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Identification and characterization of a novel outer membrane protein receptor FetA for ferric enterobactin transport in *Vibrio anguillarum* 775 (pJM1)

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Abstract

In this work we demonstrate the existence in *Vibrio anguillarum* 775 (pJM1) of two chromosomal genes encoding outer membrane proteins that operate in the transport of ferric enterobactin. One of them is a novel receptor that we named FetA and the other is the already characterized FvtA that functions in the uptake of iron complexes of both enterobactin and vanchrobactin. Ferric enterobactin transport proficiency was resumed in double mutants for these two genes when they were complemented with either *fetA* or *fvtA*, whereas only the cloned *fvtA* could complement for ferric vanchrobactin transport. Quantitative RT-PCR assays demonstrated that transcription of the *fetA* gene is regulated by FetR, that is encoded upstream and in reverse orientation from *fetA*. This gene as well as *fetA*, are up-regulated in iron limiting condition in a Fur-dependent manner. The two divergent promoters are located in the intergenic region between *fetR* and *fetA* that has a putative Fur binding site and an IrgB binding site in the overlapping promoters of *fetR* and *fetA*. FetA and FetR show high homology to *V. cholerae* IrgA and IrgB respectively and the intergenic regions *fetA–fetR* and *irgA–irgB* are also highly related suggesting a vertical transmission of the *fetA–fetR* cluster from *V. cholerae* to *V. anguillarum*.

Keywords

Iron; Siderophore; Enterobactin; Outer membrane receptor

Introduction

Vibrio anguillarum 775 (pJM1) serotype O1 is a fish pathogen that causes terminal hemorrhagic septicemia in marine and fresh water fish. Iron uptake systems mediated by siderophores and heme have been reported to be important in the survival of this bacterium under iron limiting conditions as those found in the vertebrate host and in the environment (Actis et al. 2011; Crosa 1980; Naka and Crosa 2011).

Many O1 strains of *V. anguillarum* carry the pJM1-type virulence plasmid that harbors genes for the biosynthesis of the siderophore anguibactin and its cognate transport system

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(Actis et al. 2011; Crosa 1980; Crosa and Walsh 2002; Di Lorenzo et al. 2003). Some of the genes responsible for anguibactin biosynthesis are located in the chromosome and are functional homologues of the plasmid harboring the anguibactin biosynthesis genes (Alice et al. 2005; Naka et al. 2008). It is of interest that these very same homologues are part of a biosynthesis cluster coding for the siderophore vanchrobactin (Naka et al. 2008). However, the 775 (pJM1) strain of *V. anguillarum* does not produce this siderophore. We suggested that *V. anguillarum* 775 (pJM1) originally had the ability to produce vanchrobactin, but that this strain lost that capability after acquiring the pJM1 plasmid. At that time a transposon from pJM1 might have inserted into the vanchrobactin biosynthesis gene *vabF* (Naka et al. 2008) possibly because vanchrobactin biosynthesis was no longer necessary, since anguibactin is a stronger iron chelator (Naka et al. 2008). However, although not producing vanchrobactin, the 775 strain can still take up this siderophore using the outer membrane ferric vanchrobactin receptor FvtA, first identified by our laboratory in the 775 (pJM1) strain (Naka et al. 2008), and the cognate cytoplasmic membrane transport genes. Recently it was demonstrated in the *V. anguillarum* strain RV22, a naturally-occurring pJM1-less strain and a vanchrobactin producer, that the FvtA protein might as well contribute to the transport of ferric enterobactin because a *fvtA* mutant shows a reduced uptake of ferric enterobactin (Balado et al. 2009). The fact that albeit reduced, transport still occurs, indicates that there must be another receptor to transport enterobactin in the absence of *fvtA*. In this work we report that a novel receptor, FetA, a homologue of the *V. cholerae* enterobactin receptor IrgA, functions as a ferric-enterobactin outer membrane receptor in *V. anguillarum* 775 (pJM1), but not for ferric vanchrobactin unless it is overexpressed. We also demonstrate that expression of the *fetA* gene in strain 775 is positively controlled by FetR, a LysR type regulator, and that *fetA* and *fetR* are iron regulated in a Fur dependent manner.

Materials and methods

Strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. *V. anguillarum* strains were cultured at 25°C in Trypticase soy broth supplemented with 1% NaCl (TSBS) or on agar (TSAS). As iron limiting growth conditions *V. anguillarum* strains were incubated in M9 minimal medium (Crosa 1980) supplemented with 0.2% Casamino Acids, and 5% NaCl (CM9) and in some experiments the iron chelator ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA). Ferric ammonium citrate was added into CM9 medium for iron rich growth conditions. *E. coli* strains were cultured at 37°C in LB broth or on LB agar. When necessary, antibiotics were used at following concentrations: for *V. anguillarum*, chloramphenicol 10 μg/ml and rifampicin 100 μg/ml; for *E. coli*, ampicillin 100 μg/ml and chloramphenicol 30 μg/ml.

