Identification of Fur-regulated genes in *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans is an oral pathogen that causes aggressive periodontitis as well as sometimes life-threatening, extra-oral infections. Iron regulation is thought to be important in the pathogenesis of *A. actinomycetemcomitans* infections and, consistent with this hypothesis, the *fur* gene has recently been identified and characterized in *A. actinomycetemcomitans*.

In this study, 14 putatively Fur-regulated genes were identified by Fur titration assay (Furta) in *A. actinomycetemcomitans*, including *afuA*, *dgt*, *eno*, *hemA*, *tbpA*, *recO* and *yfe* – some of which are known to be Fur regulated in other species. A *fur* mutant *A. actinomycetemcomitans* strain was created by selecting for manganese resistance in order to study the Fur regulon. Comparisons between the *fur* gene sequences revealed that nucleotide 66 changed from C in the wild-type to T in the mutant strain, changing leucine to isoleucine. The *fur* mutant strain expressed a nonfunctional Fur protein as determined by *Escherichia coli*-based ferric uptake assays and Western blotting. It was also more sensitive to acid stress and expressed higher levels of *minC* than the wild-type strain. *minC*, which inhibits cell division in other bacterial species and whose regulation by iron has not been previously described, was found to be Fur regulated in *A. actinomycetemcomitans* by Furta, by gel shift assays, and by RT-qPCR assays for gene expression.

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INTRODUCTION

Actinobacillus actinomycetemcomitans is an oral Gram-negative coccobacillus that causes periodontitis (Mombelli *et al.*, 1994; Zambon, 1994) as well as sometimes life-threatening extra-oral infections such as endocarditis (Kaplan *et al.*, 1989; Overholt, 1966; Townsend & Gillenwater, 1969). This micro-organism possesses a diverse and rather impressive array of virulence factors (Baehni *et al.*, 1981; Christersson *et al.*, 1987; Helgeland & Nordby, 1993; Meyer & Fives-Taylor, 1993; Mintz & Fives-Taylor, 1994, 1999). While it is not known which of these is involved in the pathogenesis of periodontitis, *A. actinomycetemcomitans*, like other successful pathogens, must be able to induce or repress the production of virulence factors in response to the environment. The mechanisms by which *A. actinomycetemcomitans* responds to environmental cues and the genes/proteins regulated are largely unknown.

Iron is essential for bacterial growth both as a nutrient and as a catalyst in the formation of hydroxyl radicals (Palyada *et al.*, 2004). However, iron is tightly sequestered in human hosts in order to inhibit bacterial growth and to prevent oxidative damage. Consequently, micro-organisms have evolved complex systems to efficiently capture iron.

Importantly, in order to avoid toxicity, micro-organisms achieve an effective iron homeostasis by tightly regulating the expression of genes encoding proteins involved in iron acquisition and metabolic response to iron availability (Ratledge & Dover, 2000).

In many bacteria, the Fur protein controls the transcription of these genes (Escobar *et al.*, 1999; Hantke, 1981). The main function of the Fur protein is to repress iron acquisition genes under conditions of iron sufficiency and to induce iron transport systems under conditions of iron restriction. Under iron-rich conditions, Fur can form a complex with Fe²⁺ that binds to specific consensus sequences ('Fur box') in the promoter of iron-regulated genes, leading to transcriptional repression (Bagg & Neilands, 1987). Under low-iron conditions, Fur dissociates from the promoter, allowing gene transcription. Therefore, when most Fur-regulated genes are repressed, specific genes such as those involved in iron storage or stress responses may be activated. There is growing evidence that Fur and Fur-like repressor proteins also control the expression of virulence determinants in many bacterial species (Litwin & Calderwood 1993; Ochser *et al.*, 1995; Prince *et al.*, 1993). In *Escherichia coli*, for example, the Fur protein transcriptionally regulates more than 90 genes (Escobar *et al.*, 1999). Consequently, identification of genes whose expression is controlled by Fur may lead to the discovery of as yet unknown bacterial virulence factors.

Abbreviations: Fur, ferric uptake regulator; Furta, Fur titration assay; RT-qPCR, real-time quantitative PCR.

We have cloned and characterized the *fur* gene in *A. actinomycetemcomitans* (Haraszthy *et al.*, 2002) – one of the first global regulatory genes identified in an oral pathogen. Current research in our laboratory is focused on identifying Fur-regulated genes in *A. actinomycetemcomitans*. While some of these will undoubtedly be involved in routine iron metabolism, others may control the expression of virulence factors. In this study, Fur titration assay (Furta) was used to detect Fur-regulated genes. In addition, a *fur* mutant *A. actinomycetemcomitans* strain was created by selection for manganese resistance to define the role of Fur and to confirm genes controlled by this protein.

