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Role of the pJM1 plasmid-encoded transport proteins FatB, C and D in ferric anguibactin uptake in the fish pathogen *Vibrio anguillarum*

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Summary

Vibrio anguillarum serotype O1 is part of the natural flora in the aquatic habitat, but under certain circumstances it can cause terminal haemorrhagic septicemia in marine and fresh water fish due to the action of the anguibactin iron uptake system encoded by the virulence plasmid pJM1. This plasmid harbours the genes for the biosynthesis of the siderophore anguibactin and the ferric anguibactin transport proteins FatD, C, B and A encoded in the iron transport operon. The FatA protein is the outer membrane receptor for the ferric siderophore complex and the FatB lipoprotein provides the periplasmic domain for its internalization, whereas the FatC and D proteins are located in the cytoplasmic membrane and might play a role as part of the ABC transporter for internalization of the ferric siderophore. In this work we demonstrate the essential role of these two inner membrane proteins in ferric anguibactin transport and that the lipo-protein nature of FatB is not necessary for ferric anguibactin transport.

Introduction

Vibrio anguillarum is part of the natural flora of the aquatic habitat and can also be a pathogen causing terminal haemorrhagic septicemia in marine and fresh water fish (Actis *et al.*, 1999). Some serotype O1 strains of this bacterium carry the pJM1 virulence plasmid harbouring genes encoding the siderophore anguibactin biosynthesis and transport proteins. This system is essential for *V. anguillarum* virulence as well for its survival in environments in which the iron concentration is limited (Crosa, 1980; Di Lorenzo *et al.*, 2003).

The iron transport and biosynthesis operon (ITBO) in the pJM1 plasmid of *V. anguillarum* 775(pJM1) encodes most of the genes necessary for anguibactin transport and biosynthesis (Tolmasky *et al.*, 1988). There are six genes, *fatA, B, C, D, angR* and *angT* in this operon (Fig. 1). We have previously shown that FatB, a periplasmic binding lipoprotein, and FatA, the outer membrane anguibactin-specific receptor, are essential for ferric anguibactin transport (Actis *et al.*, 1985; 1988; 1995; Lopez and Crosa, 2007; Lopez *et al.*, 2007). AngR and AngT are involved in anguibactin biosynthesis while the former also regulates the expression of the ITBO operon (Salinas *et al.*, 1989; Farrell *et al.*, 1990; Tolmasky *et al.*,

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1993; Salinas and Crosa, 1995; Chen *et al.*, 1996; Wertheimer *et al.*, 1999). Our *in silico* analysis showed that FatC and FatD are polytopic integral cytoplasmic membrane proteins that could be involved in the translocation of ferric anguibactin across the cytoplasmic membrane (Koster *et al.*, 1991). However, it has not been demonstrated as yet whether FatC and FatD are indeed involved on anguibactin transport due to the polar effect on *fatB* expression of the previously constructed mutants (Actis *et al.*, 1995).

In this work we demonstrate the essential role of these two inner membrane proteins in ferric anguibactin transport and that FatB does not need to be a lipoprotein to operate in ferric anguibactin-mediated transport.

Results and discussion

FatC and FatD are important for growth of V. anguillarum *in iron-limiting condition*

