Molecular Cloning of the fur Gene from Actinobacillus actinomycetemcomitans

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In several bacterial species, iron availability in host tissues is coordinated with the expression of virulence determinants through the fur gene product. Initial experiments showed that a cloned Escherichia coli fur gene probe hybridized to Southern blots of Actinobacillus actinomycetemcomitans strain JP2 (serotype b) chromosomal DNA. The A. actinomycetemcomitans fur gene was then cloned utilizing partial functional complementation of the fur mutant in E. coli strain H1780. Analysis of the cloned DNA sequence revealed a 438-bp open reading frame with a deduced 146-amino-acid sequence exhibiting 80% identity to Haemophilus influenzae Fur and 62% identity to E. coli Fur. The pUC Aafur gene probe (generated from JP2 serotype b) hybridized to representatives from all five A. actinomycetemcomitans serotypes as well as to two strains derived from monkeys, suggesting that fur is widely distributed in A. actinomycetemcomitans. Open reading frames having >70% identity with the E. coli and H. influenzae flavodoxin and gyrA genes, respectively, were found. Expression of the A. actinomycetemcomitans fur gene product repressed flu expression and siderophore production in E. coli. A gel shift assay demonstrated that the expressed A. actinomycetemcomitans Fur protein bound the bacterial fur consensus sequence. Further characterization of the fur gene product in A. actinomycetemcomitans may improve our understanding of its role in the pathogenesis of periodontal disease and may lead to specific therapeutic modalities.

Iron is an essential element for most biological systems (5). It is a cofactor for a variety of enzymes and redox proteins that play critical roles in important processes such as membrane energetics and DNA and RNA biosynthesis. Due to its importance and poor solubility in aerobic aqueous solutions, most organisms have developed means for mobilization, transport, and uptake of this essential element. In the vertebrate host, iron-binding proteins, such as transferrin, lactoferrin, hemoglobin, and ferritin, maintain a low free-iron concentration, inhibiting bacterial growth (29, 46).

The availability of iron appears to be an important signal that regulates expression of many virulence factors in pathogenic bacteria. The molecular basis of coordinate regulation by iron was first described for Escherichia coli (19). A number of pathogenic bacteria have been shown to regulate iron acquisition systems (23), and a number of iron-modulated bacterial systems are mediated by Fur (ferric uptake regulator). This system is regulated by a sequence-specific DNA-binding protein (23) which acts as a negative regulator of transcription in vivo by complexing with ferrous iron to repress expression of iron-regulated genes (4). In the presence of ferrous iron, the Fur protein binds the promoter-operator region of fur regulated genes and interferes with transcription. Furthermore, mutations in the fur gene result in constitutive expression of outer membrane proteins and siderophores normally repressed under iron-depleted conditions (10).

Actinobacillus actinomycetemcomitans infections in humans can cause endocarditis (30), urinary tract infection (43), brain abscesses (24), osteomyelitis, subcutaneous abscesses, and periodontal disease (49). It has been reported that A. actinomycetemcomitans can invade gingival tissues (8). While little is known about the genetic regulation of A. actinomycetemcomitans virulence factors, paradigms of other bacterial infections suggest that in order for A. actinomycetemcomitans to cause periodontal disease, it must first be able to colonize the gingival crevice. To accomplish this task, it must possess mechanisms for acquiring iron from the host. The low concentration of free iron in the human body constitutes a limiting factor for invading pathogenic bacteria by creating bacteriostatic conditions (5). As for iron in the periodontal pocket, little is known about its exact sources and concentration during periodontitis. At present no iron utilization mechanism for A. actinomycetemcomitans has been described, except for the detection of a 70-kDa iron-regulated outer membrane protein for which no function has been found (48). Previous research demonstrated that A. actinomycetemcomitans does not bind human transferrin or excrete iron-chelating compounds known as siderophores (48). A. actinomycetemcomitans does bind lactoferrin and hemoglobin (3, 15), the two iron-containing molecules that serve as iron sources in other bacterial species (29).