Construction of mutants and their complementation

DNA cloning and PCR were carried out by standard protocols (Sambrook and Russell 2001). Upstream and downstream regions of the genes to be mutated were combined by Splicing by Overlapping Extension (SOE) PCR using primers listed in Table 2 (Senanayake and Brian 1995). First PCR reactions were performed using -F and -1, and -R and -2 primers in separate tubes, while -F and -R primers were used for the second PCR (Table 2). The generated fragments were cloned into the T-vector, and subcloned into suicide vector pDM4. Conjugation of plasmids into *V. anguillarum* as well as screening of mutants was followed using procedures we previously described (Naka et al. 2008). To complement the mutations, the wild type genes containing the Shine-Dalgarno sequence were PCR amplified, cloned into pMMB208 and conjugated into *V. anguillarum* strains.

Bioassays

Bioassays were used to detect ferric-siderophore transport as described previously (Tolmasky et al. 1988). Briefly, 50 µl of overnight cultures of the strains to be tested were mixed with 20 ml CM9 melted agar (1.5% agar and ~40°C) supplemented with Cm (10 µg/ml), IPTG (500 µM) and EDDA (20 µM). After solidification, *V. anguillarum* 775 (pJM1) as an anguibactin source, *V. anguillarum* RV22 as a vanchrobactin source, purified enterobactin (1 mg/ml) (EMC microcollections GmbH, Tubingen, Germany) and ferric ammonium citrate (10 mg/ml) were streaked and/or spotted on the agar, and incubated 48 h at 25°C. The existence of growth halos around streaked or spotted iron sources were checked at the 24 and 48 h time points.

Expression analysis of *V. anguillarum* genes

Total RNAs were extracted using RiboPure-Bacteria™ kit (Ambion, Austin, TX) from *V. anguillarum* strains grown in different conditions, and treated with TURBO DNA-free™ kit (Ambion, Austin, TX) to remove contaminated residual DNA. The reverse transcription (RT) reaction was performed with SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using the Random primer (Invitrogen, Carlsbad, CA). Quantitative PCR experiments were carried out using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the procedure described in the manual.

Outer membrane extraction and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Outer membrane fractions of *V. anguillarum* were extracted using methods previously described with minor modifications (Crosa and Hodges 1981). Briefly, *V. anguillarum* was cultured in CM9 medium; late-exponential phase bacteria were harvested and pellets were resuspended into 600 µl of Tris–HCl (pH 7.6) buffer. After sonication of the suspension, cell debris was removed by centrifugation. The supernatants were treated with 1.5% sodium lauroyl sarcosinate for 2 h at 4°C, and centrifuged 1 h at 15,000 rpm at 4°C, then pellets were re-suspended in distilled water. Samples were subjected to SDS-PAGE using Criterion™ XT Precast Gel (10% Bis–Tris) (Bio-Rad, Hercules, CA, USA) for 5 h at 80 V and the gel was stained with Bio-Safe™ Commassie G-250 stain (Bio-Rad).

Results

FetA is an enterobactin receptor in *V. anguillarum* 775 (pJM1)

The serotype O1 strain 775 (pJM1) carrying the 65 kb pJM1 plasmid can take up the endogenous siderophore anguibactin as well as exogenous siderophores vanchrobactin, enterobactin and ferrichrome (Lemos et al. 1988; Naka et al. 2008). Strain H775-3, from which the pJM1 plasmid had been cured, is still able to use enterobactin as well as vanchrobactin as iron sources indicating that the specific receptor(s) for the ferric complexes of those two siderophores must be encoded on the chromosome. We recently identified in the *V. anguillarum* 775 (pJM1) strain the *fvtA* gene that shows 30% identity with the *Bordetella pertussis* enterobactin receptor gene, *bfeA* (Naka et al. 2008). We also showed that an *fvtA* mutant in an anguibactin production negative 775 (pJM1) derivative, does not transport vanchrobactin (Naka et al. 2008). Furthermore, Balado et al. (2009) demonstrated that FvtA in *V. anguillarum* serotype O2 strain RV22 could be involved in vanchrobactin as well as enterobactin transport. However the *fvtA* mutant in RV22 is still able to transport enterobactin indicating that this strain must have another enterobactin receptor. We first performed bioassays to determine whether the 775 (pJM1) *fvtA* mutant can still transport ferric enterobactin. As shown in Table 3, with this mutant we can only detect uptake of ferric enterobactin but not of ferric vanchrobactin, as compared to the control *tonB2* mutant

of this strain that cannot use any those two ferric siderophores as iron sources. Thus, there must indeed be another receptor for ferric-enterobactin in the chromosome of 775 (pJM1). Based on our whole genome sequencing data of *V. anguillarum* 775 (pJM1) (Naka et al. 2011) we identified the putative receptor gene named *fetA* that shares high similarity to the *V. cholerae irgA* gene (68% identity and 81% similarity in protein level) encoding a ferric enterobactin receptor.