The bacterial strains and primers used in this work are listed in Table 1 and Table S1 respectively. To characterize the *fatC* and *fatD* genes we first mutated them in the wild-type strain 775(pJM1). For this task we combined flanking regions of the *fatC* or *fatD* genes by SOE PCR (Senanayake and Brian, 1995) with primers containing an Eco47III site in the middle of the deletion, and thereafter we cloned the product into pGEM-T easy vector (Promega). The kanamycin resistance cassette from pKD4 with SmaI site in both ends was ligated into the Eco47III site, and the insert DNA was subcloned into pDM4 (Milton *et al.*, 1996). These plasmids were conjugated into *V. anguillarum* 775(pJM1) by triparental mating, and plasmid cointegrates were selected on tryptic soy agar with 1% NaCl (TSAS) supplemented with rifampacin (Rif) and chloramphenicol (Cm). Colonies thus obtained were cultured in tryptic soy broth with 1% NaCl (TSBS) without antibiotics. The mutants were then screened on TSAS with 15% sucrose and kanamycin (Km), and the mutations were confirmed by colony PCR and pJM1 DNA digestion. To complement the mutations the wild-type *fatC* or *fatD* genes with the upstream Shine-Dalgarno (SD) sequences were cloned into the pGEM-T easy vector and subcloned into pMMB208. The plasmids were conjugated into each of the *V. anguillarum fatC* and *fatD* mutants by triparental mating. The growth in minimal media of the wild-type and mutant strains under iron-limiting and -rich conditions was compared. Figure 2 clearly shows that under iron-limiting conditions (1 μM EDDA) both the *fatC* and *fatD* mutants grew much less than the wild-type strain whereas they behaved similarly under iron-rich conditions (10 μg ml^{-1} ferric ammonium citrate, FAC) (data not shown). Upon complementation with the wild-type genes, we observed the recovery of the mutants growth rate under iron-limiting conditions (Fig. 2). These results underscore the important role that the FatC and FatD proteins play in the ability of *V. anguillarum* to grow under iron starvation.

FatC and FatD are essential for anguibactin-mediated iron transport

To assess whether the slower growth of the *fatC* and *fatD* mutants in iron-limiting conditions is due to a defect in the transport of ferric anguibactin we performed cross-feeding bioassays. By using the suicide vector mentioned above, we constructed the same *fatC* and *fatD* mutants in an anguibactin biosynthesis-deficient and transport-proficient strain, CC9-16. As shown in Fig. 3 we did not observe any growth halo with the *fatC* and *fatD* mutants in bioassays with spots of the anguibactin-producing strain 775(pJM1), whereas the wild-type positive control showed a distinct halo. A growth halo was indeed observed when the *fatC* or *fatD* mutants were complemented with the wild-type gene *in trans*. Negative and positive controls gave the expected results. These results demonstrate that *fatC* and *fatD* are indispensable for ferric-anguibactin iron transport, and even though FatC and FatD are very highly related (45% similarity at amino acid level) they cannot be replaced by each other, suggesting specific roles for these two proteins. Furthermore, we

also performed iron uptake experiments as described previously (Crosa and Hodges, 1981;Welch and Crosa, 2005) to characterize their lack of functionality of the *fatC* and *fatD* mutants. The results in Fig. 4A (FatC) and B (FatD) clearly show that the FatC or FatD mutant does not transport ferric anguibactin while the complemented strains recover its transport. From these findings we conclude that in addition to *fatA* and *fatB, fatD and fatC* are also essential for ferric anguibactin transport since no chromosome gene can compensate for the deficiency when either of them is mutated.

The FatB lipoprotein is anchored in the cytoplasmic membrane

The *fatB* gene encoding a periplasmic binding lipoprotein is essential for anguibactin transport and attaches to the membrane by its fatty acid tail (Actis *et al.*, 1995). However, it is still unknown whether the FatB lipoprotein is attached to the outer or the cytoplasmic membrane. To answer this question total proteins from the wild-type strain were separated by sucrose gradient sedimentation. Each fraction thus obtained was used for Western blots using specific antibodies to FatB or FatA (outer membrane control). We also measured in each fraction the NADH oxidase activity, a control for cytoplasmic membrane proteins. Figure 5 shows the strongest intensity bands observed in fraction 4 for FatB and fraction 9 for FatA respectively. Furthermore, the strongest NADH oxidase activity was also observed in fraction 4 where the strongest FatB band was detected. Those results indicate that the lipoprotein FatB is anchored to the cytoplasmic membrane.

Is the lipoprotein nature of FatB essential for anguibactin transport?

