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Two ABC transporter systems participate in siderophore transport in the marine pathogen *Vibrio anguillarum* 775(pJM1)

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Abstract

ORF40 (named *fatE*) in the *Vibrio anguillarum* pJM1 plasmid encoding anguibactin iron transport systems is a homologue of ATPase genes involved in ferric-siderophore transport. Mutation of *fatE* did not affect ferric-anguibactin transport indicating that there must be other ATPase gene(s) in addition to *fatE*. By searching the genomic sequence of *V. anguillarum* 775(pJM1) we identified a homologue of *fatE* named *fvtE* on chromosome 2. It is of interest that in this locus we also identified homologues of *fatB*, *fatC* and *fatD* that we named *fvtB*, *fvtC* and *fvtD*, respectively. The *fvtE* mutant still showed ferric-anguibactin transport while the double *fatE* and *fvtE* mutation completely abolished the ferric-anguibactin transport indicating that *fatE* and *fvtE* are functional ATPase homologues for ferric-anguibactin transport. Furthermore, we demonstrate that *fvtB*, *fvtC*, *fvtD* and *fvtE* are essential for ferric-vanchrobactin and ferric-enterobactin transport.

INTRODUCTION

Vibrio anguillarum is a part of the natural flora in the aquatic environment, and some strains cause vibriosis, a terminal hemorrhagic septicemia in marine as well as fresh water fish and invertebrates (Toranzo & Barja, 1990; Aguirre-Guzmán *et al.*, 2004; Paillard *et al.*, 2004; Toranzo *et al.*, 2005). 23 serotypes of *V. anguillarum* have been reported so far, and serotypes O1, O2 and O3 are mainly causative agents of vibriosis (Sorensen & Larsen, 1986; Toranzo & Barja, 1990; Larsen *et al.*, 1994; Grisez & Ollevier, 1995; Tiainen *et al.*, 1997; Pedersen *et al.*, 1999). Many serotype O1 strains carry the pJM1-type plasmids harboring the genes involved in the siderophore anguibactin transport system that is an essential virulence factor for *V. anguillarum* (Crosa, 1980; Crosa & Walsh, 2002; Di Lorenzo *et al.*, 2003; Wu *et al.*, 2004). *V. anguillarum* biosynthesizes inside the cell a small molecular peptide iron chelator anguibactin, and secretes it to the external environment. Then, the iron-bound anguibactin, ferric-anguibactin, is transported back into the cell to utilize iron for survival under the iron limiting conditions that can be found in marine environments and inside hosts (Crosa, 1980; Actis *et al.*, 2011; Naka & Crosa, 2011b). It has been shown that the ferric-anguibactin is transported to the periplasmic space of *V. anguillarum* via the specific outer membrane receptor FatA (Lopez & Crosa, 2007). In this step, the ExbB2-ExbD2-TonB2-TtpC complex is required to transduce the energy generated from proton motive force to the FatA protein to change its conformation and enable the transport of ferric-siderophore into the periplasmic space (Stork *et al.*, 2004; Stork *et al.*, 2007; Kuehl & Crosa, 2010; Kustusch *et al.*, 2011).

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In Gram-negative bacteria, ferric-siderophores pass through the cytoplasmic membrane using ABC transport systems that rely on ATPases or MSF type siderophore transporters that depend on the proton motive force (Crosa & Walsh, 2002; Cuiv *et al.*, 2004; Raymond & Dertz, 2004; Winkelmann, 2004; Hannauer *et al.*, 2010; Reimmann, 2012). We have shown that ferric-anguibactin is transported across the cytoplasmic membrane using the ABC transport system including the periplasmic binding protein FatB and cytoplasmic membrane proteins FatC and FatD (Actis *et al.*, 1995; Naka *et al.*, 2010). However, the gene encoding an ATPase that should be part of the ABC transporter for ferric-anguibactin was still unknown, although there is a homologue (ORF40) of ATPase genes in the pJM1 plasmid (Di Lorenzo *et al.*, 2003).

