REGULATION OF EXPRESSION OF THE IRON TRANSPORT SYSTEM IN *VIBRIO ANGUILLARUM*

BY

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Dost dream of things beyond the moon,
And dost thou hope to dwell there soon?
Hast treasures there laid up in store
That all in th' world thou count'st but poor?
Art fancy sick, or turned a sot
To catch at shadows which are not?

Anne Bradstreet

To those who dare to follow their dreams.

Lives of great men all remind us
We can make our lives sublime,
And departing, leave behind us
Footprints on the sands of time;
Footprints, that perhaps another,
Sailing o'er life's solemn main,
A forlorn and shipwrecked brother,
Seeing, shall take heart again.

Henry Wadsworth Longfellow

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ABSTRACT

Vibrio anguillarum 775 outer membrane protein, FatA, the receptor for ferric-anguibactin is encoded by the virulence plasmid, pJM1, and is expressed only under iron-limiting conditions. The objective of this research is therefore: to elucidate the mechanism(s) whereby iron regulates the expression of the iron transport proteins, particularly the FatA protein, and to characterize molecularly, the iron transport region.

Transposition insertion mutants in any of the three genes upstream of the *fatA* gene, results in extremely low synthesis of FatA. Complementation studies of these mutants, located an antisense RNA (RNAα) in the *fatB* region, a region which also encodes a 40 KDa protein that is essential for iron transport. Primer extension analysis detected *fatA* mRNA species initiated from sites downstream of the start sites for RNAα as well as species with start sites located far upstream of the *fatA* translation initiation codon. The presence of these larger *fatA* RNA species and the dramatic decrease of FatA protein synthesis in the insertion mutants lend credence to the existence of a polycistronic message that encompasses the *fatA* mRNA.

RNase protection and Northern blot studies showed that the *fatA* mRNA level was only slightly reduced in the presence of RNAα, whereas the FatA protein synthesis was tremendousy reduced. The regulation of RNAα on *fatA* is therefore most likely to be at the translation level. One of the possible mechanisms may be occlusion of the ribosome binding site as result of RNAα interacting with a large

fatA transcript. In addition, RNAα may speed up the processing of the large *fatA* mRNA or the polycistronic message. Another possibility is that *fatB* may encode a positive regulator that is necessary for the translation of the *fatA* message. The antisense RNA may affect the level of FatB, and thus causes a decrease in the level of the FatA protein.

The presence of RNAα is concomitant with a decrease in the level of the *fatB* transcript. Rifampicin studies showed that initially, there was a high level of RNAα and thus *fatB* mRNA was found to be low. Later, when the RNAα level was low, there appeared to be an induction of the *fatB* mRNA, due to elongation. The action of RNAα is therefore most likely to be destabilization of the *fatB* message.

The antisense RNA is probably not the major regulatory mechanism. A Fur repressor protein is known to regulate iron transport genes in *E. coli*, and a *V. anguillarum* fur-like gene was cloned in our laboratory. The *E. coli* Fur was demonstrated to negatively regulate the transcription of *fatA*, though with low efficiency. However, the FatA protein synthesis in *E. coli* was iron regulated, and occurred independently of the presence or absence of the *E. coli* Fur. The regulation in this case was probably by RNAα, which takes over when the repressor is ineffective. However, in *V. anguillarum*, *fatA* mRNA is not synthesized under iron-rich conditions, demonstrating the effectiveness of the repressor. In this case, RNAα may shoulder less of the regulatory function.

INTRODUCTION

PART I: THE ROLE OF IRON IN INFECTIONS

Iron is an essential nutrient for all living organisms, humans or microbes. It is involved in many metabolic processes as well as in RNA and DNA synthesis. It is an important component of the electron transport chain, and many proteins require its presence for their biological activities (Barclay, 1985; Crichton et al., 1987). Many of the processes involve a change in the redox state of of the iron: Fe(II) <----> Fe(III) + e^- . Although iron is the fourth most abundant element on the earth's crust, it is not readily available to all the organisms because in an aerobic system and at the host's physiologic pH, iron exists as Fe(III), which is virtually insoluble. In physiologic conditions, Fe(III) is precipitated from solution as polymerized ferric hydroxides and oxyhydroxides and thus renders iron impossible to be assimilated (Barclay, 1985; Crichton et al., 1987). Living organisms therefore have to elaborate a series of molecules to obtain iron from the environment. In multicellular organisms such as humans, iron is absorbed in the the upper portion of the small intestine. Once internalized, iron is bound by lactoferrin in secretions and by transferrin in the blood stream, whereby it is transported to the different cell types which take up the iron by specific receptors, such as transferrin receptor. Lactoferrin is normally highly unsaturated, to permit the protein to function as an iron-withholding rather than an iron-transporting agent. Transferrin on the other hand, has the above dual functions, and therefore its mean saturation value can be as high as 69% to as low as 22%

(Weinberg, 1984). Most of the iron is within red cells as hemoglobin and the remainder is stored within the liver and spleen as ferritin in the cytoplasm and as hemosiderin in secondary lysosomes. Trace amount is made available for metabolic enzymes or in transport forms, bound to transferrin. Only 10^{-18} M exists as free iron in physiologic fluids (Bagg et al., 1987). Apart from the limitation imposed by solubility, there is a very good reason why free iron is maintained at a trace level. The reason is that, surplus iron has a propensity to catalytically promote hydroxyl radical production from superoxide and peroxide. The reaction can be represented by these equations:

$$O_2\cdot^- + Fe(III) = O_2 + Fe(II)$$

$$2O_2\cdot^- + 2H^+ = O_2 + H_2O_2$$

$$Fe(II) + H_2O_2 = Fe(III) + OH^- + OH\cdot$$

The presence of these hydroxyl radicals are detrimental to the cell. In fact, this system is employed by macrophages in the killing of bacteria (Crichton et al., 1987).

Apart from utilizing iron in the killing of bacteria, macrophages also help to create a state of hypoferremia as a mechanism of combatting infections. In normal conditions, decaying erythrocytes are phagocytized by macrophages of the reticuloendothelial system. Iron derived from the catabolism of these effete cells, combines with apoferritin which is the translation repressor of ferritin messages. The binding of iron to apoferritin derepresses the translation inhibition system, and more ferritin is synthesized. The newly synthesized ferritin normally releases its iron to apoferritin in the blood stream. However, during an infection, the inflammatory process is

accompanied by the release of leukocyte endogenous mediator, which blocks the release of iron from the macrophages. The iron level in plasma is thus decreased, and accumulates in the liver and spleen(Weinberg, 1984). The mechanism of iron withholding by macrophages or by the high affinity iron binding proteins lactoferrin and transferrin, has been demonstrated to be a very effective defence for many bacterial infections (Crichton, 1987; Payne et al., 1878; Robins-Browne et al., 1985).

PART II: IRON ACQUISITION BY BACTERIA

Just as the host requires iron for cellular functions, this metal is equally as important for the potential pathogen. Within the host's body, the concentration of available free iron is too low to support microbial growth, it is therefore of absolute necessity for the microorganism to evolve a system to capture iron from the host's proteins. Bacteria and fungi have developed low-molecular weight carriers, siderophores, with extremely high affinity and specificity for Fe(III). The Fe(III)-siderophore complexes are then internalized via specific receptors in the cell envelope, and are transported across the microbial membranes via a series of transport proteins. In addition, there is an enzymatic system for releasing the iron from the Fe(III)-siderophore complex(Bagg et al., 1987). Siderophore based iron acquisition systems have been demonstrated to be important virulence factors (Wolf et al., 1986; Payne et al., 1978; Crosa, 1980). Various

siderophore-mediated iron uptake systems have been well characterized, and will be discussed in detail in subsequent sections.

MECHANISMS NOT MEDIATED BY SIDEROPHORES

Some organisms do not have a siderophore system. In organisms such as *Legionella*, *Neisseria* and *Saccharomyces cerevisiae*, specific receptor proteins are elaborated specifically for lactoferrin or for transferrin (Neilands et al., 1987). *Neisseria gonorrhoeae* and *Neisseria meningitidis* produce iron regulated lactoferrin-receptors and transferrin-receptors in their cell membrane. Despite the similarity of the two iron-binding proteins, the reactivity between the receptors and the ligands are highly specific (Schryvers et al., 1988a; Schryvers et al., 1988b). The biochemical mechanism of release of iron from transferrin or lactoferrin is still unknown, except that after receptor binding, the transferrin and lactoferrin are not internalized. Iron is removed from the proteins, and the apotransferrin and deferrated lactoferrin are released (McKenna et al.,1988; Tsai et al., 1988).

Another mechanism by means of which organisms acquire iron is by destruction of host tissues or proteins that contain stores of iron. Red cells are a good source of iron. Some bacteria produce toxins or hemolytic enzymes to induce tissue damage. Some bacteria produce proteases that degrade transferrin and thus are able to utilize the iron that is released from the iron-transferrin complex. *Vibrio anguillarum* was shown to be able to utilize heme, hemoglobin and haptoglobin-

hemoglobin complexes as sources of iron (Mazoy et al., 1991). However, only strains that possess the siderophore system can cause infection. The siderophore system is therefore crucial for the first stage of infection. After the establishment of infection, the production of lytic enzymes or toxins, and the utilization of macromolecules such as hemoglobin as a source of iron, would augment bacterial multiplication.

Iron can also be acquired via non-specific molecules such as the ion chelators: citrate or hydroxyamino acids. The ability to utilize iron from ferric citrate has been well documented in *E. coli*, and may prove to be of importance to the pathogens, since citrate is a common metabolic intermediate in most cells (Barclay, 1985). The system consists of an outer membrane receptor protein for ferric citrate, a periplasmic protein for transport across the membranes, two very hydrophobic cytoplasmic membrane proteins for transport into the cytosol, and a hydrophilic cytoplasmic membrane protein that is the putative ATP-binding protein that provides the energy that drives the system (Presler et al., 1988; Staudenmaier et al., 1989). The system is negatively regulated by the *E. coli* repressor Fur, and is induced by ferric citrate (Braun et al., 1997). The ferric citrate system is TonB dependent. The TonB protein is located in the cytoplasmic membrane but is exposed to the periplasm, where in conjunction with the proton motive force, it may function to dislodge the transported substrate from the outer membrane receptor (Neilands, 1982).

In addition to the above mentioned mechanisms, intracellular microorganisms may acquire iron in the lysosomes in cells such as macrophages. After the release of lysosomal contents, the pH of the

phago-lysosome is low. The solubility of iron increases as pH decrease, and thus more free iron is available for the microorganism (Barclay, 1985).

PART III: SIDEROPHORE-MEDIATED IRON UPTAKE SYSTEMS

The siderophore system of iron aquisition has been evolved widely in the microbial world. It is found in Gram-positive bacteria and in Gram-negative bacteria, especially in the enteric organisms. It has been reported in fungi, in animal and plant pathogens as well as in free-living nitrogen-fixing bacteria. The single most salient feature of siderophores is their extremely high affinity for the ferric iron. The association constants for iron of enterobactin, from *E. coli* and other enteric bacteria, is reported to be 10^{52}, which is the highest value ever reported for any ferric ion-binding compound. Agrobactin from *Agrobacterium tumefaciens* has an equally high association constant. In contrast, the constant for aerobactin, also produced by enteric bacteria, is of a relatively low value of 10^{23}, when compared with the value of 10^{36} for transferrin (Neilands, 1981).

Although siderophores all bind a common ligand, the ferric ion, there is a considerable diversity in their structure. Siderophores are classified as either hydroxamates or phenolate-catecholates which are derivatives of 2,3-dihydroxybenzoic acid.

ENTEROBACTIN

The prototype of the catechol-phenolate type of siderophore is enterobactin. It is a cyclic trimer of 2,3-dihydroxy-N-benoyl-L-serine that are coordinated to bind one molecule of iron (Earhart, 1987; Bagg et al., 1987).

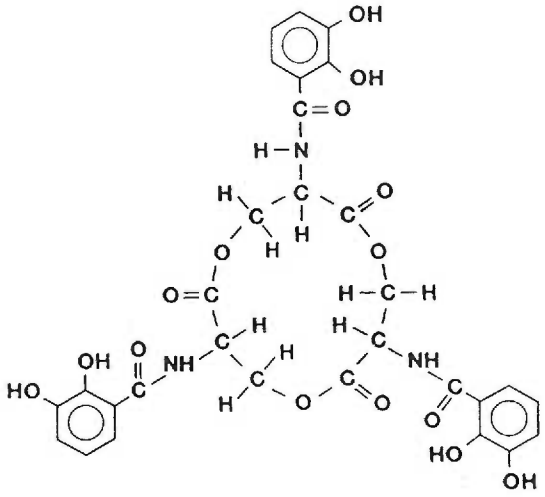

Fig. 1. Structure of enterobactin (Earhart, 1987)

The ferrienterobactin system is encoded by 11 genes located 13 min on the *E. coli* chromosome. There are 7 genes (*ent*A, *ent*B, *ent*C, *ent*D, *ent*E, *ent*F, *ent*G) involved in the biosynthesis of enterobactin (Liu et al., 1989; Nahlik et al., 1989; Ozenberger et al., 1989; Armstrong et al., 1989; Coderre et al., 1989), and 3 genes (*fep*A, *fep*B, *fep*C) encode the ferrienterobactin transport proteins (Lundrigan et al., 1986; Elkins et al., 1989; Pierce et al., 1986). The *fes* gene specifies the enzyme ferrienterobactin esterase that is necessary for the release of the siderophore-bound iron (Pettis et al., 1988).

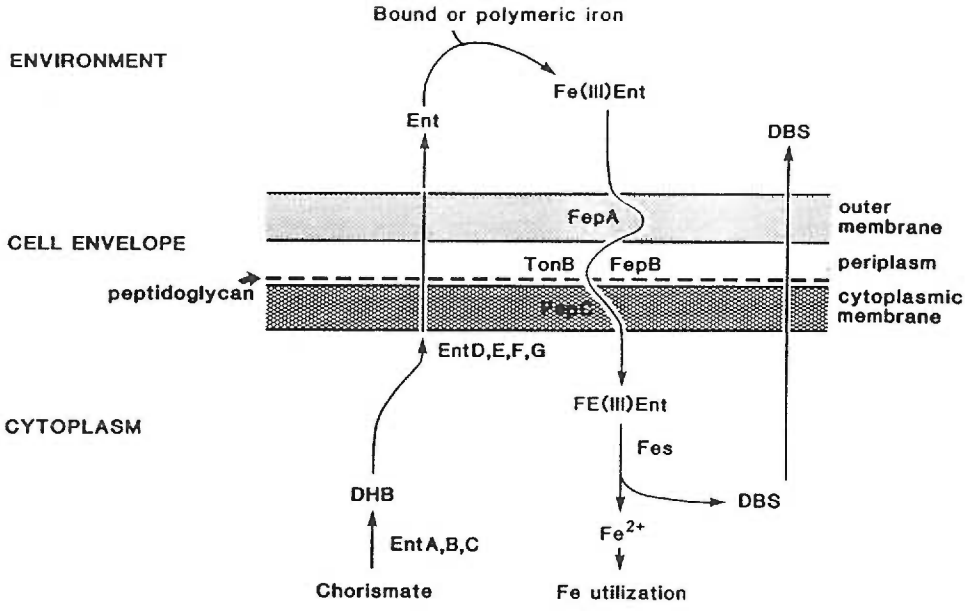

Fig. 2. The ferrienterobactin uptake system. Names and probable locations of proteins known to be necessary for enterobactin synthesis, transport and breakdown are shown. DHB, 2,3-dihydroxybenzoic acid; DBS, 2,3-dihydroxy-*N*-benzoyl-L-serine. (Earhart, 1987)

After the ferrienterobactin is transported inside the cell, the complex is hydrolysed by the esterase, and iron is released from the complex (O'Brien et al., 1971). Enterobactin is acetylated and is destroyed and excreted outside of the bacterial cell (Hartmann et al., 1980).

AEROBACTIN

The prototype of the hydroxamate form of siderophore is aerobactin, which is also encoded by the plasmid pCOLV-K30 in *E. coli*. The aerobactin operon of pCOLV-K30 is a cluster of 5 genes. Four genes (*iucA*, *iucB*, *iucC*, *iucD*) are necessary for the biosynthesis of aerobactin, and only one gene (*iutA*) is required for its transport (Neilands et al., 1987; de Lorenzo et al., 1986; de Lorenzo and Neilands, 1986; Carbonetti et al., 1984).

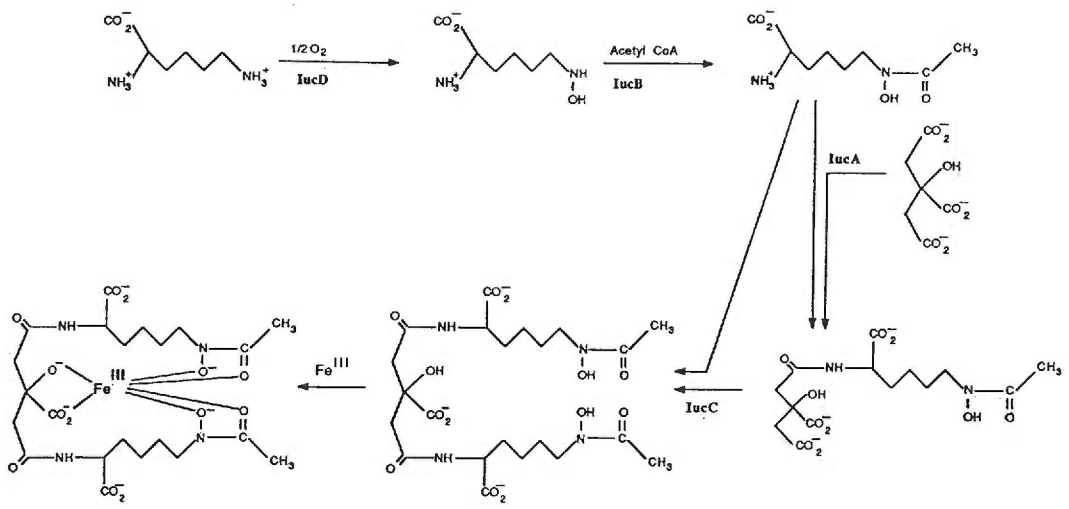

Fig. 3. Biosynthetic pathway from lysine and citrate to aerobactin. (Bagg et al., 1987)

After ferriaerobactin is internalized, the ferric iron is reduced to Fe(II). Since the siderophore has little affinity for Fe(II), iron is released from the complex. Unlike enterobactin, aerobactin is not destroyed. It is excreted into the medium and is reused (Crichton et al., 1987). This release mechanism appears to be more often employed by other siderophore-mediated iron uptake systems (Crosa, 1989)

REGULATION OF IRON UPTAKE SYSTEMS IN *E. COLI*.

The synthesis of the iron uptake proteins is observed to be iron regulated. The existence of a gene that is responsible for regulating the expression of components of enterobactin and ferrichrome uptake systems in *E. coli*, was first observed by the constitutive expression of these proteins in mutants, which were named *fur* mutants (Ernst, 1978). The term *fur* is for ferric uptake regulation. Subsequently, the repressor gene *fur* for the E. coli iron uptake systems was cloned and sequenced (Hantke, 1984; Schaffer et al., 1985). The gene encodes a 17 kDa protein which acts as a dimer, and behaves like a classic repressor in the presence of Fe(II) or other divalent cation such as Mn(II), blocking transcription of genes in the iron uptake systems as well as for toxins and hemolysin (Bagg et al., 1987; de Lorenzo, Giovannini et al., 1988; Coy et al., 1991; Stoebner et al., 1988; Calderwood et al., 1987). The consensus operator sequence is the punctated palindrome 5'-GATAATGATAATCATTATC-3' with the core sequence being 5'-TCATT-3' (de Lorenzo et al., 1987). The DNA

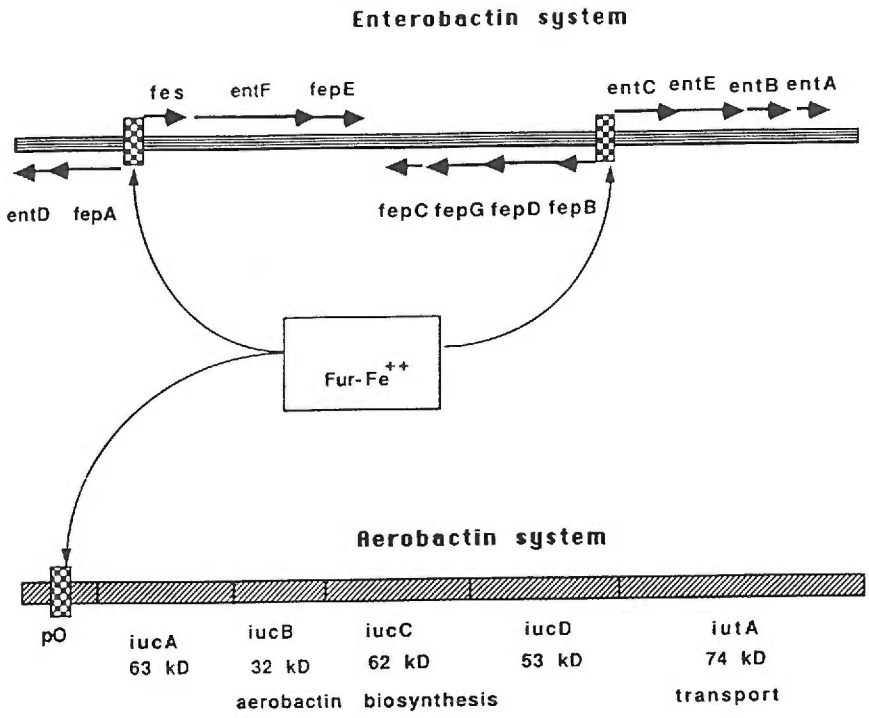

Fig. 4. Sites of Fur-Fe(II) action on the aerobactin and enterobactin systems. (Crosa, 1989)

recognition site for Fur is called "the Fur box". Iron regulated genes such as *fep*A and the promoter region of the aerobactin operon, have been demonstrated to have such a "Fur box" (de Lorenzo et al., 1987). Footprint experiments revealed that Fur also binds to the promoter region of its own gene, and is thus autoregulated (de Lorenzo et al., 1988). In addition, the catabolite-activator protein also modulates its transcription (de Lorenzo, Giovannini et al., 1988; de Lorenzo, Herrero et al., 1988). The dual control of *fur* is of importance. The autoregulation may be a mechanism to maintain the amount of intracellar Fur below a certain level. The cAMP-catabolite-activator protein regulation reflects a close correlation between the modulation

of iron absorption and the metabolic state of the organism. Recently, *fur*-like genes have been identified in other bacteria, such as *Yersinia pestis*, *Vibrio cholerae* and *Vibrio anguillarum* 775 (Staggs et al., 1991; Goldberg et al., 1990; Waldbeser, Tolmasky, Actis and Crosa, manuscript in preparation).

PART IV: IRON UPTAKE SYSTEM OF *VIBRIO ANGUILLARUM*

V. anguillarum 775 is a marine pathogen that causes hemorrhagic septicemia in salmonids, and therefore can result in serious losses among cultured fish in many parts of the world. Apart from its economic importance, our laboratory is interested in this organism because it is a natural pathogen of the fish host. The observations would therefore provide us with an accurate assessment of the host-pathogen interaction. The focus of the laboratory is on the iron-uptake systems of *V. anguillarum*. As previously mentioned, the ability to acquire iron was shown to be an important virulence factor of this marine pathogen. Two types of *V. anguillarum* iron-uptake systems have been studied. One system is mediated by the chromosome, and the other is mediated by a plasmid.

CHROMOSOME-MEDIATED IRON-UPTAKE SYSTEM OF *V. ANGUILLARUM*

The pathogenic strains of *V. anguillarum* that infect striped bass in Chesapeake Bay water, do not possess plasmids. They were shown to be able to grow under conditions of iron limitation, therefore their iron-uptake sytem was encoded by the chromosome (Toranzo et al., 1983). SDS-PAGE analysis showed that at least three novel proteins, 80 kDa, 75 kDa and 73 kDa, were induced during growth under iron-limiting conditions. Chemical analysis showed that these plasmidless strains produce a siderophore. This siderophore has very high iron binding capability, conferring upon these strains the ability to grown in iron limitations at a minimal inhibitory concentration (MIC) of the iron chelator, EDDA, three time as that of strains possessing a plasmid-mediated iron uptake system. Cell-free supernatant from these strain were able to cross feed *Salmonella typhimurium* mutants that were defective in enterobactin biosynthesis, indicated that the siderophore from these strains is functionally related to enterobactin (Lemos et al., 1988), which hitherto has only been reported in members of the family *Enterobacteriaceae* (Griffiths, 1987). DNA hybridization experiments showed that there was no homology between the enterbactin- mediated iron uptake system of *E. coli* and the plasmidless virulent *V. anguillarum* system. In addition, there was no DNA homology between the chromosome encoded siderophore system and the plasmid encoded system, and yet, supernatant from these virulent plasmid strains were able to cross feed*V. anguillarum* 775 mutants that were defective in either receptor or in plasmid

mediated siderophore synthesis (Lemos et al., 1988). At the moment, the nature of this chromosome-mediated siderophore is still an enigma. Cloning of the gene and purification of the siderophore will be necessary to determine its relationship to enterobactin.

