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Characterization of ferric-anguibactin transport in *Vibrio anguillarum*

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Abstract The fish pathogen *Vibrio anguillarum* is the causative agent of a fatal hemorrhagic septicemia in salmonid fish. Many serotype O1 strains harbors a 65 Kbp plasmid (pJM1 encoding an iron sequestering system essential for virulence. The genes involved in the biosynthesis of the indigenous siderophore anguibactin are encoded by both the pJM1 plasmid and the chromosome, while those involved in the transport of the ferric-siderophore complex, including the outer membrane receptor, are plasmid-encoded. This work describes the role of specific amino acid residues of the outer membrane receptor FatA in the mechanism of transport of ferric-anguibactin. FatA modeling indicated that this protein has a 22 stranded ß-barrel blocked by the plug domain, the latter being formed by residues 51–154. Deletion of the plug domain resulted in a receptor unable to act as an open channel for the transport of the ferric anguibactin complex.

Keywords Iron transport · Anguibactin · *Vibrio anguillarum* · Receptor · TonB2 · Plug domain

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Introduction

The ability of bacteria to cause disease depends on many parameters that work in concert to establish the pathogen in the vertebrate host. One of these factors is their ability to compete with the host organism for iron. This ion is essential for nearly all living microorganisms. However, in biological fluids it exists only as a complex with iron binding proteins, making it unavailable for bacterial use unless they possess a specific uptake system (Bullen and Griffiths 1999). Thus, invasive microorganisms must depend on their ability to use the complexed iron in order to grow and propagate within the host. Some microorganisms possess outer membrane receptors able to recognize iron-complexes of either transferrin or lactoferrin (McKenna et al. 1988; Nau et al. 1992; Simonson et al. 1982). However, most microorganism obtain iron using siderophores. These small molecular weight, high-affinity iron-binding compounds are divided into three groups distinguished by the chemical structure of the metal-binding functionality (Raymond and Dertz 2004): hydroxamates, such as ferrichrome which was the first siderophore to be identified (Neilands 1952); catechols, such as enterobactin found in *E. coli* and in other members of the Enterobacteriaceae; and hydroxycarboxylates, such as staphyloferrin and rizhoferrin (Pollack and Neilands 1970; Thieken and Winkelmann 1992). It is noteworthy

that several siderophores have been implicated in the virulence of bacteria such as *Yersinia pestis, V. cholerae, V. vulnificus* and *Pseudomonas aeruginosa* (Haag et al. 1993; Lamont et al. 2002; Litwin et al. 1996).

The fish pathogen *V. anguillarum* requires an active iron uptake mechanism mediated by the siderophore anguibactin (Crosa 1980). This peptide siderophore, ω-N-hydroxy- ω-N-[[2′-(2″, 3″-dihydroxyphenyl) thiazolin-4′-yl] carboxyl] histamine, is composed of one molecule of 2,3-dihydroxybenzoic acid (DHBA), one of L-cysteine and one of N-hydroxy-histamine (Jalal et al. 1989). In the analysis of potential virulence factors in *V. anguillarum* a correlation between the presence of the 65 Kbp low-copy number plasmid pJM1, and bacterial virulence was discovered: the strains harboring the plasmid were 10^7 fold more virulent than those in which the pJM1 plasmid was eliminated (Crosa 1980). Later it was found that the secreted anguibactin siderophore is transported into the cell cytosol via a specific transport system encoded on the pJM1 plasmid (Actis et al. 1985, 1988, 1995) (Fig. 1A). This was the first demonstration that a plasmid provides the bacteria with an iron-sequestering system crucial in overcoming nutritional immunity, one of the non-specific defense mechanisms employed by the host.

