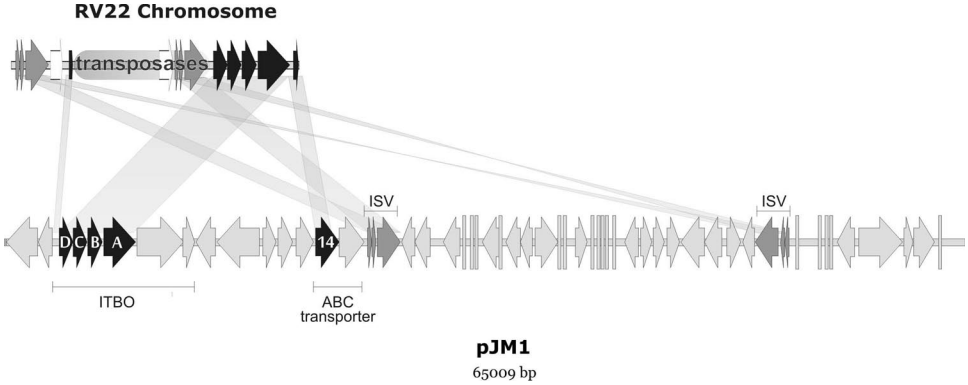

FIG. 4. Comparative analysis of the RV22 chromosomal *fatDCBA* gene cluster and genes of the pJM1 plasmid. The predicted open reading frames in RV22 showed levels of identity near 100% to different pJM1 regions corresponding to *fatDCBA* genes (DCBA), an uncharacterized putative ABC transporter gene (*orf14*) (labeled 14), and transposases (ISV), which have a different organization in RV22 and pJM1. In pJM1, *fatDCBA* form an operon (ITBO) together with the anguibactin biosynthesis genes *angR* and *angT* (11, 40) but not with the ABC transporter gene *orf14*. The open arrows indicate transposases in RV22 that are absent from pJM1. The light gray arrows indicate genes specific to one of the two molecules compared.

TABLE 3. Presence of *vab* cluster genes in a collection of *V. anguillarum* strains as determined by PCR and Southern blot hybridization

Strain	Source	Presence of:									
		Vanchrobactin biosynthesis and transport genes									RS1[a]
		vabA	*vabB*	*vabC*	*vabE*	*vabF*	*vabS*	*vabH*	*fvtA*	*orf13*	
Serotype O1											
TM14	*Oncorhynchus mykiss*, Spain	+	+	+	+	+	+	+	+	+	+
ATCC 43305	*Oncorhynchus mykiss*, Denmark	+	+	+	+	+	+	+	+	+	+
SE56.1	*Salmo* spp., Spain	+	+	+	+	+	+	+	+	+	+
SO121.1	*Salmo* spp., Spain	+	+	+	+	+	+	+	+	+	+
RI33.1	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	+
SE145.1	*Salmo* spp., Spain	+	+	+	+	+	+	+	+	+	−
PC933.1	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	+
96F	*Morone saxatilis*, United States	+	+	+	+	+	+	+	+	+	−
R82	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	+
775	*Oncorhynchus kisutch*, United States	+	+	+	+	+	+	+	+	+	+
Serotype O2											
ATCC 14181	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
PC640.1	*Solea solea*, Spain	+	+	+	+	+	+	+	+	+	−
CA3.1/04	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	−
AZ215.1	*Solea solea*, Spain	+	+	+	+	+	+	+	+	+	−
CA13.1	*Pollachius pollachius*, Spain	+	+	+	+	+	+	+	+	+	−
ACC4.1	*Scophthalmus maximus*, Portugal	+	+	+	+	+	+	+	+	+	−
PC628.1	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	−
ATCC 43306	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
RV22	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	−
43F	*Morone saxatilis*, United States	+	+	+	+	+	+	+	+	+	−
CA1.1/04	*Solea solea*, Spain	+	+	+	+	+	+	+	+	+	−
Serotype O3											
PT-493	*Plecoglossus altivelis*, Japan	+	−	+	+	+	+	+	+	+	−
11008	*Dicentrarchus labrax*, France	+	−	+	+	+	+	+	+	+	−
ATCC 43307	*Oncorhynchus mykiss*, Denmark	+	−	+	+	+	+	+	+	+	−
ET-208	*Anguilla japonica*, Japan	+	+	+	+	+	+	+	+	+	−
Serotype O4											
RPM41.11	*Scophthalmus maximus*, Spain	+	+	+	+	+	+	+	+	+	−
ATCC 43308	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O5											
ATCC 43309	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O6											
ATCC 43310	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O7											
ATCC 43311	*Anguilla japonica*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O8											
ATCC 43312	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O9											
ATCC 43313	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−
Serotype O10											
ATCC 43314	*Gadus morhua*, Denmark	+	+	+	+	+	+	+	+	+	−

[a] RS1 is an IS that disrupts the *vabF* gene.