PLASMID-MEDIATED IRON UPTAKE SYSTEM OF *V. ANGUILLARUM*

The iron uptake system of *V. anguillarum* 775 is encoded by the virulence plasmid pJM1. It was demonstrated that strains harboring the 65 kb pJM1 plasmid, or a recombinant plasmid containing the pJM1 iron uptake region, were more virulent in establishing infections in fish (Crosa, 1980; Crosa et al., 1980; Tolmasky et al., 1984). The system has two components: the extracellular siderophore anguibactin which captures the iron, and the transport component, which is made up of at least four proteins, FatA, FatB, FatC and FatD.

The siderophore anguibactin has recently been purified and its composition and structure have been identified. It has a molecular weight of 348, and it belongs to the phenolate-catecholate category of siderophores. As well as possessing a hydroxamate (N-O group) (Actis et al., 1986) X-ray diffraction studies of its anhydro derivative, proton and ^{13}C nuclear magnetic resonance spectroscopy of its deferri and Ga(III) complex, fast-atomic bombardment (FAB) mass spectrometry and chemical degradation were performed, and the compound was identified as: ω-*N*-hydroxy-ω-*N*-[[2'-(2",3"-dihydroxyphenyl)thiazolin-4'-yl]carboxy]histamine (Jalal et al., 1989).

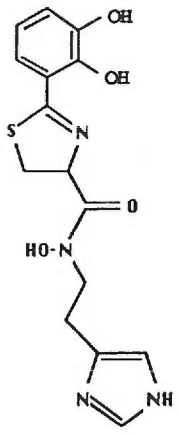

Fig. 5. Structure of anguibactin (Crosa, J. H.,

Single crystal structure determination of the Ga(III) complex of racemized anguibactin showed a 1:1 metal-ligand stoichiometry in which the *O*-hydroxy group, the nitrogen of the thiazoline ring, the hydroxamate (N-) group and the deprotonated nitrogen of the imidazole ring coordinate the metal ion (Jalal et al., 1989).

The iron uptake region of pJM1 has been cloned (Tolmasky et al., 1984). Transposition mutagenesis of the cloned region identified six genetic units: genetic units I, IV, V and VI are necessary for the biosynthesis of anguibactin. Genetic region II contains the genetic determinants for the transport proteins, and genetic region III is a region that has regulatory functions. In addition, there is a noncontiguous region that also regulates genes of the iron uptake system (Tolmasky et al., 1988). Little is known of the anguibactin biosynthetic pathway or the genes involved in the synthesis. However, the transport region (genetic region II) has been subcloned and sequenced (Actis et al., 1988; Koster et al., 1991).

The iron transport region consists of four genes, *fatD*, *fatC*, *fatB* , *fatA*, in order of direction of transcription. All four gene products are essential for the transport of iron (Actis et al., 1988; Koster et al., 1991). *fatA* encodes an 86 kDa protein (Crosa et al., 1981). Membrane iodination of total cell envelop and outer membrane, and papain treatment and iodination of whole cell, localized the FatA protein to the outer membane and being exposed to the extracellular matrix (Actis et al., 1985), where it possibly functions as a receptor for the ferri-anguibactin complex. *fatB* encodes a 40 kDa protein (Actis et al., 1988; Koster et al., 1991). By using the maxicell system, which is an *in vivo* coupled transcription-translation system, a protein of 37 kDa was identified as the gene product of *fatC*, and a 35 kDa protein was attributable to the gene *fatD* (Paper 1.). The cellular location of FatB, FatC and FatD is still to be determined. Analysis of the nucleotide sequence revealed that FatA has a region called "The TonB box", where the polypeptide interacts with the TonB protein (Koster et al., 1991). TonB is a 36 kDa hydrophilic cytoplasmic membrane protein (Postle et al., 1983), which is exposed to the periplasmic space., where together with the proton motive force, it dislodges the ligand from the outer membrane (Earhart, 1987; Braun, 1990). Analysis of the nucleotide sequence also showed that besides FatA, the other three proteins of the transport system, have various domains that are homologous to proteins of several TonB dependent iron and vitamin B^{12} transport system (Koster, 1991). Based on the homologies, FatB is expected to be a periplasmic protein, and FatC and FatD are extensively hydrophobic, cytoplasmic membrane proteins. The transport system seems to be energy dependent. It was demonstrated

that 2 mM KCN greatly inhibited the iron uptake process (Crosa et al., 1981).

POSITIVE REGULATORS FOR THE ANGUIBACTIN IRON UPTAKE SYSTEM

Existence of positive regulators was first discovered, when a recombinant plasmid containing the pJM1 iron uptake region was observed to have a twenty times higher MIC when it was present in combination with a clone containing sequences from a noncontiguous region of the pJM1 plasmid (Tolmasky et al., 1984). This region was further subcloned within a fragment of 15 kb, and the genetic determinant(s) within this fragment that conferred enhancing activities was referred to as Taf, for *trans*-acting factor. Using the *lacZ* gene that is part of the Tn*3*::HoHo1 insertion in mutants located in genetic unit II, the region that encodes the iron transport proteins, Taf was showed to increase the expression of the *lacZ* gene. In addition, the presence of the clone containing the Taf region, increased the production of anguibactin twenty fold in a *V. anguillarum* strain that was cured of the wild type plasmid and was now harboring a recombinant plasmid that contained the pJM1 iron uptake region (Tolmasky et al., 1988).

In addition to Taf, another trans-acting regulator, AngR has been identified. AngR is a 110 kDa protein that functions synergistically with Taf in the transcription activation of anguibactin biosynthetic genes (Salinas et al., 1989). The *angR* gene is 3.6 kbp, and is located immediately downstream of the gene encoding the outer membrane receptor for ferrianguibactin, *fatA*. The *angR* gene has been cloned and

sequenced. Analysis of the nucleotide sequence revealed a helix-turn-helix motif that show striking homology with other prokaryotic DNA-binding proteins, particularly the λ and P22 Cro protein (Farrell et al., 1990). A "Fur box" is located at the -35, -10 region, suggesting that AngR may be a DNA-binding protein which modulates Fe(II) regulated transcription and is itself regulated by Fe(II). Recently, AngR was found to have homology to members of the firefly luciferase family, with motif that share several characteristics with the phosphate-binding sites of phosphoproteins and nucleotide-binding proteins (Toh, 1991). The full expression of angR is in iron-limitimg conditions, and requires a *cis* region of 2.9 kbp upstream that map within the FatA coding region (Salinas et al., 1989).

NEGATIVE REGULATION OF THE ANGUIBACTIN IRON-UPTAKE SYSTEM

Much of the genetic studies were done after the iron uptake region was cloned and Tn*3*::HoHo1 insertion mutants were generated. Work is in progess in characterizing the various genetic units, using these mutants. In the iron transport region, it was demonstrated that the iron uptake deficiencies of the mutants could be complemented *in trans* (Actis et al.; Koster et al, 1991). In the process the studies led to the isolation of a negative regulator in the *fatB* region. The following papers, numbers 2 and 3 will describe the work done in characterizing the negative regulator and the possible mechanism of its mode of regulation of the *fatA* and *fatB* genes.

PAPER #1

Molecular characterization of the iron transport system mediated by the pJM1 plasmid in *Vibrio anguillarum* 775

Wolfgang L. Koster, Luis A. Actis, Lillian S. Waldbeser, Marcelo E. Tolmasky and Jorge H. Crosa

Work done by Lillian S. Waldbeser pertaining to Paper #1:

1. Complementation of mutants in the iron transport region.
2. Maxicell analysis of proteins in the iron transport region.
3. Part of the sequence analysis:
    a. Hydrophobicity analysis.
    b. Part of the domain analysis.

SUMMARY

Complementation of insertion mutants showed that the polypeptides FatD, FatC, FatB and FatA are essential for the iron-transport process encoded by pJM1. Sequence analysis followed by homology studies indicated that transport of ferric anguibactin into *V. anguillarum* 775 follows the same mechanism as reported for transport of Fe^{3+}-hydroxamates, Fe^{3+}-catecholates, ferric dicitrate, and vitamin B_{12} into *E. coli*. Homology of FatA, part of the receptor complex, to seven *E. coli* receptor proteins involved in uptake of siderophores and vitamin B_{12} support the idea of a common ancestral gene. A "TonB-Box" was found in FatA suggesting the existence of a TonB-like protein and probably *exb*B, *exb*D-like functions in *V. anguillarum*. A high homology in the primary structure of FatB to FhuD, FecB, FepB, and BtuE suggests that FatB is the anguibactin-binding protein located in the periplasmic space. FatD and FatC are polytopic integral membrane proteins. According to their homologies to other proteins from other transport systems they may be involved in the translocation of ferric anguibactin across the cytoplasmic membrane.

Abbreviations used are: Nal^r, nalidixic acid resistant; SDS-PAGE, sodium dodecil sulfate- polyacrylamide gel electrophoresis; kb, kilobases; nt, nucleotides; PBT, periplasmic binding protein-depending transport; EDDA, ethylenediamine-di(*O*-hydroxyphenyl) acetic acid.

INTRODUCTION

It has now been over 10 years since the initial discovery of the pJM1 plasmid-mediated iron uptake system and its relationship to the virulence repertoire of certain strains of *Vibrio anguillarum* (Crosa, 1980). During this time, considerable progress has been made in our knowledge of the regulatory steps leading to the expression of this system (Crosa, 1989). However, the understanding of the mechanism of iron transport into the cell cytosol has increased at a slower pace, due to inherent problems related to the complex nature of the *V. anguillarum* genetic system and the approaches that can be used in this bacterium. Previous work demonstrated that the 86-kDa OM2 protein (FatA), encoded by *fatA* (Actis et al., 1985; 1988; Tolmasky and Crosa, 1991) must be a component of the the receptor for complexes of iron with the siderophore anguibactin (Jalal et al., 1989). This protein together with products encoded upstream of *fatA* were demonstrated to be essential for the transport of iron within the cell cytosol (Actis et al., 1988). We recently published the sequences of *fatB* , which encodes a 40-kDa protein, FatB one of the essential products encoded upstream of *fatA* (Actis et al., 1988; Tolmasky and Crosa, 1991). We have now dissected the whole iron transport region and show in this work genetic and sequencing results that assign a possible role to FatB as well as to the products of the other two ORFs found upstream of *fatB*, for which the complete sequence is reported here. Furthermore, we show that the components of the *V. anguillarum* iron transport system share a remarkable homology in specific protein domains to those of other iron and vitamin transport systems described in members of the Enterobacteriaceae, phylogenetically distant from *V. anguillarum*.

MATERIALS and METHODS

Bacterial strains and plasmids.

V. anguillarum 775 (Crosa et al., 1980) was used as the source of pJM1 and anguibactin siderophore. A Nal^r derivative of the plasmid-less strain H775-3 (Crosa et al., 1980), obtained as described by Miller (1972), was used as recipient in conjugation experiments. *E. coli* strain HB101 (Boyer and Roulland-Dussoix, 1969) and JM107 (Norrander et al., 1983) were used as transformation hosts for plasmid construction and single-stranded DNA preparation, respectively. *E. coli* MM294 harboring the plasmid pRK2013 was used as helper in conjugation experiments (Figurski and Helinski, 1979; Tolmasky and Crosa, 1984). *E. coli* BN660 (Prody and Neilands, 1984) was used as maxicell host. The plasmid vectors pBR325 (Bolivar, 1978), M13mp18 and M13mp19 (Norrander et al., 1983), pACYC184 (Chang and Cohen, 1978) and pJHC-S100 (Salinas et al., 1989) were used as cloning vehicles.

General methods.

pJM1 restriction fragments were cloned as described previously (Actis et al., 1988). Tn3-HoHo1 transposition mutagenesis of pJM1 DNA was performed as described by Tolmasky et al. (1988). The insertions were localized by DNA sequencing, using as a primer a synthetic oligonucleotide complemetary to Tn3-HoHo1 DNA.

Polypeptides encoded by recombinant plasmids were identified by maxicell in vivo labeling with [^{35}S]Methionine, and subsequent analysis by SDS-PAGE (Sancar et al., 1979).

DNA sequencing was carried out by dideoxy chain-termination of single-stranded DNA obtained from M13 mp18 and M13mp19 clones

(Messing, 1983; Sanger et al., 1977). Single-stranded deletion derivatives were obtained with T4 DNA polymerase as described previously (Actis et al., 1988). DNA and protein sequence analysis was performed using the Pustell Sequence Analysis Program, International Biotechnologies, Inc.

RESULTS

Nucleotide sequence and expression of the iron transport region located upstream of *fatB*.

Fig. 1 shows the restriction endonuclease map of the pJM1 iron transport region. The 2.4 kb *Eco*RI-*Sal*I fragment was subcloned into both the M13mp18 and M13mp19 vectors. By using T4 DNA polymerase we were able to obtain overlapping deletion derivatives. From the analysis of the nucleotide sequence of both strands it was possible to find in this region two open reading frames, ORF1 and ORF2, which have the same direction of transcription as those of *fatA* and *fatB*. Fig. 2 shows the complete nucleotide sequence of these two components. For *fatD* the longest open reading frame starting with ATG at nt 115 (Fig. 2) is shown for which a potential Shine-Dalgarno (1974) sequence GGAGAG eight nucleotides upstream of *fatD* could be identified. Four codons further downstream there is a second ATG codon in frame which, however, is not preceded by a sequence showing any similarity to the Shine-Dalgarno consensus sequence (AAGGAGGT). For *fatC* we favor the start codon ATG at nt 1063 (Fig. 2) resulting in overlapping translational stop/start codons for *fatD* and *fatC*. Seven nucleotides upstream of the presumed *fatC* start codon a potential ribosome binding site was

detected: AGGGAG. This sequence resides within the coding region of FatD. A comparable situation, called "translational coupling" was reported for many prokaryotic genes organized in operons and being involved in transport processes. The translated sequence of *fatD* and *fatC* results in two proteins of 33,900 and 34,900 daltons, respectively. Hydropathy analysis, shown in Fig. 3, revealed that the polypeptides encoded by these two open reading frames are very hydrophobic. By introducing some of the clones described in Fig. 1 in the maxicell strain BN660 we were able to determine that polypeptides of the expected molecular weight were synthesized by the regions containing *fatD* and *fatC* (Fig. 4). The amount of FatC and FatD from the strain harboring pJHC-LW115 appeared to be at a reduced level (Fig. 4, lane D). The recombinant clone pJHC-LW115 contained the *Eco*RI-*Sal*I fragment of the pJM1 iron transport region, which encompassed intact *fatD* and *fatC* regions and the truncated *fatB* region. Since *fatD fatC* and *fatB* may be part of a polycistronic message (Waldbeser et al., manuscript in preparation), deletion of the 3' region of *fatB* may decrease the stability of the message, and thus resulted in reduced levels of FatD and FatC synthesis in clone pJHC-LW115.

Complementation of mutants in the transport region.

Previous work identified FatA as an essential component of the pJM1 iron transport system by complementation of mutant 14 (Actis et al., 1988). This mutant was originally mapped just outside *fatA* by restriction endonuclease analysis, however subsequent sequencing analysis demonstrated that it was indeed located inside this gene (Fig. 6). Complementation experiments using insertions 20, 17, and 15, located in *fatD*, *fatC* and *fatB*, respectively (Figs. 1 and 2) demostrated that these three are also essential for the iron transport process (Fig. 5). *V. anguillarum*

carrying either mutant 20, 17 or 15 were unable to grow under iron-limiting conditions. However, *V. anguillarum* carrying any of these derivatives together with pJHC-A179 (clone containing intact genes *fatD*, *fatC* and *fatB*) grew under these conditions, while the strain carrying pJHC-A179 alone was unable to grow under the same conditions .

Homology of FatA, FatB, FatC and FatD to other iron transport proteins.

a) ***FatA and E. coli*** receptor proteins

FatA (the OM2 protein) was previously identified as a component of the receptor for iron-anguibactin complexes by complementation and insertion mutagenesis studies (Actis et al.1985; 1988; Tolmasky et al., 1988). One of these mutants, 14 mapped within *fatA* (Figs. 1 and 6). Uptake systems of *E. coli* for different siderophores and vitamin B12 share many similar properties. Homology boxes in the sequences of several receptor proteins were recently reported (Lundrigan and Kadner, 1986; Pressler et al., 1988; Nau and Konisky, 1989; Sauer et al., 1990). It was, thus, of interest to compare the FatA primary structure with those of seven outer membrane receptor sequences from *E. coli* and one from *V. cholerae* (Fig. 6). Similarities were mainly found in regions I to IV as defined by Lundrigan and Kadner (1986). Region I contains the "Ton B-box", which is located near the N-terminus of the polypeptide chains. It is highly likely that normal energy-coupled transport activity is not dependent on the presence of specific side chains at any position in the"Ton B-box", but on the local secondary structure (Gudmundsdottir et al., 1989; Schoffler and Braun, 1989). In FatA this region reads D E S I T V Y G Q A. Eight out of ten residues are conservative exchanges or identical in most of the receptors. The highly conserved valine residue is present in FatA at position 18 of the mature protein. The "TonB-

box" is thought to be involved in a direct interaction between the receptor and the TonB protein presumably responsible for energy coupling to the outer membrane (Heller et al. 1988; Schoffler and Braun,1989). Region II is located near the C-terminus. Its function remains unclear since point mutations in FhuE in this area revealed no detectable phenotype (Sauer et al. 1990). Although region III shows the lowest grade of homology among the aligned sequences (Fig. 6), a similarity between FatA and the other proteins is detectable, specially R_{95}, G_{96} and G_{111}. Region IV represents another homology box: 45% of the FatA amino acids are found to be identical to at least two of the other proteins, three residues are highly conserved in each protein, G_{131}, G_{138} and N_{150}. Taking together the results from regions I to IV 38% of the amino acids were identical in FatA and in at least two other receptor proteins. Comparing regions I to IV of FatA with the corresponding regions of FhuA, FhuE, IutA, FecA, FepA, Cir, and BtuB (Fig. 6) the percentage of identical amino acids was in this order: 29%, 25%, 26%, 25%, 30%, 23%, and 20%. From these data it can not be estimated if FatA involved in ferric anguibactin uptake is more closely related to the receptors transporting siderophores from the hydroxamate-, phenolate-, or citrate type. When the other *E. coli* receptor proteins were compared pairwise (Fig. 6) similar values of identity were obtained; for example: FhuA/FhuE 26%, BtuB/FhuE 30%, FecA/BtuB 35%, FecA/FepA 35%, FhuA/FepA 38%, and FepA/BtuB 43%. Recently, an N-terminal portion of the *V. cholerae* irgA gene product was published. IrgA, an iron regulated 77kDa outer membrane protein, is discussed as the iron-vibriobactin receptor (Goldberg et al., 1990). In our understanding IrgA carries a typical signal sequence of 25 amino acids with SASAF/AQD as a potential signal peptidase cleavage site resulting in a known sequence up to amino acid at position 126 of the mature protein

(Goldberg et al., 1990). The sequenced portion of IrgA protein exhibits a significant homology to the correspondong regions (region I, III and part of IV) of FepA (55% identical amino acids) (Fig. 6). A higher rate of identity, however, was found by us comparing IrgA and Cir (64%). The number of identical amino acids between IrgA and other iron-regulated (or vitamin B12-regulated) outer membrane proteins was generally high, for example: IrgA/FhuA, 37%; IrgA/Iut, 43%; IrgA/BtuB, 51%. Interestingly, the number of identical amino acids between IrgA and FatA was significantly lower (19%) although the two *Vibrio* species are more closely related than *Vibrio* and *Escherichia*.

b) Homology of *FatB* to *FhuD, FecB, FepB,* and *BtuE*

The existence of a periplasmic component involved in substrate binding was reported for the uptake systems for certain siderophores such as Fe^{3+} -hydroxamates (ferrichrome, aerobactin, coprogen), Fe^{3+} -dicitrate, Fe^{3+} -enterochelin and for vitamin B_{12} (Taylor et al.,1972; Bradbeer et al.,1978; Pierce and Earhart, 1986; Elkins and Earhart, 1989; Koster and Braun, 1989; Staudenmaier et al., 1989). Recently, binding of $^{55}Fe^{3+}$ -ferrichrome to the FhuD protein was demonstrated in a FhuD overproducing strain (Koster and Braun, 1990b). Additional evidence for substrate binding to FhuD was provided by proteolytic resistance of this periplasmic protein to proteinase K in the presence of ferrichrome, aerobactin, and coprogen (Koster and Braun, 1990 b; Koster, 1991). Regions of homologous amino acid sequences representing only parts of FhuD and FecB have been published previously (Staudenmaier et al., 1989). An alignment of the complete FhuD, FecB, FepB, and BtuE primary structures revealed a strong homology along the entire length (Koster, 1991).

FatB carries a potential signal sequence and may be exported into the periplasmic space (Actis et al., 1988). When compared to the equivalent components of the "iron transport family", a striking homology over the full length was detected (Fig.7). Thirty-five percent of the amino acids were found to be identical at the same position in FatB and at least one of the other proteins. A lower rate of identity was detected after pairwise comparison. FatB compared with FhuD, FecB, FepB, and BtuE (Fig. 7) gave the following values: 16%, 17%, 20%, and 12%, respectively. These values are in a similar range compared to the other possible alignments (Fig. 7): FhuD/FecB 18%, FhuD/FepB 16%, FhuD/BtuE 10%, FecB/FepB 26%, FecB/BtuE 11%, and FepB/BtuE 13%. All binding proteins involved in iron transport (including FatB) show a better homology to each other than to BtuE which was found to be dispensable in the *btu* uptake system (Rioux and Kadner, 1989). Several characteristic motifs, often including proline residues were found in each protein at equivalent positions. They may be important for secondary and tertiary structure of the polypeptide chains. This is an important observation because no extensive homology to binding proteins of any other PBT system could be detected. From these data we conclude that FatB is the putative ferric anguibactin binding protein located in the periplasm of *V. anguillarum* 775.

Despite a similar tertiary structure, periplasmic binding proteins normally show little homology to each other (Ames et al., 1990 and references therein). An exception are those binding proteins interacting with the same intrinsic membrane protein(s), such as HisJ and LAO-BP in the histidine uptake system of *Salmonella typhimurium* (Higgins and Ames, 1981). The same holds true for LIV-BP and LS-BP of the *liv* uptake system of *E. coli*, and the LIVAT-BP of the corresponding branched-chain-amino acid uptake system of *Pseudomonas aeruginosa* (Hoshino and Kose, 1990). Therefore it is

a remarkable finding that five binding proteins from different transport systems display such a homology to each other.

c) Homology of *FatD* and *FatC* to integral membrane proteins involved in uptake of siderophores and vitamin B_{12}

Homology between integral membrane proteins of PBT systems was first observed when the primary structures of FhuB (Koster and Braun, 1986) and BtuC (Friedrich et al., 1986) were compared (Koster and Kadner, unpublished; Braun et al., 1987). Meanwhile regions of homology have been identified in FhuB, BtuC, FecC, FecD, FepD, and FepG (Staudenmaier et al., 1989; Chenault and Earhart; and McIntosh, personal communications). FhuB has twice the size of the hydrophobic components of other PBT systems and consists of two apparently duplicated halves (Koster and Braun, 1990a). All sequences published of integral membrane proteins belonging to the "iron transport family" are from *E. coli*. Therefore it was of interest to compare the known amino acid sequences with the primary structures of FatD and FatC from *V. anguillarum*. An alignment of the complete primary structures of BtuC (Friedrich et al., 1986; Rioux and Kadner, 1989), FhuB(N) and FhuB(C) (Koster and Braun, 1986; 1990a), FecC and FecD (Staudenmaier et al., 1989), and FatD and FatC is shown in Fig. 8. FatD and FatC display a striking homology to each other and to the other hydrophobic proteins. Since the similarity in the N-terminal portions is not remarkable, in the following paragraph the numbers indicating the percentage of identical amino acids refer to the sequences starting with the first residue of the second block of Fig. 8. More than 40% of the amino acid residues in FatD/FatC are present at a comparable position in at least two of the other polypeptides. This is noteworthy since the proteins originate from different organisms.