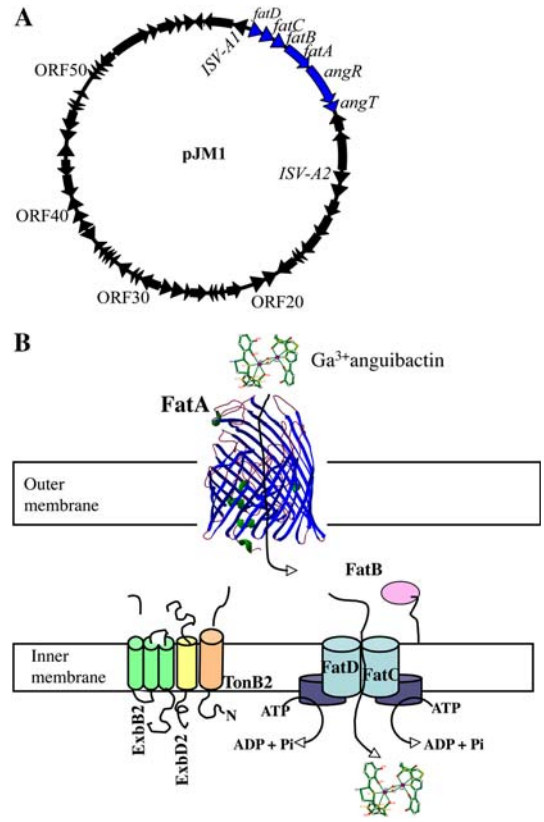

Fig. 1 (**A**) Schematic representation of the *V. anguillarum* 775 pJM1 plasmid and the ITB operon. (**B**) Scheme of the ferric-anguibactin transport components and their localizations in the cellular envelope

Results

Synthesis and transport of the siderophore anguibactin in *V. anguillarum*

Anguibactin is synthesized via a nonribosomal peptide synthetase mechanism with an assembly line organization of different catalytic and carrier protein domains whose placement and function determine the number and sequence of the amino- and carboxylic acids incorporated into the peptide product (Crosa and Walsh 2002). In *V. anguillarum* we have identified several genes encoding nonribosomal peptide synthetases harbored by the pJM1 plasmid such as: *angB/G* (Welch et al. 2000), *angM* (Di Lorenzo et al. 2004), *angN* and *angR* (Wertheimer et al. 1999). Additionally we have also identified the chromosomally encoded *aroC* gene that encodes for the isochorysmate synthetase, essential in DHBA biosynthesis (Chen et al. 1994).

Recently, we identified a novel cluster of genes in the chromosomal DNA of *V. anguillarum* 775 that shows homology to genes harbored by the pJM1 plasmid (Alice et al. 2005). We have also identified a functional chromosomal *angA* gene which codifies the only enzyme with a 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity, since the one present in the plasmid is non-functional due to a frame-shift mutation (Alice et al. 2005).

Interestingly, this chromosomal cluster differs from that of the pJM1 plasmid due to the absence of the repeated sequences found in the plasmid cluster. This finding, in combination with the abundant genes coding for other transposases in the pJM1 plasmid, would suggest that transposition

events might have occurred in the plasmid resulting in genetic rearrangements.

Transport of the ferric-anguibactin complex from the outer membrane to the cytoplasmic milieu is mediated by the iron transport–biosynthesis (ITB) operon encoded in the pJM1 plasmid. The ITB operon encodes the genes involved in this specific transport, *fatDCBA*, as well as two siderophore biosynthetic genes, *angR* and *angT* (Fig. 1A). This operon is also bracketed by the ISV-A1 and ISV-A2 insertion sequences, which are highly related to the insertion sequences found flanking various thermostable direct hemolysin genes in *V. parahaemolyticus, V. mimicus*, and non-O1 serotype *V. cholerae* (Di Lorenzo et al. 2003).

We have demonstrated that in *V. anguillarum*, as it is the case for other vibrio species, there are two TonB systems (TonB1 and TonB2). These systems are redundant with respect to the transport of heme and ferrichrome. However, only TonB2 can transport anguibactin, enterobactin and vanchrobactin, a chromosomally mediated siderophore produced by certain strains of *V. anguillarum* (Stork et al. 2004). This redundant function of the TonB proteins has also been observed in *V. cholerae* (Mey and Payne 2001; Seliger et al. 2001). The *tonB1*, *exbB1*, and *exbD1* cluster in the *V. anguillarum* 775 strain includes the heme transport genes *huvBCD* and, is regulated by the iron concentration in a Fur dependent manner (Mourino et al. 2006). This *tonB1* cluster shows between 48–72% homology to the *V. cholerae tonB1* cluster. The *V. anguillarum tonB2* cluster also shares between 62–87% homology to the *V. cholerae tonB2* cluster and is also regulated by the Fur protein.

Virulence experiments using *V. anguillarum* mutants defective in each system indicated that the *tonB2* mutant strain is severely attenuated in virulence (more than 100-fold) in rainbow trout. However, a *tonB1* mutant strain only showed a 10-fold decrease in virulence (Stork et al. 2004). As expected from these results, the double *tonB1 tonB2* mutant was more attenuated in virulence than the single *tonB2* mutant strain, suggesting that in the latter strain the ability to transport heme through TonB1 was still important for virulence.

