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Novel iron-regulated and Fur-regulated small regulatory RNAs in *Aggregatibacter actinomycetemcomitans*

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SUMMARY

Iron can regulate biofilm formation via non-coding small RNA (sRNA). To determine if iron-regulated sRNAs are involved in biofilm formation by the periodontopathogen *Aggregatibacter actinomycetemcomitans,* total RNA was isolated from bacteria cultured with iron supplementation or chelation. Transcriptional analysis demonstrated that the expression of four sRNA molecules (JA01–JA04) identified by bioinformatics was significantly upregulated in iron-limited medium compared with iron-rich medium. A DNA fragment encoding each sRNA promoter was able to titrate *Escherichia coli* ferric uptake regulator (Fur) from a Fur-repressible reporter fusion in an iron uptake regulator titration assay. Cell lysates containing recombinant AaFur shifted the mobility of sRNA-specific DNAs in a gel shift assay. Potential targets of these sRNAs, determined *in silico*, included genes involved in biofilm formation. The *A. actinomycetemcomitans* overexpressing JA03 sRNA maintained a rough phenotype on agar, but no longer adhered to uncoated polystyrene or glass, although biofilm determinant gene expression was only modestly decreased. In summary, these sRNAs have the ability to modulate biofilm formation, but their functional target genes remain to be confirmed.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a gram-negative, capnophilic bacterium associated with aggressive forms of periodontitis and less commonly with systemic diseases such as endocarditis, atherosclerosis and brain abscesses (Zambon, 1985; Kaplan *et al.*, 1989). *Aggregatibacter actinomycetemcomitans* produces *flp* bundle-forming fimbriae, extrapolymeric substance (EPS) and lipopolysaccharide (LPS), all of which have been implicated in initial colonization and biofilm formation (Scannapieco *et al.*, 1987; Rosan *et al.*, 1988; Inoue *et al.*, 1998; Kachlany *et al.*, 2000; Kaplan *et al.*, 2003; Schreiner *et al.*, 2003; Tomich *et al.*, 2007). Although environmental factors such as nutrient stress, pH, oxygen tension (Scannapieco *et al.*, 1987; Haase *et al.*, 2006) and iron concentration (Amarasinghe *et al.*, 2009) have been shown to influence the expression of fimbriae and other biofilm determinants, the molecular mechanisms that regulate biofilm formation are unknown. In other bacterial species, such as *Vibrio cholerae* and *Escherichia coli*,

biofilm formation is regulated in part by iron via short non-coding RNA molecules called small regulatory RNA (sRNA) (Mey *et al.*, 2005a,b). We hypothesize that iron-regulated sRNAs play a role in biofilm formation and dispersion in *A. actinomycetemcomitans*.

Small regulatory RNAs play an important role in the regulation of bacterial gene expression, especially under stress conditions. These sRNAs are typically 50–200 nucleotides in length and are not translated into protein (Gottesman, 2004a,b). Most bacterial sRNAs identified so far target mRNAs (in *trans*) via imperfect sequence complementary base pairing (Gottesman, 2002; Masse *et al.*, 2003). A well-known sRNA molecule in *E. coli*, RyhB, is repressed by the ferric uptake regulator (Fur) protein in *E. coli* and is expressed by the cell only during iron starvation (Gottesman, 2002; Masse *et al.*, 2003). Binding of Fur to a promoter commonly negatively regulates gene transcription. The discovery of RyhB provides a mechanism by which Fur can indirectly increase transcription from certain promoters. Under conditions of iron limitation, Fur has no binding affinity for the Fur box located near the *ryhB* promoter, allowing *ryhB* to be transcribed. RyhB sRNA promotes the degradation of *sodB* mRNA, an iron superoxide dismutase, and other target transcripts (Gottesman, 2002; Masse *et al.*, 2003). In iron-sufficient conditions, Fur assumes a conformation with high affinity for the Fur box in the *ryhB* promoter. Binding of Fur represses transcription of *ryhB*, so preventing degradation of the target mRNAs. This paper reports the presence of several iron-regulated and Fur-regulated sRNA molecules in *A. actinomycetemcomitans* that may be involved in biofilm development.