METHODS

Bacteria and growth conditions. The bacterial strains used in this study are summarized in Table 1. *A. actinomycetemcomitans* was routinely cultivated from frozen stock on tryptic soy blood agar supplemented with 0.5% (w/v) yeast extract (Difco) and 5 µg haemin ml⁻¹. *A. actinomycetemcomitans* strains were grown in trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE) in a humidified, 5% CO₂ atmosphere at 37 °C. Manganese-resistant *A. actinomycetemcomitans* strains were cultured with 10 mM MnCl₂ added to the medium and allowed to incubate at 37 °C for 48–72 h in 5% CO₂. For some experiments, *A. actinomycetemcomitans* was grown under anaerobic conditions at 37 °C in an anaerobic chamber (Coy Laboratory Products). *E. coli* strains were cultured in Luria–Bertani (LB) broth (10 g Bacto-tryptone l⁻¹, 5 g Bacto-yeast extract l⁻¹, 10 g NaCl l⁻¹, pH 7.5) at 37 °C in air. Iron-regulated *lacZ* operon fusions of *A. actinomycetemcomitans* were screened on MacConkey agar containing 30 µM FeSO₄ (van Vliet *et al.*, 2000). Antibiotics were added to LB medium as needed, at the concentrations indicated: 100 µg ampicillin ml⁻¹, 45 µg kanamycin ml⁻¹, 100 µg streptomycin ml⁻¹ and 50 µg tetracycline ml⁻¹. BHI medium with or without the iron chelator 2,2'-dipyridyl (Sigma), at a final concentration of 0.2 mM, was used to assess the effect of iron concentration on gene expression. Cells used to inoculate the iron-restricted medium were subcultured on the same medium to deplete internal iron reserves.

For growth studies, the bacteria [JP2 wild-type (*Aawt*) and the manganese-resistant *A. actinomycetemcomitans* strain (*AaMn^R*)] were cultured overnight in broth (pH 7.0). The OD₆₀₀ of overnight cultures was measured (SmartSpec 3000 spectrophotometer, Bio-Rad) and equal optical density cultures were added to new broth and cultured at

37 °C in 5% CO₂. The OD₆₀₀ was measured every 3 h for 36 h. The *AaMn^R* strain grew in aggregates; therefore the optical density was measured after 10 s vigorous vortexing. To compare cell density at OD₆₀₀ versus c.f.u. (per ml), aliquots of *A. actinomycetemcomitans* cultures were removed at various times during growth and plated in triplicate on TSBYE agar. All growth experiments were repeated at least three times with consistent results.

DNA isolation. Bacterial genomic DNA was isolated by an SDS-proteinase K method (Sambrook *et al.*, 1989). Plasmid DNA isolation (Wizard Plus Minipreps, Promega), DNA restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were used according to the manufacturers' specifications.

Preparation of bacterial cell lysates. Whole-cell protein extracts were obtained from *A. actinomycetemcomitans* cells cultured aerobically in the presence of 5% CO₂ at 37 °C under iron-chelated or iron-rich conditions. Iron-chelated conditions were obtained by the addition of 2,2'-dipyridyl. Bacterial cells were harvested by centrifugation at 12 000 g for 10 min at 4 °C, and then washed three times in ice-cold PBS, pH 7.0. The cells were lysed (Standard Cell Lysis Buffer, Amersham) and stored in aliquots at -70 °C. The protein concentration was determined by hydrolysis/amino acid analysis or by the PlusOne 2-D Quant Kit (Amersham).

Furta. Furta was performed as described by Stojiljkovic *et al.* (1994) and Tsois *et al.* (1995). Chromosomal DNA from *A. actinomycetemcomitans* was partially digested with *Sau3A*I. Fragments between 3 and 6 kb were cloned into pUC18 previously digested with *Bam*HI. *E. coli* strain H1780 was transformed with the library. The recombinants were distributed onto MacConkey agar containing 30 µM FeSO₄, supplemented with 100 µg ampicillin ml⁻¹. The cultures were incubated at 37 °C for 36 h and the Lac phenotype was recorded. *E. coli* containing plasmid pUC18 was used as a negative control and the cloned *E. coli fur* gene with its promoter region (a gift from Dr K. Hantke, Mikrobiologie II, Universität Tübingen, Germany) was used as a positive control. All experiments were repeated at least twice. The identity of the selected clones was determined by dsDNA sequencing using the dideoxy chain-termination procedure (Sanger *et al.*, 1977). Plasmid DNA was prepared (Wizard Plus Purification System, Promega) and sequenced. A universal primer, a reverse universal primer and synthetic oligonucleotide primers were synthesized to obtain the entire gene sequence. The resulting nucleic acid sequences were compared to DNA databases (Altschul *et al.*, 1990) and to annotated *A. actinomycetemcomitans* sequences (Roe *et al.*, 1999).