Structural similarities of ferric-siderophore outer membrane receptors

To date the crystal structures of several outer membrane receptors are available including the *E. coli* receptors FecA, FepA, FhuA and BtuB, and FpvA and FptA from *Pseudomonas aeruginosa* (Buchanan et al. 1999; Chimento et al. 2003; Cobessi et al. 2005a, b; Ferguson et al. 1998, 2000). In all of the cases, these receptors consist of 22 stranded ß-barrels with a pore of approximately 35–40 Å. The first three-dimensional structure obtained showed that the ß-barrel is blocked by a globular domain, which is known as plug, hatch or cork domain (Chimento et al. 2005). In this manuscript we will use the term plug domain. This domain consists of approximately 150 amino-acid residues structurally organized as a four-stranded mixed β sheet and shows the highest homology within several outer membrane receptors; however, reduced sequence conservation in the ß-strands of the ß-barrel is observed aside from certain residues located in the periplasmic turns (Chimento et al. 2005; van der Helm 2004). No homologies are observed in extracellular loops due to their involvement in specific recognition of different ferric-siderophore complexes.

In the case of FecA, the apo and holo form of the receptor indicated that upon binding of the diferric-dicitrate complex there are structural changes in loops 7 and 8 and minor changes in the loops 4, 5 and 9 (Ferguson et al. 2002). Interestingly, the ligand binding also triggers conformational changes in the plug domain (Ferguson et al. 2002; Yue et al. 2003). Since the latter structural changes are extended to the periplasmic side of the ß-barrel, that they might be responsible for the interaction of the receptor with the TonB protein and they might also regulate the opening of a putative transient channel or permeation path (Shultis et al. 2006; van der Helm 2004; van der Helm et al. 2002). Similar observations were also made for FhuA (Locher et al. 1998). However, despite the existence of crystal structures for many outer membrane receptors, it is still not clear how the ferric-siderophore complexes pass through them.

We do not have as yet the crystal structure of the *V. anguillarum* outer membrane receptor

FatA, and therefore we modeled it based on the FhuA structure (Ferguson et al. 1998; Locher et al. 1998). As is the case for the receptors mentioned above, our model also presents a 22-stranded ß-barrel and a plug domain containing a 4-stranded beta sheet (Fig. 2) (Lopez et al. 2007). As expected the sequence alignment of several receptors indicate that all the conserved amino-acid residues, mostly located in the plug domain, including those of FatA, are located below the binding sites of the different ligands.

Iron transport across the *V. anguillarum* membrane

^{55}Fe-anguibactin transport kinetics (Fig. 3) demonstrate that FatA is the only outer membrane receptor able to transport the indigenous siderophore anguibactin in *V. anguillarum*. FatB is the specific periplasmic binding lipoprotein and FatDC are the cytoplasmic membrane permeases involved in this transport (Fig. 1B) (Actis et al. 1988, 1995). Moreover, we also confirmed that ^{55}Fe-anguibactin transport is only mediated by the TonB2 system. As controls the wild type 775 strain and the *tonB1* mutant are also shown (Fig. 3). We have recently determined the dissociation constant of ferric anguibactin for this receptor and demonstrated that the binding of ^{55}Fe–anguibactin to FatA is unaffected by the absence of TonB2, indicating that the latter protein is not essential for the binding of the ferric-siderophore complex to the receptor (Lopez et al. 2007). Moreover, the absence of the FatA outer membrane protein receptor in *V. anguillarum* caused a significant overproduction of the siderophore anguibactin as expected from a receptor which does not have a N-terminal regulatory domain such as FecA in *E. coli* and FpvA in *P. aeruginosa*.

Excision of the plug domain in FatA

Removal of the plug domain in the *E. coli* ferrichrome receptor FhuA resulted in an outer membrane protein with an open channel (Braun et al. 2002). Cells expressing this truncated receptor show increased sensitivity towards antibiotics (Braun et al. 2002). Similarly, in *Serratia marcescens* expression of the hemophore receptor HasR ß-barrel increases the specific diffusion of heme in a passive way (Letoffe et al. 2005).