In addition to anguibactin, *V. anguillarum* 775 (pJM1) can transport exogenous siderophores such as vanchrobactin and enterobactin (Naka *et al.*, 2008; Balado *et al.*, 2009; Naka & Crosa, 2011a). One of them, vanchrobactin, is a chromosomally encoded siderophore produced by natural pJM1-less *V. anguillarum* O1 strains and other serotype strains (Lemos *et al.*, 1988; Conchas *et al.*, 1991; Balado *et al.*, 2006; Soengas *et al.*, 2006; Balado *et al.*, 2008). We previously showed that outer membrane proteins, FvtA and FetA, are involved in the transport of these exogenous siderophores in *V. anguillarum* 775(pJM1) (Naka & Crosa, 2011a). However, genes encoding the ABC transporter system(s) for ferric-vanchrobactin and ferric-enterobactin are still unknown. In this work, we characterized two ABC transport systems involved in ferric-siderophore uptake in *V. anguillarum* 775(pJM1): FatBCDE encoded on the pJM1 plasmid and FvtBCDE encoded in the chromosome are involved in ferric-anguibactin and ferric-vanchrobactin/enterobactin transport, respectively. The two ABC transport systems are specific for their respective siderophores except that both FatE and FvtE are functional for ferric-anguibactin transport.

MATERIALS AND METHODS

Bacterial strains, primers and media

Strains and plasmids used in the present study are listed in Table 1. The primers used in this study are listed in Table 2. *E. coli* strains were grown in LB broth with appropriate antibiotics. *V. anguillarum* strains were grown in Trypticase Soy Broth supplemented with 1.5 % NaCl (TSBS) with appropriate antibiotics [for *E. coli*: ampicillin (Amp) 100 μg/ml, kanamycin (Km) 50 μg/ml, chloramphenicol (Cm) 30 μg/ml and trimethoprim (Tp) 100 μg/ml and for *V. anguillarum*: Km 250 μg/ml, Cm 10 μg/ml, Tp 10 μg/ml and rifampicin (Rif) 100 μg/ml]. The authenticity of *V. anguillarum* strains was confirmed by oxidase test, pJM1 plasmid extraction and colony PCR using *V. anguillarum* specific primers. All strains were stored at −80 °C as glycerol stocks (TSBS supplemented with 30 % glycerol), and strains were streaked from the stock vials for each experiment.

Construction and complementation of mutants

The two flanking regions of the target genes to be mutated were PCR-amplified, and fragments thus obtained were combined by using SOE PCR (Senanayake & Brian, 1995). Then, the PCR products were ligated into pCR2.1. To mutate *fatE* and *fvtE* genes, the Km^r gene [the *Sma*I fragment from pBlue-Km-*Sma*I (Naka *et al.*, 2012)] and Tp^r gene [the *Sma*I fragment from p34E-Tp (DeShazer & Woods, 1996)] were respectively ligated in the *Eco47*III sites that are located in the middle of the deletion fragments. The fragments were subcloned into the suicide vector pDM4, transformed into *E. coli* S17-1 λ*pir* and conjugated into *V. anguillarum* as described before (Naka *et al.*, 2008). Transconjugants that show resistance to Cm (from the pDM4 plasmid) and Rif (resistance from *V. anguillarum*) were selected. The colonies obtained were inoculated into TSBS without antibiotics, cultured overnight, and plated on TSAS with 15% sucrose to select 2nd recombinants (for *fatE* and

fvtE mutants, Km and Tp were added, respectively). The colonies obtained were checked for Cm sensitivity (loss of pDM4), and the mutations were confirmed by colony PCR using primers constructed outside and inside the target genes. To complement the mutants, primers with appropriate restriction enzyme sites were used to PCR-amplify the target genes with upstream regions including the Shine-Dalgarno sequence and the start codon. The fragments thus obtained were ligated into pGEM-T Easy and subcloned into pMMB208. The plasmids were transformed into *E. coli* S17-1λ*pir* and conjugated into *V. anguillarum.*

Bioassay

Bioassay (cross feeding assay) was performed as described before (Tolmasky *et al.*, 1988). Briefly, 2x CM9 medium supplemented with 20 μg/ml Cm, 1 mM IPTG, 40 μM EDDA and an overnight culture of *V. anguillarum* strains (5 μl/ml) grown in CM9 medium, was mixed 1:1 with 3.0 % melted agar adjusted to ~50 °C. After solidification, siderophore producing bacteria grown in CM9 was spotted on the plates, and the existence of a growth halo around the spots was recorded after 24 and 48 hours incubation at 25 °C.