METHODS

Bacterial strains and culture conditions

Strains and plasmids used in this study are listed in Table 1. *Aggregatibacter actinomycetemcomitans* HK1651 rough phenotype (HK1651R) was cultured for 48–72 h anaerobically (5% CO_2, 10% H_2, 85% N_2) at 37°C from frozen stock to plates containing Bacto™ tryptic soy broth (Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.6% yeast extract, 0.04% sodium bicarbonate (TSBY), and 1.5% Bacto™ agar (Becton Dickinson and Co.). Broth cultures were grown statically overnight in TSBY at 37°C either in an anaerobic chamber or in a candle extinction jar. The spontaneous nalidixic acid-resistant strain HKR.Nal was obtained by growing a dense mid-log cell suspension on TSBY agar containing 20 μg ml^{-1} nalidixic acid followed by culture on plates containing 50 μg ml^{-1} nalidixic acid. TSBY (iron-sufficient) medium used in routine culturing of *A. actinomycetemcomitans* contains sufficient iron for robust growth. Iron-supplemented conditions were achieved by addition of $FeCl_3$ to 300 μM to TSBY just before use, while iron-limited conditions (iron-chelated) were obtained by addition of the iron chelator 2,2′-dipyridyl (Sigma, St Louis, MO) to 300 μM to the culture medium. Mutant strains of *A. actinomycetemcomitans* were grown in TSBY supplemented with 40 μg ml^{-1} kanamycin, and overexpressing strains were grown in TSBY supplemented with 2 μg ml^{-1} chloramphenicol. The *E. coli* and corresponding plasmids were maintained in Difco™ Luria-Bertani (LB) broth, Lennox (Becton Dickinson and Co.) supplemented with 50 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin, as needed.

Identification of Fur-regulated sRNA molecules

Several potentially Fur-regulated sRNA molecules were identified in *A. actinomycetemcomitans* using a bioinformatics approach based on three criteria previously reported to identify sRNA sequences in other bacterial species: (i) sequences present in InterGenic Regions (IGR), (ii) sequences ending with a ρ-independent terminator, a stem-loop followed by a run of T nucleotides, and (iii) sequences regulated directly by the Fur protein (containing a Fur box in the promoter region) (Wilderman *et al.*, 2004). The *A. actinomycetemcomitans* HK1651 genome (http://www.genome.ou.edu/act.html) (Roe *et al.*, 2006) was first partitioned into two data sets at the Oral Pathogen Sequence Database (http://www.oralgen.lanl.gov/), one that included intergenic sequences and the other consisting of all the annotated open reading frames. Using a simple pattern search based on the degenerate *E. coli* Fur-box sequence, NAT(A/T)ATNAT(A/T)ATNAT(A/T)ATN (Escolar *et al.*, 1999), several potential Fur-regulated sRNA molecules were identified in the intergenic data set. Next, the RNA structure prediction software MFOLD (Version 3.2, http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi)

