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

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Synthesis of the 86-kDa FatA outer membrane protein is repressed under iron-rich conditions. Complementation of transposition mutants derived from clones containing the pJM1 iron uptake region revealed the existence of an antisense RNA, RNAo. This RNA is only expressed under iron-rich conditions and acts as a negative regulator of FatA synthesis, with slight but discernible decrease in the steady-state level of fatA mRNA determined by RNase protection and by Northern blot analysis. Primer extension experiments revealed that the level of several possible fatA transcripts was reduced in the presence of RNAo. In addition, we found that fatA mRNA expression is slightly reduced in the presence of Escherichia coli Fur. We have identified and cloned a chromosomally encoded fur-like gene in Vibrio anguillarum.

Many factors contribute to the success of a bacterial 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 (3, 4). The possession of a system to capture iron from the host's proteins is therefore 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 M. 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, 14). One of them, fatA, encodes an 86-kDa outer membrane protein that has specific domains homologous to several outer membrane receptor proteins that are TonB-dependent (14). Expression of this pJM1 iron transport protein is negatively regulated by iron (11). In this paper we report the finding that an antisense RNA (RNAo) and a Fur-like repressor may play a role in the iron regulation of the fatA gene expression.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The wild type strain V. anguillarum 775 was used as a source of plasmid pJM1(8). V. anguillarum 775:Tn1-6 carried plasmid pHJC9-8 which was generated by Tn1 insertion of the wild type plasmid pJM1, resulting in deletion of the iron uptake region (15). The nalidixic acid resistant plasmidless mutant strain H775-3a (14) was used to detect Fur regulation of gene expression. E. coli HB1171 (17) and E. coli JM109 (18) were used as transformation hosts for plasmid construction and plasmid DNA preparation. E. coli MM294 harboring the plasmid pRK2013 (19) was used as helper in conjugal transfer of recombination into V. anguillarum. Plasmid pJHC-T7 carrying the iron uptake region of pJM1 and its Tn3-HoHo1 transposon derivatives (mutant 20, mutant 17, and mutant 15) were described previously (20, 21). pJHC-W44 contained the positive regulatory region (Taf) of pJM1 (21). pJHC-A179 and pJHC-LW115 contained pJM1 iron transport sequences cloned into the vector pJHC-S100 (22). Clone pHJC-A122 carried pJM1 iron transport sequences cloned into the vector pACYC184 (23). The fragment harbored by plasmid pHPC4 (24) consists of a copy of the ada gene (encoding streptomyacin and spectinomycin resistances) flanked by transcription termination and translation stop signals in all three reading frames. pMH15 contained the Escherichia coli fur gene cloned in pACYC184 (25). The recombinant plasmid pMET76, isolated from a V. anguillarum library (26), carries the fur-like gene. The characteristics of plasmids pRT240, containing the ompF promoter fused to an intact lacZ gene, and pSC27.1, a pRT240 derivative containing the fur operator site, were described previously (27, 28). pJHC-S300 (29) contained an 89-base pair HindIII-Stu fragment of the fatA gene cloned into the plasmid vector pBluescript KS (Stratagene, La Jolla, CA). pMET13.1 contained a 92-base pair Bad-StaI fragment of fatA cloned in pBluescript SK, pJHC-S400 (29) carried a 134-base pair Sal-I HindIII fragment of the fatB gene cloned in pBluescript SK.

General Methods—Conjugations to V. anguillarum were carried out as described previously (20). β-Galactosidase assays were performed as described by Miller (30). Hybridization of total DNA was performed under the conditions described previously (31) using 25% formamide (low stringency) or 50% formamide (high stringency). DNA probes were labeled with [α-35S]dATP (Du Pont-New England Nuclear) by the random primer method as described by Feinberg and Vogelstein (32).

RNA Isolation and Northern Blot Hybridization—For iron-rich conditions, V. anguillarum strains were grown in modified M9 minimal medium (8) containing 100 μg/ml ferric ammonium citrate until the culture reached an A600 of 0.25, then EDDHA was added to a final concentration of 10 μM, and the bacteria were allowed to grow to an A600 of 0.5. Total RNA was prepared according to the hot phenol method (33). RNAs were electrophoresed in formaldehyde-agarose gel (34) 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 (34–36). Prehybridization and hybridization were at 63 °C.