Interestingly, the number of identical amino acids in FatD/FatC is 20% which is lower than the values for FhuB(N)/FhuB(C) 27%, and FecC/FecD 36%. FatD displays a weaker homology to FatC from the same transport system than to the *E. coli* protein FhuB(N) (28%), FhuB(C) (27%), and FecC (23%), FecD (18%), and BtuC (23%). The values for FatC are lower: 17% to 20% identity with the *E. coli* integral membrane components. When the *E. coli* proteins from different systems were compared, values above 29% were found; examples are given: FhuB(N)/BtuC 30%, FhuB(N)/FecC 30%, FhuB(C)/FecC 31%, BtuC/FecD 39%. The integral membrane proteins from the *E. coli* "iron transport family" (including BtuC) show a slightly stronger homology to each other than to FatD and specially FatC from *V. anguillarum*. It is most likely that the corresponding genes derive from a common ancestor. The lower degree of similarity in the FatD and FatC polypeptides compared to the *E. coli* proteins may be the result of a divergent evolution of the two organisms.

DISCUSSION

The polypeptides FatD, FatC, FatB and FatA within the "iron uptake region" of pJM1 were identified by sequencing. Striking homologies to genes encoding proteins involved in uptake of different siderophores indicate that the uptake of ferric anguibactin into *V. anguillarum* 775 follows the same mechanism as reported for transport of Fe^{3+}-hydroxamates, Fe^{3+}-catecholates, ferric dicitrate, and vitamin B_{12} into *E. coli*.

Uptake of siderophores and vitamin B_{12} into the periplasm of *E. coli* requires highly specific receptor proteins in the outer membrane and the

TonB, ExbB, and ExbD proteins (for a review see Braun et al.,1987, 1990; Earhart, 1987; Kadner et al., 1987). FatA was previously identified as a component of the ferric anguibactin receptor in the outer membrane of *V. anguillarum* (Actis et al. 1988). Homology of FatA to seven *E. coli* receptor proteins involved in uptake of siderophores and vitamin B_{12} (Fig. 6) support the idea of a common ancestral gene. Especially noteworthy is the presence of a region similar to the "TonB-Box" in the FatA sequence. This finding suggests the existence of a TonB-like protein and probably *exb*B, *exb*D-like functions in *V. anguillarum*. In principle, the same may be true for the vibriobactin uptake system in *V. cholerae*, although only an N-terminal portion of IrgA is known up to now. Transport of siderophores across the cytoplasmic membrane of *E. coli* displays characteristics typical of a PBT mechanism (Ames, 1986; Koster and Braun, 1989; Staudenmaier et al., 1989; Earhart and McIntosh, personal communication). All PBT systems reported so far consist of: a periplasmic component presumably involved in substrate binding, one or two hydrophobic components imbedded within the inner membrane, and a peripheral cytoplasmic membrane component most likely involved in energy coupling .

The best analyzed of the PBT components are the periplasmic binding proteins. These monomeric polypeptides are organized into two globular domains forming a cleft and connected by a flexible hinge. X-ray crystallographic studies of several binding proteins suggested a "Venus-fly-trap" binding mechanism (Ames et al., 1990). In the case of several binding proteins a conformational change upon substrate binding was observed (Ames et al., 1990).

The rather hydrophilic protein FatB consists of 322 amino acids and its N-terminal region displays characteristics typical of bacterial signal sequences.

Therefore it was suggested that this protein is exported through the cytoplasmic membrane (Actis et al., 1988). A striking homology in the primary structure of FatB to FhuD, FecB, FepB, and BtuE is shown in Fig.7 These data together with the strong evidence for iron(III)hydroxamate binding to the periplasmic FhuD protein which also undergoes a conformational change upon substrate binding (Koster and Braun, 1990 b; Koster, 1991) support the idea that FatB is indeed the anguibactin-binding protein located in the periplasmic space.

No extensive homologies have been reported among integral membrane proteins of different PBT systems. Regions of strong similarity have only been observed in those hydrophobic polypeptides which are involved in transport of identical or similar substrates, such as certain sugars or siderophores (and vitamin B_{12}). Interestingly, FatD and FatC exhibit a striking homology to each other and to the equivalent proteins of the corresponding systems. With respect to these data we conclude that FatD and FatC are polytopic integral membrane proteins which are involved in the translocation of ferric anguibactin across the cytoplasmic membrane.

In all PBT systems studied so far one or two proteins peripherally associated with the cytoplasmic membrane were reported. Typical for these hydrophilic components are two regions of homology found in ATP-binding proteins of pro- and eucaryotes (Ames, 1986; Higgins et al., 1988). ATP-binding and hydrolysis concomitant with uptake of substrates were shown for several proteins of different PBT systems (Hobson et al., 1984; Mimmack et al., 1989; Ames et al., 1989; Bishop et al., 1989; Dean et al., 1989; Davidson and Nikaido, 1990). Genes encoding proteins with potential ATP-binding sites were identified as essential components in the *fhu*, *fec*, *fep*, and *btu* systems (Burkhardt and Braun, 1987; Coulton et al., 1987; Staudenmaier et al., 1989;

Friedrich et al., 1986; McIntosh, personal communication). Astonishingly, no gene encoding a polypeptide with an equivalent function was detected in the "iron uptake region" of pJM1. Therefore we suggest that this "missing gene" may be located in another region of pJM1 or on the *V. anguillarum* chromosome.

From the evolutionary standpoint it is remarkable that the iron uptake systems of *E. coli* and the anguibactin uptake system of *V. anguillarum*, as well as the vibriobactin uptake system of *V. cholerae* share many structural and functional similarities which strongly supports the idea of a common origin of these systems. Interestingly, no extensive sequence homologies could be detected between the *E. coli* iron transport proteins and those of the iron uptake system of *Serratia marcescens* (Angerer et al., 1990), although the *Serratia* system also seems to follow the PBT mechanism, and *Serratia marcescens* is much more closely related to *E. coli* than *V. anguillarum* is. It will be of interest to obtain and compare sequence data from iron transport systems from other organisms to increase our understanding of the evolution of bacterial iron uptake systems.

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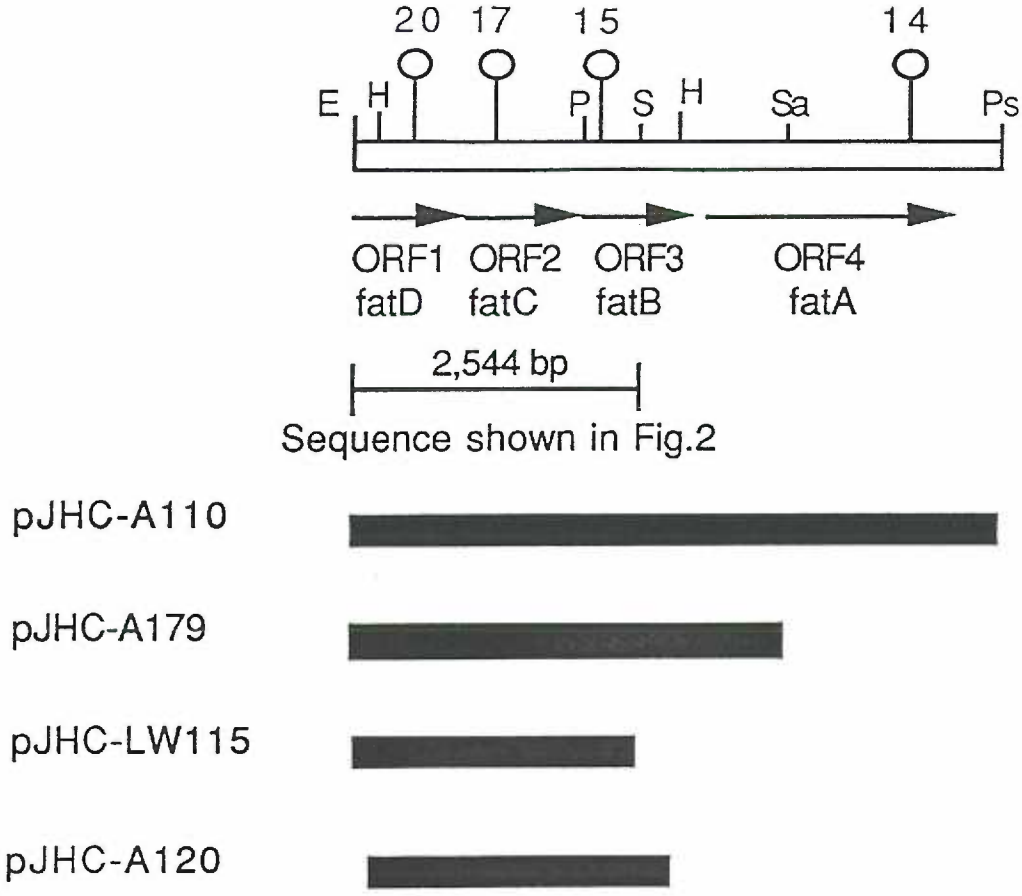
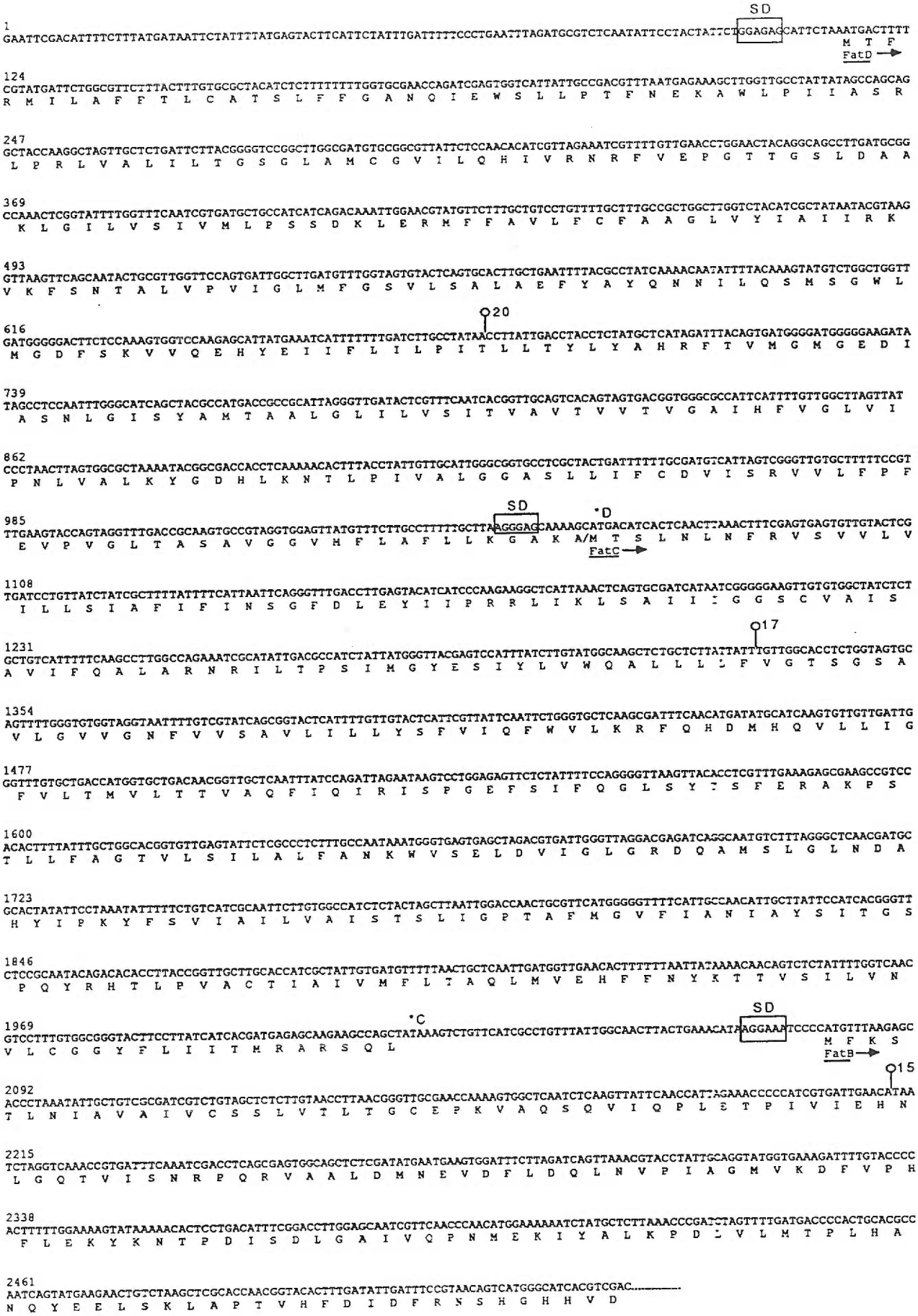


Fig. 1. Physical and genetic map of the pJM1 iron transport region and subcloned derivatives. The subcloned regions are shown below the restriction endonuclease map. E, *Eco*RI; H, *Hin*d III; S, *Sal*I; Sa, *Sac*I; P, *Pvu*I; Ps, *Pst*I. Arrows indicate the orientation of transcription. Solid bars indicate DNA regions cloned from pJM1 DNA. : Tn3-HoHo1 insertions.

Fig. 2. Nucleotide sequence of the coding region for FatD and FatC. *D and *C, translation terminations for FatD and FatC, respectively. Potential Shine-Dalgarno (SD) sequences are boxed.  , Tn3-HoHo1 insertions. The last six nucleotides shown correspond to the *Sal*I site and the dots indicate that the sequence of *fatB* is continued. The complete sequence of this gene has already been published (Actis et al., 1988).


SD
FatD
SD
FatC
SD
FatB
20
17
15
*C
*D

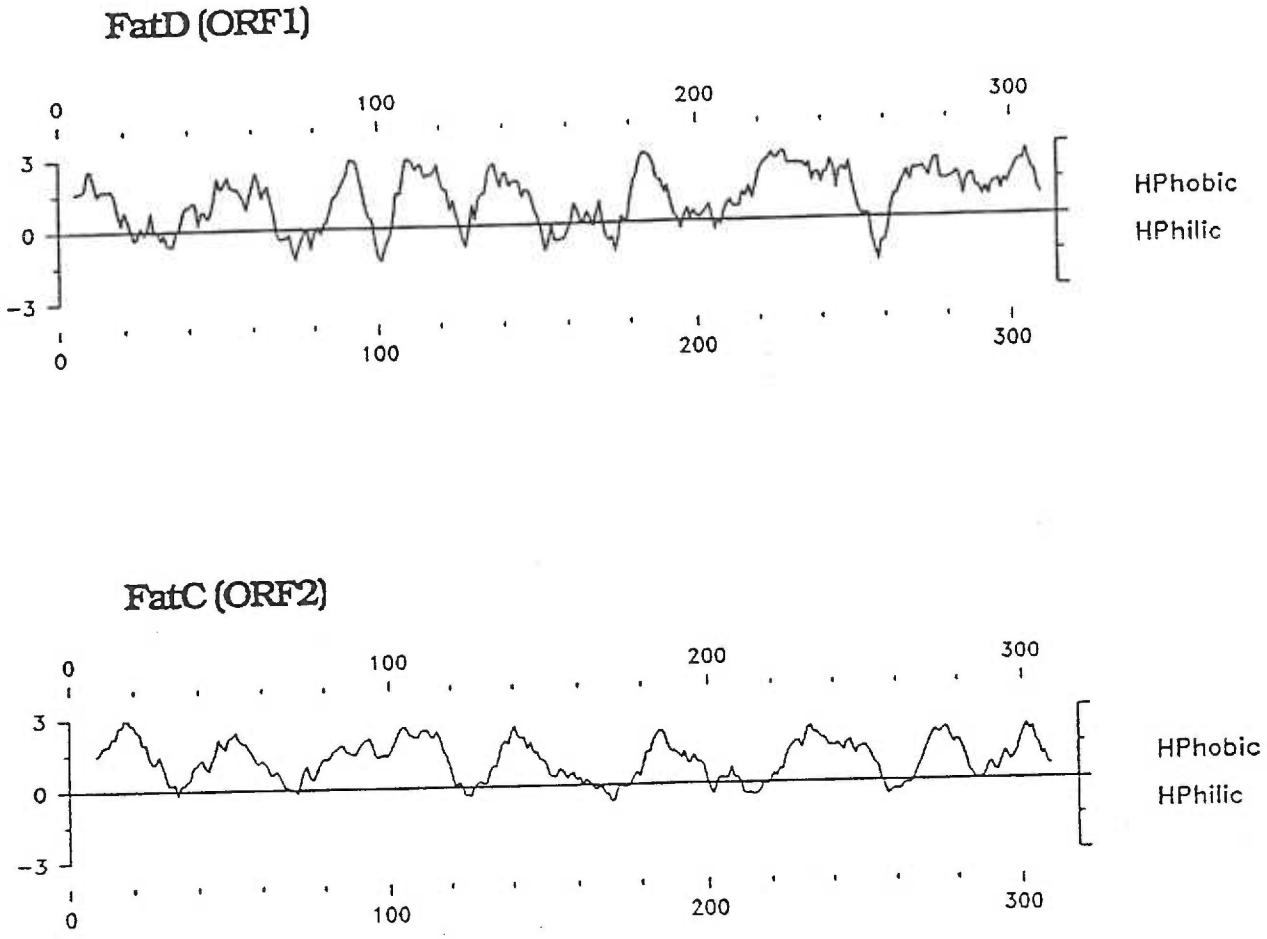


Fig. 3. Hydropathy plots for the FatD and FatC coding regions. The hydropathy plot was determined by the method of Kyte and Doolittle (1982).

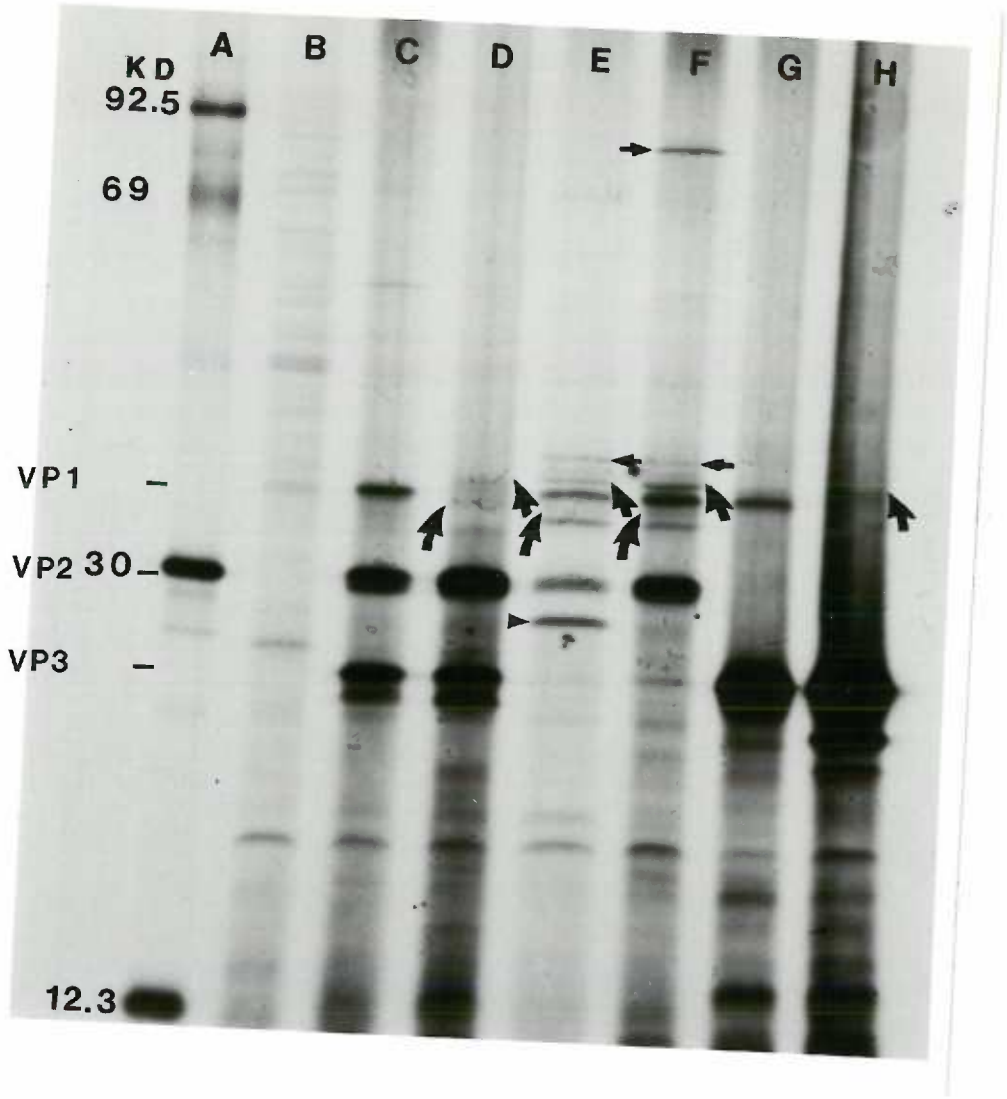


Fig. 4. Maxicell analysis of the polypeptides encoded by *fatA, fatB, fatC* and *fatD*. Autoradiograph of SDS-PAGE (15%) of the polypeptides encoded by cloned DNA segments of the iron-transport region of pJM1. Lanes B-H *E.coli* BN660 harboring: B, no plasmid; C, pJHC-S100; D, pJHC-LW115 ; E, pJHC-A179; F, pJHC-A110; G, pACYC184; H, pJHC-A120. Lane A: molecular weight standards. Horizontal arrows: pointing to the right shows FatA and pointing to the left shows FatB. Horizontal arrowhead pointing to the right shows truncated FatA. Curved arrows: pointing to the left show FatC and pointing to the right show FatD. VP1, vector encoded protein that confers resistance to tetracycline. VP2, vector encoded aminglycoside phosphotransferase. VP3, vector encoded chloramphenicol acetyltransferase.

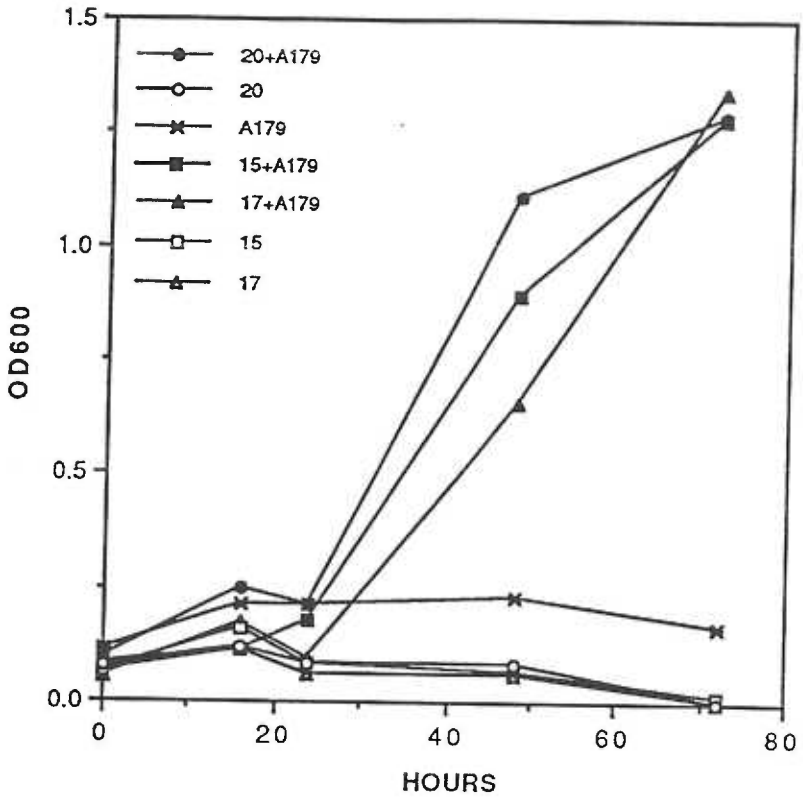


Fig. 5. Complementation analysis of mutants 15, 17 and 20 in the FatD, FatC and FatB region. The iron transport proficiency of *V. anguillarum* strains (carrying mutant 20 or 17 or 15 in the presence or absence of pJHC-A179) was determined by testing their ability to grow in M9 minimal medium containing the nonassimilable iron chelator EDDA with the addition of supernatant from *V. anguillarum* 775 (as a source of anguibactin).

Fig. 6. Alignment of homologous amino acid sequences found in FatA, IrgA of *V. cholerae* and TonB-dependent receptor proteins of *E. coli*. Regions I to IV as defined by Lundrigan and Kadner (1986) were modified and supplemented. The vertical arrow indicates the end of the signal peptide. To achieve maximum fit sequence gaps were introduced into some sequences. A dot above the sequences indicates identical residues in FatA and at least two other proteins. A colon means identical amino acids in each receptor. The numbers on the left side of the sequences give the first residue shown of the mature proteins, and the numbers on the right is the distance to the carboxyl terminus. Sequence data for FhuA, IutA, FepA, and BtuB, are taken from (Lundrigan and Kadner, 1986), and for FhuE, FecA, Cir, and IrgA from Refs. (Sauer et al., 1990; Pressler et al., 1988; Nau and Konisky, 1989a, 1989b; Goldberg et al., 1990), respectively. Dots in region IV indicate that the IrgA sequence in this region is still not known.