Table 1 List of strains used and engineered in this study

Strain	Relevant characteristic(s)	Source
Aggregatibacter actinomycetemcomitans		
HK1651R	Clinical isolate HK1651, genome project; (R) rough colony variant	ATCC
HKR.Nal	HK1651R with a spontaneous mutation (Nal^r)	This work
HKR.*fur::kan*	HKR.Nal with *fur::kan* mutation; Nal^r, Km^r	This work
HKR.*fur::kan*-pJAK16	HKR.*fur::kan* with pJAK16; Nal^r, Km^r, Cm^r	This work
HKR.*fur::kan*-pJAK*fur*	HKR.*fur::kan* with pJAK-*fur*; Nal^r, Km^r, Cm^r	
HKR(pJAK16)	HKR.Nal containing pJAK16; Nal^r, Cm^r	This work
HKR(pJAK-*fur*)	HKR.Nal containing pJAK-*fur*; *fur* overexpression strain; Nal^r, Cm^r	This work
HKR(pJAK16-JA03)	HKR.Nal containing pJAK-JA03; JA03 overexpression strain; Nal^r, Cm^r	This work
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Sm^r) *endA1 nupG* phi80*lacZ*Δ*M15*	Invitrogen
BL21(DE3)	*fhuA2* (*lon*) *ompT gal* (λ *DE3*) (*dcm*) Δ*hsdS*λ *DE3* = λ *sBamHIo* Δ*EcoRI-B int::*(*lacI::PlacUV5::T7 gene1*) *i21* Δ*nin5*	Novagen
BL21(DE3)(pAaFur)	BL21(DE3) containing pAaFur	This work
SK140	Top10 containing mobility plasmid pRK21761); Km^r	(Kachlany *et al.*, 2000)
SK140(pJAK16)	SK140 containing pJAK16; Km^r, Cm^r	This work
SK140(pJAK-*fur)*	SK140 containing pJAK16-fur; Km^r, Cm^r	This work
SK140(pJAK16-JA03)	SK140 containing pJAK16-JA03; Km^r, Cm^r	This work
H1717	*araD139* Δ(*argF-lac) U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF::*λ*plac*Mu53, Km^r, Sm^r, (*fhuF-lacZ* fusion, with *fur)*	(Hantke, 1987)
H1780	*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR fiu::*λ*plac*Mu53, (*fiu-lacZ* fusion, lacking *fur*)	(Hantke, 1987)
Plasmids		
pGEM-T	TA cloning vector; Amp^r	Promega
pGEMT-*fur*	pGEM-T containing HK1651R *fur* with engineered *Kpn*I, *Bam*HI sites; Amp^r	This work
pGEMT-*fur*::*kan*	pGEM-T containing HK1651R *fur*::*kan* with engineered *Kpn*I, *Bam*HI sites; Amp^r	This work
pGEMT-JA03	pGEM-T containing HK1651R JA03 with engineered *Kpn*I, *Bam*HI sites; Amp^r	This work
pGEMT-JA01pro	pGEM-T containing the HK1651R JA01 promoter; Amp^r	This work
pGEMT-JA02pro	pGEM-T containing the HK1651R JA02 promoter; Amp^r	This work
pGEMT-JA03pro	pGEM-T containing the HK1651R JA03 promoter; Amp^r	This work
pGEMT-JA04pro	pGEM-T containing the HK1651R JA04 promoter; Amp^r	This work
pET-30b	Novagen expression vector; Amp^r	Novagen
pAaFur	pET-30b with the HK1651R *fur* gene cloned into the *Nco*I/*Nto*I sites; Amp^r	This work
pRK21761	RK2 IncP Km^r, Amp^r, Tet^s, Lac$^+$ (*tetA*::*lacZ*) *oriT1* Mob$^+$	(Sia *et al.*, 1996)
pMB78	USS-containing cloning vector; suicide vector used for allelic exchange; Amp^r	(Bhattacharjee *et al.*, 2007)
pMfur	pMB78 containing HK1651R *fur*; Amp^r	This work
pMfur.KanEZ	pMfur with insertion in *fur* gene; Amp^r, Km^r	This work
pMJA03	pMB78 containing HK1651R JA03; Amp^r	This work
pJAK16	IncQ expression plasmid; Cm^r	(Thomson *et al.*, 1999)
pJAK-*fur*	pJAK16 containing HK1651R *fur*; Cm^r	This work
pJAK-JA03	pJAK16 containing HK1651R JA03; Cm^r	This work
Transposon		
EZ::Tn5 < KAN-2>	Tn5 transposon containing a Km^r cassette	Epicentre Biotechnologies

Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tet, tetracycline; ^{r}, resistant; ^{s}, sensitive; Sm, streptomycin; ATCC, American Type Culture Collection.

(Zuker, 2003) was used to select among the sRNAs for structurally more reliable molecules with enthalpy values ranging from −20 to −38, potential Fur boxes and terminal stem-loop structures immediately followed by a series of T nucleotides (U nucleotide for RNA).

Small RNA target prediction

Once potential Fur-regulated sRNA molecules were identified, the software program TargetRNA (Version 1.0, http://cs.wellesley.edu/~btjaden/predictions/

aggregatibacter/index.html) (Tjaden *et al.*, 2006) was used to identify potential mRNA targets. The following default parameter settings for TargetRNA were used, as previously described: the individual base-pair model of hybridization scoring was used, the putative terminator stem-loop of sRNAs was removed, the search for base-pair binding in mRNA sequences was restricted to a region from 30 nucleotides upstream of translation initiation to 20 nucleotides downstream of translation initiation, G-U base pairs were not considered when the hybridization seeds of a minimum number of consecutive base pairs were determined, and potential base-pair binding interactions were considered significant only if their *P*-value fell below 0.05 (Mellin *et al.*, 2007). Further, mRNA sequences were considered as potential targets only if their predicted interaction with sRNAs contained a hybridization seed of at least eight consecutive base pairs (Mellin *et al.*, 2007).