In the present report, we describe experiments that identify and characterize an iron-responsive regulatory circuit in A. actinomycetemcomitans that is similar to the fur system in other pathogenic bacteria and that functionally complements E. coli fur mutants.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains used in this study are summarized in Table 1. Actinobacillus species were cultured in brain heart...
infusion (BHI) broth (BBL Microbiology Systems, Cockeysville, Md.) containing hemin (5 mg/liter), sodium bicarbonate (1 g/liter), and vitamin K (0.5 mg/ml) at 37°C in 5% CO₂ in air. E. coli strains were cultured in Luria-Bertani (LB) broth (containing, per liter, 10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, and 10 g of NaCl, pH 7.5) at 37°C in air. BHI medium with or without the addition of the iron chelator 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 200 μM (23) was used to assess the effect of iron concentration on gene expression. The effect of the amount of used iron chelator could be reversed by addition of iron. Iron-regulated lacZ operon fusions of A. actinomycetemcomitans were screened on MacConkey agar plates containing 30 μM FeSO₄ (44). Chrome azurol S (CAS) plates used to assay for siderophore production were incubated at 68°C at least 6 h and then transferred to a chilled cuvette. After digestion, the DNA was extracted with phenol:chloroform and ethanol precipitated. This DNA was cloned into pUC18 vector that had been digested with Sau3A1 at an enzyme concentration predetermined to generate fragments in the range of 2 to 6 kb. After digestion, the DNA was transferred to Hybond-N⁺ membrane and blotted to Hybond-N⁺ using the sodium dodecyl sulfate-proteinase K method (35). Rapid isolation of plasmid DNA was done using Wizard Plus Minipreps DNA Purification Kit (Promega, Madison, Wis.) according to the manufacturer’s instructions. DNA restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were used according to the manufacturer’s instructions. DNA was digested with the appropriate restriction endonuclease, the reaction mixture was run on a 1% agarose gel. The DNA fragment was cut out and ethanol precipitated. This DNA was cloned into pUC18 vector that had been digested with BamHI restriction endonuclease.

Electroporation of bacterial cells. Electroporation of E. coli cells with Gene Pulser and Pulse Controller (Bio-Rad Laboratories) was done by the methods of Dower et al. (13). Forty microliters of prepared cells was removed for each transformation reaction. DNA was added, and the reaction mixture was chilled on ice for 1 min and then transferred to a chilled cuvette. After electroporation 1 ml of SOC medium (2% Bacto tryptone, 10 mM Bacto yeast extract, 2.5 mM KCl, 20 mM MgSO₄·7H₂O, 20 mM glucose) was added to the cells and they were incubated for 1 h at 37°C to allow the cells to recover and begin to express the antibiotic determinant gene(s). The recovered cells were cultured on plates with appropriate selective media.

Cloning of the A. actinomycetemcomitans fur gene. The A. actinomycetemcomitans fur gene cloned by partial functional complementation of an E. coli fur mutant. A genomic library was screened for a locus that could repress the fuslacZ in E. coli strain H1780. E. coli H1780 has an undefined mutation of the fusion fur gene. The chromosome also contains an inserted fuscZ gene that is regulated by the promoter-operator region of ferric iron uptake (fut) gene, an iron-regulated outer membrane protein (37). The mutated fur gene, under normal conditions, permits unrestricted production of β-galactosidase as indicated by bacterial colonies assuming a lac₅ (blue) phenotype on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates. However, when a plasmid containing fur is introduced into strain H1780, the cells exhibit reduced β-galactosidase activity due to the depression of the fut gene by the presence of Fur protein. When a plasmid containing fur is introduced into strain H1780, the bacterial colonies will appear white (Lac⁺) on X-Gal plates.

β-galactosidase assays. E. coli strains were grown overnight in iron-rich LB medium. A total of 10⁶ cells from this overnight culture were inoculated into M9 minimal medium supplemented with the appropriate antibiotic and either 100 μM iron or 200 μM 2,2-dipyridyl. Cells were lysed and the β-galactosidase activity was assayed and calculated as described by Miller (27) and was defined as units per units at an optical density at 600 nm (27). To examine the rate of β-galactosidase synthesis, Miller units (referred to as absorbency units at 600
nm) were plotted versus absorbance of cultures at 600 nm. This representation reflects growth rate changes of enzyme synthesis with respect to cell growth.

**Siderophore production in E. coli.** pUC Aafur and the control vector pUC were transformed into the null mutant E. coli strain SBC24. The technique developed by Schwyn and Neilands (38) was used to detect and identify siderophores. This is a universal method based on siderophore affinity for iron(III). We tested our clones on both high- and low-iron CAS agar plates.