Growth experiments

Overnight cultures of *V. anguillarum* strains in TSBS were inoculated 1:100 in CM9 broth and incubated overnight reaching an OD_{600} of 2.3 – 2.8. After incubation, the OD_{600} values of each strain were adjusted to 1 that corresponds to ~1.4×10^7 cells/ml, and 50 μl of the cultures were inoculated into 5 ml CM9 broth with or without ferric ammonium citrate (10μg/ml) or iron chelater EDDA (0, 0.1, 0.5, 1 and 5μM). The OD_{600} values were measured after 24 hours incubation at 25 °C. For streptonigrin experiments, 1 μg/ml streptonigrin (Sigma) dissolved in 10 mM Tris-HCl, pH 7.5 (1 mg/ml) was added in CM9, and the OD_{600} values were measured after 24 hours incubation at 25 °C.

RESULTS

Identification of *fatE* and *fvtE* encoding ATP binding proteins

The pJM1 plasmid encodes a homologue of ATP binding proteins of ABC transport systems (ORF40) (Di Lorenzo *et al.*, 2003). We first constructed a deletion mutant of ORF40 named *fatE* to test whether this gene is essential for ferric-anguibactin transport. The result of cross-feeding assays in Table 3 shows that the deletion of *fatE* affects neither ferric-anguibactin nor ferric-vanchrobactin/enterobactin transport. The fact that the pJM1 cured strain, H775-3, still transports ferric-vanchrobactin/enterobactin indicates that other gene(s) encoding an ATP binding protein for ferric-vachrobactin/enterobactin must exist in the chromosome of this strain, and possibly also functional for ferric-anguibactin transport. The whole genome sequencing of *V. anguillarum* strain 775(pJM1) revealed that there is an ABC binding protein homologue, *fvtE* that exhibits 84% similarity (65% identity) with the *fatE* gene (Naka *et al.*, 2011). We also found that the locus containing the *fvtE* gene also carries *fvtB*, *fvtD* and *fvtC* potentially encoding ABC transporters of ferric-siderophore (Fig. 1). We then mutated the *fvtE* gene in strain H775-3. The H775-3Δ*fvtE*::Km showed a defect in ferric-vanchrobactin/enterobactin transport while the *fvtE* mutant complemented *in trans* recovers the transport phenotype indicating that the *fvtE* gene is necessary for the ferric-vanchrobactin/enterobactin transport in H775-3 (Table 3). The *fvtE* mutation in strain 775(pJM1) (CC9-16Δ*fvtE::Km*) also caused the defect in ferric-vanchrobactin/enterobactin uptake but still could take up anguibactin. The double *fatE* and *fvtE* mutant in 775(pJM1) (CC9-16Δ*fatE::Tp*Δ*fvtE::Km*) transports neither ferric-anguibactin nor ferric-vanchrobactin/enterobactin (Table 3). Taken together, these results indicate that only *fvtE* is indispensable for ferric-vanchrobactin/enterobactin transport while both *fatE* and *fvtE* are functional for ferric-anguibactin transport. We also found that the overexpression of *fatE* in the double *fatE* and *fvtE* mutant resulted in weak ferric-vanchrobactin/enterobactin transport

only at 48 hours but not at 24 hours incubation. However, this could be an artifact due to overexpression of *fatE* from the Ptac promoter of pMMB208.

Growth effects of *fvtE* and *fatE* mutations under iron limiting conditions

We tested whether the mutation in *fatE* or *fvtE* actually affect the growth of the 775(pJM1) strain in various iron conditions. As shown in Fig. 2, under iron limitation, each *fatE* or *fvtE* mutant exhibited a similar growth rate to the wild type strain, while the double *fatE* and *fvtE* mutant showed less growth as compared with the wild type. Growth was restored close to the wild type strain level when the double *fatE fvtE* mutant was complemented with either *fatE* or *fvtE in trans*. On the other hand, we did not observe clear growth difference between any of the strains tested under iron rich conditions. These results indicate that *fatE* and *fvtE* are indeed important for the growth of *V. anguillarum* 775(pJM1) to survive under iron limiting conditions.

Streptonigrin survival test

Streptonigirin is an antibiotic that works when the iron concentration inside bacterial cells is high, thus we can compare the internal iron content by culturing the bacteria with and without this antibiotic. The wild type strain in which the internal iron concentration is high, showed much lower growth rate as compared with the *tonB2* mutant in which internal iron concentration is low (Fig. 3). The double *fatE* and *fvtE* mutant grew at a higher rate than each single mutant while the double mutant complemented with either *fatE* or *fvtE* showed a comparable growth rate to the single mutants or similar growth to the wild type (Fig. 3). These results indicate that the iron concentration inside the double mutant is lower than in the wild type and each single mutant, and both FatE and FvtE are able to increase the internal iron concentration.