Arg–L-Ser backbone could play a minor role in the recognition. A similar situation has been described for the chrysobactin receptor FctA, where recognition is mediated by the DHBA moiety (31).

Together, our results indicate that FvtA is the only route of entry for vanchrobactin (DHBA-Arg-Ser), as well as for its analogue DHBA-Orn-Ser. Elucidation of the route of entry for the latter molecule was of special interest, since this molecule possess an amino group that could be used to link antibacterial agents in the Trojan horse strategy (37).

We also tested the utilization of siderophores from other bacterial species. Results of these bioassays (Table 2) demonstrated that ferrichrome can be utilized by the parental strain and by all mutants, indicating that in *V. anguillarum* RV22 a ferrichrome receptor distinct from FvtA and ORF13 is required. Interestingly, enterobactin supported growth of the

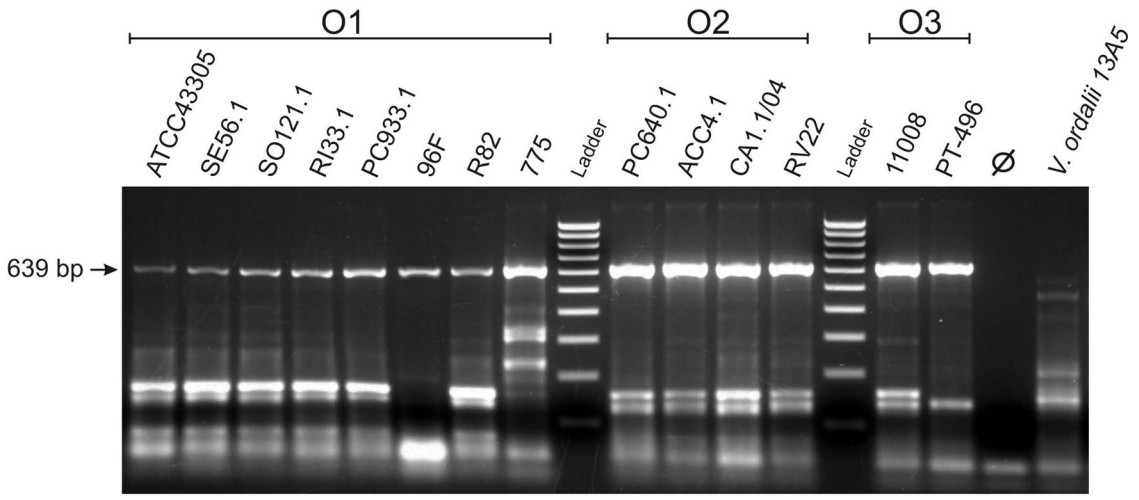

FIG. 5. Detection of *fvtA* transcripts using RT-PCR. Fourteen *V. anguillarum* strains belonging to serotypes O1, O2, and O3 were analyzed (strain designations are indicated at top). The arrow on the left indicates the expected size of the *fvtA* RT-PCR product. The same RT-PCR carried out with *Vibrio ordalii* 13A5 was negative. Negative controls using DNase-treated RNA as the PCR template to rule out the presence of contaminating DNA were negative in all cases (an example is the control for RV22 [∅]). The ladder is composed of 10 fragments whose sizes range from 100 to 1,000 bp in 100-bp increments.

Δ*fvtA* and Δ*orf13* mutant strains, which implies that an unknown *V. anguillarum* receptor is able to transport enterobactin, a catechol siderophore whose functional relationship with vanchrobactin has been suggested previously (10, 20). It is frequently found that bacteria utilize exogenous siderophores since in this way they can pirate siderophores of their competitors and escape the bacteriostatic effects caused by these compounds (33). *E. coli* K-12 possesses at least six OM receptors that enable acquisition of eight different iron-chelator complexes, four of which are exogenously produced (2).

However, amonabactin, aerobactin, and anguibactin (a siderophore produced by *V. anguillarum* strains that carry pJM1 or pJM1-like plasmids) are three siderophores that cannot be utilized by *V. anguillarum* RV22. These results are in agreement with previous observations that plasmidless *V. anguillarum* strains cannot utilize anguibactin and do not express FatA, the anguibactin OM receptor (10, 20). Surprisingly, the presence of gene sequences with high levels of similarity to *fatA* and *fatD* (the receptor and ABC transporter genes of the anguibactin system, respectively) (1, 40) was recently reported in strains that contained either no plasmids or only small plasmids (5). Interestingly, we found that the chromosome of *V. anguillarum* RV22 actually contains *fatDCBA* homologues, as well as other genes present in the pJM1 plasmid, although it has a different gene arrangement (Fig. 4). However, the expression of *fatDCBA* is likely abolished, since insertion of ca. 9 kb of transposases disrupts the first gene of the operon, from which transcription of the four genes is driven. This finding is supported by the negative results in all attempts to detect *fatDCBA* transcripts by RT-PCR (data not shown). Furthermore, Naka et al. (28) detected FatA in the OM of strain RV22 only when it was transformed with a pJM1 plasmid. These findings not only demonstrate that the FatA-mediated anguibactin acquisition system is inappropriate for use in the Trojan horse strategy but also bring up interesting questions about the origin and evolution of the vanchrobactin and anguibactin systems in *V. anguillarum*.