Based on sequence analysis and molecular modeling we propose that the putative plug domain of FatA will be included within residues 51–154 of the N-terminal domain. Therefore, to check if the *V. anguillarum* FatA receptor presents characteristics comparable to those of the other receptors we decided to delete this putative plug domain. The western blot analysis indicated that the FatA Δ51–154 mutant receptor is properly located in the outer membrane (Fig. 4A). However, the ^{55}Fe-anguibactin

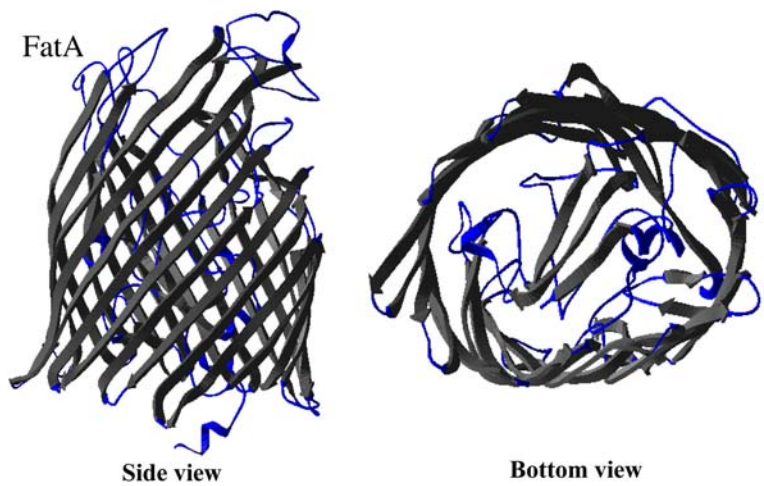

Fig. 2 Molecular modeling of the outer membrane receptor FatA. Representation of the beta barrel model of FatA viewed from one side and form the periplasmic side. For clarity some of the periplasmic loops were removed from the left figure to show the plug domain

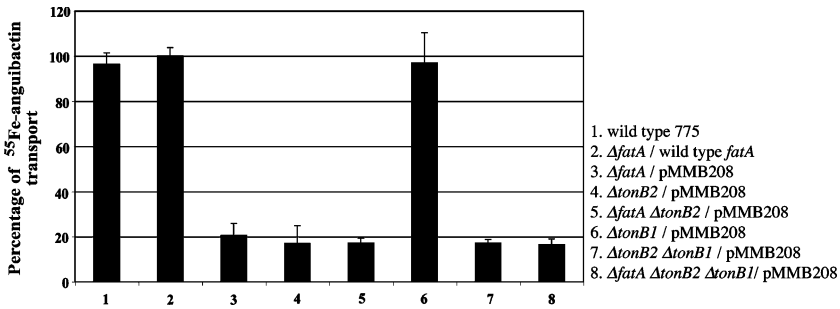

Fig. 3 ^{55}Fe-anguibactin transport in *V. anguillarum* cells. The wild type 775 strain and deletions mutants in the *fatA*, *tonB1* and *tonB2* genes were incubated for 15 min in the presence of 10 nM ^{55}Fe-anguibactin. The complementation with the wild type *fatA* gene was performed using the low copy number plasmid pMMB208. The results are shown as percentage of ^{55}Fe-anguibactin accumulation as compared to the wild type 775 strain. The values represent the mean ± standard deviation of at least three independent experiments

transport kinetics demonstrated that this truncated version of the receptor is not able to act as an open channel for the transport of ferric-anguibactin. A *V. anguillarum* Δ*fatA* Δ*tonB1* Δ*tonB2* mutant, in which the energy transduction to the outer membrane is abolished, was used as control for passive diffusion and obtained similar results (Fig. 4B). These results suggest that the external loops in this truncated receptor are protecting the *β*-barrel from acting as an open channel in the *V. anguillarum* outer membrane.

Identification of the quadrupole region of FatA

It has been described that in the *E. coli* outer membrane receptor FepA that the N-terminal and barrel domains contain a cluster of ten