Gel shift assay. Gel shift assays were performed with minor modifications (DIG Gel Shift Kit, Roche) to determine if *A.*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
<i>A. actinomycetemcomitans</i> JP2	Wild-type; highly leukotoxic	The Forsyth Institute, Boston, MA
<i>E. coli</i> H1780	<i>araD139 Δ^aargF-lacU169 rpsL150 relA1 flbB5301deoC1 ptsF25 rbsR fii-lacZ Fur⁻</i>	Hantke (1987)
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169(φ80 lacZΔM15)hsdR17 recA1 endA1 gyrA96 thi-1relA1</i>	Hanahan (1983)
Plasmids		
pUC18	Ap ^r plasmid	Messing (1983)
pUCAafur	Ap ^r plasmid containing <i>Aafur</i>	Haraszthy <i>et al.</i> (2002)
pMH15E. coli fur	Cm ^r plasmid containing <i>E. coli fur</i>	Hantke (1987)
pUCAaminC	Contains <i>minC</i> gene	This study

actinomycetemcomitans genes were able to bind A. *actinomycetemcomitans* Fur. Digoxigenin-labelled DNA fragments were generated by PCR using commercially obtained primers (Life Technologies Gibco-BRL) based on sequences obtained during this study and screened against the A. *actinomycetemcomitans* database. Primers for *minC* Forward 1 (TGAGGTTTCGAGTTGCTGATT; -69 to -49) and Reverse 1 (CTTGAGCCATCACGAAATCCAAG; +10 to -14), and Forward 2 (ATGACTGCGCTTTTCAATATAAAG; -153 to -1) and Reverse 2 (CACGAAATCCAAGAAAAAGAG; -1 to -22), generated 79 bp and 153 bp fragments, respectively. Crude whole-cell extracts were prepared from cells grown in iron-rich medium as described by de Lorenzo *et al.* (1987). Whole-cell protein isolates were tested from the following: (1) Aawt expressing the native Fur protein; (2) *E. coli* H1780 with pUCAafur expressing the cloned Fur protein; (3) AaMn^R strains expressing the mutated Fur protein; and (4) *E. coli* strain H1780 containing pUC18, which served as a negative control. Samples (approx. 20 ng) of bacterial cell extracts were mixed with the labelled DNA fragments in 5× binding buffer [100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM (NH₄)SO₄, 5 mM DDT, 10% (w/v) Tween 20, 150 mM KCl, 100 μM MnCl₂ and 1 μg poly d(I-C) nonspecific DNA]. The mixtures were incubated for 15 min at room temperature and then transferred to a 6% nondenaturing polyacrylamide gel and electrophoresed. Following electrophoretic separation, the DNA-protein complexes were blotted by capillary transfer onto positively charged nylon membranes. The digoxigenin-labelled probes were detected by enzyme immunoassay using anti-digoxigenin-AP Fab fragments. The membranes were developed using the Genius Detection Kit (Roche).

Manganese-resistant A. *actinomycetemcomitans*. Manganese selection was performed by a modification of the method of Hantke (1987). Approximately 10⁸ cells of A. *actinomycetemcomitans* JP2 were distributed on TSBYE medium containing 10 mM MnCl₂ and allowed to incubate at 37 °C for 48–72 h in 5% CO₂. The resulting colonies were restreaked onto the same medium to confirm the resistant phenotype. Genomic DNA was isolated from five Mn^R A. *actinomycetemcomitans* colonies. Each colony was obtained from separate plates, from experiments performed weeks apart. The entire *fur* gene was amplified (Expanded High Fidelity Plus PCR System, Roche) from each using *fur*-specific primers (5'-TATTTCTGTGATGCCATGGG-3'; 5'-TGCGCAATGATTAATGTCGCTA-3'), sequenced and compared to the wild-type *fur* gene.

Cloning of the A. *actinomycetemcomitans* fur mutant gene.

To compare the function of the mutant *fur* gene (AaMn^R) with that of the wild-type *fur* gene (Aawt), *E. coli* strain H1780 was used. This strain has an undefined mutation of chromosomal *fur* and a *fiu-lacZ* gene that is regulated by the promoter-operator region of an iron-regulated outer-membrane protein gene, *fiu* (Schaffer *et al.*, 1985). Under normal conditions, the mutated *fur* gene in *E. coli* H1780 permits unrestricted production of β-galactosidase, indicated by a Lac⁺ (blue) phenotype on X-Gal media. However, when a plasmid containing a functional *fur* is introduced into strain H1780, there is derepression of *fiu* by Fur and reduced β-galactosidase activity, indicated by a Lac⁻ (white) phenotype on X-Gal media. Accordingly, strain H1780 was transformed with pUC18 containing the amplified fragments of either the mutant or the wild-type *fur*. β-Galactosidase activity was assayed and calculated as described by Miller (1972). *E. coli* strains were cultured overnight in iron-rich LB medium and 10⁸ cells were inoculated into M9 minimal medium supplemented with antibiotics and either 100 μM iron or 200 μM 2,2'-dipyridyl.

Western blot analysis. Whole-cell protein extracts were prepared from bacteria grown to late exponential phase in TSBYE medium with and without 2,2'-dipyridyl. Thirty micrograms of protein extract from each strain was separated by electrophoresis on 12% SDS-polyacrylamide gels containing prestained high-molecular-mass standards (Bio-Rad). Gels were stained with Coomassie blue or

transferred onto nitrocellulose paper using a semi-dry blotting apparatus (Hoefer Scientific). Blots were blocked with 2% (w/v) BSA in PBS with azide for a minimum of 1 h. The nitrocellulose transfers were reacted with antiserum to *Pseudomonas aeruginosa* Fur (Prince *et al.*, 1993) diluted in blocking buffer. Positive reactions were detected using alkaline phosphatase. Protein concentrations were determined by the Bradford method with Coomassie protein assay reagent (Pierce).