To assess whether the *fetA* gene product is functional as a receptor for enterobactin we mutated this gene in an *fvtA* mutant background. Table 3 shows that the double *fvtA* and *fetA* mutant thus constructed, is unable to use vanchrobactin and enterobactin as iron sources even when the strain was incubated for long times. When the mutation is complemented with the *fetA* gene in *trans* we observed the recovery of ferric enterobactin transport and a very weak ferric vanchrobactin transport was also detected when it is over-expressed. These results indicate that the *fetA* protein main function must be as a ferric enterobactin receptor.

fetA is an iron regulated outer membrane protein

It was difficult to detect the *fetA* protein in the wild type strain membranes, likely due to its low expression level. Thus we needed to use a *fetA* overexpressing strain to test whether FetA is located in the outer membrane fractions. The predicted size of FetA is similar to FatA and FvtA, thus to detect FetA we constructed the H775-3Δ*fetA*Δ*fvtA* strain that does not express *fetA*, *fatA* nor *fvtA*. The *fetA* gene was overexpressed from pMMB208-*fetA* in this strain. The H775-3Δ*fetA*Δ*fvtA* strain carrying empty pMMB208 was used as a negative control. The SDS-PAGE of the outer membrane preparations shown in Fig. 1 provides the correlation between the presence of an outer membrane protein band of 69.57 kDa that corresponds to the mature FetA protein in the H775-3Δ*fetA*Δ*fvtA* (pMMB208-*fetA*) while the negative control did not show this band indicating that FetA actually localize in the outer membrane of *V. anguillarum*.

A regulator of *fetA* expression

Analysis of the upstream region of *fetA* unveiled the presence of a gene that we named *fetR* that is a homologue (72% identity and 83% similarity in protein level) of the *V. cholerae irgB* gene belonging to the LysR family of transcriptional activators (Fig. 2). In *V. cholerae* IrgB is a positive regulator of the *irgA* gene, and *irgA* and *irgB* were both iron regulated in a Fur-dependent manner (Goldberg et al. 1990a, b, 1991; Watnick et al. 1998). The comparison of the putative promoter region of *fetA* and *fetR* with the corresponding region of *V. cholerae* revealed that they are highly conserved including a ferric uptake regulator (Fur) and IrgB binding sites identified between *irgA* and *irgB* in *V. cholerae* (Watnick et al. 1998) (Fig. 2). The similarity of both genes as well as the promoter region motivated us to test whether they have similar regulation mechanisms in both bacteria. First, we determined whether *fetR* and/or *fetA* are regulated by iron concentration and/or Fur using quantitative RT-PCR (qRT-PCR). As shown in Fig. 3, the expression of both *fetR* and *fetA* is clearly down regulated under iron rich condition while expression patterns in a *fur* mutant were similar in both iron-rich and iron-limiting conditions. These results indicate that *fetR* as well as *fetA* are iron regulated in a Fur-dependent manner. Furthermore, we examined the expression of *fetA* in the *fetR* wild type and mutant backgrounds using qRT-PCR (Fig. 4). Transcription of *fetA* was dramatically decreased in the *fetR* deletion mutant demonstrating that *fetR* is required for the expression of *fetA*. Complementation of the *fetR* mutant in *trans* restored the *fetA* expression similar to the wild type level. These results clearly demonstrate that *fetR* encoded upstream and in reverse orientation from *fetA*, is required for *fetA* expression at the level of transcription.

Discussion

Little is known about the mechanisms by which *V. anguillarum* cells recognize the ferric enterobactin complexes and subsequently internalize the essential iron into the cell cytosol. In this work, we initiated the identification and characterization of the components of the ferric enterobactin transport system. The experimental evidence indicates that FetA must be a receptor for ferric enterobactin transport. However it was also clear that the inability to grow in the presence of enterobactin under iron limitation conditions only occurred if the *V. anguillarum* cells were impaired in the expression of both *fetA* and *fvtA*. When these double mutants were complemented with either *fetA* or *fvtA*, ferric enterobactin transport proficiency was resumed indicating that FetA and FvtA can both operate as ferric enterobactin outer membrane receptors. Furthermore, our bioassay experiments demonstrated that FetA does not act as a vanchrobactin receptor at the chromosomal expression level. Our recent whole genome sequencing study revealed that both FvtA and FetA exist in all of sequenced *V. anguillarum* strains such as O1 serotype pJM1-carrying strain 775 (pJM1), O1 serotype pJM1-less strain 96F and O2 serotype pJM1-less strain RV22 while *V. ordalii*, that is a very closely related species to *V. anguillarum* only harbors *fvtA* but not *fetA* (Naka et al. 2011).