The lipoprotein nature of periplasmic binding proteins for siderophore transport is uncommon in Gram-negative bacteria. In addition to our finding with FatB, it was also shown in *V. cholerae* ViuP for vibriobactin as well as enterobactin transport (Wyckoff *et al.*, 1999) and in *Campylobacter jejuni* and *C. coli* CeuE for enterobactin transport (Park and Richardson, 1995; Richardson and Park, 1995). In Gram-positives, such as the *Bacillus subtilis* FhuD protein required for the internalization of ferric-hydroxamates, is a lipoprotein that anchors to the outside of the cytoplasmic membrane, possibly due to the lack of an outer membrane (Schneider and Hantke, 1993). Bacterial lipoproteins are recognized by the type II signal peptidase that has a 'lipobox' recognition motif that is typically L_3-[A/S/T]$_{-2}$-[G/A]$_{-1}$-C_{+1}, where the signal peptidase cleaves in front of the +1 cysteine. The +1 cysteine is absolutely conserved in all bacterial lipoproteins (Braun and Wu, 1994; Hutchings *et al.*, 2009). The thiol group of the cysteine is crucial to the lipoprotein biogenesis as the diacylglycerol lipid anchor is added in a thioether linkage (Braun and Wu, 1994). The sequence of the carboxy-terminal end of the FatB signal peptide is L-T-G-C, and the cysteine (position 23) residue should serve as the diacylglycerol lipid anchor (Actis *et al.*, 1995). To test whether the lipoprotein nature of FatB is necessary for ferric anguibactin transport we replaced this cysteine for alanine. The wild-type *fatB* gene with its upstream region was cloned into the T-vector, and C23A *fatB* was created by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The wild-type *fatB* and C23A *fatB* were, respectively, subcloned into pMMB208, and subsequently conjugated into the *fatB* null mutant constructed by inserting the Km resistance cassette in the SalI site located in the middle of the *fatB* gene. To determine the localization of C23A FatB, we prepared total proteins, periplasmic proteins and membrane proteins from the *fatB* null mutant complemented with pMMB208 carrying either *fatB*, C23A *fatB*, or the empty vector and then identified with Western blots with anti-FatB serum. Figure 6 shows that C23A FatB fractionates now in the periplasm, whereas the wild-type FatB is not detected in this fraction. The wild-type FatB as well as C23A FatB can also be found in membrane fractions. Furthermore, in the total protein fraction C23A FatB has a higher molecular weight than the wild-type FatB possibly due to the lack of processing by the signal peptidase II. From these results we can speculate

that the loss of the signal peptidase II recognition site due to the C23A mutation might have resulted in signal peptidase I cleavage of the C23A FatB N-terminal ends as reported in various systems (Cavard *et al.*, 1987; Kempf *et al.*, 1997; Yerushalmi *et al.*, 2005). However, it is clear that the signal peptidase I cleavage is not perfect leading to the detection of C23A FatB in membrane factions. The prediction of the signal peptidase recognition site using the lipoP program (Juncker *et al.*, 2003) showed that the change from C23 to A could lead to the creation of a signal peptidase I cleavage site between position 23 and 24 in C23A FatB.