Growth at acidic pH. To determine whether mutation of the *fur* gene affected the growth of A. *actinomycetemcomitans* under acidic conditions, Aawt and AaMn^R strains were cultured in broth under aerobic conditions ranging from pH 6.0 to 7.2 in increments of 0.1 unit (Clyne *et al.*, 1995). The broth was adjusted to the desired pH with HCl. To ensure that all strains were in the same growth phase, the bacteria were grown to an OD₆₀₀ of approximately 0.1 (SmartSpec 3000 spectrophotometer) in pH 7.0 broth and diluted in the test medium to an OD₆₀₀ of 0.05. Bacterial growth was monitored by measuring the OD₆₀₀ until stationary phase was reached, after which no increase in optical density was observed. The pH of the medium was monitored by pH electrode (accumet basic AB15, Fisher Scientific) and did not change by more than 0.5 unit during the course of the experiments.

To compare cell density at OD₆₀₀ versus c.f.u. (per ml), aliquots of cultures were removed at various times during growth and plated in triplicate on TSBYE agar. All growth experiments were repeated at least three times with consistent results.

RNA isolation. RNA was extracted from mid-exponential-phase cells of Aawt and AaMn^R strains grown in iron-rich or iron-chelated medium (RNAqueous Extraction Kit, Ambion) according to the manufacturer's instructions. RNA yields were analysed spectroscopically.

Real-time quantitative PCR (RT-qPCR). One-step RT-qPCR was performed to quantify gene expression. PrimerExpress software (version 2.0; Applied Biosystems) was used to design primers and probes. The primer sequences were examined (BLAST) to ensure that they were specific to the target gene. Primers had *T_m* values of between 58 and 60 °C. Probes did not overlap the primers and had *T_m* values 10 °C higher than the primers. The fluorescent reporter was attached to the 5' end of the probes. TaqMan probes had 5'-FAM and 3'-TAMRA modifications. The quencher was attached to the 3' end of the probes. The primers and probes used for real-time PCR (Applied Biosystems) are listed in Table 2. Reaction mixtures (20 μl) contained 20 ng template RNA, 1× TaqMan Master Mix, 0.25 units MultiScribe reverse transcriptase μl⁻¹, 0.4 units RNase inhibitor μl⁻¹, TaqMan probe (Applied Biosystems), at a concentration of 0.25 μM, and forward and reverse gene-specific primers at a concentration of 0.6 μM. All reactions were run in triplicate and repeated three times with freshly isolated RNA obtained from separately grown cells. Positive controls included (1) standard RT-PCR amplification with a known template producing a single amplicon of the expected size; and (2) amplification with a primer/probe set for *afuA*, a gene known to be Fur regulated. Negative controls included (1) amplification with a primer/probe set for 16S rRNA, which is not Fur regulated; (2) amplification with a reaction mixture without a template; and (3) amplification without the reverse transcriptase. ROX was used as an internal (passive) reference dye to normalize the signal of the reporter. Since the reference dye is added to each reaction in equal amounts, this controls for non-amplification-based variability in the results. Reaction plates were processed on an Applied Biosystems 7900HT Sequence Detection System. The reverse transcription reaction was performed at 48 °C for 30 min. The AmpliTaq Gold polymerase was activated at 95 °C for 10 min followed by 40 cycles of denaturation for 15 s at 95 °C and annealing and extension for 60 s at 60 °C. Amplification data were analysed with the ABI Prism SDS 2.1 software (Applied Biosystems). Relative

Table 2. Primers and probes for RT-qPCR

Function	Primer or probe	Sequence
Control	16S rRNA-F	TGGTGAATCTGGCGGGTTAT
	16S rRNA-R	TCATTGGATCCGGTTTCCA
	16S rRNA-probe	CATCTCGGTCACCGTTCCCATGG (complementary strand)
Ferric uptake protein	<i>afuA</i> -F	TGTGGATTTCTTAGCCGGTAAAA
	<i>afuA</i> -R	CAGGACGTAGCGGGTATTCTG
	<i>afuA</i> -probe	CGCACAGCAGTAAAGGCTTCCTGACCT (complementary strand)
Cell division inhibitor	<i>minC</i> -F	CTACGCCAAAAACAGCGATT
	<i>minC</i> -R	CCGTCTGCCGCCACTTC
	<i>minC</i> -probe	CCATGCTCCACATCACCGTGAATGAT

quantification of gene expression was performed by the C_t method (Winer *et al.*, 1999), with 16S rRNA expression serving as an endogenous control to normalize expression within each sample. By comparing the threshold cycle between the wild-type and mutant strain, the relative expression of a given gene could be determined. Statistical significance was determined by ANOVA and Fisher's multiple comparison analysis.