**RNase Protection**—RNase protection studies were performed as described by Krieg and Melton (35). Total RNA and labeled riboprobes were allowed to hybridize at 45 °C overnight. RNase A (Boehringer Mannheim) and RNase T1 (Sigma) treatment was at 34 °C for 30 min. Proteinase K (Boehringer Mannheim) treatment was at 37 °C for 15 min. Treated samples were extracted with phenol, chloroform-isomyl alcohol, and ethanol precipitated. Samples were analyzed in 6% urea-acrylamide gel. Labeled riboprobes were made by in vitro transcription of linearized plasmid templates as described by Melton et al. (36), using T3 or T7 RNA polymerases (Bethesda Research Laboratories) and [γ-32P]UTP. Labeled HindIII-digested pBR322 fragments were used as molecular weight markers.

**Primer Extension**—A synthetic oligonucleotide, 5' -TACCTGAT-3' complementary to the 5' terminus of fatA was end labeled using T4 polynucleotide kinase (New England Biolabs) and [α-32P]dATP (Du Pont-New England Nuclear), as described by Sambrook et al. (34). The end-labeled oligonucleotide was annealed with 30 μg of total RNA at 42 °C, and primer extension was performed using avian myeloblastosis virus reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) as described by Ghosh et al. (37). 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. (38).

**Immunoblot Analysis**—Total cell membranes were prepared from *V. anguillarum* grown under iron-rich or iron-limiting (M9 medium with 1-5 μM EDDHA) conditions, as described previously (11). The membrane proteins were subjected to electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gels (11) and transferred to nitrocellulose membranes. The presence of FatA protein was detected using absorbed polyclonal anti-FatA antibody and horseradish peroxidase-labeled using T4 polynucleotide kinase (New England Biolabs) as described by Krieg and Melton (35). Total RNA and labeled riboprobes were made by in vitro transcription of linearized plasmid templates as described by Melton et al. (36), using T3 or T7 RNA polymerases (Bethesda Research Laboratories) and [γ-32P]UTP. Labeled HindIII-digested pBR322 fragments were used as molecular weight markers.

**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 genes, respectively (21) (Fig. 1a) in the recombinant plasmid pJHC-T7, which carried the iron uptake region of *pJM1* (20). These derivatives were then mobilized by conjugation into *V. anguillarum* H775–3a which is a nalidixic-resistant derivative of *V. anguillarum* 775 which has been cured of the plasmid *pJM1*. *V. anguillarum* strains harboring all three mutants were transport-deficient and expressed a reduced amount of FatA (21). Since transposition mutations were located upstream of *fatA*, the data suggest that *fatA* may be within a polycistronic message. In the process of carrying out complementation studies for iron transport we found that clones pJHC-A122 and pJHC-A179 (Fig. 1a) had an inhibitory effect on *fatA* expression in mutant 17 (Fig. 2a, compare lanes D and E with F). We have also found that the recombinant clone pJHC-LW115 (Fig. 1a) had no inhibitory effect on mutant 17 (data not shown). The data suggest that pJHC-A122 and pJHC-A179 encode a negative regulator of *fatA*, and the expression of this regulator requires the region 3' of the *SalI* site.

**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 *SalI* site. Alternatively, the negative regulator could be a protein encoded within the *fatB* region. The negative regulator could function directly as a repressor or it could inhibit the expression of *fatA* by titrating an activator of transcription or translation. Another possibility is that a DNA sequence that may be the binding site for a negative regulator of *fatA* may occupy the *fatB* region that is upstream of the HindIII site in *fatB* and encompasses the *SalI* site. The lack of inhibition by the clone pJHC-LW115 may be caused by the truncation of the recognition sequence for the negative regulator or the actual truncation of the negative regulatory genetic determinants.

To investigate these possibilities, constructs pJHC-LW95, pJHC-LW87, and pJHC-LW96 were made by insertion of the Δ fragment, containing transcription and translation stop signals, in pJHC-A122 either at the HindIII site in *fatD*