The comparative analysis of the intergenic sequences between *V. anguillarum fetA* and *fetR* and *V. cholerae irgA* and *irgB* revealed that the IrgB binding site as well as Fur binding site identified in *V. cholerae* (Watnick et al. 1998) are highly conserved in *V. anguillarum* suggesting similar expression mechanisms in those two systems. qRT-PCR assays using *V. anguillarum* 775 (pJM1) confirmed that transcription of the *fetA* gene is regulated by FetR, which is encoded upstream and in reverse orientation from *fetA* and that *fetA* as well as *fetR* are iron regulated in a Fur dependent manner. Based on the similarity of genes as well as possession of similar regulation mechanisms, we can speculate on the vertical transmission of the *fetA–fetR* cluster from *V. cholerae* to *V. anguillarum*.

It is intriguing that strain 775 (pJM1) is able to transport ferric vanchrobactin although this strain lost the ability to biosynthesize this ancestral siderophore due to interruption of the *vanF* gene (Naka et al. 2008). The FvtA receptor also can be used as a ferric enterobactin receptor, and *V. anguillarum* carries the additional enterobactin receptor FetA. We speculate that *V. anguillarum* 775 (pJM1) can utilize ferric vanchrobactin or ferric enterobactin in environmental conditions where those exogenous ferric siderophores are available. It has been demonstrated that some *Aeromonas* strains produce enterobactin, and *V. anguillarum* pJM1-less O1 and O2 serotype strains and *Vibrio* sp. DS40M4 produce vanchrobactin (Andrus and Payne 1983; Lemos et al. 1988; Massad et al. 1991; Sandy et al. 2010). These bacteria could be candidates for the source of the two exogenous siderophores since they are inhabitants of the aquatic environments where *V. anguillarum* pJM1-carrying strains can be found. We are currently performing experiments to assess the particular environmental conditions in which FvtA and/or FetA are necessary to enhance the growth of *V. anguillarum* pJM1-carrying strains and how this organism can interact with the enterobactin and/or vanchrobactin-producing bacteria.

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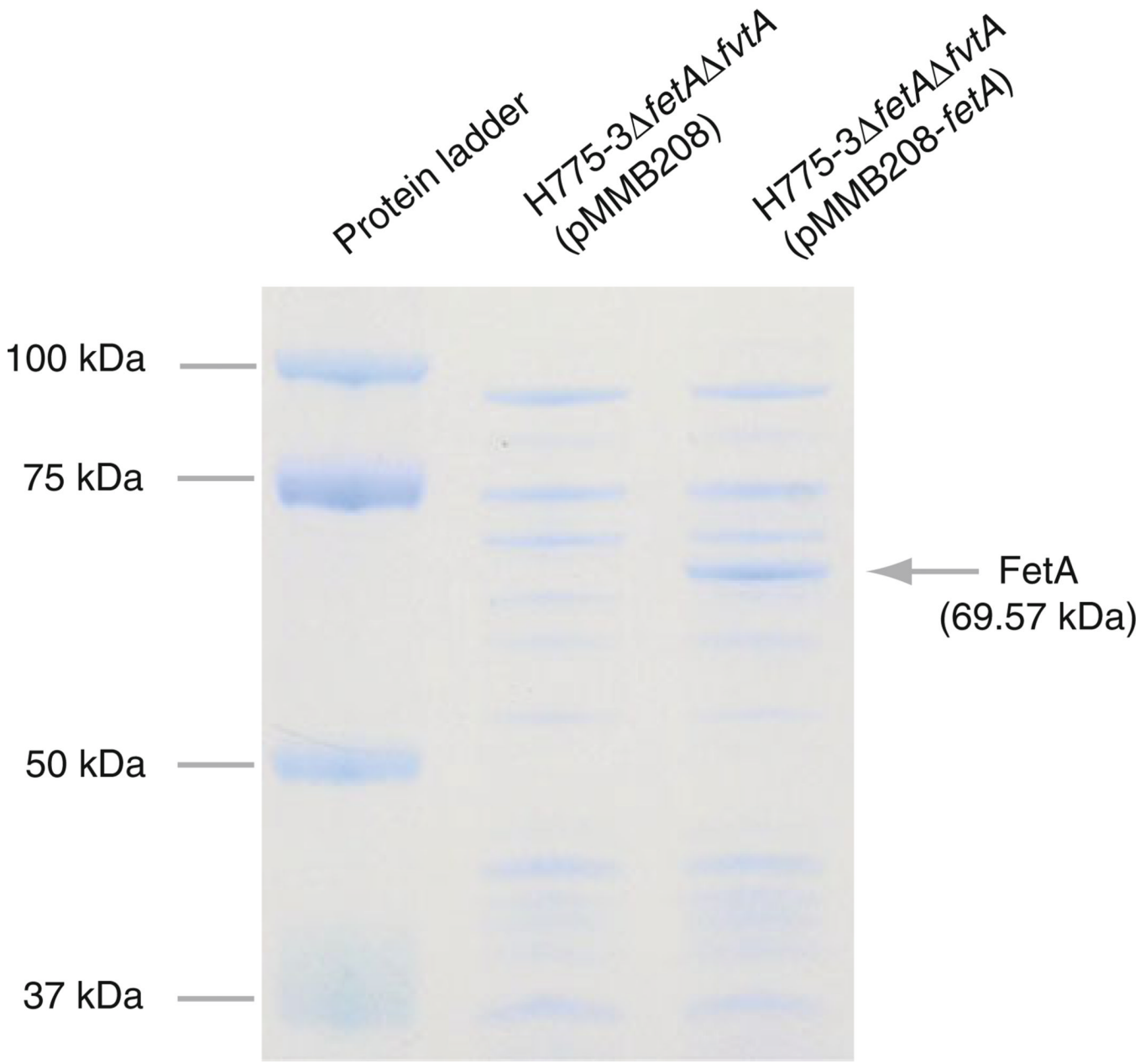