**Gel shift assay.** For the DNA mobility shift assay, the technique described by de Lorenzo et al. (12) was used with minor modifications. In order to test whether the cloned Fur protein functions as a DNA-binding protein, we used a 39-bp synthetic double-stranded DNA fragment composed of the exact consensus sequence motif reported for E. coli fur. The ends were labeled using Klenow enzyme and [γ-32P]ATP. Crude whole-cell extracts were prepared from cells grown in high-iron media as described by de Lorenzo et al. (12). We tested the cloned and expressed Fur protein from A. actinomycetemcomitans and the native Fur protein from whole-cell isolates of A. actinomycetemcomitans strain JP2. E. coli fur was used as a positive control. The radiolabeled fur consensus sequence was mixed with proteins isolated from crude extracts of whole cells. The gels were run at constant voltage, dried, and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.).

The DIG Gel Shift Kit (Roche Molecular Biochemicals) was used with minor modifications to determine whether the selected A. actinomycetemcomitans DNA fragment was Fur regulated. This kit detects sequence-specific DNA binding. The proteins were digoxigenin-labeled probes. DNA fragments were generated by PCR, and the digoxigenin label (Roche Molecular Biochemicals) was incorporated during the reaction. Primers for the PCR were selected based on sequences determined during this study and commercially obtained (Life Technologies Gibco BRL Custom Primers). Oligonucleotide primers for the PCR included (i) A. actinomycetemcomitans fur gene Forward (AGGCCAAGTGTCAAAGAAA CTTGACAT) and Reverse (AAATGCAAACCTCCGAGAATGTA CCAAGGCG); (ii) generating an 82-bp fragment, and (iii) E. coli fur gene (29) Forward (CAAGGGGCTT ACGATTGTTTCC) and Reverse (ATGATCTGGAGATCTGTTCTG), generating a 124-bp fragment.

To facilitate DNA binding studies, approximately 20 ng of the various cell extracts were mixed with the labeled DNA fragments. The 5% binding buffer for the protein-DNA incubation contained 100 mM HEPES, pH 7.6; 5 mM EDTA; 50 mM Na2SO4; 5 mM 1,10-phenanthroline; 2,2,2-trichloroethanol (DTT); Tween 20, 10% (wt/vol); 150 mM KCl; 100 mM MnCl2; and 1 M glycerol (45) nonspecific DNA. The mixtures were incubated for 15 min at room temperature, then transferred onto a 6% nondenaturing polyacrylamide gel and electrophoresed. Following electrophoretic separation, the DNA-protein complexes were blotted by capillary transfer onto BM (Roche Molecular Biochemicals) positively charged nylon membranes (42). The digoxigenin-labeled probes were detected by enzyme immunoassay using anti-digoxigenin-AF, Fab-fragments. The membranes were developed using the Genius Detection Kit (Roche Molecular Biochemicals).

**Sequencing the fur gene.** The nucleotide sequence of the fur gene and the 5’ and 3’ flanking regions were determined on double-stranded DNA using the dyeodeoxy chain termination procedure (36). Plasmid DNA was prepared using the Wizard Plus Midi DNA Purification System (Promega). Double stranded DNA sequencing was carried out using the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio). A universal primer, a reverse universal primer, and synthetic oligonucleotide primers were synthesized to obtain the sequences for the entire region of the gene. The resulting nucleic acid and deduced amino acid fur sequence alignments were compared to protein and DNA databases (2).

**RNA analysis.** RNA was extracted from mid-log phase cells of A. actinomycetemcomitans strain JP2 grown in BHI media using Trireagent (Sigma) according to the manufacturer’s instructions. Northern blot analysis was performed with equivalent amounts of RNA (20 μg per lane) electrophoresed through a 1.5% formaldehyde agarose gel and transferred to a charged nylon membrane (Roche Molecular Biochemicals). The membrane was reacted with the digoxigenin-labeled PCR-amplified A. actinomycetemcomitans fur gene. The 5’ terminus of a specific fur transcript was determined by primer extension analysis. The primer was complementary to +37 to +59 on the fur gene. The primer extension assay was performed according to the manufacturer’s instructions (GeneAmp Thermoscript Reverse Transcriptase RNA PCR kit; Perkin-Elmer). First, the size of the product was estimated by comparison to a pBR322 DNA/BstBI (Hinfl) marker (Fermentas Inc., Hanover, Md.). Next, the cloned fur gene DNA was sequenced with the same primer using the dyeodeoxy chain termination method and the Sequenase kit (version 2.0; U.S. Biochemical Corp.), with [32P]ATP (U.S. Biochemical Corp.).