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**Fig. 1.** Panel a, physical and genetic map of *pJM1* iron transport region and of regions subcloned. E, EcoRI; H, HindIII; S, SalI; C, ClaI; SaI; St, StuI; P, PstI. The orientation of the arrows indicates the direction of the transcripts. 15, 17, and 20 are Tn3-HoHo1 insertions. nt, nucleotides. Panel 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 0 fragment insertions in derivatives pJHC-LW95, pJHC-LW87, and pJHC-LW96. H, HindIII; P, PstI; S, SalI.
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**FIG. 2. Analysis of FatA protein and RNAα in the complementation of mutant 17.** Bacteria were grown under iron-limiting conditions. All strains contained mutant 17. Lanes A, with pJHC-LW87; lanes B, with pJHC-LW96; lanes C, with pJHC-LW95; lanes D, with pJHC-A122; lanes E, with pJHC-A179; lanes F, mutant 17 alone. Panel a, immunoblot analysis of FatA protein. All lanes are loaded with equal amount of total membrane proteins. Panel b, RNase protection analysis of RNAα. The riboprobe was made from pJHC-S400 (Fig. 1a) linearized with HindIII and transcribed by T7 RNA polymerase. Lane G, riboprobe without RNase treatment. nt, nucleotides.

(gcngenerating pJHC-LW95) or at the PvuI site at the 5′ terminus of fatB (generating pJHC-LW87) or at the HindIII site at the 3′ end of fatB (generating pJHC-LW96) (Fig. 1b). The recombinant clones pJHC-LW95, pJHC-LW87, and pJHC-LW96 were then mobilized by conjugation into V. anguillarum H775–3a harboring the mutant 17 plasmid. FatA synthesis was determined by immunoblot of total membrane proteins from strains grown under iron limitation, since FatA is only synthesized in iron-limiting but not in iron-rich conditions (11, 12). We observed that the clones pJHC-LW87 and pJHC-LW95 still showed an ability to cause inhibition of FatA synthesis (Fig. 2a, lanes A and C). However, the inhibitory effect was abolished when the Ω fragment was inserted at the fatB HindIII site as in pJHC-LW96 (Fig. 2a, lane B). The abrogation of inhibition by the Ω insertion at the fatB HindIII site (in pJHC-LW96) ruled out the possibility of the existence of a cis binding sequence for a negative regulator. The other possibility, that the inhibitor could be a small peptide was ruled out since not only we were unable to identify any open reading frame encoded within fatB in either strain which would include the SalI site, but also no new peptides in addition to those already known were detected in in vitro transcription-translation experiments using as template a plasmid carrying the fatB region (39). Therefore, the Ω insertion studies strongly suggested that an antisense RNA may be transcribed in the the fatB region, from a promoter downstream of the SalI site, and that this antisense RNA was likely to affect the expression of the fatA gene negatively.

RNase protection studies were also performed. A riboprobe designed to detect transcripts from the noncoding strand was constructed from the SalI-HindIII fragment at the 3′ region of fatB (Fig. 1a). Where there was a decrease of the FatA protein synthesis, antisense transcripts were detected in the strains H775–3a (mutant 17 together with either pJHC-A179, pJHC-A122, pHJC-LW95, or pJHC-LW87) grown in iron-limiting medium (Fig. 2b, lanes E, D, C, and A). In strain H775–3a (mutant 17 and pHJC-LW96) where inhibition of FatA synthesis was abrogated (Fig. 2a, lane B), the antisense transcript was not detected (Fig. 2b, lane B). Under iron starvation the amount of RNAα made by mutant 17 is too low to be detected in the gel (Fig. 2b, lane F); however, there may be enough basal amount for some inhibition to occur. Comparison with lane B may not be as straightforward because the construct with the Ω fragment, although impaired in the production of RNAα, still makes sense RNA which can interact and take out of circulation the basal level of RNAα made by mutant 17 leading to a relief of repression and an increase in the FatA level. These data confirmed that the negative regulator was indeed an antisense RNA, which was designated RNAα. Under the control of its own promoter, as in mutant 17, RNAα is observed to be expressed only in iron-rich condition but not in iron limitation (29). Since RNAα was detected in strains H775–3a (mutant 17 together with either pJHC-A179, pJHC-A122, pJHC-LW95, or pJHC-LW87) under iron-limiting conditions, the RNAα transcripts were probably constitutively transcribed via a vector promoter.