Characterization of *fvtB*, *fvtC* and *fvtD*

The genome sequencing of *V. anguillarum* 775(pJM1) revealed that *fvtE* is located in the cluster containing genes potentially encoding ferric-siderophore ABC transporter proteins (Fig. 1). *fvtB*, *fvtC* and *fvtD* are homologues of *fatB* (31 % identity and 54 % similarity in amino acid sequence), *fatC* (38 % identity and 68 % similarity in amino acid sequence) and *fatD* (32 % identity and 57 % similarity in amino acid sequence), respectively. Since *fvtE* is involved in ferric-vanchrobactin and ferric-enterobactin transport as described above, we were interested in analyzing the requirement of *fvtB*, *fvtC* and *fvtD* for the ferric-siderophore transport. Our bioassay results indicate that the mutation in each gene abolishes the ferric-vanchrobactin or ferric-enterobactin transport while ferric-anguibactin transport is not affected with these mutations (Table 4). The ferric-vanchrobactin or ferric-enterobactin transport was recovered when these mutations were complemented *in trans* with each wild type gene (Table 4). Taken together, *fvtB*, *fvtC* and *fvtD* are specifically involved in ferric-vanchrobactin or ferric-enterobactin transport.

CONCLUSIONS

In this work, we have identified two genes, *fatE* and *fvtE*, encoding ATP binding proteins involved in ferric-anguibactin transport. *fatE* is specific to ferric-anguibactin transport while *fvtE* is functional for both ferric-anguibactin and ferric-vanchrobactin/enterobactin transport. Furthermore, we identified homologues of ABC transport proteins for ferric-vanchrobactin/enterobactin. FvtB is a homologue of a periplasmic binding protein while *fvtC* and *fvtD* are homologues of cytoplasmic membrane proteins. We showed that *fvtB, fvtC* and *fvtD* are essential for ferric-vancrhobactin/enterobactin transport but not for ferric-anguibactin transport. Our previous report showed that *fatB* encoding a periplasmic binding protein, and *fatC* and *fatD* encoding cytoplasmic membrane proteins are only functional for ferric-