Vanchrobactin biosynthesis and transport genes are ubiquitous in *V. anguillarun* strains. It is not known if the vanchrobactin transport system is widely distributed in *V. anguillarum* strains. This question is of special interest because in order for the vanchrobactin analogues to be employed in the development of novel antimicrobials, their route of entry into the cell should be widespread in the species. PCR and Southern hybridization were used to assay the presence of the *fvtA*, *orf13*, and *vab* genes in a collection of 33 strains that represented the 10 main serotypes (serotypes O1 to O10) of *V. anguillarum*. The results indicate that all strains contain *fvtA* and *orf13* and that *vabB* is absent from some serotype O3 isolates (strains PT-4933, 11008, and ATCC 4330) (Table 3). Thus, all *V. anguillarum* strains seem to harbor a copy of the *fvtA* gene.

It appears from the present results that the vanchrobactin biosynthesis and transport gene system is ubiquitous in *V. anguillarum* strains. A question that arises is why not all of these strains produce vanchrobactin. The presence of the pJM1 plasmid, containing most genes necessary for anguibactin production and transport, has been associated with the lack of production of vanchrobactin. Naka et al. (28) showed that *vabF* in the *V. anguillarum* 775 (a strain carrying pJM1) chromosome is disrupted by the insertion sequence (IS) RS1. In the present study we detected the presence of RS1, an insertion that abolishes vanchrobactin biosynthesis, in *vabF* in all strains that carry pJM1 or pJM1-like plasmids (Table 3). RS1 encodes a transposase that is 100% identical to the RS1 (*orf21*) transposase originally described for the pJM1 plasmid (11), suggesting that this IS could have transposed from the plasmid to the chromosome. The genes encoding the proteins of the anguibactin-iron uptake system on pJM1-like plasmids are flanked by ISs ISV-A1 and ISV-A2 in a transposon-like struc-

ture, and transposition of ISV-A2 at a frequency of 7.2×10^{-6} was recently demonstrated (21).

The vanchrobactin system was proposed to be the ancestral siderophore in *V. anguillarum* (28), a hypothesis reinforced by our finding that vanchrobactin biosynthesis genes are present in all strains of *V. anguillarum* tested, as well as by the dependence of anguibactin biosynthesis on some vanchrobactin biosynthesis elements that complement pJM1 pseudogenes (1). *fvtA* is cotranscribed with *vabD* as a polycistronic mRNA, and we know that these genes are essential for vanchrobactin transport and biosynthesis, respectively (3). The sequence of *fvtA* homologues in anguibactin-producing strains is 100% identical to the sequence of RV22 *fvtA*. In addition, *V. anguillarum* 775 (an anguibactin-producing strain) can also use vanchrobactin (37), and in this strain *vabD* is a functional gene (28). These two observations clearly suggest that *fvtA* (from which promoter *vabD* gene transcription is driven) is expressed not only in vanchrobactin-producing strains but also in anguibactin-producing strains. To verify this hypothesis, we used RT-PCR to detect the presence of *fvtA* transcripts in RNA samples from *V. anguillarum* strains grown under low-iron conditions. Figure 5 shows the expression of *fvtA* in 14 selected strains representing the three most relevant pathogenic serotypes (serotypes O1, O2, and O3) (Table 3). Sequencing of the PCR amplicons confirmed that the 639-bp band detected corresponded to *fvtA* (data not shown).

Therefore, when siderophore uptake machinery is used to internalize siderophore analogues coupled to antimicrobial agents against *V. anguillarum*, the strategy of choice should be to use the vanchrobactin entry pathway, since it is the only pathway present in all strains, especially strains of the three main pathogenic serotypes.

Conclusions. In this work we have determined that FvtA is the OM transporter for vanchrobactin and its analogues in *V. anguillarum*. In addition, we have demonstrated that *fvtA* is present in both vanchrobactin- and anguibactin-producing strains, where it is a functional gene. The vanchrobactin analogue DHBA-Orn-Ser, of special interest for attaching antimicrobial ligands, is optimally transported by FvtA. FvtA would be an optimum route of entry for new antibacterial compounds using the Trojan horse strategy, due to its ubiquity in this species and to the wide range of vanchrobactin analogues transported.

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