The next question was whether C23A FatB is still able to operate in ferric anguibactin transport. Mutation of the cysteine residue, indispensable for the lipoprotein nature, has been reported in other systems as well. In some cases the cysteine mutation affects the function of the protein (Cavard *et al.*, 1987; Luirink *et al.*, 1988; Fernandez *et al.*, 1996), while in other cases the mutation does not affect or partially affect the function of proteins (Pugsley and Cole, 1987; Kornacker *et al.*, 1991; Zhang *et al.*, 1992; Yerushalmi *et al.*, 2005). pMMB208 harbouring the wild-type *fatB* and C23A *fatB* were, respectively, conjugated into the null *fatB* mutant in a CC9-16 genetic background and bioassays were conducted. We observe in Fig. 3 that C23A *fatB* and the wild-type *fatB* complemented the null *fatB* mutant, showing growth halos around the spots with anguibactin. Negative and positive controls gave the expected results (see Fig. 3). From this experiment it is clear that C23A FatB is still able to function in ferric anguibactin transport. To further demonstrate that the FatB wild-type and C23A proteins behaved similarly at the transport level, we performed an iron uptake experiment as described previously (Crosa and Hodges, 1981; Welch and Crosa, 2005). The results in Fig. 4 clearly demonstrate that the wild-type FatB and C23A FatB transported the radioactive ferric anguibactin with similar kinetics. From these results we conclude that the lipoprotein nature of FatB is not essential for ferric anguibactin transport.

It is not clear what is the reason for the lipoprotein nature of FatB and its binding to the cytoplasmic membrane of the Gram-negative *V. anguillarum*. We recently demonstrated that the high affinity pJM1-encoded siderophore anguibactin system might have been acquired at some point during evolution, possibly horizontally (Alice *et al.*, 2005; Naka *et al.*, 2008), thus the FatB lipoprotein could have originated in bacteria in which the embedding of the periplasmic domain as a lipoprotein is essential for their life cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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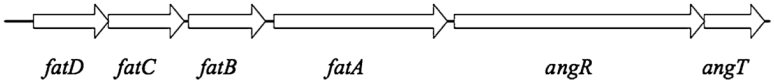

Fig. 1.

The iron transport and biosynthesis operon (ITBO) in the pJM1 plasmid of *V. anguillarum* 775(pJM1). FatC and FatD are polytopic integral cytoplasmic membrane proteins. FatB is a periplasmic binding lipoprotein. FatA is a ferric-anguibactin outer membrane receptor. AngR is involved in anugibactin biosynthesis and regulates the expression of the ITBO. AngT is involved in anguibactin biosynthesis.

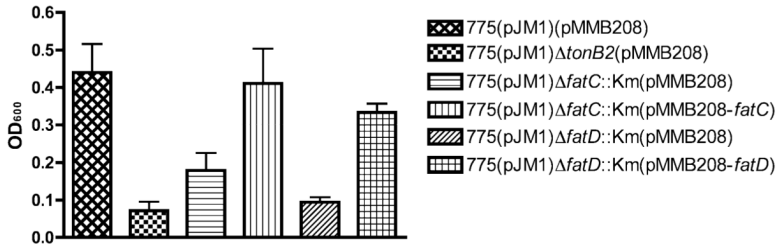

Fig. 2.

Effect of mutations in either *fatC* or *fatD* on growth under iron-limiting conditions. *Vibrio anguillarum* strains were grown first in TSBS and then in CM9 minimal medium. A 40 μl aliquot of an overnight culture in CM9 (adjusted OD_{600} to 1) was inoculated into CM9 supplemented with 10 μg ml^{-1} Cm and 0.5 mM IPTG. EDDA (1μM) was added to achieve iron-limiting conditions. OD600 was measured after 24 h incubation at 25°C. Experiments were carried out three times, and the error bar shows the standard deviation.