anguibactin transport but not for ferric-vanchrobactin/enterobactin transport (Naka *et al.*, 2010). Taken together, we conclude that two ferric-siderophore ABC transporters, pJM1-encoded FatDCB-FatE and chromosome 2-encoded FvtBDCE, play a role in ferric-anguibactin and ferric-vanchrobactin/enterobactin, respectively, and FvtE is also functional for ferric-anguibactin transport. These results are quite different from the finding in *Vibrio cholerae*. In *V. cholerae*, it has been shown that two sets of ABC transport systems encoded by *vctPDGC* and *viuPDGC* are functionally redundant for the transport of both, the endogenous siderophore vibriobactin and the exogenous siderophore enterobactin, recognized by different specific outer membrane receptors (Wyckoff *et al.*, 1999; Mey *et al.*, 2002). *fvtB*, *fvtD*, *fvtC* and *fvtE* are homologues of *vctP*, *vctD*, *vct*G and *vct*C, respectively, and the genetic organization of *V. anguillarum fvtBDCE* and *V. cholerae vctPDGC* is very similar. The region encompassing *vctPDGC* that includes the *hly* region containing the hemolysin gene *hlyA* and lipase genes *lipAB*, was proposed to be a pathogencity island encoding products capable of damaging host cells and/or involved in nutrient acquisition (Ogierman *et al.*, 1997). The homologous locus of the *V. cholerae hlyA* region has been identified in *V. anguillarum* (Rock & Nelson, 2006). Our analysis of genomic data of *V. anguillarum* 775(pJM1) indicates that the *fvtBDCE* cluster is also located adjacent to the *V. anguillarum hly* region. Based on these facts, we hypothesize that *fvtBDCE* in *V. anguillarum* and *vctPDGC* in *V. choleare* might have originated from the same ancestral ABC transporter while *fatBCD-fatE* in *V. anguillarum* and *viuPDGC* in *V. cholerae* could be horizontally acquired after these bacteria were separated from the common ancestor. We previously proposed that the pJM1 plasmid was possibly acquired by *V. anguillarum* to obtain a stronger anguibactin siderophore system by replacing the chromosomal-encoded siderophore vanchrobactin (Naka *et al.*, 2008). In addition to pJM1-encoded FatE, the existence of another functional ATP binding protein, FvtE, for ferric-anguibactin transport could probably easily have facilitated the acquisition of the pJM1 plasmid during evolution ensuring the ability to take up ferric-anguibactin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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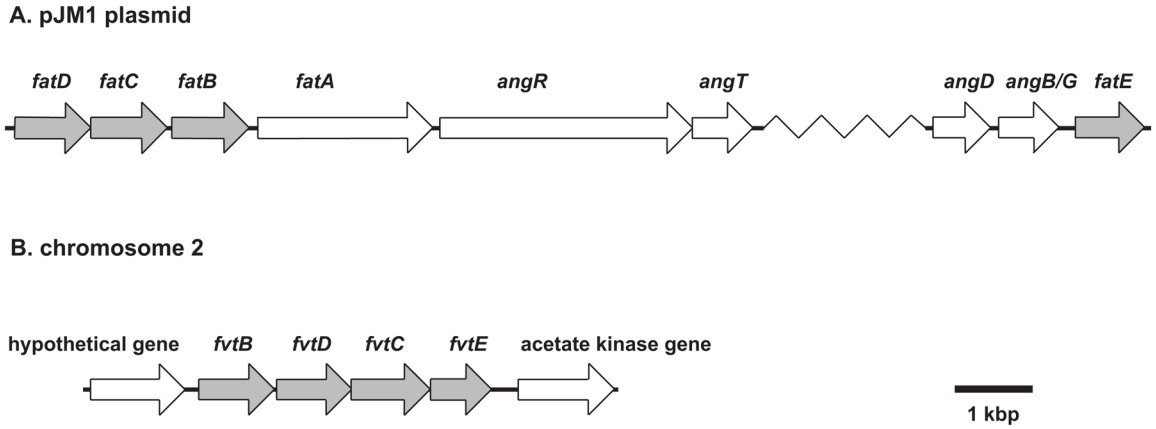

Fig 1. Two ABC transporter clusters identified in *V. anguillarum* 775(pJM1)

Schematic maps of the *fatDCB-fatE* locus on the pJM1 plasmid (panel A) and of the *fvtBDCE* on the chromosome 2 (panel B).

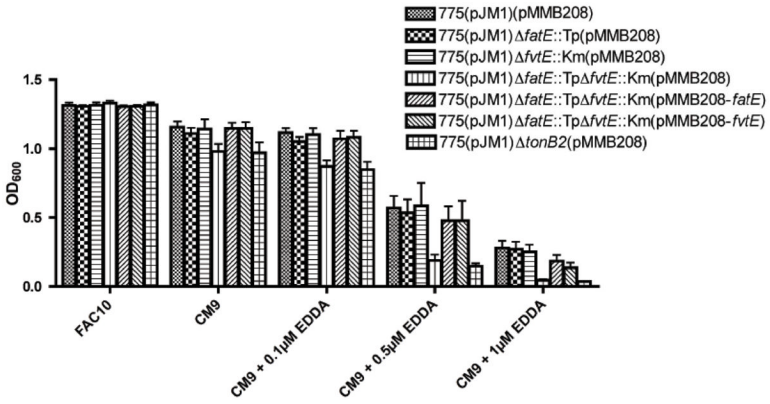

Fig 2. FatE and FvtE are important for the survival of *V. anguillarum* 775(pJM1) under various iron conditions

Overnight culture of *V. anguillarum* in CM9 was adjusted to an OD_{600} of 1.0, and 50 μl of the culture was inoculated into 5 ml CM9 broth either with or without addition of ferric ammonium citrate (10 μg/ml) or iron chelator EDDA (0, 0.1, 0.5, 1 and 5μM). The growth of each strain (OD_{600}) was measured after 24 hours incubation at 25 °C. Experiments were repeated five times.