**RESULTS**

Southern blot analysis indicates the presence of a fur gene locus on the A. actinomycetemcomitans chromosome. In our initial experiments, an E. coli fur gene probe was employed to screen A. actinomycetemcomitans genomic DNA for the presence of a fur locus. EcoRI-digested DNA from A. actinomycetemcomitans strain JP2 was electrophoresed on 1% agarose gels. Southern blotted, and hybridized with an E. coli fur oligonucleotide probe. The fur probe hybridized to a 4-kb restriction fragment suggesting that A. actinomycetemcomitans might contain a fur gene.

**Cloning of the A. actinomycetemcomitans fur gene by functional complementation.** Initial attempts to clone the A. actinomycetemcomitans fur gene using standard DNA hybridization screening techniques were unsuccessful due to high background hybridization. Consequently, an alternative approach was employed utilizing partial functional complementation of the fur mutant in E. coli strain H1780 (19, 20). A. actinomycetemcomitans DNA was partially digested with Sau3A1 restriction endonuclease to produce DNA fragments of 2.0 to 6.0 kb. These fragments were then ligated into plasmid pUC18 using the BamHI site and electroporated into E. coli strain H1780. E. coli strain H1780 has an iron-inducible, Fur-repressible fiu (ferric ion uptake) promoter fused to the β-galactosidase gene. Because of this construct, iron-replete conditions cause high expression of β-galactosidase, leading to dark blue colonies on medium containing X-Gal. However, a fur gene in trans leads to repression of the fiu promoter and clones have a white phenotype. Approximately 30,000 individual colonies were screened before isolating a single white colony. Plasmid DNA was isolated from this clone and the phenotype confirmed by retransformation into E. coli strain H1780. The plasmid, designated pUC Aafur, contained a 2.0-kb insert and the putative fur clone (Fig. 1). Restriction of pUC Aafur with EcoRI produced a 1.0 kb fragment while a HindIII digested produced a 300-bp fragment. Both fragments failed to hybridize to the E. coli fur gene probe. Restriction of pUC Aafur with an EcoRI-HindIII digest produced three fragments: a 1.0-kb EcoRI/EcoRI fragment, a 0.3-kb HindIII/HindIII fragment, and a 0.7-kb EcoRI/HindIII fragment (Fig. 1a). Although the 700-bp EcoRI/HindIII fragment hybridized to the E. coli fur gene (Fig. 1b) on Southern blot analysis, the subcloned fragment could not complement for the fur mutation in our system. This indicated that the subcloned DNA fragment did not include the entire gene that is needed for the expression of a functional Fur protein. Orientation of the fragments from the EcoRI/HindIII digest is shown in Fig. 1a and 1c.

**The magnitude of lacZ repression in pUC Aafur transformed H1780 (fur minus) bacteria was quantitated (Fig. 2) and found to be 17 units, which is less than the activity of**
normal controls (361 units), indicating that the clone could complement the fur mutation in this *E. coli* strain.

**Nucleotide sequence and analysis of pUC Aafur.** pUC Aafur was sequenced from both directions including 402 bases downstream of the fur stop and 405 bases upstream from the putative start codon (Fig. 3). An open reading frame (ORF) of 438 bp was contained in the sequenced DNA with a start codon located at nucleotide 406 to 409 and termination codon at positions 844 to 847. A potential Shine-Dalgarno polypurine tract was located 10 bp upstream (positions 395 to 400) of the putative start codon. Both genes are transcribed in the same direction.

A deduced amino acid sequence of the pUC Aafur ORF showed high similarity with other Fur proteins in the database (data not shown) with the highest identity to *H. influenzae* (80%) and the least to *B. subtilis* (36%). Considerable conservation is observed in the N-terminal two-thirds of Fur proteins. When compared with *E. coli* Fur, the areas of conservation are localized to regions encoding critical functions such as DNA binding (9, 33). Sequence conservation was markedly reduced at the extreme N-terminal end and more importantly near the C terminus.