RESULTS AND DISCUSSION

Detection of iron- or Fur-regulated genes

To detect Fur/iron-regulated genes in *A. actinomycetemcomitans*, Furta was used. Originally established in *E. coli* (Stojiljkovic *et al.*, 1994), Furta utilizes *E. coli* strain H1780. This strain carries *fhuF-lacZ*, a Fur-regulated gene fusion sensitive to changes in Fur concentration. Fur-binding promoter regions carried on a high-copy-number plasmid can be identified by binding Fur in *E. coli*, preventing Fur binding to the *lacZ* construct located on the chromosomal DNA and allowing transcription of the β -galactosidase gene. High levels of β -galactosidase expression result in red *E. coli* colonies on MacConkey medium, indicating that the plasmid carries a Fur-binding fragment, whereas reduced β -galactosidase expression results in a pale colony indicating that the plasmid does not carry a Fur-binding fragment (Stojiljkovic *et al.*, 1994; Tsolis *et al.*, 1995). Furta is a powerful tool for identifying Fur-regulated genes, but it is extremely sensitive to both iron and Fur concentrations. *A. actinomycetemcomitans fur* has 62 % homology to *E. coli fur* gene and the cloned *A. actinomycetemcomitans fur* gene was able to complement the *fur* mutation in *E. coli* (Haraszthy *et al.*, 2002). However, the assay probably targets clones containing Fur boxes with a high degree of similarity to the *E. coli* Fur box since the *E. coli* Fur protein will bind those sites preferentially. This would suggest that mainly *A. actinomycetemcomitans* promoters recognizing *E. coli* Fur were detected in this study. In the future we intend to use more sensitive techniques such as proteomics to detect most of the Fur-regulated genes in *A. actinomycetemcomitans*.

Fifty potentially Fur-regulated clones were selected after screening 30 000 clones from an *A. actinomycetemcomitans* plasmid bank by Furta. When these 50 clones were sequenced, 14 were identified as containing potentially

Fur-regulated genes and the remainder were identified as duplicates or false-positive clones. Clones were considered false positive if a 'Fur box'-like sequence was not identified in their promoter region or the clone did not include the promoter region of the sequenced gene. Although it is possible that some of these genes might be Fur regulated by an unknown method they will be the subject of future studies. As shown in Table 3, several different types of genes were noted among the 14 potentially Fur-regulated genes. These include a previously described Fur-regulated gene, *afuA*, encoding a periplasmic ferric uptake protein belonging to the metal-transporting, binding-protein-dependent ABC systems (Willemsen *et al.*, 1997) and another iron acquisition gene, *hemA*. A transferrin-binding protein gene, *thpA*, also appeared to be Fur regulated. Three genes encoding outer-membrane proteins were identified, including a putative outer-membrane protein with no known function, (clone 2), a TonB-dependent outer-membrane siderophore receptor (clone 3) and a possible transcriptional regulator (clone 4). Putative genes encoding an adhesion molecule (clone 1), an ABC transporter (clone 6) and a possible permease (clone 7) were identified. A putative iron-transport gene similar to the *yfe* system of *Yersinia pestis* (Bearden & Perry, 1999) was also detected. Genes for various enzymes appear Fur regulated such as *recO* (DNA repair) and *dgt* (deoxyguanosine triphosphate triphosphohydrolyase). A cell division inhibitor gene, *minC*, was also among the potential iron/Fur-regulated genes as was *eno*, encoding the glycolytic enzyme enolase, known to be important in the pathogenesis of streptococcal infections (Pancholi & Fischetti, 1998; Pancholi, 2001).

Manganese-resistant *A. actinomycetemcomitans*

The Fur regulon can best be detected using *A. actinomycetemcomitans* mutants deficient in the Fur protein. The altered Fur generated in this study may still retain some function. Attempts in a number of species to obtain *fur* mutants by gene replacement have been unsuccessful (Prince *et al.*, 1993; Berish *et al.*, 1993; Funahashi *et al.*, 2000); however, it is possible to produce *fur* mutants using manganese selection (Prince *et al.*, 1993; Lam *et al.*, 1994; Tolmasky *et al.*, 1994; Benson *et al.*, 2004). The Mn^R Fur mutants are generated using high concentrations of

Table 3. Putative Fur-regulated genes

Gene	Function	Reference
<i>afuA</i>	Ferric uptake protein	Willemsen <i>et al.</i> (1997)
Clone 1	Putative adhesion protein	Fine <i>et al.</i> (2005)
Clone 2	Iron-regulated outer-membrane protein	Komatsuzawa <i>et al.</i> (2002)
Clone 3	TonB-dependent outer-membrane siderophore receptor	Hoegy <i>et al.</i> (2005)
Clone 4	Iron-regulated outer-membrane protein (possible transcriptional regulator)	Komatsuzawa <i>et al.</i> (2002)
Clone 6	ABC transporter	Rhodes <i>et al.</i> (2005)
Clone 7	Permease	Grass <i>et al.</i> (2005)
<i>dgt</i>	Deoxyguanosine triphosphate triphosphohydrolase	Wurgler & Richardson (1990)
<i>eno</i>	Enolase	Pancholi (2001)
<i>hamA</i>	Haemoglobin-binding gene	Hayashida <i>et al.</i> (2002)
<i>minC</i>	Cell division inhibitor	de Boer <i>et al.</i> (1990)
<i>tbpA</i>	Transferrin-binding protein	Loosmore <i>et al.</i> (1996)
<i>recO</i>	DNA repair	Merrell <i>et al.</i> (2002)
<i>yfe</i>	ABC transporter	Bearden & Perry, (1999)