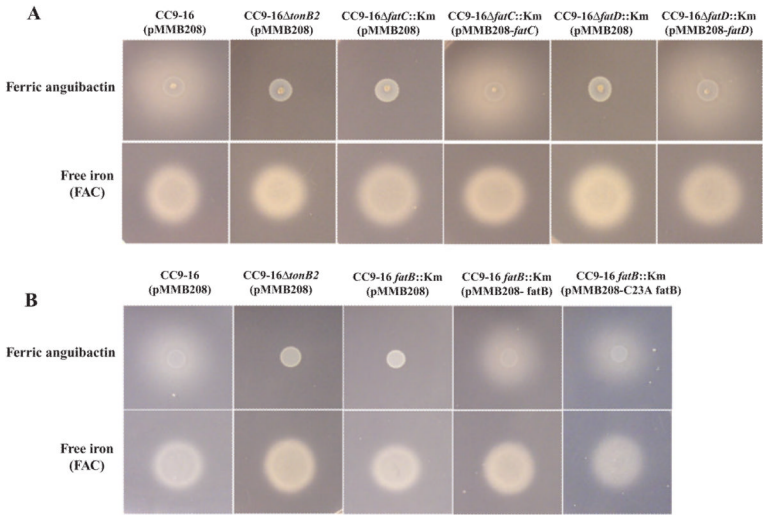

Fig. 3. Bioassay for anguibactin uptake

A. Bioassay to assess whether *fatC* or *fatD* is necessary for ferric anguibactin transport. B. Bioassay anguibactin uptake of strains with the wild-type FatB and C23A FatB. Indicator strains were grown in TSBS, and then CM9 minimal medium. A 50 μl aliquot of an overnight culture was mixed with 20 μl of melted CM9 1.5% agar, 20 μM EDDA, 500 μM IPTG and 10 μg μl^{-1} Cm. After the agar became solid 5 μl of *V. anguillarum* 775(pJM1) culture (labelled as ferric anguibactin) and 1 μl of 1 mg ml^{-1} ferric ammonium citrate [labelled as free iron (FAC)] were spotted on each plate as sources of anguibactin and free iron respectively. Plates were incubated at 25°C for 24 h. The experiments were repeated three times, with consistent results. Strain CC9-16(pMMB208) was used as a positive control; strain CC9-16Δ*tonB2*(pMMB208), constructed as described (Lopez *et al.*, 2009), was used as a negative control.

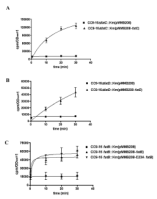

Fig. 4.

Kinetics of ^{55}Fe-anguibactin uptake by *V. anguillarum.* Characterization of FatC (A), FatD (B) and FatB (C). *Vibrio anguillarum* strains grown to exponential phase (OD_{600} = 0.3–0.6) in CM9 were washed twice and resuspended in casamino acid-free CM9 containing the chelator sodium nitrilotriacetate at a concentration of 100 μM. The anguibactin siderophore was loaded with ^{55}Fe by incubation with $^{55}FeCl_3$(1 μCi ml^{-}1) for 6 h and then mixing it with an equal volume of *V. anguillarum* in CM9 salts. At each time point 1 ml of mixture was withdrawn, filtered through a 0.45 μm filter (Millipore Corporation) and immediately washed twice with10 ml of 100 mM Sodium citrate. The filters were air-dried and the radioactivity was measured in a liquid scintillation counter. The values were normalized to an OD (OD_{600} = 1), and results were fitted using the GraphPad Prism4 program.