Several iron-binding regions believed to be important for the activity of the Fur protein were noted. First was a His-X-His-X-Cys-X2-Cys-X2-Cys (residues 87 to 98) (6) motif which compares favorably with the *E. coli* sequence. It has been shown by alkylation studies that this region is essential for *E. coli* Fur activity (11). The second suggested iron binding region, a Cys-X-Y-Cys-Gly motif (9) (residues 94 to 98) was also perfectly conserved and it overlaps with the His-X-His motif is absent.

Another ORF was identified upstream of the fur gene (bp 1 to 383) (Fig. 3). This region contains the 3′ end of a gene that shares 70 and 74% sequence identity with the flaD (flavodoxin) genes from *E. coli* and from *H. influenzae*, respectively. The gene stop codon was located 23 bp upstream of the putative fur start codon. Both genes are transcribed in the same direction. Downstream of the fur stop codon another ORF begins at 1133 bp. Analysis of this sequence revealed the 5′ end of a gene that shares 82% and 89% identity to gyrA (gyrase) genes from *E. coli* and *H. influenzae*, respectively.

**pUC Aafur gene product represses siderophore production in E. coli.** To determine whether the repressive activity associated with pUC Aafur was confined to *flacZ* expression or whether it could play a role in iron regulatory pathways, we assessed the ability of pUC Aafur to repress the production of siderophore in *E. coli*. *E. coli* siderophore repression is mediated through *fur* under iron replete conditions. Therefore, a *fur* null mutant (*E. coli* strain SBC24; Schwyn and Neilands [38]) results in constitutive siderophore production.

To confirm that the cells were producing siderophores constitutively, a plate assay for siderophore production was used. The cells were examined on chrome azurol blue agar plates originally described by Schwyn and Neilands (38). In this assay, siderophores produced by bacteria growing on the plates remove iron complexed to the highly colored dye, which can be seen as a color change. When *E. coli* SBC24 containing pUC

**FIG. 1.** Restriction map of the plasmid, designated pUC Aafur. (a) Restriction of pUC Aafur with an EcoRI-HindIII enzyme. (b) Southern blot analysis of the subcloned 700-bp EcoRI-HindIII fragment which hybridized to the *E. coli* fur gene. (c) Physical map of the *A. actinomycetemcomitans* gene sequence located on a 2.0-kb EcoRI-HindIII fragment. The coding region of the gene is boxed. Relevant restriction sites are shown. The location of the flavodoxin gene and the gyrA gene is indicated upstream and downstream of the fur gene, respectively. Orientation of the fragments from the EcoRI-HindIII digest is shown.

**FIG. 2.** Galactosidase activity in *E. coli* strain H1780 (fur mutant). Cultures are as follows: H1780, H1780 plus pUC18, H1780 plus pMH15 *E. coli* fur, H1780 plus pUC Aafur, and two controls, X-Gal and ONPG (o-nitrophenyl-β-D-galactopyranoside). The cells were grown either under low-iron (open bars), or high-iron (closed bars) conditions. Each column represents the average from three separate experiments. Error bars, standard deviations.
alone was tested, since there was no fur gene product present to repress the siderophore production, there was no color change on either the high- or low-iron plates. By contrast, E. coli SBC24 containing pUC Aafur expresses a functional Fur protein which was able to interact with the consensus Fur binding sequences located upstream of the E. coli siderophore gene thereby inhibiting siderophore production (Fig. 4).

Gel retardation assay. In examining the function of A. actinomycetemcomitans Fur as an iron-dependent repressor, its DNA binding activity was confirmed using a synthetic double-stranded oligonucleotide composed of the exact consensus sequence motif reported for E. coli Fur. A 39-bp DNA fragment containing the iron box showed reduced mobility in gel retardation assays in the presence of A. actinomycetemcomitans Fur protein (Fig. 5A). In the absence of divalent metal ions such as Mg²⁺/H⁺ and Mn²⁺/H⁺, no reduced mobility was observed, even at high protein concentrations. These results indicated that A. actinomycetemcomitans Fur protein is able to efficiently bind to the E. coli iron box consensus element in the presence of metal.
FIG. 4. Chrome azurol blue agar plates showing siderophore production by bacteria grown under high-iron conditions. The yellow color of the right side of the agar plate indicates that *E. coli* SBC24 containing pUC is unable to inhibit siderophore production even under high-iron conditions. The green color on the left side of the agar plate indicates that *E. coli* SBC24 containing pUC *Aafur* inhibits siderophore production. Minor levels of iron removal from the CAS dye are seen in the heavy growth at the left side of this figure. This is probably due to the mass of cells found at this point on the agar removing iron from the CAS dye by something like low pH, not siderophore production.