Fig. 1.

Localization of FetA in the outer membrane fractions. H775-3Δ*fetA*Δ*fvtA*(pMMB208), HNVA-9 carrying pMMB208 and H775-3Δ*fetA*Δ*fvtA*(pMMB208-*fetA*), HNVA-9 carrying pHN9 were grown in CM9 broth supplemented with 5 μg/ml Cm and 500 μM IPTG and outer membrane fractions were extracted by using sarkosyl. Samples were subjected to SDS-PAGE using Criterion™ XT Precast Gel (10% Bis–Tris) for 5 h at 80 V. Protein ladder, Precision Plus Protein Standards (Bio-Rad)

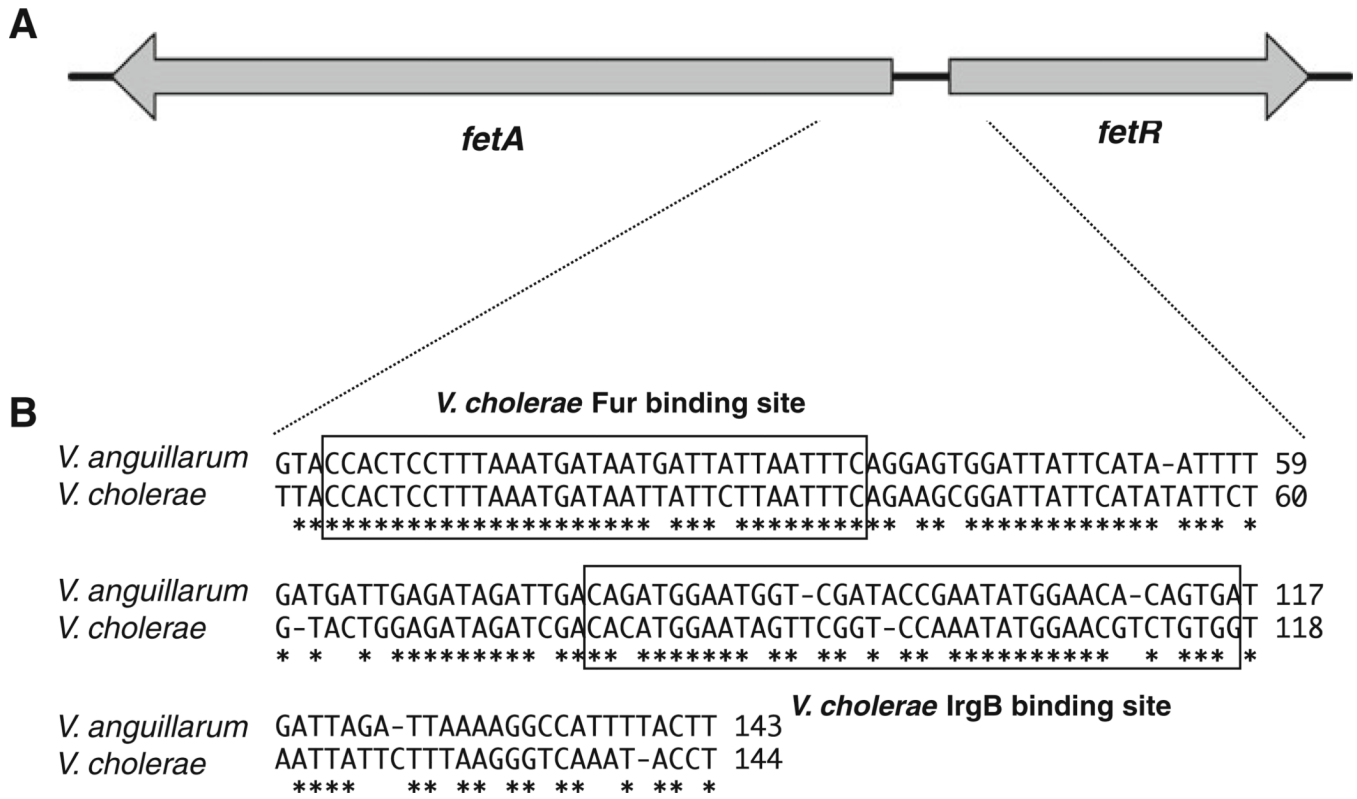

Fig. 2.