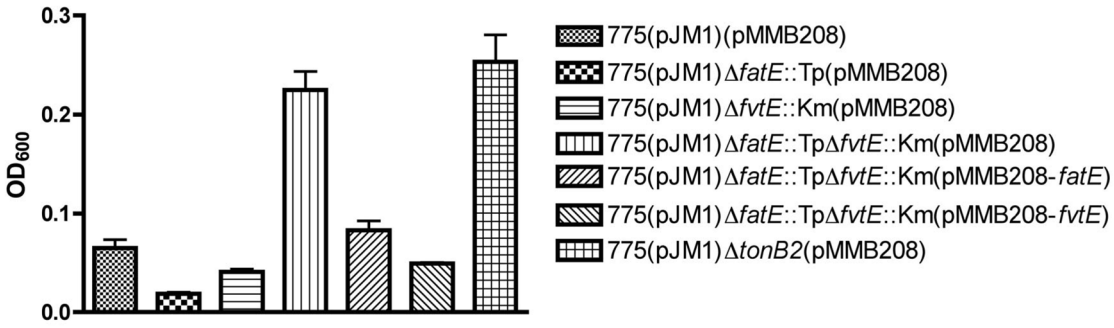

Fig 3. The double *fatE fvtE* mu\tant contains less iron inside the cell

Overnight culture of *V. anguillarum* strain in CM9 was adjusted to an OD_{600} of 1.0, and 50 μl of the culture was inoculated into 5 ml CM9 broth supplemented with 1 μg/ml streptonigrin, and the growth of each strain (OD_{600}) was measured after 24 hours incubation at 25 °C. Experiments were repeated at least three times.

Table 1

Strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference or source
V. anguillarum strains		
775(pJM1)	Wild type, Washington (serotype O1, pJM1), isolated from coho salmon (*Oncorhynchus kisutch*)	(Crosa, 1980)
H775-3	Plasmidless derivative of 775(pJM1)	(Crosa, 1980)
HNVA-10	H775-3Δ*fvtA::Km*	This study
775(pJM1)-pMMB	775(pJM1) harboring pMMB208	(Naka *et al.*, 2008)
CC9-16	775 (pJM1) derivative of anguibactin deficient, anguibactin transport system proficient	(Walter *et al.*, 1983)
HNVA-11	CC9-16Δ*fatE::Tp*	This study
HNVA-12	CC9-16Δ*fvtE::Km*	This study
HNVA-13	CC9-16Δ*fatE::Tp*Δ*fvtE::Km*	This study
HNVA-14	CC9-16Δ*fvtB*	This study
HNVA-15	CC9-16Δ*fvtC*	This study
HNVA-16	CC9-16Δ*fvtD*	This study
96F-pMMB	vanchrobactin producer (serotype O1, plasmidless) harboring pMMB208	(Naka *et al.*, 2008)
E. coli strains		
DH5α	*F*$^-$, *φ80lacZ*ΔM15, *endA1*, *recA1*, *hsdR17*, ($r_K^- m_K^+$), *supE44*, *thi-1*, *gyrA96*, *relA1*, Δ(*lacZYA-argF*)*U169*, λ$^-$	Laboratory stock
S17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR hsdM+recA* RP4 2-Tc::Mu-Km::Tn*7*(Tp^r Sm^r)	(Simon *et al.*, 1983)
Plasmids		
pCR2.1	Amp^r, Km^r, PCR cloning vector	Invitrogen
pGEM-T Easy	A vector for the cloning of PCR products with blue/white screening, Ap^r	Promega
pBlue-Km-*Sma*I	Source of the Km resistance cassette with *Sma*I recognition sites in both sides	(Naka *et al.*, 2012)
p34E-Tp	Source of the Tp resistance cassette with *Sma*I recognition sites in both sides	(DeShazer & Woods, 1996)
pDM4	Suicide plasmid *sacB* gene, R6K origin, Cm^r	(Milton *et al.*, 1996)
pHN15	pDM4 harboring Δ*fatE::Tp* of *V. anguillarum* 775(pJM1)	This study
pHN16	pDM4 harboring Δ*fvtE::Km* of *V. anguillarum* 775(pJM1)	This study
pHN17	pDM4 harboring Δ*fvtB* of *V. anguillarum* 775(pJM1)	This study
pHN17	pDM4 harboring Δ*fvtC* of *V. anguillarum* 775(pJM1)	This study
pHN17	pDM4 harboring Δ*fvtD* of *V. anguillarum* 775(pJM1)	This study
pMMB208	A broad-host-range expression vector; Cm^r *IncQ lacI*q *Ptac*; polylinker from M13mp19	(Morales *et al.*, 1991)
pHN18	pMMB208 harboring *V. anguillarum* 75(pJM1) *fatE*	This study
pHN19	pMMB208 harboring *V. anguillarum* 775(pJM1) *fvtE*	This study
pHN20	pMMB208 harboring *V. anguillarum* 775(pJM1) *fvtB*	This study
pHN21	pMMB208 harboring *V. anguillarum* 775(pJM1) *fvtC*	This study
pHN22	pMMB208 harboring *V. anguillarum* 775(pJM1) *fvtD*	This study