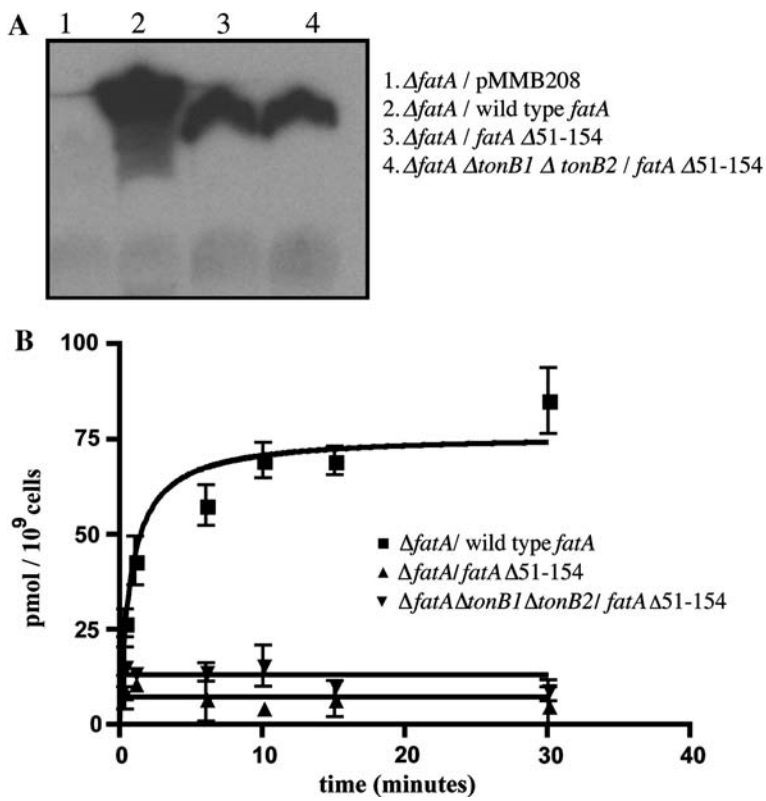

Fig. 4 Characterization of the FatA Δ51–154 mutant. **(A)** Western blot of the isolated outer membrane factions of several *V. anguillarum* mutants. As negative control the Δ*fatA* mutant harboring the empty vector is shown in line 1. **(B)** 10 nM ^{55}Fe-anguibactin transport. The values represent the mean ± standard deviation of at least three independent experiments

residues named the lock region (Chakraborty et al. 2003). Moreover, four of these conserved residues form a quadrupole that consists of the arginine 75 located at the end of strand β3, glutamic acid 511 located in the strand 14 of the β-barrel, glutamic acid 567 in strand 16 of the β-barrel, and arginine 126 located at the end of the strand 5 of the mixed β sheet ((Chakraborty et al. 2003) and Fig. 5). It has also been demonstrated by both structural analysis and biochemical studies, that these specific four residues form hydrogen bonds with each other. Interestingly, these residues are structurally conserved in location and orientation in other *E. coli* siderophore receptors such as FecA (residues R150, R196, E541 and E587) and FhuA (residues R93, R133, E522, E571) (Fig. 5). In addition to these specific residues, other residues also support this cluster: some of them are serine or aspartic acid, proline and phenylalanine or tyrosine. The three dimensional structure of these receptors showed that the conserved residues of the quadrupole form two separate points of contact between the globular domain (plug domain) and the β-barrel. These interactions are important for the correct positioning of the globular domain within the β-barrel.

Biochemical studies performed in the *E. coli* receptor FepA indicated that substitution of residue E567 for glutamine results in a receptor mutant with defects in transport when compared with substitutions in E511 (Chakraborty et al. 2003). However, in the case of the ferrichrome receptor FhuA, substitutions of the conserved glutamates E522 and E571 to alanine did not show any defect in transport (Endriss et al. 2003). Similar results were obtained when the E541 residue of FecA was substituted with alanine. Conversely, a substitution of glutamate E587 in FecA by alanine resulted in reduced transport rates as compared to the wild type FecA (Sauter and Braun 2004). Regarding the other conserved residues it has been observed in the case of FepA, that when the conserved R75 was changed to glutamine the resulting mutant showed a strong defect in transport, suggesting that it must be essential for this region. In contrast substitution of R133 to alanine in the *E. coli* ferrichrome receptor FhuA did not show any effect on transport (Endriss et al. 2003).

Our FatA model, together with the sequence alignments, suggest that the residues belonging to the quadrupole would consist of the charged amino acid residues R95, K130, E505 and E550 (Fig. 6A) (Lopez et al. 2007). To analyze the role of these residues in ferric-anguibactin transport we mutated these residues to alanine and glutamine.