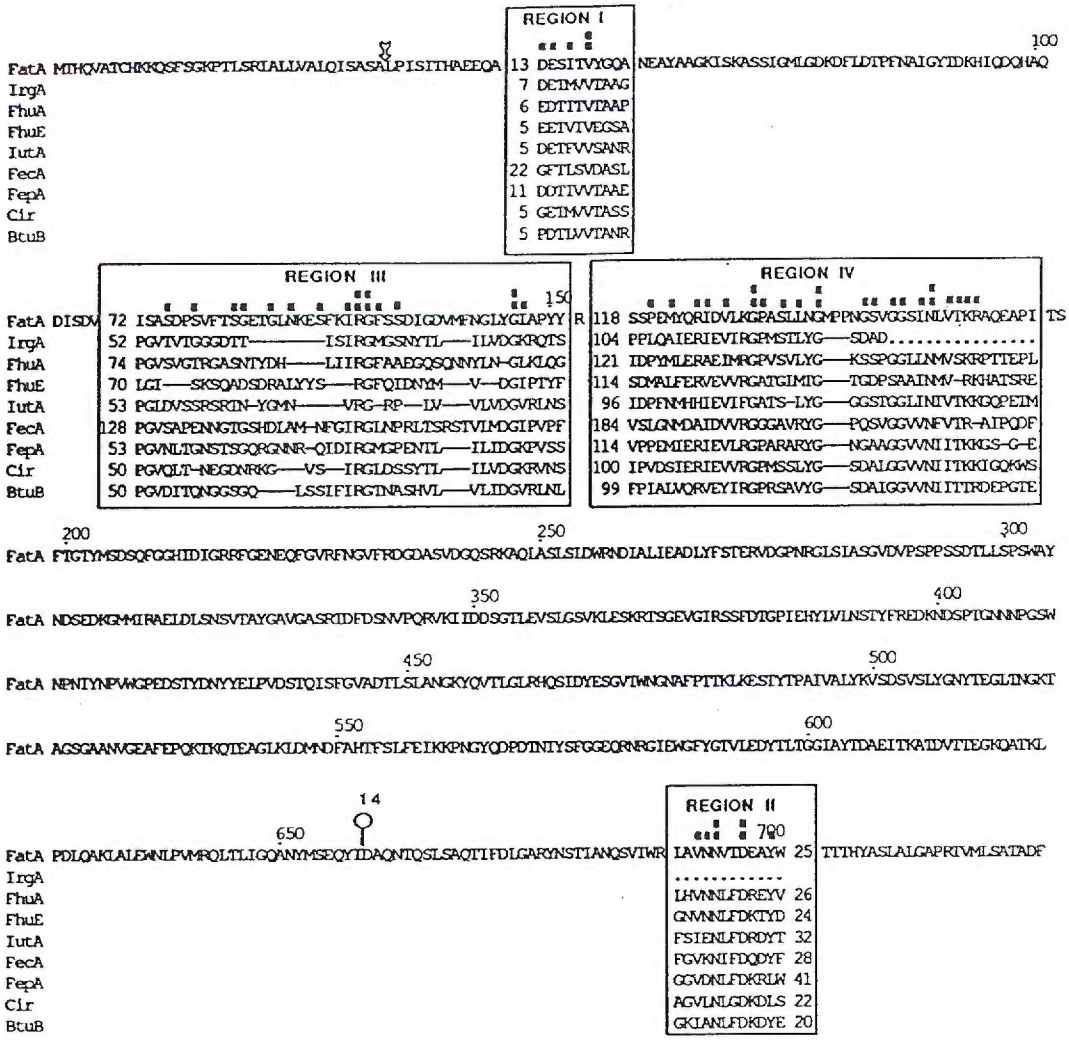

REGION I
REGION III
REGION IV
REGION II
FatA
IrgA
FhuA
FhuE
IutA
FecA
FepA
Cir
BtuB

Fig. 7. Alignment of the FatB, FhuD, FecB, FepB, and BtuE primary structures. Identical amino acids in FatB and at least one other protein are marked by dots. Highly conserved residues are indicated by colons above the sequences. Sequence comparison data are from Koster (1991).

```
                                .                    . .   .      :.. ..
FatB    MFKSTLNIAVAIVCSSLVTLTGCEPKVAQSQVIQPLETPIVIEHNLGQTVISNRPQRVAAL
FhuD                          MSGLPLISRRRLLTAMALSPLLWQMNTAHAAAIDPNRIVAL
FecB                        MLAFIRFLFAGLLLVISHAFAAMVQDEHGTFTLEKTPQRIVVL
FepB                 MRLAPLYRNALLLTGLLLSGIAAVQAADWPRQITDSRGTHTLESQPQRIVST
BtuE                       MQDSILTTVVKDIDGEVTTLEKFAGNVLLINVASKCGLTPQYEQLE

            .. .  . ..          .   .     . .  ...     . . .:. ...
FatB    DMNEVDFLDQLNVP---------IAGMVKDFVPHF-LEKYKNTPDISDL-GAIVQPNMEKI
FhuD    EWLRVEVLLALGIV---------PYGVADTINYRLWVSEP-PLPDSVIDVGLRTEPNLELL
FecB    ELSPADALAAVDVS---------PIGIADDNDAKRILPEVRAHLKPWQSVGTRAQPSLEAI
FepB    SVTLTGSLLAIDAPVIASGATTPNNRVADDQGFLRQWSKV-AKERKLQR-LYIGEPSAEAV
BtuE    NIQKAWVDRGFMVL---------GFPCNQFLEQEPGSDEEIKTYCTTTWG--VTFPMFSKI

         ...... .       ..   .. :.  ::.  .             .     .    .
FatB    YALKPDLVL---MTPLHA-NQYEELSKLAPTVHF--------DIDFRNSHGHHVDII-KQH
FhuD    TEMKPSFMVWSAGGYG---PSPEMLARIAPGRGFNFSDGKQPLAMARKSLTEMADLLNLQS
FecB    AALKPDLII--ADSSRHA-GVYIALQQIAPVLLL-------------KSRNETY-AENLQS
FepB    AAQMPDLILISATGGDSALALYDQLSTIAPTLII-----------------NYDDKSWQS
BtuE    EVNG---------EGRH--PLYQKLIAAAPTAVAPEESGFYARMVSKGRAPLYPDDILWNF

            ....  .   .. . .  .        .   .     . ..   . ..     :
FatB    VID-LGEIFNKQTLAQKKVAEID--AKVDEVQALTAERSEKALVVMHNNGSFSSFGIEERY
FhuD    AAE-THLAQYEDFIRSSKPRVFKRGRPLLLTTL----IDPRHMLVFGPNSLFQEILDE---
FecB    AAI-IGEMVGKKREMQARLEQHKERMAQWASQL---PKGTRVAFGTSREQQFNLHTQETWT
FepB    LLTQLGEITGHEKQAAERIAQFDKQLAAAKEQIKLPPQPVTAIVYTAAAHSANLWTPESAQ
BtuE    EKF-LVGRDGKVIQRFSPDMTPED--PIVMESIKLALAK

        .     ..     ..    ..   .  .. .   .       .      .   .
FatB    GFVFDVLGV--KPASTEIAAS-LHGQPISSEFINQANPDILYIIDRTAVMEGKPVIDAEHL
FhuD    --------------YGIPNAWQGETNFWGSTAVSIDRLAAYKDVDVLCFDHDNSKDMDALM
FecB    GSVL---------ASLGLNV-PAAMAGASMPSIGLEQLLAVNPAWLLVAHYREESIVKRWQ
FepB    GQMLEQLGFTLAKLPAGLNASQSQGKRHDIIQLGGENLAAGLNGESLFLFAGDQKDADAIY

        ..::.    .  .. .  ...  ..    .     .. :  . .
FatB    ANPLLRQTKAWKNGKVIFVDADAWYITSASITSLKIVIDDIIKGYQS
FhuD    ATPLWQAMPFVRAGRFQRVPAVWFYGATLSAMHFVRVLDNAIGGKA
FecB    QDPLWQMLTAAQKQQVASVDSNTWARMRGIFAAERIAADTVKIFHHQPLTVVK
FepB    ANPLLAHLPAVQNKQVYALGTETFRLDYYSAM---QVLDRLKALF
```