Table 3

Bioassay to assess whether *fatE* or *fvtE* is necessary for ferric siderophore transport

Indicator strains	Iron sources			
	anguibactin	vanchrobactin	enterobactin	FAC
H775-3	−	+	+	+
H775-3Δ*fvtE::Km*	−	−	−	+
CC9-16 (pMMB208)	+	+	+	+
CC9-16Δ*fatE::Tp* (pMMB208)	+	+	+	+
CC9-16Δ*fvtE::Km* (pMMB208)	+	−	−	+
CC9-16Δ*fatE::Tp*Δ*fvtE::Km* (pMMB208)	−	−	−	+
CC9-16Δ*fatE::Tp*Δ*fvtE::Km* (pMMB208-*fatE*)	+	−*	−*	+
CC9-16Δ*fatE::Tp*Δ*fvtE::Km* (pMMB208-*fvtE*)	+	+	+	+

A 50 μl aliquot of an overnight culture of indicator strains in CM9 broth was mixed with 20 ml of melted CM9 1.5% agar (adjusted to ~50°C) supplemented with 20 μM EDDA, 500 μM IPTG and μ0 mg ml^{-1} Cm. After the agar became solid, 5 μl of *V. anguillarum* 775(pJM1)(pMMB208) overnight culture as a source of anguibactin, 5μl of *V. anguillarum* 96F(pMMB208) overnight culture as a source of vanchrobactin, 1μl of 1 mg ml^{-1} purified enterobactin from EMC microcollections GmbH, and 1 μl of 1 mg ml^{-1} ferric ammonium citrate (FAC) were spotted on each plate. The existence of growth halos around the spots were recorded after 24 hours incubation at 25 °C.

+, growth;

−, no growth;

−*, a weak halo was observed after 48 hours incubation, possibly due to overexpression of *fatE* from the Ptac promoter in pMMB208.

H775-3, pJM1-cured strain of 775(pJM1); CC9-16, 775(pJM1) derivative of a Tn*1* insertion mutant able to utilize ferric-anguibactin complexes but unable to synthesize anguibactin (Walter *et al.*, 1983).

Table 4

Bioassay to assess whether *fvtBCD* are necessary for ferric siderophore transport

	Iron sources			
Indicator strains	**anguibactin**	**vanchrobactin**	**enterobactin**	**FAC**
CC9-16 (pMMB208)	+	+	+	+
CC9-16Δ*fvtB* (pMMB208)	+	−	−	+
CC9-16Δ*fvtB* (pMMB208-*fvtB*)	+	+	+	+
CC9-16Δ*fvtC* (pMMB208)	+	−	−	+
CC9-16Δ*fvtC* (pMMB208-*fvtC*)	+	+	+	+
CC9-16Δ*fvtD* (pMMB208)	+	−	−	+
CC9-16Δ*fvtD* (pMMB208-*fvtD*)	+	+	+	+

A 50 μl aliquot of an overnight culture of indicator strains in CM9 broth was mixed with 20 ml of melted CM9 1.5% agar (adjusted to ~40°C) supplemented with 20 μM EDDA, 500 μM IPTG and 10 μg ml^{-1} Cm. After the agar became solid, 5 μl of *V. anguillarum* 775(pJM1)(pMMB208) overnight culture as a source of anguibactin, 5μl of *V. anguillarum* 96F(pMMB208) overnight culture as a source of vanchrobactin, 1μl of 1 mg ml^{-1} purified enterobactin from EMC microcollections GmbH, and 1 μl of 1 mg ml^{-1} ferric ammonium citrate (FAC) were spotted on each plate. The existence of growth halos around the spots were recorded after 24 hours incubation at 25 °C.

+, growth

−, no growth.

CC9-16, 775(pJM1) derivative of a Tn*1* insertion mutant able to utilize ferric-anguibactin complexes but unable to synthesize anguibactin (Walter *et al.*, 1983).