The transport kinetics using ^{55}Fe-anguibactin indicated that the R95 and K130 residues are both essential for ferric-anguibactin transport (Lopez et al. 2007). Interestingly, mutations of these residues to either alanine or glutamine resulted in the absence of transport (Fig. 6B). In the case of the conserved glutamic acid E505 on FatA, when mutated to alanine or glutamine, no defect

Fig. 5 Sequence alignment of sequences harboring part of the lock region of 19 different outer membrane receptor proteins. Conserved residues are shown in bold font

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FepA   IDIRGMGP---ENTLILIDGKPVSSRNSVRQGWRGERDTRGDTSWVPPEMIERIEVLRGP 128
PfeA   IDIRGMGP---ENTLILVDGKPVSSRNSVRYGWRGERDSRGDTNWVPADQVERIEVIRGP 130
BfeA   VDIRGMGP---ENTLILIDGKPVTSRNAVRYGWNGDRDTRGDTNWVPAEEVERIEVIRGP 134
RumA   VGIRGLPARLSPRSTILLDGIPLAAAPYGQPQLSMSPLSLG--------SISSIDVMRGA 126
FecA   FGIRGLNPRLASRSTVLMDGIPVPFAPYGQPQLSLAPVSLG--------NMDAIDVVRGG 198
FpvA   YYARGFSIN-----NFQYDGIPST---------ARNVGYSAGNTLSDMAIYDRVEVLKGA 209
PupB   YWSRGFAIQ-----NYEVDGVPTS---------TRLDNYSQS-----MAMFDRVEIVRGA 209
PupA   IYSRGSAIN-----IYQFDGVTTY---------QDNQTRNMPSTLMDVGLYDRIEIVRGA 210
FhuE   YYSRGFQID-----NYMVDGIPTY---------FESR-WNLGDALSDMALFERVEVVRGA 128
FptA   YYVRGFKVD-----SFELDGVPAL---------LGN----TASSPQDMAIYERVEILRGS 124
PbuA   FYSRGFRMSG----QYQYDGVPLD---------IGSSYVQADSFNSDMAIYDRVEVLRGA 208
FcuA   YRIRGYN----------LDGDDIS---------FGGLFGVLPRQIVSTSMVERVEVFKGA 157
FhuA   LIIRGFAAEG----QSQNNYLNGLK--------LQGN--FYNDAVIDPYMLERAEIMRGP 135
FoxA   VALRGFHG-G----DVNNTFLDGLR--------LLSDGGSYNVLQVDPWFLERIDVIKGP 129
FyuA   ISLRGVSSAQDFYNPAVTLYVDGVP----------QLSTNTIQALTDVQSVELLRGP 114
AleB   QTLRGRGML------VLLDGIPLN----------TNRDSARNLANIDPALVERVEVLRGS 215
IutA   MNVRGRPLV------VLVDGVRLN----------SSRTDSRQLDSIDPFNMHHIEVIFGA 110
ViuA   PTIRGIDGSGPSVGGLASFAGTSPRLNMSIDG-RSLTYSEIAFGPRSLWDMQQVEIYLGP 128
FatA   FKIRGFS----------SDIGDVM---------FNGLYGIAPYYRSSPEMYQRIDVLKGP 132
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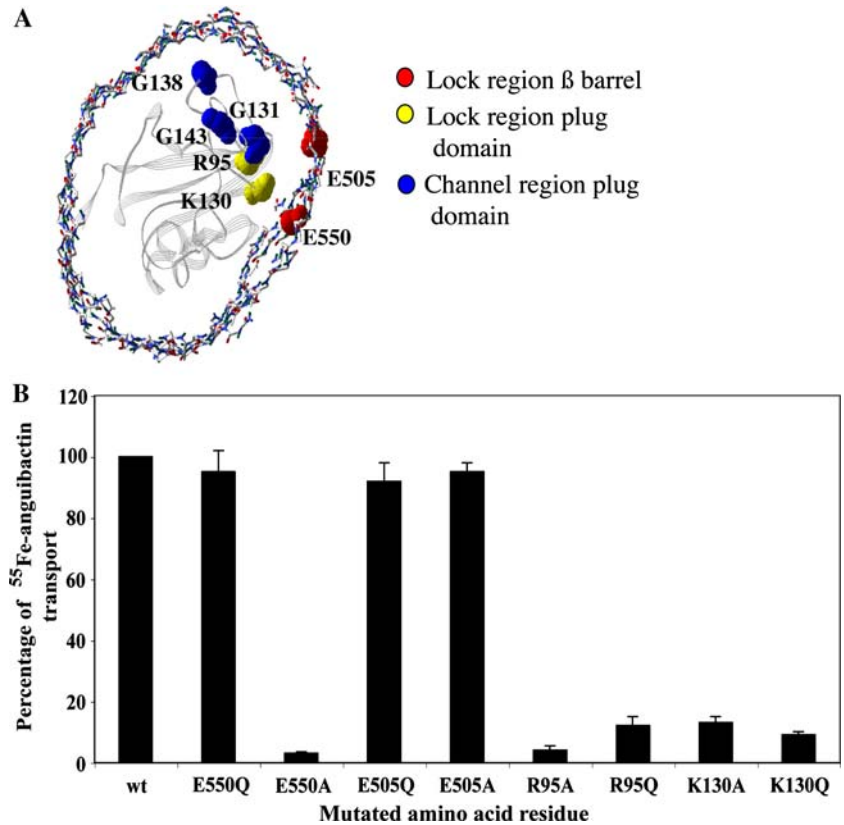