Fig. 8. Primary structures of FatD and FatC compared with amino acid sequences of integral membrane proteins BtuC, FhuB, FecC, and FecD. Identical residues in FatD/FatC and at least two other proteins are marked by dots above the sequences, whereas colons point to identical amino acids in all polypeptides compared. Sequence data are taken from Friedrich et al. (1986), Koster and Braun (1986), and Staudenmaier et al. (1989).

```
FatD                          MTFRMILAFFTLCATSLFFGANQIEWSLLPTFNEKAWLPIIAS
FatC                               MTSLNLNFRVSVVLVILLSIAFIFINSGFDLEYIIPR
FhuB(N)       MSKRIALFPALLLALLVIVATALTWMNFSQALPRSQWAQAAWSPDIDVIEQMIFHYS
FhuB(C)        MKVNDRVAAERQHVLAFALAGGVLLLMAVVVALSFGRDAHGWTWASGALLEDLMPW
FecC         MTAIKHPVLLWGLPVAALIIIFWLSLFCYSAIPVSGADATRALLPGHTPTLPEALVQNL
FecD                MKIALVIFITLALAGCALLSLHMGVIPVPWRALLTDWQAGREHYYVLMEY
BtuC             MLTLARQQQRQNIRWLLCLSVLMLLALLLSLCAGEQWISPGDWFTPRGELFVWQI

                 ..... .  . :  ..  ..  : . .:   .:   :    . ..    ... ..
FatD          RLPRLVALILTGSGLAMCGVILQHIVRNRFVEPGTTGSLDAAKLGILVSIVMLPSSDKLE
FatC          RLIKLSAIIIGGSCVAISAVIFQALARNRILTPSIMGY-ESIYLVWQALLLLFVGTSGSA
FhuB(N)       LLPRLAISLLVGAGLGLVGVLFQQVLRNPLAEPTTLGVATGAQLGITVTTLWAIPGAMAS
FhuB(C)       RWPRIMAALFAGVMLAVAGCIIQRLTGNPMASPEVLGISSGAAFGVVLMLFLVPGNAFGW
FecC          RLPRSLVAVLIGASLALAGTLLQTLTHNPMASPSLLGINSGAAWLWRYQRAESDADCRLF
FecD          RLPRLLLALFVGAALAVAGVLIQGIVRNPLASPDILGVNHAASLASVGALLLMPSLPVM-
BtuC          RLPRTLAVLLVGAALAISGAVMQALFENPLAEPGLLGVSNGAGVGLIAAVLLGQGLTPNW

                  .           .. . .        ...   . . .. :  ..
FatD          RMFFAVLFCFAAGL-VYIAI-IRKVKFSNTALVPVIGLMFGSVLSALAEFYAYQNNI---
FatC          VLGVVGNFVVSAVLILLYSFVIQFWVLKRFQHDMHQVLLIGFVLTMVLTTVAQF-IQI-R
FhuB(N)       QFAAQAGACVVGLIVFGVAWG------KR--LSPVTLILAGLVVSLYCGAINQLLVIFHH
FhuB(C)       LLPAGSLGAAVTLLIIMIAAG-------RGGFSPHRMLLAGMALST-AFTHLLMMLQASG
FecC          SVVIAACGGGVSWLLVMTAGG-----GFRHTHDRNKLILAGIALSAFCMGLTRITLLLAE
FecD          VLPLLAFAGGMAGLILLKMLA------KTH--CPMKLALTGVALSA-CWASLTDYLMLSR
BtuC          ALGLCAIRGALIITLILLRFA------RRH-LSTSRLLLAGVALGIICSALMTWAIYFST

                  .       .  :..              .    ..  ...      . ..   ..   :
FatD          LQ---SMSGWLMGSFSKV----VQEHYEIIFLILPITLLTYLYA-HR-FTVMGMGEDIAS
FatC          IS--PGEFSIFQG-LSYTSFERAKPSTLLFAGTVLSILALFANKWVSELDVIGLGRDQAM
FhuB(N)       DQLQ-SMFLWSTGTLTQTDWGGVERLWPQLLGGVMLTLLLL-----RPLTLMGLDDGVAR
FhuB(C)       DPRMAQVLTWISGSTYNATDAQVWRTGIVMVILLAIT-PLC----RRWLTILPLGGDTAR
FecC          DHASYGIFYWLAGGVSHARWQDVWQLLPVVVTAVPVVLLL-ANQ----LNLLNLSDSTAH
FecD          PQDVNNALLWLTGSLWGRDWSFVKIAIPLMILFLPLSLSFC-----RDLDLLALGDARAT
BtuC          SVDLRQLMYWMMGGFGGVDWRQSW----LMLALIPVLLWICC-QS-RPMNMLALGEISAR

                 .:.    .    . .   ...  .   :.. : :.. . ..    :    .  ... ..
FatD          NLGISYAMTAALGLILVSITVAVTVVTVGAIKFVGLVIPNLVALKYG-DHLKNTLPIVAL
FatC          SLGLNDAHYIPKYFSVIAILVAISTSLIGPTAFMGVFIANIAYSITGSPQYRHTLPVACT
FhuB(N)       NLGLALSLARLAALSLAIVISALLVNAVGIIGFIGLFAPLLAKML-GARRLLPRLMLASL
FhuB(C)       AVGMALTPTRIALLLLAACLTATATMTIGPLSFVGLMAPHIARMM-GFRRTMPHIVISAL
FecC          TLGVNLTRLRLVINMLVLLLVGACVSVAGPVAFIGLLVPHLARFWAGF-DQRNVLPVSML
FecD          TLGVSVPHTRFWALLLAVAMTSTGVAACGPISFIGLVVPHMMRSITGG-RHRRLLPVSAL
BtuC          QLGLPLWFWRNVLVAATGWMVGVSVALAGAIGFIGLVIPHILRLC-GLTDHRVLLPGCAL

                 .. ... ..    .  ... . . . ... . :   ..  . . .
FatD          GGASLLIFCD-VISRVVLFPFEVPVG-LTASAVGGVMFLAFLLKGAKA
FatC          IAIVMFLTAQLMVEHF--FNYKTTVSILVMVLCGGY-FLITTMRARSQL
FhuB(N)       IGALILWLSDQIILWLTRVWMEVSTGSVIA-LIGAPLLLWLLPRLRSISAPD
FhuB(C)       VGGLLLVFAD-WCGRMVLFPFQIPAGLLST-FIGAPYFIYLLRKQSR
FecC          LGATLMLLAD-VLARALAFPGDLPAGAVLA-LIGSPCFVWLVRRRG
FecD          TGALLLVVAD-LLARIIHPPLELPVGVLTA-IIGAPWFVWLLVRMR
BtuC          AGASALLLAD-IVARLALAAAELPIGVVTAT-LGAPVFIWLLLKAGR
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PAPER #2

Two Mechanisms for the Negative Regulation by Iron of the FatA Outer Membrane Protein Expression in *Vibrio anguillarum* 775

Lillian S. Waldbeser, Marcelo E. Tolmasky, Luis A. Actis and Jorge H. Crosa

SUMMARY

Synthesis of the 86 kDa outer membrane protein FatA is repressed under iron rich conditions. Complementation studies of transposition mutants derived from clones containing the pJM1 iron uptake region revealed the existence of an antisense RNA, RNAα, that acts as a negative regulator of FatA synthesis, and is only expressed under iron-rich conditions. We found that the presence of RNAα, constitutively synthesized from a fusion construct that used an iron-insensitive promoter, led to a dramatic reduction of FatA synthesis under iron limitation, with no significant decrease in the steady state level of *fatA* mRNA. However, primer extension experiments revealed that the level of several possible *fatA* transcripts was reduced in the presence of RNAα. In addition to the regulation by RNAα, we also found that *fatA* mRNA expression is slightly reduced in the presence of *E. coli* Fur, suggesting that *E. coli* Fur could function but very inefficiently as a repressor for *V. anguillarum fatA* gene. Since we have identified and cloned a chromosomally-encoded *V. anguillarum fur*-like gene, a Fur-like repressor system must exist in *V. anguillarum*.

Abbreviations: AMV, avian myeloblastosis virus; bp, basepair(s); EDDA, ethylene-diamine-di(*o*-hydroxyphenyl) acetic acid; kb, kilobase(s); kbp, kilo-basepair; kDa, kilodalton; mRNA, messenger RNA; Nal^r, nalidixic acid resistant; nt, nucleotide(s); SDS, sodium dodecyl sulfate.

INTRODUCTION

Many factors contibute to the success of a pathogen in establishing an infection. The ability to harness iron from the host's microenvironment has been recognized as an important virulence factor because bacteria require iron as cofactor for enzymes involved in general metabolism, replication, as well as in the electron transport chain (1-4). Although iron is abundant in nature, it is not readily available in the vertebrate host where it is bound by high-affinity iron-binding proteins, such as transferrin, lactoferrin, and heme containing proteins. Therefore, the possession of a system to capture iron from the host's proteins is crucial for the bacterial survival (5-7).

The iron uptake system of the pathogen *Vibrio anguillarum* 775 is encoded by the 65 kb virulence plasmid pJM1(8). The system consists of anguibactin, a siderophore of Mr 348 that captures iron (9,10), and an energy-dependent transport system whereby iron is internalized (11, 12). The transport region of pJM1 is approximately 5.7 kb and contains four genes, *fatA*, *fatB*,*fatC* and *fatD* (13). One of them, *fatA*, encodes an 86 kDa outer membrane protein, which has specific domains homologous to several outer membrane receptor proteins that are TonB dependent (14-17). Particularly, FatA has in common with the other proteins, a TonB box, a region where it putatively interacts with the TonB protein for the energy coupled transport process. FatB shows homology in specific domains with periplasmic transport proteins, and FatC and FatD with cytoplasmic membrane proteins, of other transport systems (14). Expression of these pJM1 iron transport proteins is negatively regulated by iron(11). In this

communication, we report the finding that iron regulates the expression of the *fatA* gene by possibly two mechanisms. One of them is via a Fur-like repressor (18-22), and the other uses an antisense RNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The wild type strain *Vibrio anguillarum* 775 was used as a source of plasmid pJM1 (8). *V. anguillarum* 775::Tn*1*-6 carried plasmid pJHC9-8 which was generated by Tn*1* insertion of the wild type plasmid pJM1, resulting in deletion of the iron uptake region (23). The Nal^r plasmid-less*V. anguillarum* strain H775-3a (14) was used as recipient for the recombinant plasmids in conjugation experiments. *E. coli* BN4020 (24) was used to detect Fur regulation of gene expression. *E. coli* HB101 (25) and *E. coli* JM109 (26) were used as transformation hosts for plasmid construction and DNA preparation. *E. coli* MM294 harboring the plasmid pRK2013 (27) was used as helper in conjugative transfer of recombinant clones into *V. anguillarum*. Plasmid pJHC-T7 carrying the iron uptake region of pJM1 and its Tn*3* ::HoHo1 transposition derivatives (mutant 20, pJHC-T7::20; mutant 17, pJHC-T7::17 and mutant 15, pJHC-T7::15) were obtained as described (28, 29). pJHC-W44 contained the positive regulatory region (Taf) of pJM1 (29). pJHC-A179 and pJHC-LW115 contained pJM1 iron transport sequences cloned into the vector pBR325 containing the kanamycin resistance fragment from pUC4K (30).

Clone pJHC-A122 carried pJM1 iron transport sequences cloned into the vector pACYC184 (31). Plasmid pHP45 (32) was the source of the Ω fragment. pMH15 contained the *E. coli. fur* gene cloned in pACYC184 (18). pMET67 was a pVK102 derivative containing the *V. anguillarum* H775-3a *fur* gene. The characteristics of plasmids pRT240 and pSC27.1 were previously described (33, 34). pJHC-S300 contained a 85 bp *Hin*dIII-*Stu*I fragment of the *fatA* gene cloned into the plasmid vector pBluescript KS (Stratagene). pMET13.1 contained a 94 bp *Cla*I-*Sac*I fragment of *fatA* cloned in pBluescript SK (Stratagene). pJHC-S400 carried a 135 bp *Sal*I-*Hin*dIII fragment of the *fatB* gene cloned into pBluescript SK.

General methods

Conjugations to *V. anguillarum* were carried out as described previously (28). β-galactosidase assays were performed as described by Miller (35). Hybridization of total DNA was performed under the conditions described previously (36) using 25% formamide (low stringency) or 50% formamide (high stringency). DNA probes were labelled with [α^{32}P]dATP by the ramdom primer method as described by Feinberg, et al. (37). Preparation of a *V. anguillarum* gene bank was already described (38).

RNA Isolation and Northern Blot Hybridization

For iron-rich conditions,*V. anguillarum* strains were grown in M9 minimal media containing 100 μg/ml ferric ammonium citrate until the culture reached OD_{600} of 0.5. Under iron-limiting conditions, the bacterial were grown in M9 media till OD_{600} of 0.25, then EDDA was

added for the final concentration to be 10 uM, and the bacteria were allowed to grow till OD_{600} of 0.5. Total RNA were prepared according to the hot phenol method as described by van Gabian et al. (39). RNAs were electrophoresed in formaldehyde-agarose gel and transferred to nylon membranes. The membranes were stained with methylene blue to check for even loading and proper transfer of the RNAs. The Northern blots were analyzed with riboprobes or with DNA probes as described by Sambrook, et al. (40). Prehybridization and hybridization were at 63°C for riboprobes and at 42°C for DNA probes.

RNase Protection

RNase protection studies were performed as described by Krieg, et al. (41). Total RNA and labelled riboprobes were allowed to hybridize at 45°C overnight. RNase A (Boehringer Mannheim Biochemicals) and RNase T1 (Sigma Chemical Co.) treatment was at 34 °C for 30 mins. Proteinase K (Boehringer Mannheim Biochemicals) treatment was at 37 °C for 15 mins. Treated samples were extracted with phenol, chloroform-isoamyl alcohol and ethanol prepicipitated. Samples were analyzed in 6% urea acrylamide gel. Labelled *Hin*fI digested pBR322 fragments were used as molecular weight standards. Labelled riboprobes were made by in vitro transcription of linearized plasmid templates as described by Melton, et al. (42), using T3 or T7 RNA polymerases (Bethesda Research Laboratories) and [α^{32}P]UTP.

Immunoblot

Total membranes were prepared from *V. anguillarum* grown under iron-rich (M9 medium with 100 μg/ml ferric ammonium citrate) or iron-limiting (M9 medium with 1-5 μM EDDA) conditions, as previously described (11). The membrane proteins were subjected to electrophoresis in 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The presence of FatA protein was detected using absorbed polycloning anti-FatA antibody and horseradish peroxidase-conjugated protein A as described previously (12).

Primer Extension

A synthetic oligonucleotide, 5'-TACCTGATGAGTCAT-3' complementary to the 5' terminus of *fatA* was end labelled using T4 polynucleotide kinase (New England Biolab) and [γ^{32}P]dATP, as described by Sambrook, et al. (40). The end-labelled oligonucleotide was annealed with 30 μg total RNA at 42°C, and primer extension was performed using AMV reverse transcriptase (Life Sciences Inc.) as described by Ghosh et al. (42). The same oligonucleotide was used as primer to generate sequencing ladders of the region immediately 5' of the *fatA* translation site by the dideoxy chain termination method of Sanger et al. (44).

RESULTS

Inhibition of FatA expression by insertion mutagenesis and by complementation.

Transposition mutants 20, 17 and 15, were generated by Tn3 :: HoHo1 insertions into the *fatD*, *fatC* and *fatB* respectively in pJHC-T7 (a recombinant clone carrying the pJM1 iron uptake region) (28, 29) (Fig. 1a). All three mutants were transport deficient (29), and immunoblot analysis of these mutants revealed a concomitant reduction of FatA expression (Fig. 2). Since these insertions were located upstream of *fatA* , and transposition mutations are generally strongly polar, these genetic results suggest that *fatA* may be included within a polycistronic message. We also carried out complementation studies for iron transport and found that recombinant clone pJHC-A179 (Fig. 1a) was able to complement these mutants in iron transport (14). However, the presence of this recombinant plasmid inhibited the expression of *fatA* (Fig. 3a). We also found that clone pJHC-A122 (containing a truncated *fatD*, intact *fatC* and a majority of the coding region of *fatB*) also had an inhibitory effect on *fatA* expression. On the other hand, pJHC-LW115 (containing intact *fatD* and *fatC* and the 5' region of *fatB*) did not affect the expression of *fatA* (Fig. 3a). Therefore, truncation of *fatB* at the *SalI* site abolished the inhibition phenomenom. Thus, pJHC-A179 and pJHC-A122 must, encode a negative regulator of *fatA*. Expression of this regulator requires the 3' region of *fatB*.

Identification of the negative regulator.

An attractive possibility is that the negative regulator is an antisense RNA that is transcribed from the *fatB* region, 3' of the *Sal*I site, or the negative regulator could be a protein encoded by the other strand. Another possibility is that a DNA sequence which may be the binding site for a negative regulator of *fatA*, may occupy the *fatB* region that is upstream of the *Hin*dIII site but encompass the *Sal*I site. The lack of inhibition by the clone pJHC-LW511 may be due to the truncation of the recognition sequence for the negative regulator or to actual truncation of the negative regulatory genetic determinants.

To investigate these two possibilities, constructs pJHC-LW95, pJHC-LW87 and pJHC-LW96 were made with Ω fragment, a DNA fragment containing transcription termination signals and translation stop signals, inserted in clone pJHC-A122 either at the *Hin*dIII site in *fatD* (generating pJHC-LW95), or at the *Pvu*I site at the 5' terminus of *fatB* (generating pJHC-LW87), or at the *Hin*dIII site at the 3' end of *fatB* (generating pJHC-LW96) (Fig. 1b). We observed that the clones pJHC-LW95 and pJHC-LW87 still showed ability to cause inhibiton of FatA synthesis (Fig. 3a, lanes A, B, E and F). However, the inhibitory effect was abolished when the Ω fragment was inserted at the *fatB* *Hin*dIII site as in pJHC-LW96 (Fig. 3a, lanes C and D). The abrogation of inhibition by the Ω insertion at the *fatB* *Hin*dIII site (as in pJHC-LW96) also ruled out the possibility of the existence of a *cis* binding sequence for a negative regulator. Another possibility, that the inhibitor could be a small peptide was ruled out since we were unable to identify any open reading frame encoded within *fatB* in either strand that would include the *Sal*I site. Therefore, the Ω insertion

studies strongly suggested that an antisense RNA may be transcribed from the the *fatB* region, downstream of the *Sal*I site, and that this antisense RNA was likely to negatively affect the expression of the *fatA* gene.

RNase protection studies were performed using RNA harvested from the strains that were used in the immunoblot analysis. A riboprobe designed to detect transcripts from the non-coding strand was constructed from the *Sal*I-*Hin*dIII fragment at the 3' region of *fatB* (Fig. 1a). Divergent transcripts were detected in the strain H775-3a (pJHC-T7::17) grown in iron-rich medium, and in H775-3a (pJHC-T7::17 and pJHC-A122, or pJHC-LW87, or pJHC-LW95) also grown in iron-rich media (Fig. 3b). In strain H775-3a (pJHC-T7::17, pJHC-LW96) where inhibition of FatA synthesis was abrogated (Fig. 3a, lanes C and D), the counter transcript was not detected (Fig. 3B, lanes C and D). This data confirmed that the negative regulator encoded by the *fatB* region was an antisense RNA, which was designated RNAα.

To analyze the effect of the antisense RNAα, in the wild type context, where the FatA protein is abundantly expressed when compared with mutant 17, RNase protection studies were performed using RNA harvested from the wild type *V. anguillarum* 775, and from the plasmidless strain H775-3a which now harbored the clone pJHC-T7. The antisense RNA was observed in RNA harvested from both the wild type strain 775 and H775-3a (pJHC-T7), but only when the cells were grown in iron-rich media (Fig. 4a). RNAα was not detected when these strains were grown in iron-limiting conditions. RNAα was found in both iron-rich and iron-limiting conditions in the strain carrying both pJHC-T7 and pJHC-A122 which harbors the determinants for

RNAα cloned at the *Hin*dIII site of pACYC184, within the tetracycline resistance gene. In this case the antisense transcript must be driven by promoters of the *tet* gene, which are insensitive to the iron concentration of the medium, resulting in the constitutive synthesis of RNAα. In this clone, approximately 350 nt of RNAα was deleted from the 5' end, and the remainder of RNAα started at the *Hin*dIII site. Although the wild type RNAα is 650 nt long, the results indicated that with less than 50% of this antisense RNA is sufficient for the inhibitory activity.

Regulation of *fatA* gene expression by the antisense RNAα.

The levels of *fatA* mRNA were determined on the same RNA samples harvested from the above mentioned strains by using RNase protection assays. The riboprobe designed to detect the *fatA* message was constructed using the *Hin*dIII-*Stu*I fragment from the 3' region of *fatA* (Fig. 1a). The *fatA* message was observed only in RNA harvested from cells grown under iron-limiting conditions. When compared with H775-3a (pJHC-T7), there was a slight decrease in the level of *fatA* mRNA from the strain H775-3a (pJHC-T7, pJHC-A122) in which RNAα was synthesized constitutively (Fig. 4b). These observations were substantiated by Northern blot analysis of the same RNA preparations (Fig. 5). The *fatA* mRNA was again slightly decreased in the strain carrying both pJHC-T7 and pJHC-A122. It is also notable that the *fatA* mRNA was at the same level in the strain H775-3a harbouring either of the clones with the Ω insertion (pJHC-LW95, pJHC-LW96, or pJHC-LW87) (Fig. 5, lanes H, J and L). The same RNA samples were tested for the presence of RNAα and we found that the level of RNAα was