FIG. 5. (A) Binding of the *A. actinomycetemcomitans* Fur protein to bacterial iron box. The DNA used in this assay was radiolabeled with $^{32}$P and contained the iron box consensus sequence 5'-GATAATGATAATCATTATC-3'. The protein samples used were extracts of *E. coli fur* strain H1780 containing plasmid pUC18. Lane 1, Fur box DNA; lane 2, pUC18 plus H1780; lane 3, *Aafur*; lane 4, *E. coli fur*. (B) Binding of the *A. actinomycetemcomitans* Fur protein to the *A. actinomycetemcomitans* fur promoter region. Lane 1, *E. coli fur*; lane 2, *Aafur*; lane 3, empty; lane 4, pUC18 plus H1780; lane 5, *A. actinomycetemcomitans* fur box DNA (82 bp from the upstream region of *Aa fur* gene).
derived from monkeys suggesting that fur is widely found in A. actinomycetemcomitans (data not shown).

Regulation of fur. The sequencing analysis indicated that the flavodoxin gene was located 23 bp upstream from the methionine start codon. In a number of microorganisms, the flavodoxin gene has been shown to be regulated by the fur gene product (1). In order to test the possibility that the fur gene is transcribed as part of an operon with the regulatory element located upstream of the flavodoxin gene, we performed a Northern blot analysis (Fig. 6A). RNA was prepared from strain JP2 following growth in both low- and high-iron media, electrophoresed through 1.5% formaldehyde gels and blotted onto nylon membranes. The membranes were probed with a digoxigenin-labeled PCR-generated fragment, which contained the full length of the fur gene. An approximately 580-bp fragment was identified. Our sequence data indicates that the fur message is 438 bases long and that the remaining bases of the message were not sufficient for the regulatory element of the fur gene to be located upstream of the flavodoxin gene.

Primer extension analysis to localize the start site of A. actinomycetemcomitans fur transcription. The transcriptional initiation site for fur was mapped by primer extension analysis (Fig. 6), using a primer within the coding region of fur and total RNA prepared from A. actinomycetemcomitans strain JP2. Two major bands were repeatedly present, which were precisely mapped by running appropriate sequencing ladders along with the primer extension products. Primer extension analysis determined a putative transcriptional start point of fur to be at the C (+1) that was 149 bp upstream from the initiation codon and within the flavodoxin gene (Fig. 3). Another putative transcriptional start point was seen, as noted by the smaller band, at A (+1) that was located 92 bp upstream from the initiation stop codon.

Identification of an A. actinomycetemcomitans iron box sequence. The iron boxes of iron-regulated genes generally exist in close proximity to their promoter region (17). Upstream of the transcription start site, possible −35 and −10 (Fig. 3) sequences were identified. Immediately downstream of the transcriptional start site, a putative iron box was identified (GACAATGATACCTTTGTCGG). This sequence shares 68% identity with the E. coli consensus iron box. These results indicate that the iron box-like sequence was not located immediately upstream of the putative −35 and −10 sequences as it is in the majority of Fur-regulated genes reported so far.

In order to examine whether the fur gene is autoregulated, we tested the promoter region of the gene for possible Fur regulation. FURTA and gel retardation assays were employed to test the putative iron box along with its flanking regions. The FURTA utilized E. coli strain H1780 with fhuF: lacZ, a Fur-regulated gene fusion sensitive to changes in repressor concentration (44). In this strain, a functional Fur binding sequence is present on a high copy number plasmid resulting in iron-dependent repression and β-galactosidase expression. E. coli cells containing the pUC18 vector were used as a negative control.
control, and *E. coli* cells containing the cloned *E. coli* iron box were used as positive control. *E. coli* H1780 was transformed with the cloned *A. actinomycetemcomitans* fur gene in an attempt to confirm the DNA sequence data. The colonies containing the *A. actinomycetemcomitans* fur gene appeared red on the indicator plate along with the positive control cells. Next, we confirmed our findings in a gel retardation assay. From the promoter region of *A. actinomycetemcomitans*, we amplified an 81-bp DNA fragment, which included the possible binding site. The mobility of the 81-bp fragment was retarded when mixed with the *A. actinomycetemcomitans* Fur protein. These experiments suggested that the *A. actinomycetemcomitans* fur gene might be indeed autoregulated (Fig. 5B).