Next we analyzed the effect of the antisense RNAα in the wild type context, where the FatA protein is abundantly expressed when compared with mutant 17. We found that in the presence of plasmid pJHC-A122, which encoded RNAα, the level of FatA was dramatically decreased in V. anguillarum 775, carrying the wild type plasmid pJM1, (Fig. 3, compare lanes C and D), and in the strain H775–3a harboring the recombinant clone pJHC-T7, which contained the iron uptake region of pJM1 (Fig. 3, compare lanes A and B). RNase protection studies were performed using RNA harvested from these strains. The antisense RNA was observed in RNA harvested from strains 775 (pJM1) and H775–3a (pJHC-T7), but only when the cells were grown in iron-rich media (Fig. 4a, lanes D and F). RNAα was found in both iron-rich and iron-limiting conditions in the strain carrying both pJHC-T7 and pJHC-A122, which encoded determinants for RNAα cloned at the HindIII site of pACYC184, within the tetracycline resistance gene (Fig. 4a, lanes H and I). In this case the antisense transcript must be driven by promoters of the tet gene, resulting in the constitutive synthesis of RNAα. In this clone, approximately 350 nucleotides of RNAα were deleted from the 5′ end, and the remainder of RNAα started at the HindIII site. Since wild type RNAα is 650 nucleotides long (29), our results demonstrate that less than 50% of the RNA sequence is sufficient for the inhibitory activity.

**Regulation of fatA Gene Expression by RNAα—**We used RNase protection assays to determine the levels of fatA mRNA. The riboprobe designed to detect the fatA message was constructed using the HindIII-StuI fragment from the 3′ region of fatA (Fig. 1a). The fatA mRNA was observed only in RNA harvested from cells grown under iron-limiting conditions (Fig. 4b, lanes F, H, and J). The level of fatA mRNA from the strain H775–3a (pJHC-T7, pJHC-A122), in which RNAα was synthesized constitutively was slightly but discernibly decreased in comparison with that from H775–3a (pJHC-T7) (Fig. 4b, compare lanes J and H). These observations were corroborated by Northern blot analysis of the same RNA preparations (Fig. 4c, compare lanes F and D). However, the slight decrease in the fatA mRNA level cannot

**FIG. 3. Immunoblot analysis of FatA protein.** Total membrane proteins were prepared from bacteria grown under iron-limiting conditions. The same amount of proteins were loaded in all lanes. Lane A, V. anguillarum H775–3a (pJHC-T7, pJHC-A122); lane B, strain H775–3a (pJHC-T7); lane C, V. anguillarum 775 (pJM1, pJHC-A122); lane D, strain 775 (pJM1).
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Fig. 4. RNase protection and Northern blot studies of RNAa and fatA transcripts. Panel a, detection of antisense RNAa using riboprobe made from pJHC-S400 linearized with HindIII. Lane A, molecular weight markers; lane B, riboprobe without RNase treatment; lane C, riboprobe treated with RNase; lanes D, F, and H, RNA harvested from strains grown in iron-rich media; lanes E, G, and I, RNA harvested from strains grown under iron-limiting conditions; lanes D and E, V. anguillarum 775 (pJM1); lanes F and G, strain H775–3a (pJHC-T7); lanes H and I, strain H775–3a (pJHC-T7, pJHC-A122). nt, nucleotides. Panel b, detection of fatA mRNA using riboprobe made from pJHC-S300 (Fig. 1a) linearized with HindIII. Lane A, molecular weight marker; lane B, riboprobe without RNase treatment; lanes C, E, G, and I, RNA harvested from strains grown in iron-rich medium; lanes D, F, H, and J, RNA harvested from strains grown under iron-limiting conditions; lanes C and D, V. anguillarum 775–3a (plasmidless); lanes E and F, strain 775 (pJM1); lanes G and H, strain H775–3a (pJHC-T7); lanes I and J, strain H775–3a (pJHC-T7, pJHC-A122). Panel c, Northern blot analysis of fatA transcripts. Riboprobe used for hybridization was made from pMET13.1 (Fig. 1a) linearized with ClaI. Lanes A, C, and E, RNA harvested from bacteria grown in iron-rich medium; lanes B, D, and F, RNA harvested from bacteria grown under iron-limiting conditions; lanes A and B, V. anguillarum 775–3a (plasmidless); lanes C and D, H775–3a (pJHC-T7); lanes E and F, H775–3a (pJHC-T7, pJHC-A122).

account for the tremendous decrease of FatA protein synthesis.