dramatically reduced when the Ω insertion occured at the *fatB* *Hin*dIII site (as in pJHC-LW96), while the RNAα levels were not changed in the case of Ω insertions at the *fatD* *Hin*dIII site (as in pJHC-LW95) or at the *Pvu*I site (as in pJHC-LW87) (Waldbeser et al., manuscript in preparation). The data implied that *fatA* mRNA level was not significantly affected by the level of the antisense RNAα. However, the slight difference in *fatA* mRNA levels could not account for the tremendous decrease of the FatA protein generated by the presence of the RNAα clone in H775-3a (pJHC-T7, pJHC-A122) as well as in the strain 775 (pJM1, pJHC-A122) (Fig. 6) .

Mapping of the 5' end of the *fatA* message.

The *Hin*dIII site where RNAα begins in the inhibitory clone pJHC-A122, is 484 bp upstream of the translation start codon of *fatA*. Primer extension was therefore performed to map the 5' end of the *fatA* message. Using an oligonucleotide that encompassed the *fatA* start codon, we obtained numerous RNA species. There were two major RNA species with only one nucleotide difference in size, commencing from start sites at Ma (MaI and MaII), which were 103 and 102 bases upstream of the *fatA* start codon. (Figs. 7 and 8). These species of RNA were decreased in strain H775-3a harboring the clone pJHC-T7 and the recombinant plasmid pJHC-A122 that encoded RNAα. We also noticed that new RNA species appeared in this strain (Fig. 7, lane 3, indicated by arrows). The Ma sites are 22 and 23 nt downstream of the RNAα start site,Ra, that was mapped previously (Salinas et al., manuscript in preparation). We also detected two secondary start sites: Mb and Mc, 243 nucleotides and 362 nt

respectively, 5' of the *fatA* start codon, and therefore Mb is 118 nt upstream (in the sense direction) of the RNAα start site Ra while Mc coincides with the RNAα start site Rb. The results in this section suggest that the secondary *fatA* mRNA species starting at sites Mb and Mc, but not the major species from site Ma, overlap and thus can interact with the antisense RNA species initiated from start site Ra.

We also detected *fatA* mRNA molecules with start sites at MHd and MHe (Fig 7) that were distinctly present in strain H775-3a (pJHC-T7, pJHC-A122), and were barely discernable in the wild type strain 775. Start sites MHd and MHe were approximately 593 and 713 nt respectively from the *fatA* start codon. These higher molecular weight *fatA* mRNA species were also present in the strain harboring pJHC-T7 alone, but in such low abundancy that we were unable to reproduce them in photographs.

The primer extension results suggested the possible existence of a large *fatA* transcript that may start much further upstream of start site Mc. If the *fatA* message were part of a polycistronic message, insertions upstream of *fatA* should affect the expression of *fatA* . We had previously shown in this paper, that insertions upstream of *fatA* lead to a reduction in FatA synthesis. The primer extension studies and the genetic data therefore strongly support the existence of a polycistronic message, and that *fatA* is part of the *fatDCBA* message.

Fur regulation of *fatA* expression.

The presence of RNAα does not lead to an appreciable reduction of *fatA* mRNA levels. However, under iron-rich conditions there is a complete shut-off in the synthesis of *fatA* mRNA. It is therefore

possible that in addition to RNAα inhibition, *fatA* expression may be affected by another regulatory mechanism that is responsive to the concentration of iron. It is possible that a Fur-like repressor protein, as already described in other bacteria (19, 21, 22, 45, 46, 47), may be the responsible for this phenomenom. To investigate this possibility, pJHC-T7 was introduced into *E. coli* BN4020 (Fur^-), and into the isogenic strain which was also harboring pMH15, a plasmid carrying the *E. coli fur* gene. We observed that, although poorly expressed in *E. coli*, the *fatA* mRNA levels were found to be the same in iron-rich and iron-limiting conditions in *E. coli* BN4020 (Fur^-) (Fig. 9, lanes H and I). However, when the *fur* clone pMH15 was present in BN4020, the *fatA* mRNA was present at a lower level in RNA harvested from cells grown in iron-rich media than under iron-limiting conditions (Fig. 9, lanes J and K). Therefore, the data suggested that *E. coli* Fur repressor could function, though very inefficiently, as a repressor in the regulation of the *V. anguillarum fatA* gene. This observation was supported by the fact that the FatA protein was iron regulated in *E. coli* BN4020. Fig. 10, lanes A-D, showed that FatA was expressed under iron-deficient conditions but not in iron-rich medium, either in the presence or absence of an active *E. coli fur* gene. However, we observed that in the presence of Fur, the synthesis of FatA was at a much greated level (Fig. 10, lane A) than when Fur was absent (Fig. 10, lane C).

Genetic and functional detection of *fur* in *Vibrio anguillarum*

Because under iron-rich conditions, no *fatA* mRNA was detected, it is possible that a *fur*-like gene that encodes a more efficient product

exists in *V. anguillarum*.To detect the presence of a Fur-like repressor in *V. anguillarum* H775-3a, we introduced into this bacterium the plasmid pSC27.1 which has a Fur-binding sequence between the *ompF* promoter and the *lacZ* gene (33). Therefore, β-galactosidase production will be repressed in iron-rich conditions if a Fur-like protein exists. The control was plasmid pRT240 (33, 47) which is identical to pSC27.1 without the Fur-binding sequence. *V. anguillarum* H775-3a (pRT240) produced β-galactosidase constitutively while*V. anguillarum* H775-3a (pSC27.1) showed a 0.24 inhibition ratio of β-galactosidase activity when grown under iron-rich as compare to iron-limiting conditions (Table 1). These results indicated that *V. anguillarum* must have a chromosomally encoded Fur-like element.

Physical detection of a *fur*-like gene in *V. anguillarum* was performed by Southern blot hybridization under low stringency conditions of *Hin*dIII-digested chromosomal DNA, using as a probe the *E. coli fur* gene obtained from plasmid pMH15 (18). The probe hybridized with two bands of 6.6 and 3.5 kbp (data not shown). Hybridization was not observed under high stringency conditions, indicating a degree of divergency at the nucleotide level between the *E. coli* and the *V. anguillarum fur* genes.

Cloning of the *fur*-like gene from *V. anguillarum*

Isolation of a recombinant clone, containing the *V. anguillarum* *fur*-like gene, was performed using a gene bank of the plasmidless *V. anguillarum* H775-3 in the vector pVK102 (38). We obtained the recombinant plasmid pMET67 which contained the 6.6 and 3.5 Kbp *Hin*dIII fragments that hybridized with the *E. coli fur* probe, as

mentioned in the previous section. The Fur activity of this recombinant clone was tested by using the Fur-binding site *lacZ* plasmids described in the previous section. *E. coli* BN4020 (Fur^-) already carrying pRT240 or pSC27.1 were transformed with either pMET67 or pMH15 and β-galactosidase activity was determined in cultures grown under iron-rich conditions. The Table 2 shows that the presence of the *V. anguillarum fur*-like gene (pMET67) generated an inhibition of the β-galactosidase activity produced by plasmid pSC27.1 similar to that observed for a clone carrying the *E. coli fur* gene (pMH15). Neither pMET67 nor pMH15 modified significantly the β-galactosidase activity mediated by plasmid pRT240. The results in this section suggest that pMET67 carries a functional *fur*-like gene from the *V. anguillarum* chromosome.

DISCUSSION

In this work, we demonstrated that at least two mechanisms may play an important role in the repression of the expression of the *fatA* gene, when *V. anguillarum* 775 is grown under iron-rich conditions. One of these mechanisms is mediated by an antisense RNA (RNAα), that is induced under iron-rich conditions, and the other may be via a Fur-like protein that fuctions as a repressor in the presence of iron. In the first case, the presence of the antisense RNA leads to a dramatic reduction of FatA biosynthesis.

Northern blot and primer extension experiments showed that the level of the steady state *fatA* transcripts were slightly, but reproducibly reduced by the presence of the clone that encoded RNAα. Primer

extension experiments also revealed the existence of various *fatA* mRNA initiation sites (MaI, MaII, Mb, Mc, MHd and MHe), therefore the 2.35 kb *fatA* message detected in the Northern blots is probably a family of several species of *fatA* mRNA. The *fatA* mRNA molecules (initiated from MaI and MaII sites) do not overlap the RNAα start sites Ra, Rb or Rc, but those starting from Mb would overlap RNAα from start site Ra. However, the inhibitory recombinant clone pJHC-A122 encoded only the 300 nt from the 3' end of RNAα, a region that is upstream of *fatA* start site Mc. In order for this truncated antisense RNA to interact with *fatA* RNA, the *fatA* transcript had to be initiated at a site upstream of Mc, such as from MHd or MHe sites or from further upstream of *fatB*. In that case, *fatA* mRNA must be part of a polycistronic message. The immunoblot analysis of mutants 20, 17 and 15, demonstrated that Tn*3*::HoHo1 insertions in *fatD*, *fatC* and *fatB*, all upstream of *fatA*, resulted in a decrease in the synthesis of FatA protein. The polarity of the insertion mutations on FatA expression further supported the existence of a polycistronic message encompassing *fatA*.

In the cleavage of the polycistronic *lacZYA* message, it was demonstrated that cleavage at the start of the upstream message (*lacY*) inactivated the ability of the distal message (*lacA*) to participate in the formation of an initiation complex with ribosomes (49 and 53). Similar mechanism of message inactivation were implicated for the polycistronic gal, trp and mal messages (48, 50-52). In the *fat* operon, we may have a similar phenomenon. The 2.35 Kb f*atA* mRNA was found to be present with and without the presence of RNAα. However, when RNAα was present, little or no Fat A protein was synthesized, inspite

of a good level of the 2.35 Kb *fatA* RNA. A plausible explanation for this phenomenon is that this population of *fatA* mRNA, which must be distal to the cleavage sites, may be functionally inactivated for translation in the same manner as that reported for the *lacA* message in the *lac* operon.

The decreased FatA level as assessed by immunoblot analysis may be due to secondary structure modifications generated by interaction between *fatA* mRNA and RNAα. Changes in secondary structure could lead to a decrease in translational efficiency, for example, by leading to inaccessibility of the ribosome binding site of *fatA* mRNA starting at sites upstream of Ma, since transcripts from sites MaI and MaII do not overlap with RNAα. Since pJHC-A122 encoding the truncated RNAα was able to exert such a profound effect on the translation of *fatA*, the sequences involved in generating the changes in the secondary structure must be located 5' of Mc and upstream of the *Hin*dIII site in *fatB*.

It was of interest that primer extension of RNA obtained from the V. anguillarum strains harboring the iron uptake clone (pJHC-T7) together with the RNAα clone (pJHC-A122) produced novel RNA species, in addition to those described in the strain harboring only pJHC-T7. It is conceivable that the interaction between the large *fatA* mRNA molecules (initiated from start sites upstream of Ma) and the antisense RNA, resulted in conformational changes in the *fatA* mRNA molecule. As a consequence of these conformational changes, various RNA endonuclease sites would then become exposed, supporting the appearance of the extra RNA bands. From all of the above, we concluded that RNAα may regulate *fatA* expression primarily at the

level of translation, although we cannot rule out the possibility that the rate of processing of the full length *fatA* transcript may also be affected. If *fatA* mRNA is not part of a polycistronic message, RNAα may possibly affect the expression of *fatA*, by interacting with a positive regulator that may be essential for the translation of the *fatA* message.

However, the antisense RNA is not the major regulatory mechanism, since *fatA* mRNA is not detected under iron-rich conditions. In this vein, we were also able to demonstrate that *V. anguillarum* has a chromosomally mediated Fur-like protein that could recognize the *E. coli* Fur binding site. The *V. anguillarum* Fur-like product must be homologous to the *E. coli* Fur protein because it could be expressed and it was functional in *E. coli* as well as in *V. anguillarum*. However, the *V. anguillarum fur*-like gene hybridized with that from *E. coli* only under low stringency conditions indicating that there must be considerable divergency at the nucleotide level between the *E. coli* and the *V. anguillarum* genes. Furthermore, the regulation of *fatA* mRNA by *E. coli* Fur was not very dramatic, and immunoblot analysis showed that FatA protein synthesis was iron regulated, and was synthesized independently of the presence of the *E. coli* Fur. We believe that in this case, the regulation was afforded by RNAα encoded in the same clone. Therefore, the Fur product of *E. coli* may not be as effective in the regulation of the *V. anguillarum fatA* gene. It remains to be demonstrated whether the *V. anguillarum fur* gene product is responsible for the dramatic reduction of *fatA* mRNA and thus FatA protein synthesis when *V. anguillarum* 775 is grown under iron-rich conditions. Our present evidence suggests that the

antisense RNA might function as a fine-tuning mechanism in the iron regulation of the pJM1 *fatA* gene, by controlling the expression of the transcripts that have already been initiated.

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TABLE 1. β-galactosidase activities of *V. anguillarum*

Strain[a]	β-galactosidase activity (U)[b]		Inhibition ratio
	+ 30 μM FeCl3	+ 1 μM EDDA	Fe/EDDA
H775-3a (pRT240)	245	177	1.38
H775-3a (pSC27.1)	109	452	0.24

[a]Both strains are *V. anguillarum*

[b]Miller units (Miller, 1972)

TABLE 2. β-galactosidase activities of *E. coli* BN4020 derivatives[b]

Plasmids	β-galactosidase activity (U)[a]
pSC27.1	1190
pSC27.1 + pMET67	440
pSC27.1 + pMH15	370
pRT240	1550
pRT240 + pMET67	1310
pRT240 + pMH15	1490

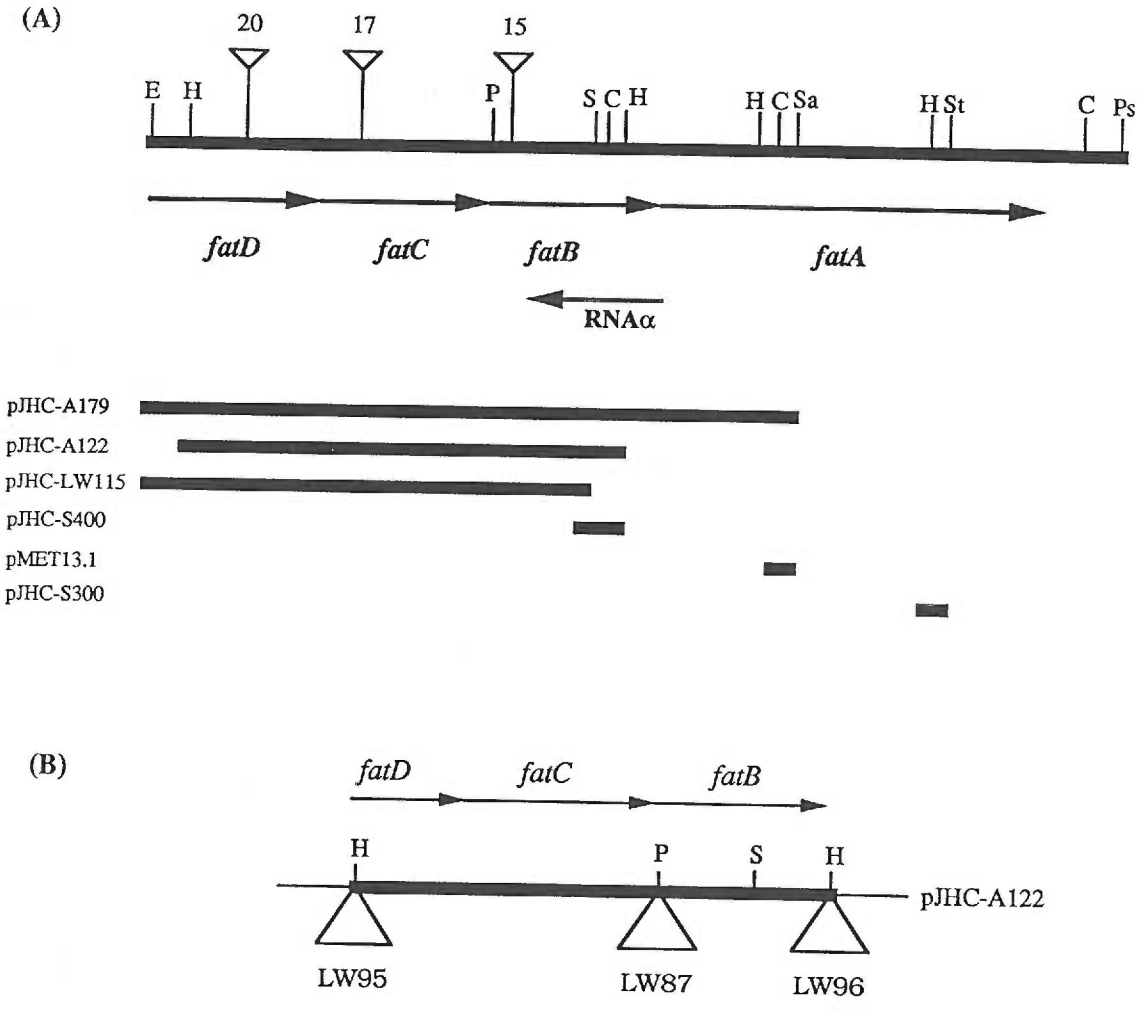
[a] Miller units (Miller, 1972)

[b] *E. coli* BN4020 derivatives were grown in minimal medium with the addition of 30 μM $FeCl_3$.

Fig. 1. (A) Physical and genetic map of pJM1 iron transport region and of regions subcloned. E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; C, *Cla*I; Sa, *Sac*I; St, *Stu*I, Ps, *Pst*I. The orientation of the arrows indicates the direction of the transcripts. 15, 17, 20 are Tn*3*-HoHo1 insertions.

(B) Restriction map of cloned pJM1 regions in pJHC-A122 and derivatives. Solid bar represents pJM1 DNA; thin line represents pACYC184 DNA. Open triangles are Ω fragment insertions in derivatives pJHC-LW95, pJHC-LW87 and pJHC-LW96. H, *Hin*dIII; P, *Pvu*I; S, *Sal*I.

Fig. 1


(A)
20
17
15
E H
P
S C H
H C Sa
H St
C Ps
fatD
fatC
fatB
fatA
RNAα
pJHC-A179
pJHC-A122
pJHC-LW115
pJHC-S400
pMET13.1
pJHC-S300
(B)
fatD
fatC
fatB
H
P
S
H
pJHC-A122
LW95
LW87
LW96

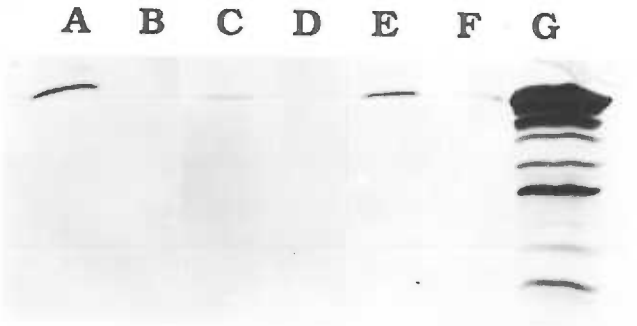
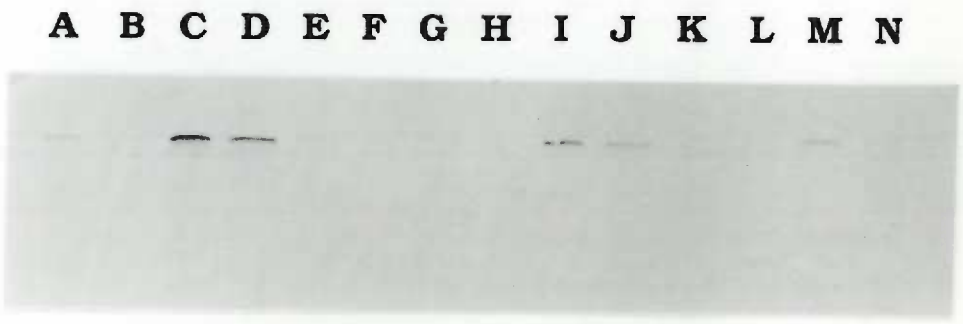


Fig. 2. Immunoblot analysis of FatA protein in insertion mutants. Lanes: A, C, E, G, bacteria grown under conditions of iron-limitation; B, D, F, bacteria grown in iron-rich media; A and B, mutant 20; C and D, mutant 17; E and F, mutant 15; G, *V. anguillarum* 775.

Fig. 3. Analysis of FatA protein and RNAα in the complementation of mutant 17.
(A) Immunoblot analysis of FatA protein. Lanes: A, C, E, G, I, K, M, bacterial strains grown under iron-limiting conditions; B, D, F, H, J, L, N, bacteria grown in iron-rich media. All lanes are from *V. anguillarum* H775-3a harboring mutant 17. Lanes: A and B, with pJHC-LW87; C and D, with pJHC-LW96; E and F, with pJHC-LW95; G and H, with pJHC-A122; I and J, with pJHC-LW115; K and L, with pJHC-A179; M and N, mutant 17 alone.
(B) RNase protection analysis of RNAα. The 133 nt riboprobe was made from pJHC-S400 linearized with *Hin*dIII and transcribed by T7 RNA polymerase. Lanes: A, C, E, G, I, K, bacterial strains grown under iron-limiting conditions; B, D, F, H, J, L, bacteria grown in iron-rich media, A - J are from *V. anguillarum* H775-3a harboring mutant17; A and B, with pJHC-LW87; C and D, with pJHC-LW96; E and F, with pJHC-LW95; G and H, with pJHC-A122; I and J, mutant 17 alone; K and L, *V. anguillarum* H775-3a (plasmidless); M, riboprobe without RNase treatment.

(A)



(B)

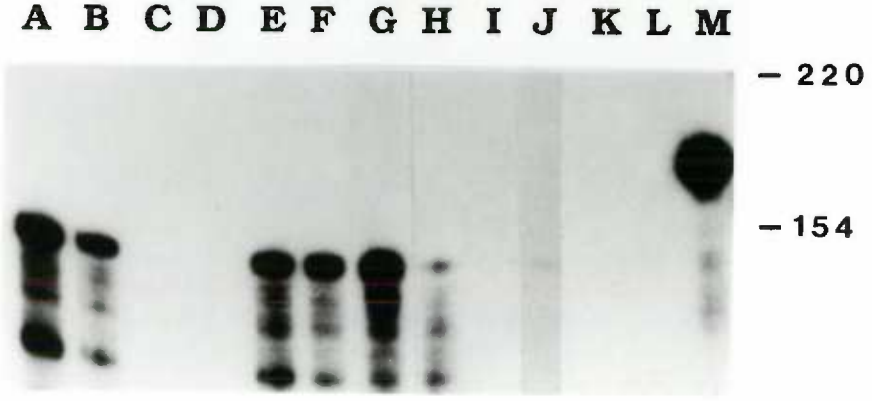
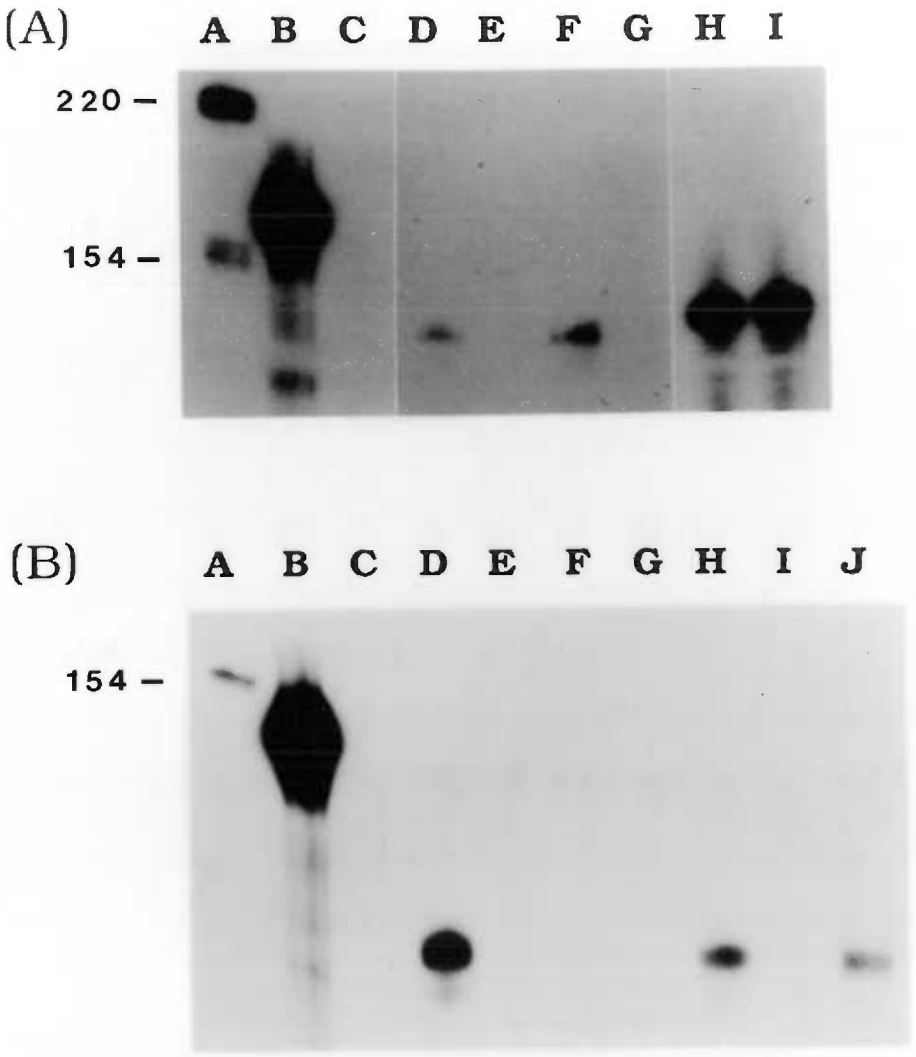


Fig. 4. RNase protection studies.

(A) Detection of antisense RNA, RNAα using a 133 nt riboprobe made from pJHC-S400 linearized with *Hin*dIII and transcribed by T7 RNA polymerase. Lane: A, molecular weight standard; B, riboprobe without RNase treatment; C, riboprobe treated with RNase; D, F, H, RNA harvested from strains grown in iron-rich media; E, G, I, RNA harvested from strains grown under iron-limiting conditions; D and E, *V. anguillarum* strain 775 (pJM1); F and G, strain H775-3a (pJHC-T7); H and I, strain H775-3a (pJHC-T7, pJHC-A122).

(B) Detection of *fatA* mRNA using a 84 nt riboprobe made from pJHC-S300 linearized with *Hin*dIII and transcribed by T3 RNA polymerase. Lane: A, molecular weight standard; B, riboprobe without RNase treatment; C, E, G, I, RNA harvested from strains grown in iron- rich media; D, F, H, J, RNA harvested from strains grown under iron-limiting conditions; C and D, *V. anguillarum* 775 (pJM1); E and F, strain H775-3a; G and H, strain H775-3a (pJHC-T7); I and J, strain H775-3a (pJHC-T7, pJHC-A122).


(A)
A B C D E F G H I
220 –
154 –
(B)
A B C D E F G H I J
154 –

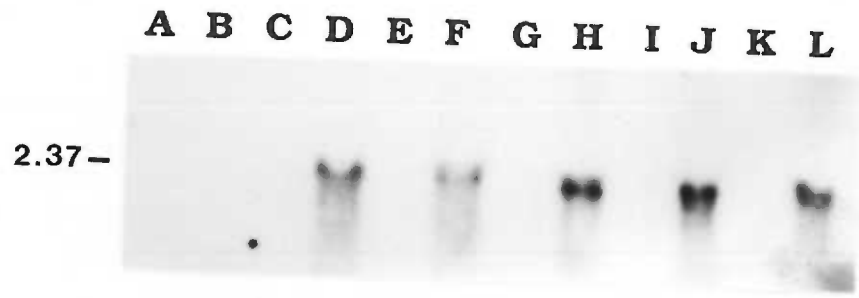


Fig. 5. Northern blot analysis of *fatA* transcripts. Riboprobe used for hybridization was made from pMET13.1 linearized with *ClaI* and transcribed by T3 RNA polymerase. Lanes: A, C, E, G, I, K, RNA harvested from bacteria grown in iron-rich media;B, D, F, H, J, L, RNA harvested from bacteria grown under iron-limiting conditions; A and B, *V. anguillarum* H775-3a (plasmidless); C and D, H775-3a (pJHC-T7); E and F, H775-3a (pJHC-T7, pJHC-A122); G and H, H775-3a (pJHC-T7, pJHC-LW95); I and J, H775-3a (pJHC-T7, pJHC-LW96); K and L: H775-3a (pJHC-T7, pJHC-LW87).

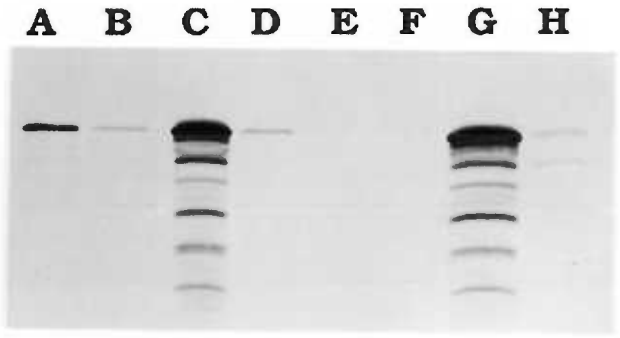
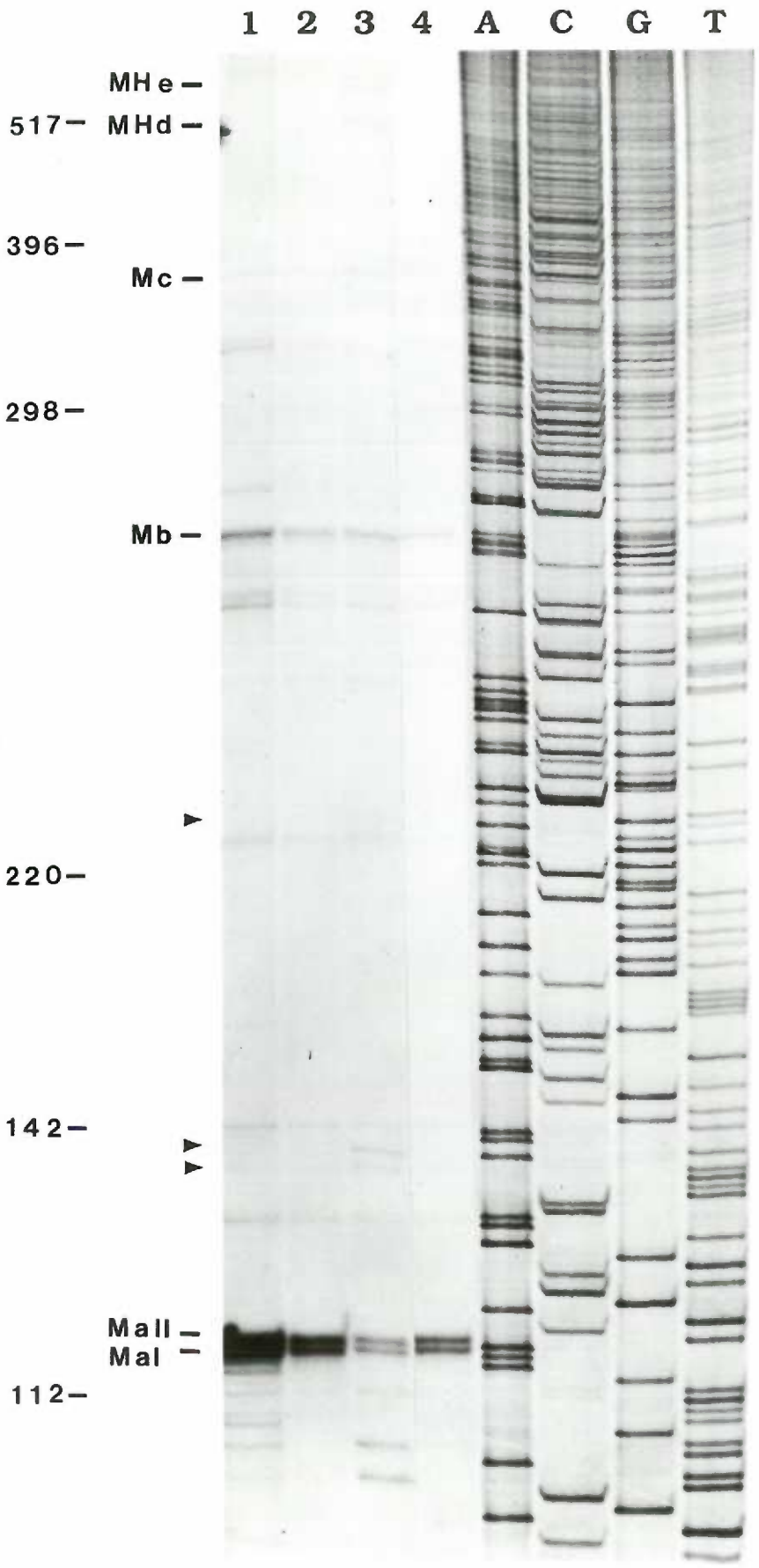


Fig. 6. Immunoblot analysis of FatA protein. Lanes: A, C, E, G, bacteria were grown under iron-limiting conditions; B, D, F, H, Bacteria were grown in iron-rich media; A and B, *V. anguillarum* H775-3a (pJHC-T7, pJHC-A122); C and D, strain H775-3a (pJHC-T7); E and F: *V anguillarum* 775 (pJM1, pJHC-A122); G and H, strain 775 (pJM1).