manganese. Mn^R *A. actinomycetemcomitans* were obtained by culturing strain JP2 on TSBYE medium containing 10 mM $MnCl_2$. Comparison of the *fur* gene from five Mn^R colonies (*AaMn^R*) with the wild-type (*Aawt*) revealed the same single point mutation in each. The colonies were picked from several different plates originating from separate cell cultures. There was a change in nucleotide 66 from C to T, changing leucine to isoleucine. Single point mutations and small insertions have been reported for other Mn^R *fur* mutants (Lam *et al.*, 1994). The majority of the mutations are clustered at the N- and C-terminal regions of the protein. The *AaMn^R* mutant contains a single point mutation in the amino-terminal domain where the helix–turn–helix domain is located, which may be important for DNA binding (Kolade *et al.*, 2001), resulting in a nonfunctional Fur protein.

Manganese selection is a relatively specific method for isolating *fur* mutations in many species of bacteria (Brickman *et al.*, 1995; Lam *et al.*, 1994; Prince *et al.*, 1993; Hantke, 1987). The molecular mechanism of manganese selection is not known, but it has been suggested that the ability of Mn^{2+} to activate Fur for DNA binding may be a source of manganese toxicity, perhaps by inappropriately repressing iron uptake functions (Bsat & Helmann, 1999). Another possibility is that the *fur* gene is highly sensitive to the mutagenic action of manganese (Prince *et al.*, 1993). Manganese mimics iron by binding to the Fur protein to form a Fur– Mn^{2+} complex instead of the Fur– Fe^{2+} complex and repressing iron uptake genes. As a result, bacteria with the wild-type *fur* gene repress iron uptake systems and starve for iron in the presence of manganese, while *fur* mutants fail to repress iron-uptake systems and survive. The Fur protein in these mutants is thought to retain some function, explaining why this particular class of mutations is not lethal. It has been also suggested that some gene products that are required for viability may be produced only in the presence of Fur. It is possible that some of the iron acquisition genes are turned off in the *AaMn^R* strain either directly by Fur or

by another so far unidentified pathway to prevent the accumulation of iron inside the cells. It is also possible that the *AaMn^R* strain survived iron starvation by acquiring a mutation which at least partially derepresses the iron uptake system (Thomas & Sparling, 1996). Alternatively, constitutive expression of all of the Fur-repressed genes may be detrimental to the growth of the bacteria because of the resulting intracellular iron overload. This type of explanation is supported by the work of Touati *et al.* (1995), who observed that *fur* mutants of *E. coli* produced iron overload, leading to oxidative stress and DNA damage. In our future studies we aim to create a *fur* null mutant *A. actinomycetemcomitans* strain to further define the Fur regulon. By comparing the two *fur* mutant strains we will be in a position to identify genes whose expression differs between these strains. Those genes could be of potential interest in defining the role of Fur in *A. actinomycetemcomitans* virulence.

The activity of the *fur* gene from *AaMn^R* was tested in a *fur*-deficient *E. coli* strain H1780 by cloning it into pUC18. Since the *fur* gene of H1780 has been inactivated, the *fiu–lacZ* reporter gene is unregulated and constitutive. Introduction of a clone encoding an active Fur protein into H1780 re-establishes Fe-dependent regulation of the *fiu–lacZ* reporter gene, leading to a phenotype change. The *fur* gene cloned from the *AaMn^R* strain was not able to regulate the *fiu–lacZ* reporter gene. The Lac^+ (blue) phenotype of the clones indicated that the gene with the point mutation encoded a Fur protein that was unable to bind to the Fur-regulated promoter in *E. coli*. This indicates that the *fur* gene mutation in the *AaMn^R* strain results in a non-functional Fur protein under aerobic conditions.

Effect of iron limitation on the growth of *AaMn^R*

The effect of *fur* mutation on growth rate was examined by comparing the growth of *AaMn^R* and *Aawt* in iron-rich and iron-chelated media. Similar to previously studied *fur* mutant strains (Litwin & Calderwood, 1994) the *AaMn^R*

Fig. 1. Effect of *fur* mutation on the growth rate of *A. actinomycetemcomitans*. Growth was monitored by measuring OD₆₀₀ over 30 h. Samples were randomly plated onto TSBYE plates in order to compare the growth of AaMn^R cells (Δ) to that of Aawt cells (■) under iron-rich conditions: AaMn^R cells grew more slowly than Aawt cells under these conditions. Under iron-chelated conditions the growth of AaMn^R cells (◇) was very similar to that of Aawt cells (▽) type growth were very similar. The results are means of three independent growth experiments done in triplicate.

strain grew more slowly than the Aawt strain under iron-rich conditions (Fig. 1). In the iron-chelated medium both strains had a very similar growth rate. This indicated that the Fur protein in the mutant strain is unable to respond to changes in environmental iron concentrations. The constitutive high-level expression of iron uptake systems in the AaMn^R mutant may be an unneeded energy drain in the presence of sufficient iron and may, therefore, impede growth.