Mapping of the 5' End of the fatA Message—Since the HindIII site where RNAa begins in the inhibitory clone pJHC-A122 is 484 base pairs upstream of the translation start codon of fatA (Fig. 1a), the fatA transcript must commence at a location 5' of the fatB HindIII site. Primer extension was therefore performed to map the 5' end of the fatA message. Using an oligonucleotide that encompassed the fatA start codon, we detected numerous RNA species, but we have only labeled the more abundant species (Mal, MalI, and Mb) and the higher molecular weight species (Mc, MHe, and MHe). There were two major RNA species with only one nucleotide difference in size, with their 5' termini located at Ma (Mal and MalI), which were 103 and 102 nucleotides upstream of the fatA start codon (Figs. 5 and 6). These species of RNA were decreased in strain H775–3a harboring both the clone pJHC-T7 and the recombinant plasmid pJHC-A122, which encoded extra copies of a truncated RNAa (Fig. 5, lane 3). We also noticed that new RNA species appeared in this strain (Fig. 5, lane 3, indicated by arrows). The Ma sites are 22 and 23 nucleotides downstream of the RNAa start site, Ra, which was mapped previously (29). We also detected two minor fatA mRNA species, with 5' termini at Mb and Mc, 243 and 362 nucleotides respectively, 5' of the fatA start codon, and therefore Mb is 118 nucleotides upstream (in the sense direction) of the RNAa start site Ra, whereas Mc coincides with the RNAa start site Rb. The results in this section support the hypothesis that the secondary fatA mRNA species, with 5' ends 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 5' termini mapped to sites at MHe and MHe, which were distinctly present in strain H775–3a (pJHC-T7, pJHC-A122) (Fig. 5,
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Fig. 6. Physical and genetic map of pJM1 iron transport region and of fatA and RNAα transcripts from 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, and MHe, location of 5' termini of fatA transcripts. E, EcoRI; H, HindIII; P, PvuI; Sa, SacI; St, StuI; Ps, PstI.

Fig. 7. RNase protection study of the regulation of fatA expression by Fur. Riboprobe was made from pMET13.1 linearized with CiaI and transcribed by T3 RNA polymerase. Lane A, molecular weight marker; lane B, riboprobe without RNase treatment; lane C, riboprobe treated with RNase; lanes D, F, H, and J, RNA harvested from strains grown in iron-rich medium; lanes E, G, I, and K, RNA harvested from strains grown under iron-limiting conditions; lanes D and E, V. anguillarum strain H775-3a (plasmidless); lanes F and G, strain H775-3a (pJHC-T7); lanes H and I, E. coli BN4020 (pJHCT7); lanes J and K, strain BN4020 (pJHC-T7, pMH15).

lane J), and were barely discernible in the wild type strain 775. Sites MHD and MHe were approximately 593 and 713 nucleotides, 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 abundance that we were unable to reproduce them in photographs.

Fur Regulation of fatA Expression—The presence of RNAα is concomitant with a slight reduction of fatA mRNA levels. However, under iron-restricted 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 acts at the transcription level, that is responsive to the concentration of iron, for example a Fur-like repressor protein, as already described in other bacteria (40–45). To investigate this possibility, pJHC-T7 was introduced into E. coli BN4020 (fur::Tn5) (16) and into the isogenic strain which also harbored pMH15 (25), 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 the Fur− strain E. coli BN4020 (Fig. 7, lanes H and I). However, when the fur clone pMH15 was also present in this strain, the level of fatA mRNA was lower in RNA harvested from cells grown in iron-rich medium as compared with those grown under iron-limiting condition (Fig. 7, lanes J and K). The data suggest that the E. coli Fur repressor could function, although very inefficiently, as a repressor in the regulation of the V. anguillarum fatA gene.

Genetic and Functional Detection of fur in V. anguillarum—Because under iron-rich conditions no fatA mRNA was detected, it is possible that a fur-like gene that encodes a more efficient protein 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 (27). In this construct β-galactosidase production will be repressed in iron-rich conditions if a Fur-like protein exists. The control was plasmid pRT240 (27, 45), which is identical to pSC27.1 without the Fur binding sequence. V. anguillarum H775-3a (pRT240) produced β-galactosidase constitutively, whereas V. anguillarum H775-3a (pSC27.1) showed a 0.24 inhibition ratio of β-galactosidase activity when grown under iron-rich as compared with iron-limiting conditions (Table I). These results indicate that V. anguillarum must have a chromosomally encoded Fur-like element.

Cloning of the fur-like Gene from V. anguillarum—Physical detection of a fur-like gene in V. anguillarum was performed by Southern blot hybridization of HindIII-digested chromosomal DNA using as a probe the E. coli fur gene obtained from plasmid pMH15 (25). Under low stringency conditions the probe hybridized with two bands of 6.6 and 3.5 kb. Hybridization was not observed under high stringency conditions, indicating a degree of divergence at the nucleotide level between the E. coli and the V. anguillarum fur genes.