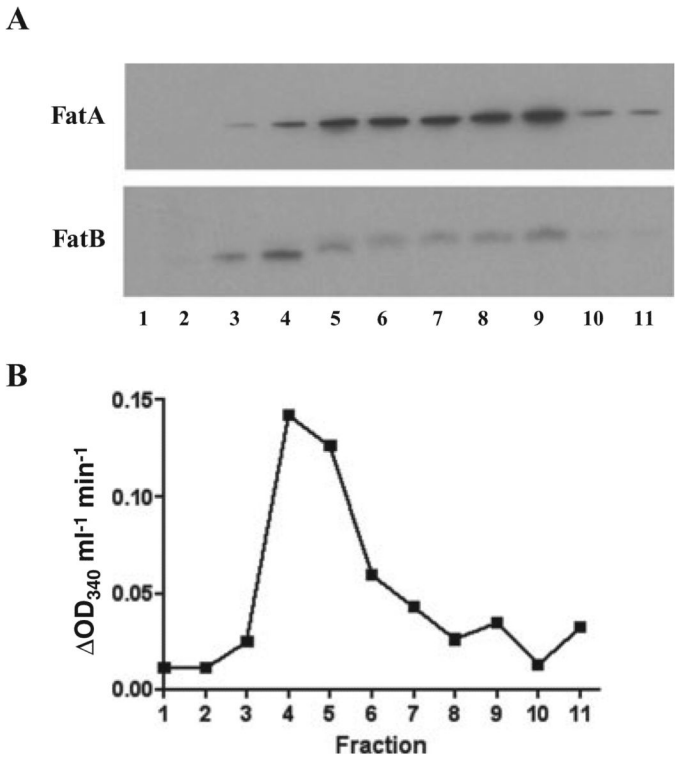

Fig. 5.

The FatB protein attaches to the cytoplasmic membrane. A. Western blots of proteins separated by sucrose density gradient centrifugation. Sucrose density gradient centerifugation was conducted as described by Nikaido (1994) with modification. One litre of *V. anguillarum* 775 overnight culture in CM9 broth was harvested and resuspended in 20 ml Hepes buffer (pH 7.4). After French pressure treatment and centrifugation, 1 ml of total protein was layered on top of a sucrose gradient (0.25 ml of saturated sucrose, 1.5 ml of 2.02 M sucrose, 5 ml of 1.44 M sucrose and 3 ml of 0.77 M sucrose) in the 14 × 89 mm polyallomer centrifuge tubes (Beckman). After 20 h ultracentrifugation at 4°C in the SW28 rotor (Beckman) at 100 000 *g* each 1 ml of fractions was collected from the top of the tube, and 3 μl (for FatA detection) and 15 μl (for FatB detection) of samples were used for Western bots. B. NADH oxidase activity of fractions obtained from sucrose gradient sedimentation. The NADH oxidase activity was measured as described by Osborn and colleagues (1972) with modification. Samples (30 μl) was mixed with 240 μl of assay buffer (50 mM Tris-HCl (pH 7.5), 0.2 mM dithiothreitol). After 5 min incubation at 25°C, 30 μl of 1.2 mM NADH was added, and the decrease in OD_{340} was measured at 25°C.

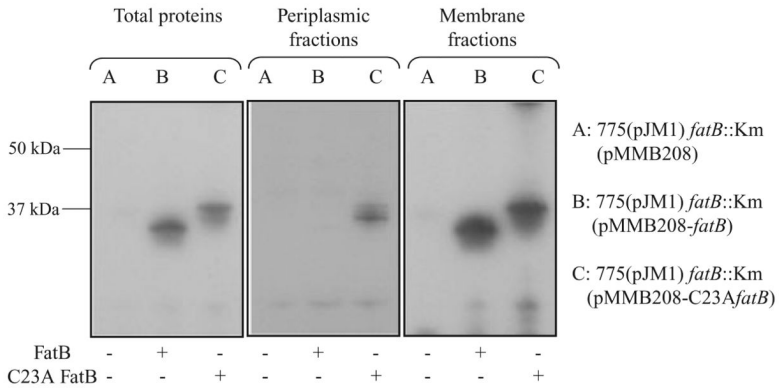

Fig. 6.

Western blot detection of FatB in cell fractions of *V. anguillarum* carrying either the wild-type FatB or C23A FatB. Periplasmic proteins were extracted as described by Wunderlich and colleagues (1993) with some minor modifications. Briefly, an exponential phase bacterial culture in CM9 minimal medium with 10 μg ml^{-1} Cm and 0.5 mM IPTG was centrifuged. The pellets were washed with CM9 minimal medium and resuspended in 2 ml BBS/EDDA (200 mM boric acid/NaOH, pH 8.0, 160 mM NaCl, 1 mM EDTA) per gram cell. The suspension was incubated for 45 min at 4°C with gentle agitation, and centrifuged (27 000 *g*, 1 h, 4°C) to pellet the spheroplasts. The supernatant containing periplasmic proteins was carefully transferred into new tubes. To obtain total protein the bacterial cells prepared as described above were resuspended into 10 mM Tris buffer (pH 7.6), sonicated 5 × 5 s and centrifuged at 15 000 r.p.m. at 4°C for 5 min. Supernatants were transferred into new tubes and used as total proteins. Membrane proteins were obtained by centrifuging the total proteins for 1 h at 30 000 *g*, and after resuspension this step was repeated. The presence of FatB or C23A FatB in the periplasm fractions and in total proteins was determined by Western blotting using anti-FatB serum.

Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Phenotype	Source or reference
Vibrio anguillarum strains		
775(pJM1)	Wild-type, Washington (serotype O1, pJM1)	Laboratory stock
775(pJM1)-pMMB	775(pJM1) harbouring pMMB208	Naka *et al.* (2008)
HNVA-1	775(pJM1)Δ*fatC*::Km	This study
HNVA-2	775(pJM1)Δ*fatD*::Km	This study
CSL-71	775(pJM1) *fatB*::Km	This study
CSL-48	775(pJM1)Δ*tonB2*	Lopez *et al.* (2009)
CC9-16	Anguibactin deficient, anguibactin transport system deficient	Walter *et al.* (1983)
HNVA-3	CC9-16Δ*fatC*::Km	This study
HNVA-4	CC9-16Δ*fatD*::Km	This study
HNVA-5	CC9-16 *fatB*::Km	This study
HNVA-6	CC9-16Δ*tonB2*	This study
Escherichia coli strains		
S17-1 λpir	λ-*pir* lysogen; *thi pro hsdR hsdM*$^{+}$ *recA RP4 2-Tc:Mu-Km:Tn7(Tp*^{r} *Sm*^{r}*)*	Simon *et al.* (1983)
DH5α	*F*$^{-}$, ϕ*80lacZ*ΔM15, *endA1, recA1, hsdR17, (*$r_K{}^{-}m_K{}^{+}$*), supE44, thi-1, gyrA96, relA1,* Δ*(lacZYA-argF)U169,* λ^{-}	Laboratory stock
Plasmids		
pGEM-T easy	Cloning vector Amp^{r}	Promega
pCR2.1	Cloning vector Amp^{r}, Km^{r}	Invitrogen
pDM4	Suicide plasmid *sacB* gene, R6K origin, Cm^{r}	Milton *et al.* (1996)
pKD4	Template plasmid, Km^{r}, Amp^{r}	Datsenko and Wanner (2000)
pHN1	pDM4-Δ*fatC*::Km	This study
pHN2	pDM4-Δ*fatD*::Km	This study
pCL23	pDM4-*fatB*::Km	This study
pMMB208	A broad-host-range expression vector; Cm^{r} *IncQ lacI*^{q} *Ptac*; polylinker from M13mp19	Morales *et al.* (1991)
pHN3	pMMB208-*fatC*	This study
pHN4	pMMB208-*fatD*	This study
pCL24	pMMB208-*fatB*	This study
pCL25	pMMB208-C23A *fatB*	This study

Vibrio anguillarum strains were grown at 25°C in Tryptic soy broth (TSBS) or on agar (TSAS) supplemented with 1% NaCl, or in CM9 minimal medium (Crosa, 1980). Either ferric ammonium citrate (FAC) or ethylenediamine-di-(*o*-hydroxyphenyl acetic) acid (EDDA) was supplied in the CM9 medium to obtain iron-rich or -limiting conditions respectively. *Escherichia coli* were grown at 37°C in LB broth or agar. Antibiotics were used as following concentrations: 100 μg ml^{-1} rifampicin (Rif), 10 μg ml^{-1} chloramphenicol (Cm) and 200 μg ml^{-1} kanamycin (Km) for *V. anguillarum*, and 50 μg ml^{-1} ampicillin (Amp), 30 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} kanamycin for *E. coli*.