Fig. 6 **(A)** Schematic representation of the FatA beta barrel viewed from the top. The extracellular loops were removed from this scheme. The plug domain is shown with secondary structure. Residues of the lock region and the conserved glycines of the ß5–ß6 loop are also shown. **(B)** Percentage of ^{55}Fe-anguibactin transport in the wild type and site directed mutants of the lock region. The values represent the mean ± standard deviation of at least three independent experiments

in transport was observed, suggesting that this residue is not as essential as those mentioned above. However, mutation of residue E550 supports its essential role during ferric-anguibactin transport (Fig. 6B) (Lopez et al. 2007). Thus substitutions of glutamic acids with glutamines would be permissive for the formation of hydrogen bonds. It is therefore possible that these hydrogen bonds are replacing the salt bridges present in the wild type FatA receptor. Nevertheless, a substitution of these residues by an alanine, would not result in the formation of hydrogen bonds.

The biochemical results obtained for the *V. anguillarum* FatA receptor together with those obtained with the *E. coli* receptors FepA, FhuA and FecA suggest that the quadrupole region of outer membrane receptors have differences regarding their role during the transport process. While some of the results imply that the transport mechanism is comparable for both *V. anguillarum* and *E. coli* receptors, others suggest that only some of the residues of the quadrupole region are essential for the transport of a specific ferric-siderophore complex. As suggested by Shultis et al. (Shultis et al. 2006), these differences might be related not only to the chemical properties of the different ferric-siderophore complexes but also to their molecular size.

Is a transient channel created during ferric-siderophore transport?

As mentioned above, several studies have been performed to elucidate the mechanism of transport in ferric-siderophore outer membrane receptors. However, it is still a matter of controversy how the ferric-siderophore complexes are being transported through the lumen of these receptors since the N-terminal plug domain is located within the lumen of the receptors. To date two theories are being evaluated. In one of them it has been postulated that the plug domain should remain within the beta-barrel. Therefore, during ferric siderophore transport the plug is rearranged due to allosteric transitions in such a

way that resulted in the opening of a channel or permeation pathway through which the complex permeates into the periplasm (Ferguson et al. 2002). On the other hand it has also been postulated that the plug domain should be completely removed from the barrel during the passage of the ferric-siderophore complex. Recent reports concerning the three dimensional structure of the *E. coli* TonB protein in complex with the cobalamine receptor BtuB (Shultis et al. 2006) and the ferrichrome receptor FhuA (Pawelek et al. 2006) suggest that a conformational change or localized unfolding of the plug should happen to open a permeation path. There are two studies in FhuA that would indicate that a partial unfolding of the plug during transport through outer membrane receptors. These studies were performed using ^{55}Fe-ferrichrome transport kinetics (Endriss et al. 2003) as well as using a ferrichrome analogue ^{55}Fe-ferricrocin transports together with cross-linking assays of engineered cysteines (Eisenhauer et al. 2005). In both publications the authors suggest that fixation of the plug domain to the beta-barrel prevents the allosteric movements of the globular domain and therefore this domain is not displaced from the beta-barrel while the ferric-siderophore complex is transported.

In the case of the *E. coli* enterobactin receptor FepA it was suggested that some conserved glycines located within the ß5–ß6 loop of the plug domain play an important structural role, which may be crucial for the formation of the transient channel previously mentioned (G127 i and G134 i + 7) (Chakraborty et al. 2003). These conclusions were based on biochemical results obtained by using ^{55}Fe-enterobactin transport kinetics. A double G127A/G134A mutant shows a 12-fold increase in the K_M value without affecting binding suggesting that a hinge motion around these two glycines is important for the formation of the transient channel (Chakraborty et al. 2003). On the other hand, and more recently, Ma et al (2007) demonstrated in FepA by determining the susceptibility of engineered cysteine residues to modification by fluorescein maleimide during Fe-enterobactin transport, that the transport mechanism would be more consistent with a model in which the plug domain is removed from the beta-barrel. In the latter case it was speculated that the fluorophore used in the assays might not be fully accessible to interact with some of the engineered cysteines due to its size (427 Da) or as suggested from the same authors, an unexpected conformational change or steric factors within the barrel might prevent the interaction of specific cysteine residues with the fluorescein maleimide. Whether the differences observed concerning the mechanism of transport through FepA are due to the type of techniques used in the different studies remains to be answered. The removal of the plug domain during the TonB-dependent transport is unlikely to occur because of the large amount of intramolecular polar contacts with the beta-barrel, according with Faraldo-Gómez et al. (2003); however, the extensive solvation of the barrel-plug interface might offer a solution to this problem.