Isolation of a recombinant clone containing the V. anguillarum fur-like gene was performed using a gene library (26) of the plasmidless V. anguillarum H775-3 in the vector pVK102. Fig. 8, a and b, shows an example of hybridization

| Table I | β-Galactosidase activities of V. anguillarum |
|---------|------------------------------------------|
| Strains | β-Galactosidase activity* | Inhibition by Fe/EDDA |
|         | +30 μM FeCl₃ | +1 μM EDDA |
| Miller units |
| H775-3a (pRT240) | 245 | 177 | 1.38 |
| H775-3a (pSC27.1) | 109 | 452 | 0.24 |

* In Miller units (Miller, 1972).


**Figure 8.** Identification of a recombinant clone harboring the *V. anguillarum* fur gene. Panel a, agarose gel electrophoresis of the products of HindIII digestion of five recombinant clones from the *V. anguillarum* H775-3 gene library (26). Panel b, autoradiography of Southern blot hybridization of the gel shown in panel a, using as probe the HindIII-BglII fragment from pMH15 which contained the *E. coli* fur gene (25).

Table II

| Plasmids      | β-Galactosidase activity* |
|---------------|---------------------------|
|               | Miller units              |
| pSC27.1       | 1,190                     |
| pSC27.1 + pMET67 | 440                      |
| pSC27.1 + pMH15 | 370                      |
| pRT240        | 1,550                     |
| pRT240 + pMET67 | 1,310                    |
| pRT240 + pMH15 | 1,490                    |

* In Miller units (Miller, 1972).

Experiments carried out to detect a recombinant clone harboring the fur-like gene. We obtained the recombinant plasmid pMET67, which included the 6.6- and 3.5-kb HindIII fragments that hybridized with the *E. coli* fur probe. The Fur activity of this recombinant clone was tested as described in the previous section. *E. coli* BN4020 (Fur*) already carrying pRT240 or pSC27.1 was transformed with either pMET67 or pMH15, and β-galactosidase activity was determined in cultures grown under iron-rich conditions. Table II shows that the presence of the *V. anguillarum* fur-like gene (pMET67) resulted in 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 functions 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, whereas Northern blot and primer extension experiments showed that the level of the steady-state *fatA* transcripts was only slightly decreased.

Primer extension experiments also revealed the existence of various *fatA* mRNA with different 5' termini mapped to 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 300 nucleotides from the 3' end of RNAα, a region that is upstream of *fatA* start site Mc. For this truncated antisense RNA to interact with *fatA* RNA, the latter 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 Tn3::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*. Our previous *in vitro* transcription-translation analysis indicated that a promoter may exist 3' of the *SalI* site, since insertion of an Ω fragment at the *SalI* site of an EcoRI-PstI fragment still produced FatA protein, albeit at a barely detectable level (39). The *fatA* mRNA from this construct must be either at a very low level or is functionally impaired for translation and therefore plays a very small part in the overall expression of *fatA*. Our data indicate that *fatA* is most likely to be predominantly transcribed as part of a polycistronic message.

In the analysis of the *E. coli* lac mRNAs, it was found that the lac messages were transcribed as a polycistronic lacZYA message, which was subsequently cleaved to form six individual lac mRNAs (46, 47). It was demonstrated that for the lacZYA transcript, 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 (48). In the *V. anguillarum* fat operon, we may have a similar phenomenon. The level of the 2.35-kb *fatA* mRNA was only very slightly decreased in the presence of extra RNAα, encoded by pJHC-A122. However, in this strain, little or no FatA protein was synthesized. A plausible explanation for this phenomenon is that this population of *fatA* mRNA may be functionally inactivated for translation in the same manner as that reported for the lacA message in the lac operon.

One of the mechanisms for functional inactivation of messages is a change in the secondary structure of the leader sequence 5' of the ribosome binding site of the mRNAs. It has been shown that changes in the translation initiation region influenced the translation efficiency of the fnr gene and of genes of the gal, trp, and mal operons (49–52). In this study the decreased FatA synthesis, as assessed by immuno-
The iron concentration of the medium shifts from a low to a high level is mainly controlled at the transcriptional level. 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. The mechanism is most likely to be mediated by a Fur-like repressor protein. The antisense RNA then functions as a fine-tuning mechanism in the iron regulation of the pJM1 fatA gene, by inactivating the transcripts that have already been initiated. Inactivation is primarily at the translational level, although evidence suggests that RNAa may also contribute to the destabilization of the fatA messages.

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