Western blot analysis

The amount of Fur produced in Aawt and AaMn^R strains was examined in Western blots. Total cellular proteins were extracted and equal amounts were loaded to each lane. The Western blots were probed with antiserum to the *P. aeruginosa* Fur protein (a gift from Dr M. L. Vasil, Department of Microbiology, University of Colorado Health Sciences Center, Denver, CO, USA) that was previously shown to react with both the *E. coli* and the *A. actinomycetemcomitans* Fur protein (Haraszthy *et al.*, 2002). As expected, the wild-type *A. actinomycetemcomitans* strain responded to available iron by expressing significantly more Fur protein under iron-rich conditions than under iron-chelated conditions (Fig. 2). The AaMn^R mutant strain was unable to respond to available iron and expressed equal amounts of Fur protein under both iron-rich and iron-chelated conditions (Fig. 2). These data are, therefore, consistent with the idea that the point mutation in the *fur* gene results in a non-functional or partially functional Fur protein, unable to respond to varying iron concentrations under the test conditions.

Fig. 2. Western blot analysis showing *A. actinomycetemcomitans* whole-cell isolates probed with antibody against *P. aeruginosa* Fur. Lanes: 1 and 3, Aawt; 2 and 4, AaMn^R. Cells in lanes 1 and 2 were grown in iron-rich medium; those in lanes 3 and 4 were grown in iron-chelated medium. A similar level of Fur was detected in AaMn^R cells regardless of iron availability while Aawt cells reacted to the iron level, expressing less Fur in iron-chelated medium.

The effect of *fur* mutation on growth at acidic pH

To characterize growth inhibition at low pH, AaMn^R and Aawt strains were cultured in TSBYE broth at pH ranging from 6.0 to 7.2 in increments of 0.1 pH unit (Fig. 3) until stationary phase was reached. The Aawt strain and the AaMn^R mutant had similar growth rates in pH 7.0 broth but the growth rate of the AaMn^R strain at pH 6.8 was reduced compared to that of the parent strain. The wild-type *A. actinomycetemcomitans* adapted well to acidic conditions and was able to grow even at pH 6.0, indicating that it can withstand and adapt to acidic environments. The AaMn^R strain grew poorly in acidic media, indicating that Fur is important in the ability of *A. actinomycetemcomitans* to adapt to an acidic environment. Relatively little is known about the mechanisms involved in the acid resistance of *A. actinomycetemcomitans*. The fact that this bacterium can persist and propagate in the oral cavity suggests that it is able to grow at mildly acidic pH. The ability to adapt to acidic environments could be important in the pathogenesis of *A.*

Fig. 3. Effect of acidic pH on the growth of Aawt (■) and AaMn^R (○) after 24 h (stationary phase) in broth adjusted to pH from 6.1 to 7.2 in increments of 0.1 unit. The parent strain was able to achieve limited growth at pH 6.1 but the *fur* mutant was not able to grow at that pH. Growth was restored at pH 6.9 for the AaMn^R strain while Aawt grew at 6.5 pH at its normal rate.

actinomycetemcomitans infections since there are frequent episodes of high acidity in the human oral cavity (Bijlsma *et al.*, 2002). Generally, bacteria growing at acidic pH respond by inducing changes in lipopolysaccharide composition (McGowan *et al.*, 1998; Moran *et al.*, 2002), increasing expression of chaperone-like proteins (Huesca *et al.*, 1998), and expressing several genes at the transcriptional and/or translational level (Merrell *et al.*, 2003; Ang *et al.*, 2001). However, the exact role in acid resistance of many of these factors is largely unknown. Since iron is essential for growth, an environmental shift from high to low iron concentrations serves as an important regulatory signal to the cell. Because iron solubility is a function of pH, it is reasonable to speculate that Fur may sense acid indirectly in terms of iron availability. Therefore, Fur may play a role in adaptation to acidic environment by sensing acid stress and subsequently regulating the expression of some genes in an iron-independent fashion. The role of Fur in acid resistance should be the subject of future studies.

Characterization of the *minC* gene

Sequence analysis of one of the Furta-positive clones revealed a 562 bp ORF encoding a 20 kDa protein. Comparison of the 562 bp sequence to the annotated *A. actinomycetemcomitans* genome identified the clone as containing a *minC* gene. In *E. coli*, *minC* inhibits septum formation and cell division (de Boer *et al.*, 1990, 1991). *E. coli* cells transformed with the pUCAaminC clone formed long chains, suggesting that the cells were unable to divide properly. This clone also grew slowly (data not shown).

A potential Fur box (AATAATTCTTTACTTATTA) was detected upstream of the *minC* start site. It contained 11 of 19 bases or 57.9 % identity to the *E. coli* Fur box. To test the potential regulatory effect of Fur, a 79 bp and a 153 bp fragment were amplified from the promoter region of the gene. Both of these fragments included the putative Fur box with flanking regions of various sizes. *A. actinomycetemcomitans* Fur retarded the mobility of the putative Fur consensus sequence in the gel mobility shift assays (Fig. 4). The Fur protein from the AaMn^R strain or from *E. coli* containing pUC18 alone was not able to bind to the promoter region of the *minC* gene and retard its mobility. This experiment suggested that the gel shift seen in the binding reaction containing the promoter region of the *minC* gene resulted from the formation of a Fur protein–*fur* consensus probe complex, illustrating that *minC* does indeed contain a Fur box and that Fur binds to *minC*.