Schematic map of *V. anguillarum fetA* and *fetR* (**a**), and the comparison of its intergenic region with that between *V. cholerae irgB* and *irgA* (**b**). **a** The orientation of the *fetA* and *fetR* was shown as *arrows*. **b** The nucleotide sequence of the intergenic region of *V. anguillarum fetA–fetR* as compared with that between *V. cholerae irgA* and *irgB*. *Asterisks* designate conserved nucleotide sequences, and the Fur binding site and the IrgB binding site previously identified in *V. cholerae* (Watnick et al. 1998) are shown in the *box*

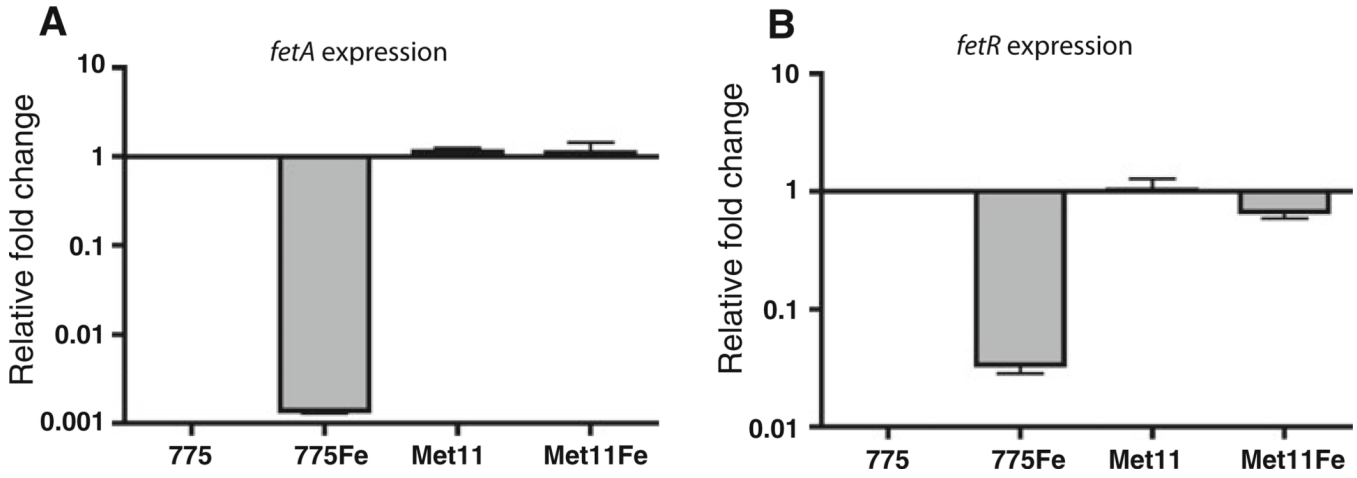

Fig. 3.

Iron and Fur regulation of *fetA* and *fetR*. The expression of *fetA* (**a**) and *fetR* (**b**) was evaluated by qRT-PCR. 775, 775 (pJM1) grown in CM9; 775Fe, 775 (pJM1) grown in CM9 with 10 μg/ml ferric ammonium citrate (FAC); Met11, 775MET11 (a *fur* mutant of 775) grown in CM9; Met11Fe, 775Met11 grown in CM9 with 10 μg/ml FAC. *aroC* was used as an internal control, and expression levels are expressed relative to 775 samples. The data represents mean value of at least three independent experiments (*error bars* indicate the standard error of the mean)