**DISCUSSION**

The presence of a Fur protein has not been shown in any of the periodontal pathogens; however, studies have indicated that *A. actinomycetemcomitans* does respond to iron starvation by changing the repertoire of expressed outer membrane proteins (48). The iron concentration in the oral cavity can be highly variable. As a result, *A. actinomycetemcomitans* has to be able to withstand situations of both iron limitation and iron overload. In this paper, we report the cloning and initial characterization of a fur gene from *A. actinomycetemcomitans* that is both structurally and functionally similar to several other cloned bacterial fur genes. The close homology of the *A. actinomycetemcomitans* fur gene to *E. coli* fur permitted us to use an internal DNA fragment from *E. coli* fur gene as a probe to identify the *A. actinomycetemcomitans* fur gene by partial complementation. The *A. actinomycetemcomitans* fur complemented the ability of the *E. coli* fur mutant to repress siderophore production. The *A. actinomycetemcomitans* fur can bind to the iron transport (*fhu*) promoter. Together this suggests that the *A. actinomycetemcomitans* fur may be a global regulator in *A. actinomycetemcomitans* as fur is in other bacteria (14, 21).

*A. actinomycetemcomitans* Fur protein also shows a high level of identity to other known Fur proteins. The degree of similarity of the *A. actinomycetemcomitans* Fur protein with respect to its counterparts is reflected both antigenically, by its reactivity in Western blots, and functionally, by the high level of repression of the *E. coli* fhu promoter. The ORF of 146 amino acids exhibits 80% identity to *H. influenzae* and 62% to *E. coli* Fur protein. Homology analysis indicated the conservation of several iron-binding motifs. When compared with fur genes from other bacteria, the most conserved regions are located near the N terminus and are markedly reduced near the C terminus (9, 21, 33, 34). When *A. actinomycetemcomitans* fur sequences were aligned with the other published fur sequences, analysis of sequences upstream of the fur gene indicated that the deduced amino acid sequences shared identity with *flaD* (flavodoxin) gene from both *E. coli* (70%) and *H. influenzae* (74%). The flavodoxin gene stop codon was located 23 bp upstream of the fur putative start codon. The presence of the flavodoxin gene upstream of the fur gene has also been reported in *E. coli*, *H. influenzae*, and recently in *Klebsiella pneumoniae* (1). An intervening sequence of 28 bases separating the flavodoxin and fur genes is seen in *H. influenzae* while longer intervening sequences between the two genes are reported in *E. coli* (286 bp) (1) and in *K. pneumoniae* (287 bp). The genes are transcribed in the same direction as the fur gene. Downstream of the fur stop codon, another ORF was found, which showed identity to gyrase A genes from *E. coli* (82%) and *H. influenzae* (89%).

Previous work with *E. coli* suggests that transcription of the fur gene is autoregulated by the Fur protein, as well as by the catabolite activator protein (12). It has been shown that in *E. coli* (1) the flavodoxin gene is Fur regulated. In *A. actinomycetemcomitans* the fur gene and the flavodoxin gene are very close; therefore, they both could have been transcribed as part of an operon with an iron regulatory element located upstream of the flavodoxin gene. This possibility was ruled out by primer extension experiments demonstrating that the fur transcriptional start site was located 149 bases upstream of the start codon and within the flavodoxin gene. We identified two possible start sites. Repeated experiments gave the same results. Therefore, it is possible that the fur is transcribed from two promoters as it has been shown in *E. coli* (50) and *Campylobacter jejuni* (45). In *E. coli* the regulation of expression of fur is in response to several stimuli, including superoxide and peroxide stress (50).