Expression analysis of *minC* by RT-qPCR

In order to determine if gene regulation occurs at the transcriptional level, RT-qPCR was performed. Willemsen *et al.* (1997) found that *A. actinomycetemcomitans* *afuA* gene expression increased under iron-chelated conditions and that the *fur* gene promoter region contains a Fur box able to bind the Fur protein. We used this gene in our experiments as a positive control. Consistent with previous studies, our

Fig. 4. Gel shift assay for binding of *A. actinomycetemcomitans* Fur protein to the *minC* gene promoter region. Crude whole-cell extracts were prepared from cells grown in iron-rich medium. Lanes: 1, DNA (promoter region of the *minC* gene); 2, Aawt (native Fur protein); 3, *E. coli* H1780 + pUCAafur (cloned Fur protein); 4, AaMn^R (mutated Fur protein); 5, *E. coli* H1780 + pUC18.

studies also showed increased *afuA* gene expression under iron-chelated conditions. There was an eightfold increase in the copy number of *afuA* transcripts in Aawt cultured under iron-chelated conditions compared to Aawt cultured under iron-rich conditions. In the AaMn^R mutant, there was a five- to sevenfold increase in the copy number of the *afuA* transcripts compared to Aawt when both were cultured under iron-rich conditions (Fig. 5).

RT-qPCR analysis of total RNA from AaMn^R and Aawt cells grown in iron-rich and iron-chelated media showed that Aawt grown in iron-chelated medium expressed higher level of *minC*, indicating that the gene is iron regulated. AaMn^R cells showed increased expression of *minC* compared to Aawt strains grown in iron-rich medium regardless of iron availability, indicating Fur regulation (ANOVA $P=0.05$). Fisher multiple comparison showed that all three groups were significantly different from the control group. The copy number of the *minC* transcript in the AaMn^R mutant increased threefold relative to that of the transcript in Aawt grown under iron-rich conditions ($P=0.013$ iron-rich, $P=0.028$ iron-chelated). The assays were repeated three times with RNA isolated from separately grown cells. The cell numbers were carefully adjusted by comparing OD₆₀₀ to c.f.u. Aawt grows faster than the AaMn^R strain; therefore even though the cell count was adjusted before RNA extraction and the assays were done with carefully measured RNA the cells might be at different growth stages. The expression of certain genes is affected by the growth stage of the cells (Beyer-Sehlmeyer *et al.*, 2005), which would be a possible explanation why the relative gene expression varies somewhat with *afuA*. *minC* expression was at the same level in the AaMn^R strain regardless of iron availability, and very close in iron-chelated medium, since these cells grew at a similar rate. The results from this experiment indicate that *minC* is iron regulated through the Fur protein. Thus, the Furta, the gel shift assays, and the RT-qPCR assays for gene

Fig. 5. Relative expression of the *minC* gene as determined by RT-qPCR. The *afuA* gene was used as a positive control. Values were obtained as described in Methods and represent the mean of three individual RT-qPCR experiments using RNA isolated from separately grown cultures in iron-rich and iron-chelated media. The gene expression was compared to that of Aawt cells grown in iron-rich medium. Relative quantification of gene expression was performed by the C_t method, with 16S rRNA expression serving as an endogenous control to normalize expression within each sample.

expression all indicate that *A. actinomycetemcomitans minC* is Fur regulated. To our knowledge this is the first report of Fur regulation of the *minC* gene.

Conclusions

This study identified 14 putative Fur-regulated genes in *A. actinomycetemcomitans*. Many of these genes are known to be iron regulated in other bacterial species but one gene, *minC*, not previously described as being iron regulated, was found to be iron/Fur regulated in *A. actinomycetemcomitans*. A manganese-resistant *fur* mutant of *A. actinomycetemcomitans* was generated and shown to produce a non-functional Fur protein in our assays. The expressed protein might have retained some function; this will be the subject of future studies. The mutant strain was more sensitive to acid stress and expressed higher levels of *minC* than the wild-type strain.

The importance of these studies will be to improve our understanding of the nature of iron availability with regard to the pathogenesis of periodontal disease. In the long term, these studies will provide a firm foundation on which future investigations may be based that lead to the development of treatment to prevent or retard the progression of periodontal disease. It is possible that bacterial iron metabolism can be exploited as a new approach to antimicrobial chemotherapy. An intriguing aspect of this work is that, because iron is absolutely essential for bacteria, it could make the development of bacterial resistance very difficult for any organism deprived of iron. Chelation of iron in order to inhibit growth of invading bacteria could be developed into a therapy against periodontal infection. Another, even more

intriguing possibility is that the identification of Fur-regulated proteins and their role in the pathogenesis of *A. actinomycetemcomitans* may facilitate the development of antibacterial drugs based on concepts such as delivering siderophore-antibiotic conjugates (Ghosh *et al.*, 1996) with high affinity and specificity, through bacterial iron transport systems.

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