In the case of FatA we propose that a conformational change within the beta-barrel should occur during transport to facilitate the opening of a transient channel. Therefore, we decided to study several site directed mutants in which specific glycine residues located in the ß5–ß6 loop of the plug domain of FatA (G131 i, G138 i + 7 and G143) were mutated to alanine ((Lopez et al. 2007) and Fig. 6A). If these glycine residues are important during ferric-anguibactin transport, an alanine substitution would make this structural rearrangement more difficult or even impossible. To test our hypothesis, single, double and triple mutants were constructed to examine the contribution of each residue to ferric-anguibactin transport. Single mutations in the conserved glycines of FatA did not affect transport. Furthermore, as shown in Fig. 7 double mutations either did not affect transport (G131A/G138A and G138A/G143A) or abolished it (G131A/G143A). As expected, the triple mutant also showed a defect in transport. From our point of view, these biochemical results on FatA are consistent with the model in which the conserved glycines located in the *β5-β6* loop allowed the rotation of these strands to form a transient channel. Moreover, since a glycine to alanine mutation will only result in small local changes in the structure it is unlikely that the mutation could obstruct the removal of the plug domain. How-

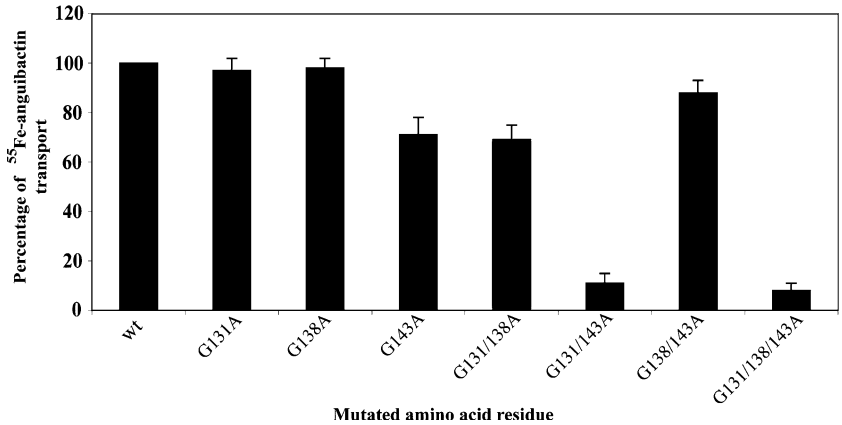

Fig. 7 Percentage of ^{55}Fe-anguibactin transport in the wild type and site directed mutants of the conserved glycines. The values represent the mean ± standard deviation of at least three independent experiments

ever, whether a partial removal of the plug occurs during ferric-anguibactin transport, remains to be elucidated.

Conclusion.

Based on sequence alignments and structural homologies we have demonstrated the existence of highly conserved amino-acid residues of FatA as compared with other outer membrane receptors, being some of them essential for ferric-anguibactin transport such as R95, K130 and E550. Among these residues, we established that they form two separate points of contact between the plug domain (R95 and K130) and the beta barrel (E505 and E550). Interestingly, although they are structurally conserved in location and orientation as compared to the *E. coli* receptors FepA, FecA, FhuA and BtuB, their importance is dissimilar possible due to the type of siderophore being transported.

Regarding the mechanism of transport, two different theories explain the passage of the ferric-siderophore complexes through the outer membrane receptors. Both theories are in agreement with various biochemical data obtained using site directed mutagenesis in FepA, FecA and FhuA. In the case of FatA, the biochemical data obtained with site directed mutations of conserved glycines residues located in the $\beta5$–$\beta6$ loop of the plug domain, suggest the existence of a conformational change or partial unfolding of the plug during ferric-anguibactin transport. It remains to be investigated whether the differences concerning the mechanism of ferric-siderophore transport observed in the studies performed with several other outer membrane receptors are due to the size and/or chemical properties of each ferric-siderophore complex.

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