Fig. 7. Primer extension analysis of *fatA* mRNA. RNA samples were harvested from bacteria grown under iron-limiting conditions. Lane: 1, *V. anguillarum* 775; 2, *V. anguillarum* H775-3a (pJHC-T7); 3, strain H775-3a (pJHC-T7, pJHC-A122); 4, strain H775-3a (pJHC-T7, pJHC-LW96); A, C, G, T, sequencing ladders for bases A, C, G, T respectively. Arrow heads, RNA species present in strain harbouring pJHC-T7 and pJHC-A122, but absent in other strains. MaI, MaII, Mb, Mc, MHd and MHe are start sites of *fatA* RNA molecules.


1
2
3
4
A
C
G
T
MHe
517
MHd
396
Mc
298
Mb
220
142
MaII
MaI
112

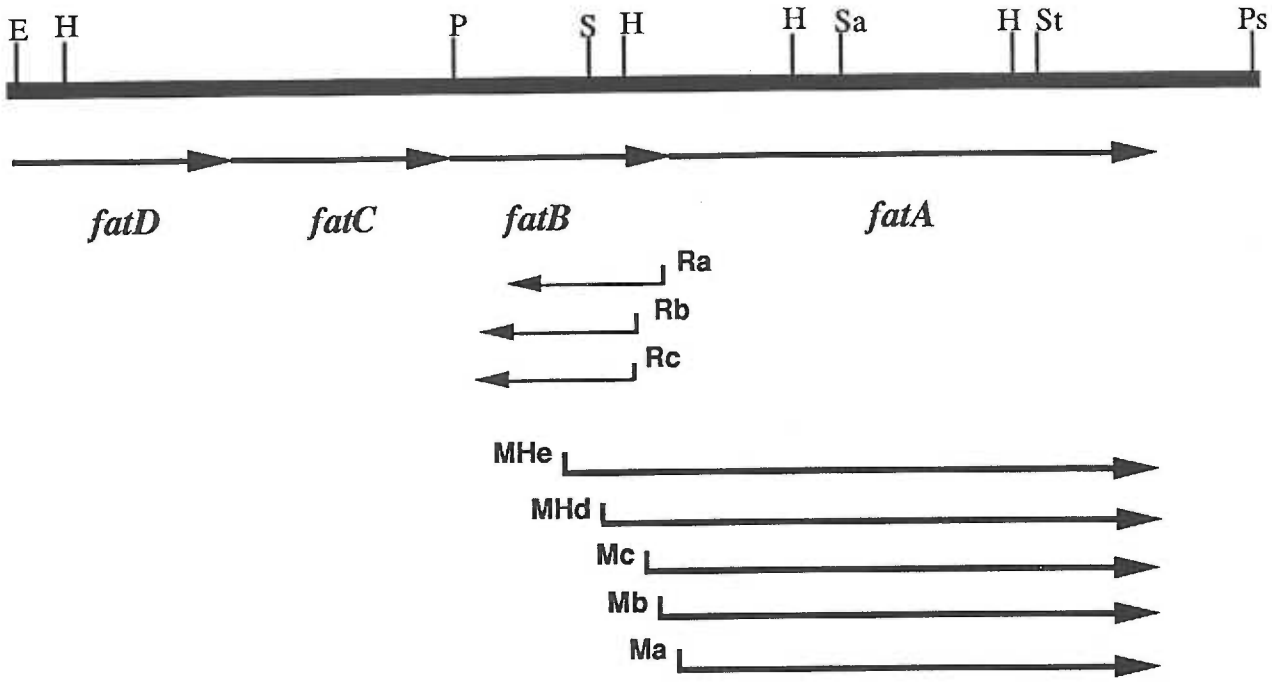


Fig. 8. Physical and genetic map of pJM1 iron transport region and of *fatA* and RNAα transcripts from start sites determined by primer extension.The orientation of the arrows indicates the direction of the transcripts. Ra, Rb, Rc: RNAα start sites. Ma, Mb, Mc, MHd, MHe: *fatA* start sites. E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I; Sa, *Sac*I; St, *Stu*I; Ps, *Pst*I.

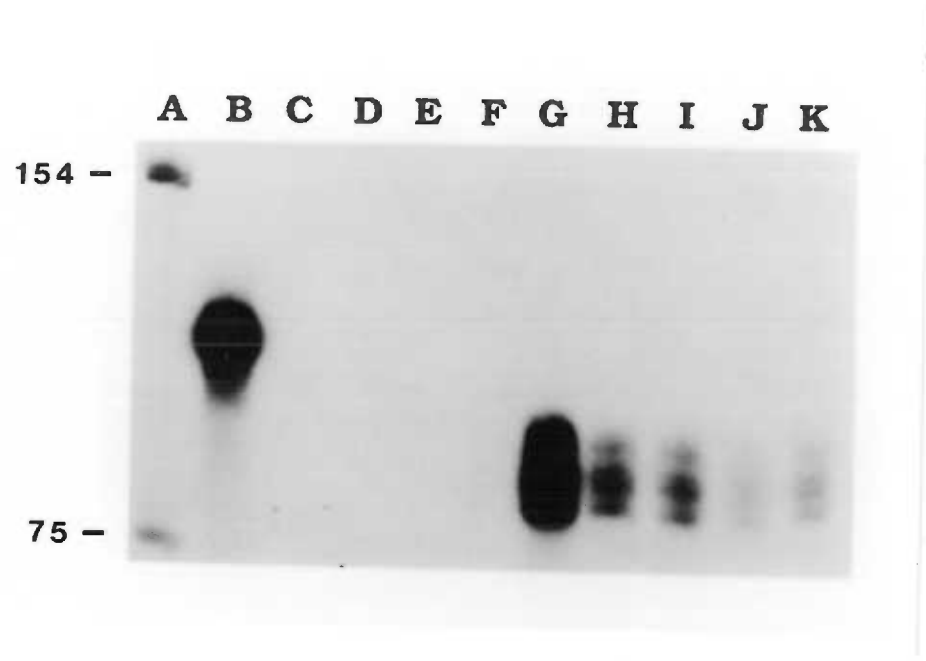


Fig. 9. RNase protection study of the regulation of *fatA* expression by Fur. Riboprobe of 94 nucleotides was made from pMET13.1 linearized with *Cla*I and transcribed by T3 RNA polymerase. Lane: A, molecular weight marker; B, riboprobe without RNase treatment; C, riboprobe treated with RNase; D, F, H, J, RNA harvested from strains grown in iron-rich media; E, G, I, K, RNA harvested from strains grown under iron-limiting conditions; D and E, *V. anguillarum* strain H775-3a (plasmidless); F and G: Strain H775-3a (pJHC-T7); H and I, *E. coli* BN4020 (pJHC-T7); J and K, strain BN4020 (pJHC-T7, pMH15).

Fig. 10. Immunoblot analysis of FatA protein in *V. anguillarum* and in *E. coli*. Lanes A, C and E, bacteria grown under iron-limiting conditions. Lanes B, D and F, bacteria grown in iron rich media. Lanes A and B, *E. coli* BN4020 (pJHC-T7, pMH15). Lanes C and D, *E. coli* Bn4020 (pJHC-T7). Lanes E and F, *V. anguillarum* H775-3a (pJHC-T7).

PAPER #3

ANTISENSE RNA REGULATION OF THE IRON TRANSPORT PROTEIN GENE *fatB* IN *VIBRIO ANGUILLARUM*

Lillian S. Waldbeser and Jorge H. Crosa

SUMMARY

The expression of *fatB*, an essential iron transport gene, is repressed under iron-rich conditions. Furthermore, the level of *fatB* transcripts is found to be inversely proportional to that of an antisense RNA, RNAα that is homologous to two-thirds of the *fatB* coding region, and is synthesized under iron-rich conditions. We have cloned and characterized the iron regulated RNAα promoter by fusing the 5' terminus of RNAα and the upstream sequences, to a promoterless chloramphenicol transacetylase gene. This construct has promoter activity only on the antisense strand,we therefore used it for determination of RNAα start sites by primer extension because there was a question that the site mapped previously may have been detecting endonuclease sites instead of start sites. Kinetic studies in the presence of rifampicin demonstrated that an important step in the regulation of *fatB* expression by the antisense RNA is at the transcript level.

Abbreviations: bp, basepair(s); EDDA, ethylene-diamine-di(O-hydroxyphenyl) acetic acid; kDa, kilodalton(s); MIC, minimal inhibitory concentration; min, minute(s); mRNA, messenger RNA; Nalr, nalidixic acid resistant; nt, nucleotide(s).

INTRODUCTION

The pJM1 plasmid-encoded iron uptake system of *Vibrio anguillarum* 775 specifies four proteins (FatA, FatB, FatC and FatD) that are essential for the transport of iron from the environment into the bacterial cytosol(1, 2, 3). FatB is a 40 kDa protein which is the putative periplasmic component of the iron transport system. Analysis of the *fatB* nucleotide sequence revealed striking homology to its counterpart of the vitamin B12 and other TonB dependent iron transport systems, such as ferrichrome, aerobactin, coprogen, dicitrate and enterchelin (3).

The synthesis of the outer membrane protein, FatA , is known to be expressed under iron-limiting conditions (4), and recently it was found that iron represses the expression of *fatA* and *fatB* at the RNA level (Salinas, Waldbeser and Crosa, manuscript in preparation). Subsequently, complementation studies identified a negative regulator, an antisense RNA (RNAα) that is encoded in the *fatB* region. RNAα was found to inhibit the expression of *fatA* post-transcriptionally, primarily by inhibiting the translation of the *fatA* messages (Waldbeser et al., manuscript submitted for publication).

The start sites for the 650 nt RNAα were mapped to the 3' terminus of *fatB* (Salinas et al., manuscript in preparation). RNAα was homologous to approximately two-thirds of the *fatB* gene. In this paper, we report the isolation of the RNAα promoter and show that RNAα negatively regulates the expression of the *fatB* gene.

RESULTS

Iron concentration versus the level of RNAα in the regulation of *fatB*

We have recently identified an antisense RNA that is expressed in iron-rich conditions (Salinas, Waldbeser and Crosa, manuscript in press). This antisense RNA, RNAα, is encoded in the *fatB* region, and it acts as a negative regulator of *fatA* expression (Waldbeser et al., manuscript in preparation). To understand the relationship between iron, *fatB* mRNA and RNAα, we analyzed the *fatB* mRNA and RNAα level by RNase protection in RNA prepared from *V. anguillarum* strain H775-3a (pJHC-T7) containing the pJM1 iron-uptake region, grown in various concentrations of ferric ammonium citrate. We found that in M9 minimal medium, no RNAα transcript was detected, but as increasing amount of ferric ammonium citrate was added, the level of RNAα increased in direct proportion to the amount of ferric ammonium citrate in the medium (Fig. 2a). Conversely, using the riboprobe made from the 3' region of *fatB* (pJHC-S400), *fatB* messages were found to be at a very high level from cells grown in M9 minimal media. As ferric ammonium citrate was added, the *fatB* mRNA started to decrease rapidly (Fig.2b). Therefore increasing iron concentration led to an increase in RNAα level, and a concomitant decrease of *fatB* mRNA.

RNAα regulation of *fatB* expression

In order to examine the effect of the antisense RNA on *fatB* expression, we made a series of plasmids and transferred them to the*V. anguillarum* strain H775-3a which harbors the plasmid pJHC-T7

containing pJM1 iron transport region. Plasmid pJHC-A122 contained RNAα sequences (Fig. 1a), cloned at the *Hin*dIII site immediately downstream of the *tet* promoter of pACYC184. The RNAα transcripts were driven by the *tet* promoter and are thus not regulated by iron (Fig. 3b, lanes G-N). Plasmids pJHC-LW87, pJHC-LW95 and pJHC-LW96 were derivatives of pJHC-A122. Plasmid pJHC-LW95 contained an Ω fragment, with transcription termination signals and translation stop signals, inserted at the *Hin*dIII site in *fatD* (Fig. 1b). Plasmid pJHC-LW87 had the Ω fragment inserted at the *Pvu*I site of *fatB*, while pJHC-LW96 had the Ω fragment inserted at the *Hin*dIII site of *fatB*. RNA were prepared from strains H775-3a (pJHC-T7), H775-3a (pJHC-T7, pJHC-A122), H775-3a (pJHC-T7, pJHC-LW87), H775-3a (pJHC-T7, pJHC-LW95) and H775-3a (pJHC-T7, pJHC-LW96). RNase protection experiments were performed using riboprobe from the 3' region of *fatB* (pJHC-LW210) that detected *fatB* messages, and from pJHC-S400 that detected RNAα transcripts (Fig. 1a). *fatB* transcripts were detected only from RNA harvested from strain H775-3a (pJHC-T7), grown in iron-limiting medium, where RNAα was not detected. Whereas under iron-rich conditions, RNAα was observed (Figs. 3a and 3b, lanes E and F) but *fatB* was not expressed. In strains H775-3a (pJHC-T7, pJHC-A122), H775-3a (pJHC-T7, pJHC-LW95) and H775-3a (pJHC-T7, pJHC-LW87), where an excess of RNAα was made, the *fatB* mRNA level was greatly diminished (Figs. 3a and 3b. lanes G, H, I, J, M and N). Conversely, the level of *fatB* mRNA was high in strian H775-3a (pJHC-T, pJHC-LW96) where the transcription of RNAα from pJHC-LW96 was blocked by an Ω fragment inserted between the *tet* promoter and the pJM1 sequences (Figs. 3a and 3b, lanes K and L).

These RNase protection studies with RNAα under the control of an external promoter, demonstrated that indeed,it is the presence of RNAα that is concomitant with a decrease in the level of *fatB* transcripts. We also noticed that in strains H775-3a (pJHC-T7, pJHC-A122) and H775-3a (pJHC-T7, pJHC-LW96), the *fatB* expression was no longer iron regulated (Fig. 3a, lanes G, H, K and L). A possible explanation is that a portion of the *fatB* transcripts may be made from the pJHC-A122 clone and from its derivative pJHC-LW96. In pJHC-LW95 and pJHC-LW87, the transcription of *fatB* was blocked by the Ω fragment, and the *fatB* mRNA observed (transcribed from pJHC-T7) was iron regulated.

Primer extension was therefore performed to map the start sites of *fatB* mRNA. We detected six species of RNA molecules, with the major species starting at a site that was 124 nucleotides from the *fatB* translation start codon (Fig. 4). The start site of the smallest species was 44 nucleotides from the *fatB* translation start codon, and the largest species started 152 nucleotides from the *fatB* translation start codon.

Determination of RNAα start sites and cloning of its promoter

Possible mechanisms by which RNAα can lead to a decrease of *fatB* mRNA may necesitate the interaction of *fatB* mRNA and RNAα through hybrid formation. We have recently determined the probable start sites for RNAα as Ra, Rb and Rc (Salinas et al., manuscript in preparation, and also shown in Fig. 4). These start sites were determined from RNA harvested from a strain harboring a plasmid that contained both *fatB* and RNAα sequences. Since *fatB* mRNA-RNAα

interaction may expose ribo-endonuclease sites along the hybrid structure, the RNAα start sites that mapped previously, may be in reality ribonuclease cleavage sites.

In order to assess whether this was the case, and to locate and characterize the RNAα promoter, we constructed a recombinant plasmid pJHC-LW217. The plasmid contained an 857 bp pJM1 *Hin*dIII fragment, which extended from the *Hin*dIII site at the 3' end of *fatB* to the *Hin*dIII site at the 5' region of *fatA* (Fig. 1). This fragment was cloned immediately upstream of a promoterless chloramphenicol transacetylase (*cat*) gene in the vector pKK232-8. Promoter activity was assessed by the ability of *E. coli* HB101 to grow in media containing chloramphenicol. The strains were grown in M9 minimal media with 30 μg/ml chloramphenicol and varying concentrations of ferric ammonium citrate or EDDA. Promoter activity was detected from the nonsense strand in the strain carrying pJHC-LW217 where the DNA orientation was from *Hin*dIII (*fatA*) to *Hin*dIII (*fatB*) to *cat*. The *E. coli* HB101 (pJHC-LW217) strain was able to grow in M9 media with chloramphenicol. However, we observed no difference in growth when ferric ammonium citrate or EDDA was added (Table 1). E. coli HB101 (pJHC-LW218) did not grow in media containing chloramphenicol (Table 1), which meant no promoter activity from the sense strand i.e. from the direction of *Hin*dIII (*fatB*) to *Hin*dIII (*fatA*) to *cat*, when the fragment was cloned in the opposite direction. We also tested these strains in M9 media with 1.5 mg/ml ampicillin and without any antibiotics added to the media. The strain harboring pJHC-LW218 was show to be viable in the media by its ability to grow

in the M9 media with or without ampicillin, and with ferric ammonium citrate or with EDDA.

By using this construct, in which the sense RNA is not transcribed, we were able to verify by primer extension,the accuracy of the locations of RNAα start sites mapped previously. Using RNA harvested from *V. anguillarum* H775-3a (pJHC-LW217) (Fig. 1A) we were able to map the start sites to within six nucleotides from the previously mapped location for site A, and to within one nucleotide for sites B and C (Fig. 4).

Kinetics of RNAα levels in the presence of rifampicin

Since at steady state, the level of *fatB* transcripts are decreased in the presence of RNAα, there is a possibility that the interaction with RNAα may lead to the instability of *fatB* mRNA. To investigate this possibility, RNA was prepared from rifampicin treated cultures of *V. anguillarum* strains H775-3a (pJHC-T7), H775-3a (pJHC-T7, pJHC-A122) and H775-3a (pJHC-T7, pJHC-LW96). Using a riboprobe from the 3' region of *fatB* (pJHC-LW210) (Fig. 1a), we found that in strain H775-3a harboring the plasmid pJHC-T7, with the pJM1 iron transport region, and the strain H775-3a that harbored pJHC-T7 and the plasmid pJHC-LW96, where the RNAα transcription was blocked by the Ω fragment, the *fatB* transcripts were at a high level at time 0. The level increased very slightly for 4 min., then it started to decrease gradually, with those from H775-3a (pJHC-T7, pJHC-LW96) decreasing more rapidly (Fig. 5a). The *fatB* transcripts from the strain H775-3a (pJHC-T7, pJHC-A122) on the other hand, was at a much lower level at time 0. The level rose very sharply between 0 - 2 min,

and peaked at 4 min. Then it began to decline gradually at the same rate as that from strain H775-3a (pJHC-T7, pJHC-LW96). The data from time points after 4 min demonstrated that RNAα did not cause instability of the *fatB* transcript. The *fatB* mRNA levels from 0 - 4 min. indicate that RNAα may interfere with the transcription of *fatB*. However, the analysis of RNAα in these rifampicin treated samples does not support this contention. We found that RNAα was at a high level at time, 0. Then between 0 - 2 min, it plummetted to one tenth of the level at time 0, and it was maintained at a steady level thereafter. This sharp difference between RNAα at time 0 and at 2 min., may account for the low level of *fatB* transcripts at 0 min. and a dramatic increase between 0 - 2 min.

Another possibility for the low *fatB* transcript level in the interval 0 - 2 min. may be due to the high level of RNAα competing with the probe for the duplex formation with *fatB* mRNA. RNA from rifampicin treated H775-3a (pJHC-T7, pJHC-A122) cells, were subjected to formaldehyde agarose gel electrophoresis. The Northern blot was probed with a riboprobe from the 5' terminus of *fatB* (pJHC-LW260) (Fig. 1a). This probe is from a region just upstream of where RNAα terminates. We found that the *fatB* transcript level was indeed low at time 0 and then increased gradually and peaked at 5 min (Fig, 6). These data showed that the initial low *fatB* mRNA level was not due to the presence of large amount of RNAα out competing the probe for the *fatB* mRNA in hybrid formation. The low level that we observed in the RNase protection analysis was therefore valid.

Fur regulation of *fatB* expression

We have recently shown that there is a chromosomal *fur*-like gene in *V. anguillarum* (Waldbeser et al., manuscript in preparation). Since *fatB* transcripts in the wild type are only seen under iron-limiting conditions, it is possible that the iron responsive Fur-like repressor mechanism may also be employed to regulate the *fatB* gene. To investigate this possibility, RNase protection studies were conducted to detect *fatB* mRNA levels in RNA samples prepared from strains*V. anguillarum* H775-3a harboring pJHC-T7, the clone encoding the pJM1 iron transport region, *E. coli* BN2040 (Fur^-) harboring pJHC-T7 and *E. coli* BN2040 harboring pJHC-T7 and pMH15, a plasmid containing the *E. coli fur* gene. The bacteria were grown in iron-rich and in iron-limiting conditions. In the Fur^- strain, *E. coli* BN2040, the *fatB* transcripts were at the same level in RNA from cells grown in iron-rich or in iron-limiting conditions. In the Fur^+ strain, *E. coli* BN2040 (pMH15), there was a slight decrease in the *fatB* mRNA level from cells grown in iron-rich medium, when compared with that from cells grown in iron-limiting medium (Fig. 7). We feel that the slight difference in these *fatB* mRNA levels is not significant, and that the *E. coli* Fur does not function efficiently in the regulation of *fatB* expression.

DISCUSSION

Although we have cloned the *V. anguillarum* chromosomal *fur*-like gene, we found that the *E. coli fur* product did not significantly repress the transcription of *fatB* mRNA in the presence of iron. However, both the ferric ammonium citrate studies and the RNase protection analysis of *fatB* and RNAα transcripts in the constructs where RNAα was directed by an external promoter, clearly demonstrated that it is the presence of RNAα and not iron that is responsible for a decrease in the *fatB* mRNA level. Therefore the regulation of *fatB* expression by iron appears to be largely due to the presence of an antisense RNA, rather than a Fur type of regulatory mechanism.

RNase protection studies showed that the *fatB* transcript was expressed only in iron-limitation conditions, while RNAα was transcribed only in iron-rich conditions. In order to study the effect of RNAα, the *fatB* region which also encodes RNAα, was cloned downstream of the *tet* promoter for RNAα to be expressed independently of the iron concentration. It can be clearly seen that the Ω fragment at the *fatD Hin*dIII site or at the *fatB Pvu*I site did not block the transcription of RNAα., and the *fatB* mRNA was low in these samples. In the samples where the transcription of RNAα is blocked, the *fatB* messages returned to a high level. The data clearly demonstrate that RNAα inversely affect the *fatB* mRNA level.

From the kinetics of *fatB* mRNA level in rifampicin treated cells, at first glance, the data suggested that RNAα may affect *fatB* at the transcription initiation level. The kinetics of RNAα levels in the same rifampicin treated samples, presented us with a more plausible

alternative. RNAα may actually affect the *fatB* mRNA stability, therefore the *fatB* transcript level was low when RNAα was in abundance.When RNAα level fell to one tenth of its initial level, its effect was no longer observable. It is also possible that the antisense RNAα interacts with the *fatB* message that is in the process of being transcribed, causing the latter to terminate prematurely or preventing it from elongating. The apparent induction effect at the time 0-2 mins, in the strain harboring pJHC-T7, the clone containing the pJM1 iron transport region, and the clone pJHC-A122 which encodes RNAα, may be because the *fatB* messages were elongating without being destabilized. In conclusion, it is possible that RNAα could affect the *fatB* mRNA level by interfering with the transcription of *fatB* but the kinetics if the rifampicin treated strain showed that the mechanism is more likely to be via message instability or via prevention of elongation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

V. anguillarum strain H775-3a was a Nal^r derivative of the plasmid-less strain H775-3 (13) generated by the method described by Miller (14), was used as recipient for the recombinant plasmids. *V. anguillarum* strain 775::Tn*1*-6 carried plasmid pJHC9-8 which was generated by Tn*1* insertion of the wild type plasmid pJM1, resulting in deletion of the iron uptake region (15). *E. coli* BN4020 (AB1157 Δ*lacU169 gal*K *fur*::Tn*5* Km^r) (16), *E. coli* HB101 (17) and JM109 (18) were used as