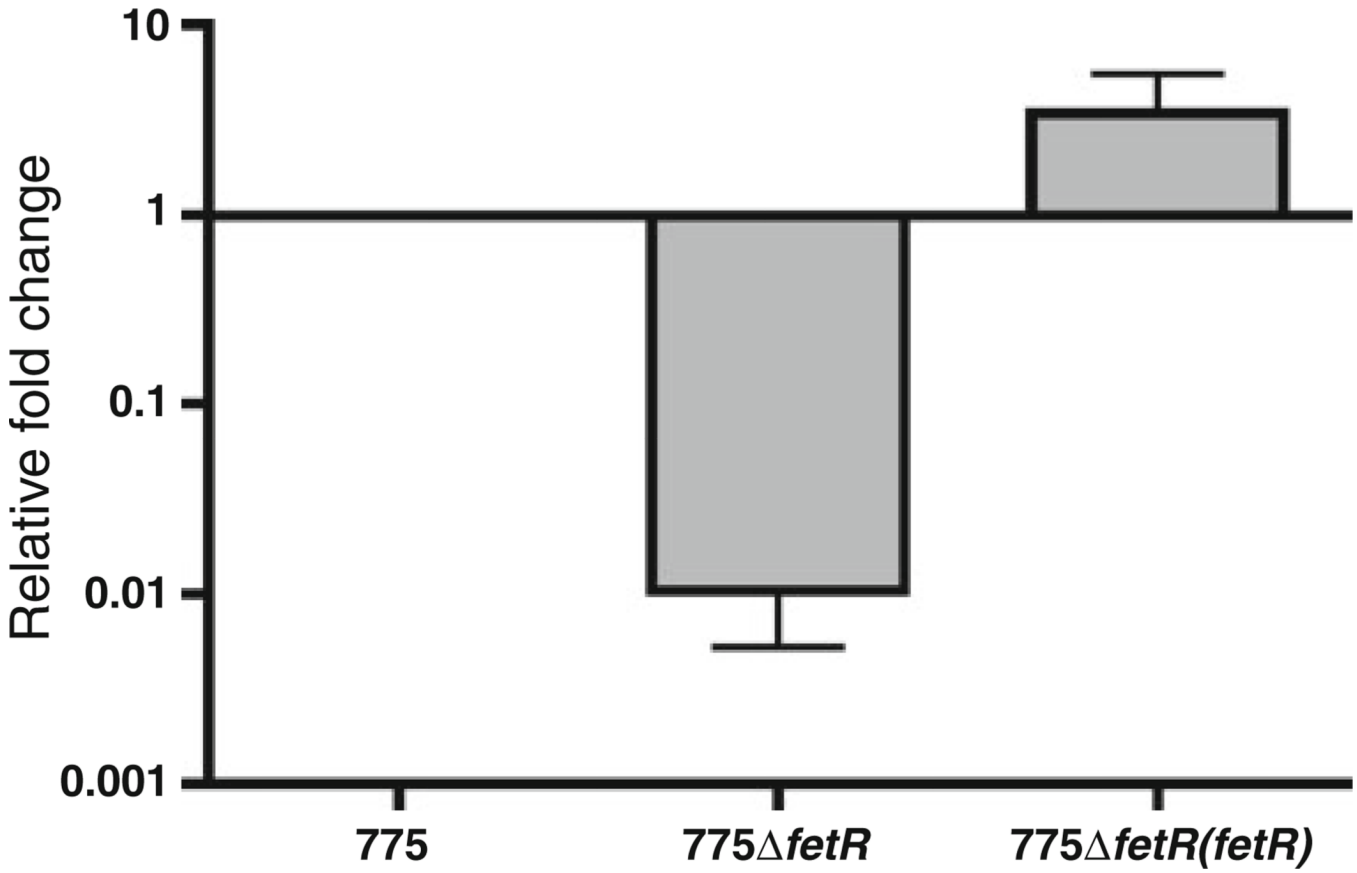

Fig. 4.

FetR positively regulates the expression of *fetA*. 775, 775 (pJM1) carrying pMMB208; 775Δ*fet*R, HNVA-10 carrying pMMB208; 775Δ*fetR*(*fetR*), HNVA-10 carrying pHN10. RNAs were extracted from exponential phase cultures of *V. anguillarum* strains grown in CM9. RT reactions were performed using random primers. *aroC* was used as an internal control. Data were normalized to *aroC* and compared with 775 data. The data represents mean value of at least three independent experiments (*error bars* indicate the standard error of the mean)

Table 1

Strains and plasmids used in this study

Strain or plasmid	Phenotype	Source or reference
V. anguillarum strains		
775 (pJM1)	Wild-type, Washington (serotype O1, pJM1)	Crosa (1980)
775 (pJM1)-pMMB	775 (pJM1) harboring pMMB208	Naka et al. (2008)
HNVA-10	775 (pJM1)Δ*fetR*	This study
RV22	Wild type, Spain (serotype O2, plasmidless)	Lemos et al. (1988)
RV22-pMMB	RV22 harboring pMMB208	This study
CC9-16	775 (pJM1) derivative of anguibactin deficient, anguibactin transport system deficient	Walter et al. (1983)
CC9-16Δ*fvtA*	CC9-16Δ*fvtA*	Naka et al. (2008)
HNVA-6	CC9-16Δ*tonB2*	Naka et al. (2010)
HNVA-7	CC9-16Δ*fetA*	This study
HNVA-8	CC9-16Δ*fvtA*Δ*fetA*	This study
H775-3	Plasmidless derivative of 775 (pJM1)	Crosa (1980)
HNVA-9	H775-3Δ*fetA*Δ*fvtA*	This study
775MET11	775 *fur* point mutant isolated in the presence of 10 mM $MnCl_2$	Tolmasky et al. (1994)
E. coli strains		
Top10	F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Invitrogen
	φ80*lacZ*Δ*M15* Δ*lacX74 recA1*	
	*araD139*Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	
S17-λkpir	λ-*pir* lysogen; *thi pro hsdR hsdM+ recA* RP4-2 Tc:Mu-Km:Tn7(Tp^r Sm^r)	Simon et al. (1983)
Plasmids		
pCR2.1	Amp^r, Km^r, PCR cloning vector	Invitrogen
pMMB208	A broad-host-range expression vector; Cmr *IncQ lacI*q *Ptac*; polylinker from M13mp19	Morales et al. (1991)
pHN5	pDM4 harboring Δ*fvtA*	This study
pHN6	pDM4 harboring Δ*fetA*	This study
pHN7	pDM4 harboring Δ*fetR*	This study
pHN8	pMMB208 harboring *fvtA*	This study
pHN9	pMMB208 harboring *fetA*	This study
pHN10	pMMB208 harboring *fetR*	This study