The site at which Fur binds to DNA has been termed an "iron box" or a "fur box," and comparison of these fur boxes has generated a 19-bp consensus sequence that shows dyad symmetry (41). Binding of Fur to the iron box has been shown experimentally for several genes, including fur itself. Furthermore, the iron box can confer iron regulation on a reporter gene (41). Analysis of the upstream region of the fur gene revealed the presence of a 19-bp sequence, which showed 63% identity with *E. coli* iron box consensus sequence. The putative iron box-like sequence was located 141 bases upstream of the start site. In order to determine whether the putative iron box-like sequences can function as a binding site for the Fur protein, we introduced the fur gene located on a multicopy plasmid into an *E. coli* strain H1780. This strain can be used as a convenient genetic screen for Fur regulated genes (41). The method is based on the use of a chromosomal iron-regulated lacZ fusion to the fhuF gene. This fusion is exceptionally sensitive to small changes in iron concentration because of the weak affinity of the fhuF promoter for the Fur–Fe²⁺ repression complex. Introduction of a multicopy plasmid carrying Fur-binding sites into the test strain appears to deplete the intracellular Fur pool. This gives rise to the dissociation of the repressor from the fusion promoter, thereby allowing lacZ transcription. In the presence of 30 μM iron, the clone containing the fur promoter region was able to deplete the pool of Fur protein allowing the transcription of the fhuFlacZ. This derepression can be observed on MacConkey plates supplemented with iron by the lacZ color reaction. The clones containing the fur promoter region appeared red on these indicator plates as opposed to pale colonies containing just the pUC vector itself. This indicated that the *E. coli* Fur protein recognized an iron box in the promoter region, which suggests that the fur gene might be autoregulated. When we tested the promoter region from *A. actinomycetemcomitans* in a gel shift assay, we found that the Fur protein was able to retard the mobility of the DNA fragment tested in the presence of Mg²⁺, indicating that the promoter of the fur gene contains a protein binding region. Several controls suggested that the gel shift
seen in the binding reaction containing the promoter region of the fur gene was due to the Fur protein binding and not to a nonspecific DNA-binding protein in the extract. The Northern blot analysis suggested that the expression of fur might be regulated at transcriptional level since the presence of 2,2-dipyridyl, a membrane-permeable iron chelator, resulted in the increase in the amount of fur mRNA. Under iron sufficient conditions, a steady state of fur expression may be maintained.

In a previous study by Willemsen et al. (47) a Fur-regulated periplasmic protein was identified. A putative iron box was detected in the promoter region of the gene coding for the protein. When the iron box was mutated at two bases, bases 5 and 16, the gene was no longer iron regulated (47). The putative iron box identified in the present study showed some similarity to this previously identified iron box. The two iron boxes in A. actinomycetemcomitans are not highly conserved. The previously identified sequences important for the Fur binding function were conserved in these putative iron boxes. These two promoter regions were tested in our study, and both were able to bind the Fur protein, resulting in a band shift.

Multiple virulence factors are involved in bacterial pathogenesis with specific factors being implicated at different points in pathogenesis. Likewise, there is growing evidence that several putative virulence factors may contribute to A. actinomycetemcomitans pathogenicity. Such candidate virulence factors may include those in the iron/Fur-regulated protein group. Initial studies showed that A. actinomycetemcomitans responds to environmental iron availability (48) while the present study shows that A. actinomycetemcomitans contains genes which code for a protein homologous to the Fur protein of E. coli. Fur and Fur-like repressor proteins control the expression of a number of bacterial virulence determinants in several different bacterial species (22, 23, 31, 39, 40). For example, in E. coli, the Fur protein transcriptionally regulates more than 36 genes (6). The identification of genes whose expression is repressed by Fur in response to iron availability may lead to the discovery of as yet unknown virulence factors required for bacterial infection. Current research in our laboratory is focused on identifying all Fur-regulated genes in this bacterium. While some of these Fur-dependent genes will undoubtedly be involved in routine iron metabolism, it is likely that the fur regulator may have additional roles in A. actinomycetemcomitans, including controlling expression of genes involved in virulence.

Recently it has been reported that iron levels affect the in vivo pathogenicity of another oral pathogen, Porphyromonas gingivalis. In the study by Grenier et al. (16), the authors showed that pathogenic strains of P. gingivalis could multiply under iron-limiting conditions, while nonpathogenic strains could not. When an excess of hemin or ferric chloride was added to serum, significant growth of the nonpathogenic strains occurred. Under iron-limiting conditions, the pathogenic strains of P. gingivalis had a much lower requirement for human iron-loaded transferrin and hemin. Similarly, it is possible that strains of A. actinomycetemcomitans having a low iron requirement may be more pathogenic in the human oral cavity.

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