transformation hosts for plasmid construction and for DNA preparation. *E. coli* MM294 harboring the plasmid pRK2013 (19) was used as helper in conjugative transfer of recombinant clones into *V. anguillarum*. Plasmid pJHC-T7 carrying the iron uptake region of pJM1 has been described (20). pJHC-A122 contained pJM1 iron transport sequences cloned into the *Hin*dIII site of the vector pACYC184 (21). pJHC-LW87, pJHC-95 and pJHC-LW96 were Ω fragment insertion derivatives of pJHC-A122. Plasmid pHP45 (22) was the source of the Ω fragment. pMH15 contained the *E. coli fur* gene (23) cloned in pACYC184. pJHC-LW217 had the 857 bp HindIII fragment cloned immediately upstream of the promoterless cat gene in pKK232-8 (28). pJHC-S400 carried a 135 bp *Sal*I-*Hin*dIII fragment from the 3' region of the *fatB* gene, cloned into pSK (Stratagene). pJHC-LW210 was a *Bal*31 derivative of pJHC-S400. pJHC-LW260 contained a 191 bp *Tha*I-*Rsa*I fragment from the 5' terminus of the *fatB* gene, cloned into pSK.

RNA Isolation and Northern Blot Hybridization

V. anguillarum strains were grown in M9 minimal media containing 100 mg/ml ferric ammonium citrate til 1 OD_{600} at 0.5, or for iron-limiting condition the bacteria were grown in M9 media till OD_{600} at 0.25, then 10 μM EDDA was added and the bacteria were allowed to grow till OD_{600} at 0.5. Total RNA were prepared according to the hot phenol method as described by van Gabian et al. (24). RNA were subjected to 1% formaldehyde-agarose gel electrophoresis and

transferred to nylon membranes. The blots were analysed with riboprobes as described by Sambrook. Fritsch and Maniatis (25). Prehybrization and hybridization were at 63°C.

RNase Protection

RNase protection studies were performed as described by Krieg (26). Total RNA and labelled riboprobes were allowed to hybridize at 45°C overnight. RNase A (Boehringer Mannheim Biochemicals) and RNase T1 (Sigma Chemical Co.) treatment was at 34°C 30 min. Proteinase K (Boehringer Mannheim Biochemicals) treatment was at 37°C 15 min. Treated samples were extracted with phenol, chloroform-isoamyl alcohol and ethanol precipitated. Samples were analyzed in 6% urea acrylamide gel. Labelled *Hin*fI digested pBR322 fragments were used as molecular weight markers.

Riboprobes

Labelled riboprobes were made by in vitro transcription of linearized plasmid templates as described by melton (27), using T3 or T7 RNA polymerases (Bethesda Research Laboratories) and [α^{32}P]ATP.

Primer Extension

For RNAα, a synthetic oligonucleotide 5'-CGTATTGTTACCGAG-3' complementary to the region near the *Hin*dIII site in the *fatB* gene was used. For *fatB*, a synthetic oligonucleotide 5'-GGTGCTCTTAAACAT-3' complementary to the 5' terminus of *fatB* was used. The oligonucleotides were end labelled using T4 polynucleotide kinase (New England Biolab.) and [γ^{32}P]ATP, as described by Sambrook,

Fritsch and Maniatis (25). The end-labelled oligonucleotides were annealed with 30μg total RNA at 42°C, and primer extension was perfermed as described by Ghosh (29), using avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.). The same olignucleotides were used as primers to generate sequencing ladders by the dideoxy chain termination method of Sanger (30).

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The authors wish to thank Drs. E. W. Barklis and S. M. Landfear for helpful discussions. Many thanks to Dr. L. A. Actis for suggestions and for proof-reading the manuscript, and to J. Comer for helping with computer programs.

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Table 1. Antibiotic resistance of *E. coli* HB101 (pJHC-LW217) and (pJHC-LW218)

	Number of bacterial colonies					
	pJHC-LW218			pJHC-LW217		
	0AC	Ap	Cm	0AC	Ap	Cm
50 μg/ml FAC	692	540	0	626	680	418
25 μg/ml FAC	372	518	0	446	444	284
10 μg/ml FAC	429	580	0	400	590	336
M9 only	522	554	0	652	794	370
2 μM EDDA	415	448	0	580	468	300
4 μM EDDA	353	193	0	416	542	328
6 μM EDDA	203	40	0	326	240	0
8 μM EDDA	0	0	0	240	0	0
10 μM EDDA	276	180	0	58	0	0
20 μM EDDA	0	0	0	41	0	0
40 μM EDDA	0	0	0	0	0	0

0AC, no ampicillin or chloramphenicol added.

Ap, 1.5 mg/ml ampicillin added.

Cm, 30 μg/ml chloramphenicol added.

FAC, ferric ammonium citrate.

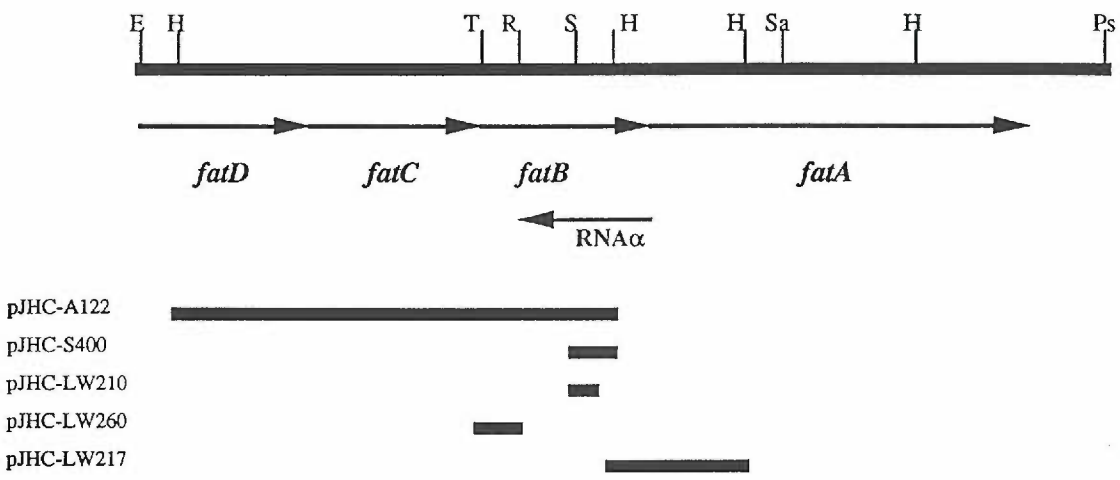
EDDA, ethylenediamine-di(*O*-hydroxyphenyl) acetic acid.

FIG. 1. (A) Physical and genetic map of pJM1 iron transport region and of regions subcloned. E, *Eco*RI; H, *Hin*dIII; T, *Tha*I; R, *Rsa*I; S, *Sal*I; Sa, *Sac*I; Ps, *Pst*I. The orientation of the arrows indicates the direction of the transcripts.

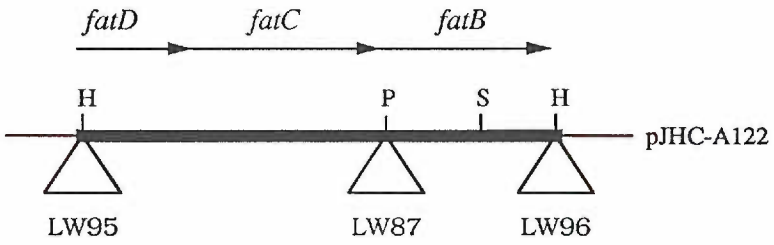
(B) Restriction map of cloned pJM1 regions in pJHC-A122. Solid bar represents pJM1 DNA; thin line represents pACYC184 DNA. Open triangles are Ω fragment insertions. H, *Hin*dIII; P, *Pvu*I; S, *Sal*I.

Fig. 1

(A)


E H T R S H H Sa H Ps
fatD fatC fatB fatA
RNAα
pJHC-A122
pJHC-S400
pJHC-LW210
pJHC-LW260
pJHC-LW217

(B)


fatD fatC fatB
H P S H
pJHC-A122
LW95 LW87 LW96

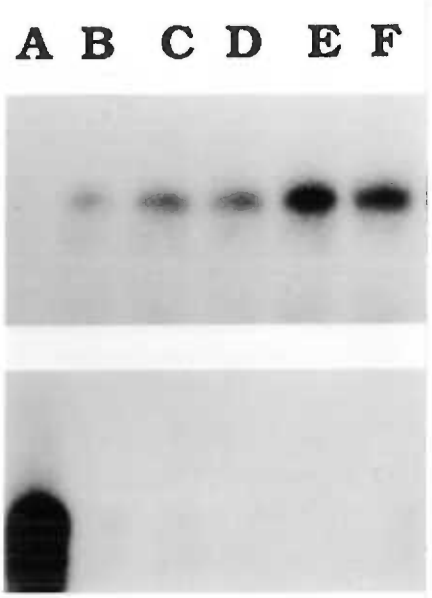


Fig. 2. Analysis of RNAα and fatB mRNA in varying levels of iron concentration.
(A) Detection of RNAα by RNase protection method, using a 135 nucleotide riboprobe made from pJHC-S400 linearized with *Hin*dIII and transcribed by T7 RNA polymerase.
(B) Dectection of *fatB* mRNA by RNase protection method, using a 133 nucleotide riboprobe made from pJHC-S400 linearized with *Sal*I and transcribed by T3 RNA polymerase.
RNA were harvested from *V. anguillarum* H775-3a (pJHC-T7). Lanes: A, bacteria grown in M9 minimal medium only; B-H, bacteria grown in M9 minimal media with 2, 4, 8, 10, 15, 20 and 25 μg/ml ferric ammonium citrate respectively.

Fig. 3. RNase protection studies.

(A) Detection of *fatB* mRNA using a73 nucleotide riboprobe made from pJHC-LW210 linearized with *SalI* and transcribed by T3 RNA polymerase.

(B) Detection of antisense RNA, RNAα using a 135 nucleotide riboprobe made from pJHC-S400 linearized with *Hin*dIII and transcribed by T7 RNA polymerase.

Lanes: A, riboprobe without RNase treatment; B, riboprobe treated with RNase; C, E, G, I, K, M, RNA harvested from bacteria grown in iron-rich media; D, F, H, J, L, N, RNA harvested from bacteria grown in iron-limiting media; C and D, *V. anguillarum* 775::Tn*1*-6 (pVK102); E and F, V. anguillarum H775-3a (pJHC-T7); G and H, H775-3a (pJHC-T7, pJHC-A122); I and J, H775-3a (pJHC-T7, pJHC-LW95); K and L, H775-3a (pJHC-T7, pJHC-LW96); M and N: H775-3a (pJHC-T7, pJHC-LW87).

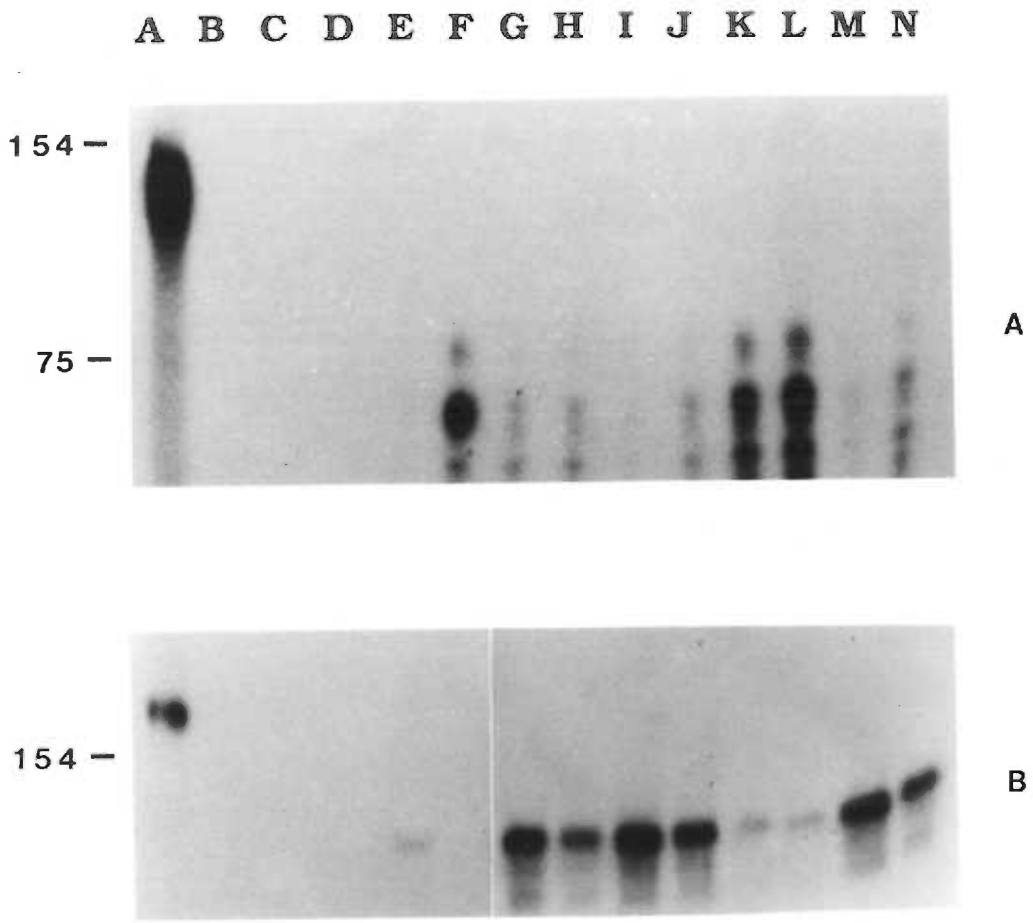

A B C D E F G H I J K L M N
154
75
A
154
B

Fig. 4. RNAα start sites mapped with *Hin*dII fragment and *fatB* start sites as determined by primer extension. Fa, Fb, Fc, Fd, Fe and Ff are *fatB* start sites. Ra, Rb and Rc are RNAα start sites previously determined. ^: RNAα start sites mapped with *Hin*dIII fragment. Boxed sequence is the coding region for FatB. Direction of arrows is the direction of the transcripts. SD, Shine-Dalgarno sequence.

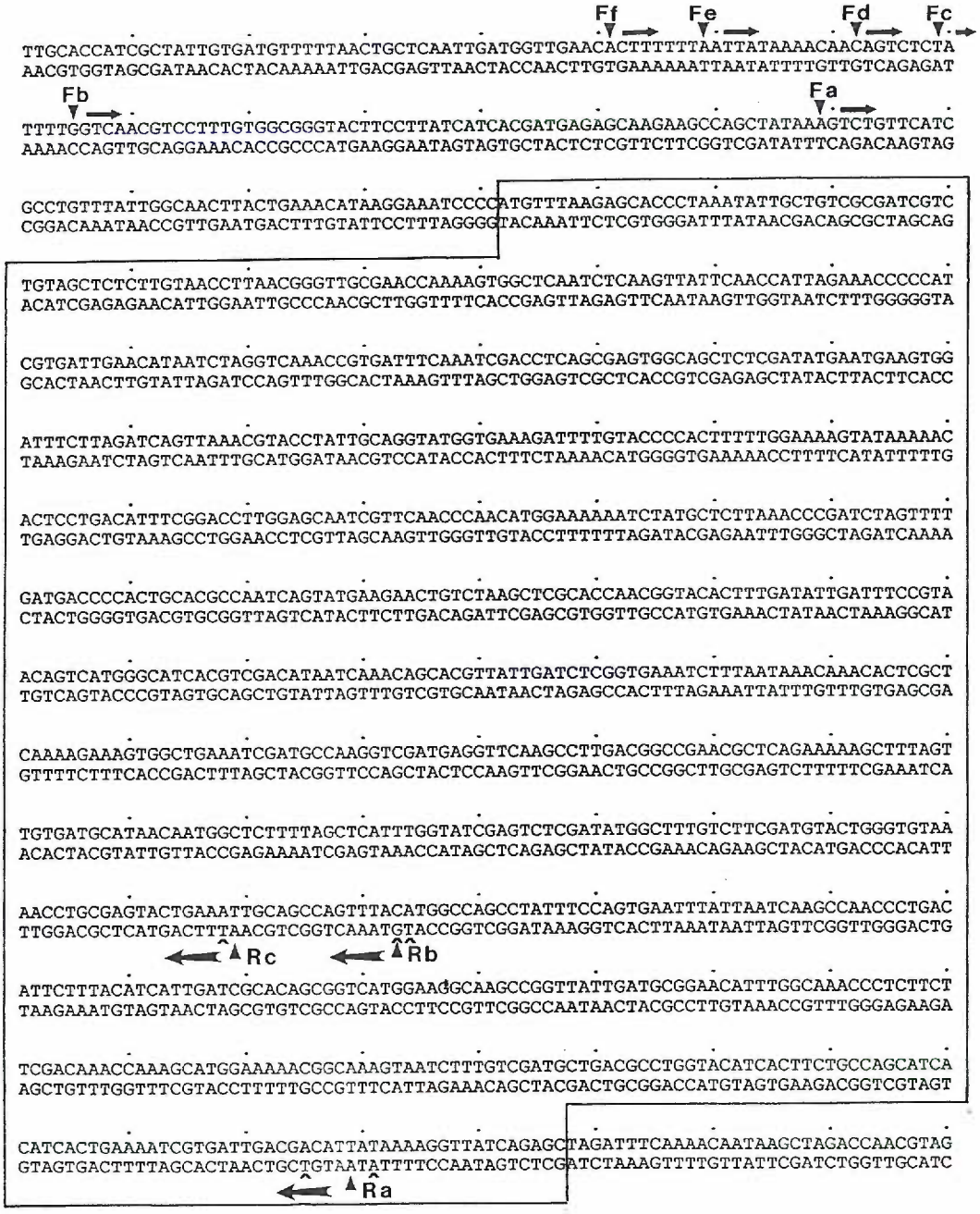
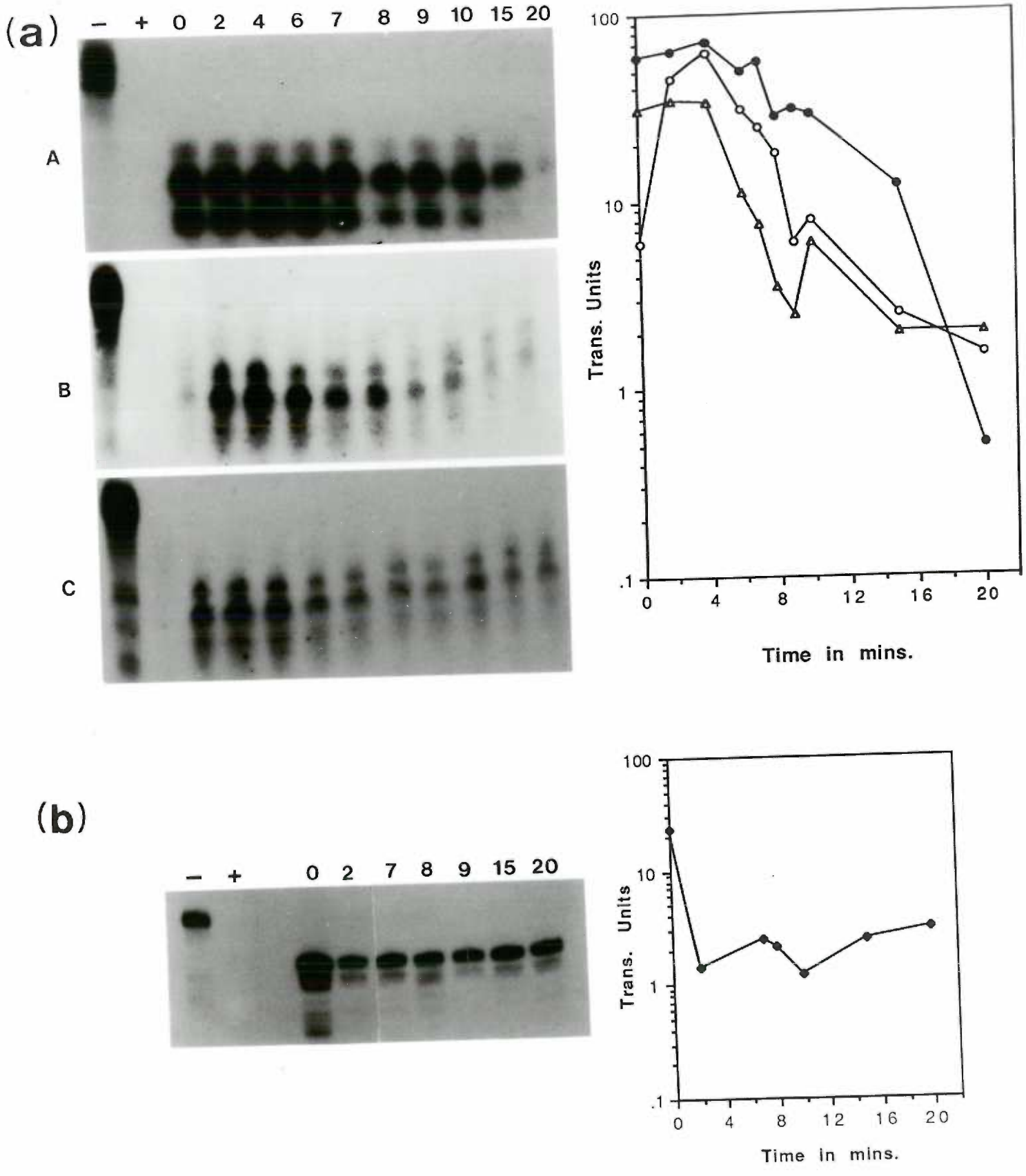

Ff
Fe
Fd
Fc
Fb
Fa
Rc
Rb
Ra

Fig. 5. Rifampicin studies.

(A) Detection of *fatB* mRNA by RNase protection method, using a 73 nucleotide riboprobe made from pJHC-LW210 linearized with *SalI* and transcribed by T3 RNA polymerase. RNA were harvested from bacteria grown in iron-limiting conditions. Lane -, riboprobe without RNase treatment. Lane +, riboprobe subjected to RNase treatment. Lanes 0-20, the number of minutes after rifampicin added. Panel A, RNA from *V. anguillarum* H775-3a (pJHC-T7). Panel B, RNA from H775-3a (pJHC-T7, pJHC-A122). Panel C, RNA from H775-3a (pJHC-T7, pJHC-LW96). The RNase protection experiments were performed four separate times. The data shown are from one of the experiments, and are representative of data from the other experiments. The autoradiographs were quantitated using a densitometer. The graph with solid circles, data for half-life of *fatB* mRNA in H775-3a (pJHC-T7); open circles, in H775-3a (pJHC-T7, pJHC-A122); open triangles, in H775-3a (pJHC-T7, pJHC-LW96).

(B) Detection of RNAα by RNase protection method, using a 135 nucleotide riboprobe made by linearizing pJHC-S400 with HindIII and transcribed with T7 RNA polymerase. The experiment was performed two separate times, once by RNase protection and once by northern blot. RNA was harvested from *V. anguillarum* H775-3a (pJHC-T7, pJHC-A122) grown in iron-rich medium. Lanes - and +, riboprobe without and with RNase treatment. Lanes 0-20, the number of minutes of rifampicin treatment. Solid diamonds, the densitometer values of the autoradiograph were plotted to determine the half-life of RNAα.


(a)
– + 0 2 4 6 7 8 9 10 15 20
A
B
C
100
10
1
.1
Trans. Units
0 4 8 12 16 20
Time in mins.
(b)
– + 0 2 7 8 9 15 20
100
10
1
.1
Trans. Units
0 4 8 12 16 20
Time in mins.

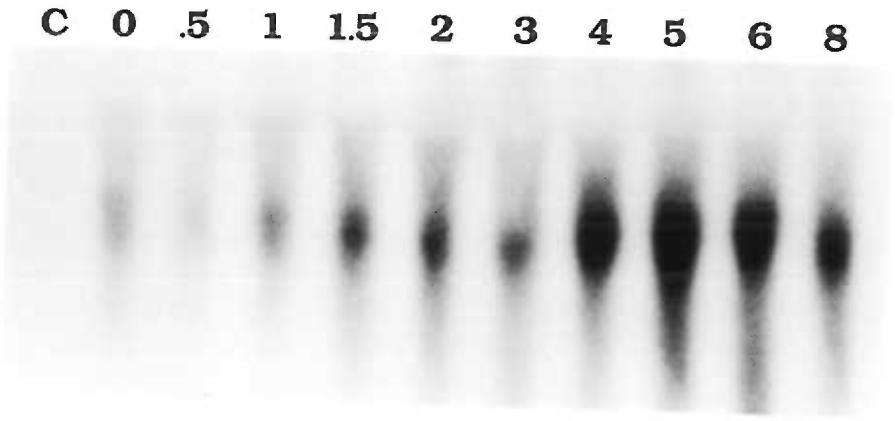


Fig. 6. Northern blot analysis of *fatB* levels. RNA harvested from *V. anguillarum* H775-3a (pJHC-T7, pJHC-A122) grown in iron-limiting condition. The 191 nucleotide riboprobe used was made from pJHC-LW260 linearized with *Psf*I and transcribed by T3 RNA polymerase. Lane C, RNA from V. anguillarum 775::Tn1-6 (pVK102). Lanes 0-8, RNA harvested after 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 8 minutes of rifampicin treatment.

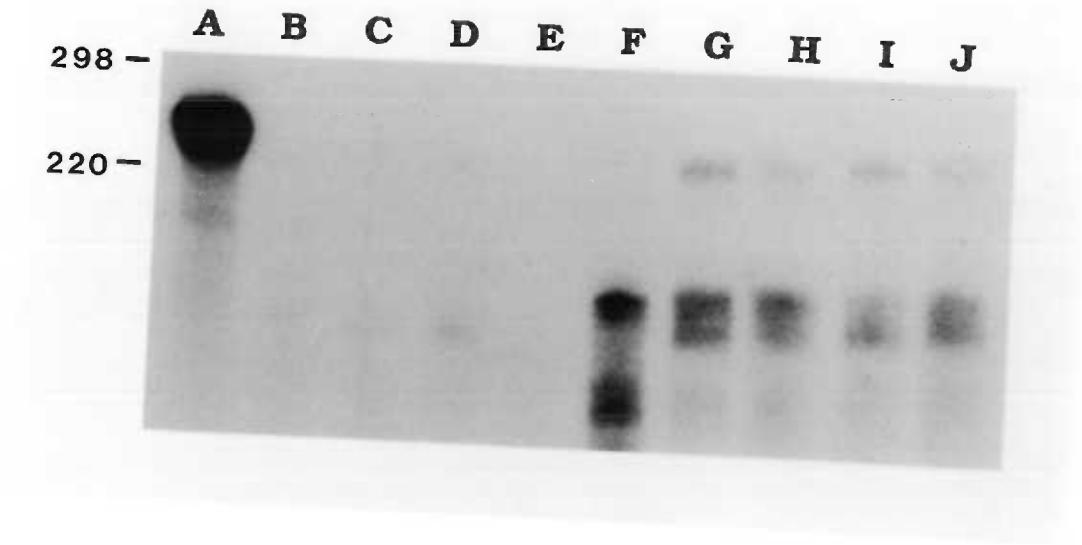


Fig. 7. RNase protection study of the regulation of *fatB* expression by Fur. Riboprobe of 191 nucleotides was made from pJHC-LW260 linearized with PstI and transcribed by T3 RNA polymerase. Lanes: A, riboprobe without RNase treatment; B, riboprobe treated with RNase; C, E, G, I, RNA harvested from bacteria grown in iron-rich media; D, F, H, J, RNA harvested from bacteria grown in iron-limiting conditions; C and D, *V. anguillarum* strain H775-3a (plasmidless); E and F, strain H775-3a (pJHC-T7); G and H, E. coli BN4020 (pJHC-T7); I and J, strain BN4020 (pJHC-T7, pMH15).

DISCUSSION AND CONCLUSIONS

The ability to acquire iron has been demonstrated to be a very important virulence factor (Payne et al, 1978; Ronins-Browne et al., 1985), and in *V. anguillarum*, the association of virulence with the plasmid pJM1 which encodes an iron-uptake system, was established (Crosa, 1980; Tolmasky et al., 1984; Wolf et al., 1986). The synthesis of the FatA outer membrane protein of the pJM1 iron uptake system was shown to be regulated by iron (Crosa et al., 1981; Actis et al., 1985), and a negative regulator of *fatA* and of *fatB*, the gene encoding the periplasmic protein of the iron transport system was located in the *fatB* region (Waldbeser et al., manuscript in preparation). Subsequently, the negative regulator was demonstrated to be an antisense RNA (Waldbeser et al., Paper 2, manuscript in preparation; Salinas et al., manuscript in preparation). The objective of my research was to study how iron regulates the expression of iron transport proteins, particularly the FatA protein, and to determine the mechanism(s) employed by RNAα in the regulation of *fatA* and *fatB*.

Complementation studies demonstrated that all four genes of the iron transport region are essential for iron uptake. The FatA protein was previously isolated as an outer membrane protein of 86 kDa, and FatB was identified as a 40 kDa protein (Actis et al., 1988). Using maxicell analysis, a 37kDa protein was identified as the product of *fatC* and a 35 kDa protein as FatD. Based on the analysis of the nucleotide sequences, the polypeptides of the *V. anguillarum* pJM1 encoded transport proteins appear to have many domains that are

homologous to several iron and vitamin B_{12} transport proteins that are TonB dependent, and therefore are members of the periplasmic-binding-protein-dependent transport (PBT) family (Ames, 1986). The transport of ferric anguibactin might therefore be very similar to those reported for the transport of ferric hydroxamate, ferric catecholate, ferric dicitrate, and vitamin B12 in *E. coli*. In addition, a "TonB box", which is present in the receptor protein of the systems (Nau et al., 1989), was also found in FatA, therefore a TonB-like energy coupling system also functions in *V. anguillarum*.

Analysis of FatA sequence revealed typical signal sequences at the amino terminal, suggesting that this protein is exported through the cytoplasmic membrane. An alignment of the complete periplasmic proteins, FhuD of the coprogen system, FecB of the citrate system, FepB of the enterobactin system and BtuE of the vitamin B12 transport system, revealed a strong homology along the entire length of the proteins with FatB and therefore further confirming FatB being a periplasmic protein. This homology is very exceptional, because inspite of the similarities in their tertiary structure, periplasmic proteins usually do not show homology to each other.

Alignment of the complete protein sequence of FatC and FatD, with integral membrane proteins of the vitamin B12 transport system, the coprogen system and the ferric enterobactin transport system, showed that FatC and FatD have extensive homology to each other and to the other hydrophobic integral membrane proteins. In certain regions, FatC and FatD have more than 50% homology to at least two of these protein when amino acids at equivalent positions are compared. This homology is remarkable, considering usually there is little

homology among integral membrane proteins of different PBT systems (Adams et al., 1989).

With the available information, one can derive a transport model that is similar to that described for other PBT proteins (Ames, 1986). The model would then place FatA as the outer membrane receptor for the ferric-anguibactin complex. After receptor binding, the ferric-anguibactin complex passes through the outer membrane into the periplasmic space where it encounters FatB. After being bound to the ferric-anguibactin complex, the FatB protein may undergo a conformational change, and is then able to interact with the two cytoplasmic membrane proteins, FatC and FatD. FatC and FatD probably form a channel for the ferric-anguibactin to pass through to the cytosol.

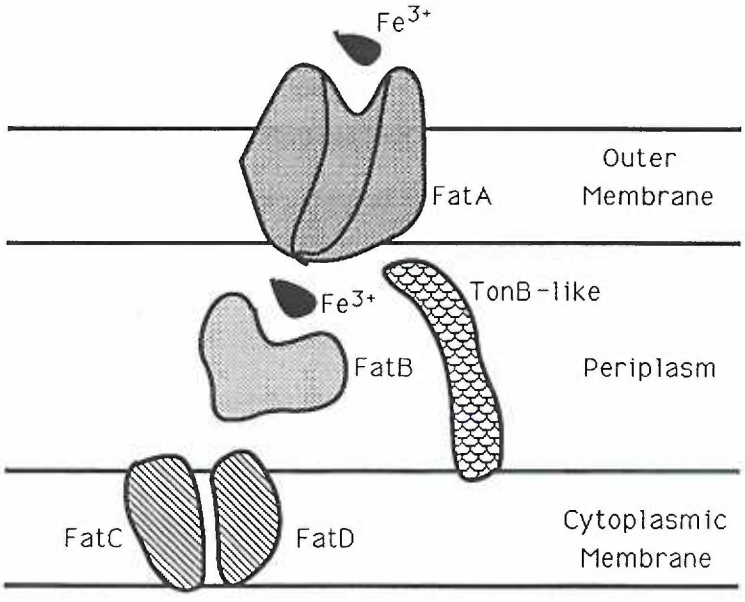


Fig. 1 A proposed model for ferric-anguibactin transport.

In all PBT systems that have been studied, ATP is required to provide energy that drives the transport system (Hobson et al., 1984; Bishop et al., 1989; Dean et al., 1989). One or two hydrophilic proteins, with ATP-binding regions, are usually associated with the integral membrane proteins. However, we were not able to locate sequences suggestive of ATP-binding sites in the pJM1 iron transport region. It is conceivable that such a gene exists and that it may be encoded in another part of the pJM1 plasmid or it may be chromosome-mediated.

We have demonstrated that iron regulates the expression of *fatA* and *fat*B genes at the RNA level. The *E. coli* repressor Fur has been reported to function in the presence of iron to negatively regulate the transcription of genes in various iron uptake systems (de Lorenzo, Giovannini et al., 1988), as well as genes encoding other virulence factor. Despite the fact that we cloned a functional *V. anguillarum* chromosomally mediated *fur*-like gene, that has some homology to the *E. coli fur*, we found that the *E. coli fur* product did not efficiently regulate the transcription of *fatA* or *fatB*.

Using a clone in which the RNAα transcription is under the control of an external promoter we demonstrated that the presence of an increased amount of RNAα leads to a decrease in synthesis of FatA. In the presence of RNAα, the steady state *fatA* mRNA level is not significantly decreased, when compared with the dramatic decrease in FatA synthesis. These 2.35 kb species of *fatA* mRNA may therefore be incapacitated for translation. Primer extension studies showed that the two major species of *fatA* mRNA were decreased in the presence

of RNAα, but the fall in the mRNA level was insufficient to account for the large decrease in the protein synthesis. The implication is therefore, that RNAα regulates *fatA* at the translation level.

Studies of *fatA* expression in the presence of increased levels of a truncated RNAα, suggests that sequences at the 5' region of RNAα are not necessary for its inhibitory function, and that for this truncated RNAα to interact with the *fatA* message, the transcript has to have initiated far upstream of the *fatA* translation start codon. The *fatA* mRNA could therefore be part of a large or a polycistronic message, that includes *fatD*, *C*, *B* and *A* genes. Studies of FatA synthesis by transposon insertion mutants revealed that insertion in any of these three genes upstream of *fatA*, results in a dramatic decrease in FatA synthesis. These data lend support to the existence of a polycistronic message. However, we have not been able to detect this large transcript. The reason may be that, like the *trp*, *lac*, *mal* and *gal* messages (Murakawa et al., 1991; Higgins et al., 1988; Kennell, 1986; Achord et al., 1974; Forchhammer et al., 1972), if ribosomes are not engaging the messages in translation, the *fat* messages may start to be processed as soon as they are being transcribed. However, if *fatA* mRNA were not part of a polycistronic message, RNAα may affect the expression of *fatA* by interacting with a positive regulator that may be essential for the translation of the *fatA* messages.

RNAα is homologous to approximately two-thirds of the coding region of *fatB*. The level of *fatB* mRNA is inversely proportional to the level of RNAα. Studies using the truncated RNAα that was under the

control of an external promoter that was not regulated by iron, showed that *fatB* is responsive to the presence of RNAα rather than the iron concentration of the medium.

The kinetic studies of *fatB* mRNA and RNAα level in bacteria treated with rifampicin, showed that the *fatB* mRNA is low initially, when RNAα level is the highest. The implication is that RNAα does affect the stability of the *fatB* message, and the effect is most noticeable when there is an abundance of the antisense RNA. The other possibility is that RNAα could inhibit the elongation of the *fatB* message that is in the process of being transcribed, or cause it to terminate prematurely. The conclusion is therefore, that RNAα may regulate *fatB* by destabilizing the *fatB* message or by inhibiting its elongation.

The conclusion of this part of my thesis studies on the regulation by iron of the iron transport genes, *fatA* and *fatB* is that iron regulates these genes at a post transcriptional level, through the action of the antisense RNA, RNAα, which is expressed in iron-rich conditions. RNAα regulates *fatA* primarily at the translation level, mostly likely by hybridizing with sequences upstream of the ribosome binding site. The subsequent conformational changes may render the ribosome binding site inaccessible. However, we cannot rule out the posibility that RNAα may also regulate the expression of *fatA* by interacting with factor(s) that may be involved in the synthesis of FatA. RNAα regulates *fatB* at the RNA level, by destabilizing the *fatB* message. At the transcription level, we have not been able to test the function of the *V.*

anguillarum Fur-like repressor system, primarily because we need a "*fur*$^{-}$" strain of *Vibrio* in order to test the system accurately, since the *E. coli* Fur system was a poor substitute for the *V. anguillarum* Fur.

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