Table 2

Primers used in this study

Primers name	Nucleotide sequences
Construction of mutants	
fvtA mut-F	TTTTGAGCCAGTGGATCAGC
fvtA mut-R	CGTGTTTGTCAACATGGATCAG
fvtA mut-1	CTAGAACTGGTAATCAGCGACATCCATGGGCTTCATGACCT
fvtA mut-2	AGGTCATGAAGCCCATGGATGTCGCTGATTACCAGTTCTAG
fetA mut-F	TCAAGATGCGACTCTTCTGC
fetA mut-R	CTCAATGTGCTCCAAACCATC
fetA mut-1	CCTAACCAATAACGACGTCCATTCAGCGGATTGAGTCTGG
fetA mut-2	CCAGACTCAATCCGCTGAATGGACGTCGTTATTGGTTAGG
fetR mut-F	TTCAATGGCTTGCAGTGGTG
fetR mut-R	ACCAAGTGGAATCAGCCGTC
fetR mut Cla1-1	GGTGCTTTCCATTCCTCAGGATCGATGGAAAGCTTTGATCGCACTG (underline, *Cla*I site)
fetR mut Cla1-2	CAGTGCGATCAAAGCTTTCCATCGATCCTGAGGAATGGAAAGCACC (underline, *Cla*I site)
Complementation	
fvtA comp *Sph*I F	GCATGCCGATAATGGTTGCAGCAACATC (underline, *Sph*I site)
fvtA comp *Xba*I R	TCTAGACTAGAACTGGTAATCAGCGAC (underline, *Xba*I site)
fetA com *Sph*I F	GCATGCCAAAATTATGAATAATCCACTCCTG (underline, *Sph*I site)
fetA com *Eco*RI R	GAATTCTTAAAAATTAACGTCAATACCTAACC (underline, *Eco*RI site)
fetR com *Sph*I F	GCATGCACAGATGGAATGGTCGATACCG (underline, *Sph*I site)
fetR com *Xba*I R	TCTAGATTATGGTGCTTTCCATTCCTCAG (underline, *Xba*I site)
qRT-PCR	
fetA-QRT-F	TCACCTCATCACACGTGTTC
fetA-QRT-R	ATTTGGTCGAGTTTGACGCG
fetR-QRT-F	AAGGCAACCGTCTAACTTTGAC
fetR-QRT-R	TCCAGCCTTGTTGAGCAAAC
aroC-QRT-F	GTCGAATCCACCTTATGTGG
aroC-QRT-R	ACGGAATGTGGTCGTATTGG

Table 3

Bioassay to test ferric-siderophore utilization

Indicator strains	Iron sources			
	Anguibactin	**Vanchrobactin**	**Enterobactin**	**FAC**
Wt (pMMB208)	+	+	+	+
Δ*tonB2* (pMMB208)	−	−	−	+
Δ*fvtA* (pMMB208)	+	−	+	+
Δ*fetA* (pMMB208)	+	+	+	+
Δ*fvtA*Δ*fetA* (pMMB208)	+	−	−	+
Δ*fvtA*Δ*fetA* (pMMB208-*fvtA*)	+	+	+	+
Δ*fvtA*Δ*fetA* (pMMB208-*fetA*)	+	$+^{w}$	+	+

50 μl of overnight cultures of indicator strains in CM9 broth was mixed with 20 ml of melted CM9 agar (adjusted to ~40°C) supplemented with 1 mM IPTG and 20 μM EDDA. After solidification iron sources such as anguibactin from *V. anguillarum* 775 (pJM1)-pMMB, vanchrobactin from *V. anguillarum* RV22-pMMB and 1 μM of purified enterobactin from EMC microcollections GmbH was spotted on the plates, and growth halos around spots were checked after 24 or 48 h incubation at 25°C. Ferric ammonium citrate (FAC) was used as a positive control. +, growth at 24 and 48 h; $+^{w}$, weakly growth only at 48 h; −, no growth at 24 and 48 h. Wt, CC9-16; Δ*tonB2*, HNVA-6; Δ*fetA*, HNVA-7; Δ*fvtA*Δ*fetA*, HNVA-8