RNA isolation and analysis

Total RNA was prepared from *A. actinomycetemcomitans* by a modification of the sodium dodecyl sulfate (SDS) lysis/CsCl cushion procedure, as previously described (Haase *et al.*, 2003). Briefly, bacterial cells were grown anaerobically to late log to early stationary phase (~21 h), scraped from the vessel walls, and harvested by centrifugation. The cells were washed with cold lysis buffer (50 mM Tris–HCl, pH 7.5), and resuspended in lysis buffer containing RNAprotect™ (Qiagen, Valencia, CA). The suspension was passed through a 20-gauge needle several times to break up aggregated cells. One milliliter of 20% SDS was added to the cell suspension, then vortexed briefly and incubated at 37°C with vortexing once every minute until the cells were completely lysed. This was followed by addition of 4 g solid CsCl to the suspension, with slow mixing by gentle inversion for 2 min. An additional 8 ml cold lysis buffer was added and mixed, and the precipitate was removed by centrifugation at 15,000 ***g*** for 10 min. The supernatants were carefully layered onto 4 ml 5.7 M CsCl cushions. Total RNA was pelleted by ultracentrifugation (Beckman L8-M with a SW28 rotor) at 102,000 ***g*** at 20°C for 28 h. Supernatants were removed by inversion, the pellets were dissolved in 100 μl diethyl pyrocarbonate (DEPC)-treated water, 3 volumes 95% ethanol were added and the RNA was pelleted by centrifugation, pellets were reconstituted in 100 μl DEPC-treated water, divided into aliquots and stored at −70°C. RNA was evaluated for quantity by measurement of absorption at 260 nm and 280 nm, using a Nanodrop spectrophotometer, and for integrity by using formaldehyde agarose gel electrophoresis.

RNA isolation for Northern blots

Total RNA was isolated from *A. actinomycetemcomitans* during late log to early stationary growth phase [optical density at 600 nm (OD_{600}) ~ 0.12]. Briefly, cells were grown in 20 ml TSBY in a tissue culture flask (Falcon, Becton Dickinson and Co.) for about ~ 21 h at 37°C in a candle jar. Cells were scraped from the vessel walls and pelleted by centrifugation for 5 min at 6000 ***g*** at 4°C. Pellets were resuspended in cold lysis buffer (50 mM Tris–HCl, pH 7.5) containing RNAprotect™ (Qiagen), incubated at room temperature for 5 min before centrifugation in a swinging bucket rotor at 4000 ***g*** at 20°C. The RiboPure™-Bacteria Kit (Ambion, Applied Biosystems, Carlsbad, CA) was used to recover total RNA according to the manufacturer's instructions with the following modifications. After the addition of lysis buffer (RNAwiz), the pellet was mixed by pipetting up and down several times for 1 min, and then vortexed for 1 min to break up micro-colonies before adding to a tube containing Zirconia beads. The tubes were placed on a Mini-Beadbeater 8 (BioSpec Products, Bartlesville, OK) and pulsed for 1 min at 4°C followed by resting for 1 min; the bead beating cycle was repeated a total of three times. Total RNA was recovered from the lysate by extraction with chloroform. To enrich for sRNA, one-third volume of 100% ethanol was added to the aqueous layer and mixed thoroughly before applying to a spin column filter from the *mir*Vana™ miRNA Isolation kit (Ambion, Applied Biosystems). Filtrate was applied to another spin column and eluted following the manufacturer's protocol. RNA samples were treated with Turbo-DNase-free (Ambion, Biosystems), quantified on the Nanodrop spectrophotometer, and integrity assessed by agarose gel electrophoresis.

Northern blot

Small RNA was isolated from *A. actinomycemcomitans* as described. The sRNA samples (10 μg) were

combined with gel loading buffer II (*mir*Vana miRNA kit) and incubated at 95°C for 5 min before loading. Gels of 6% AccuGel™ 19 : 1 acrylamide (National Diagnostics, Atlanta, GA) and 7 M urea were prepared in 1 × Tris–borate EDTA (TBA; National Diagnostics) and pre-run in 1 × TBE for 1 h at 400 V. After loading, gels were run for ~ 1 h at 200 V in 1 × TBE. RNA size markers with transcripts ranging from 1000 to 100 bases (RNA Century-Plus ladder, Applied Biosystems) were used to estimate transcript size. DNA (2 μg) corresponding to each sRNA used to generate the Northern blot probe was used as an additional control. RNA was transblotted (Hoefer Scientific, Holliston, MA) to Hybond N+ membrane (Amersham, GE Healthcare, Piscataway, NJ) in 1 × TBE for 75 min at 200 V, and UV cross-linked. Blots were prehybridized and hybridized overnight at 42°C in ULTRAHyb® buffer (Ambion, Applied Biosystems) according to the NorthernMax® protocol (Ambion, Applied Biosystems). To generate double-stranded DNA probes complementary to candidate sRNA, chromosomal DNA from strain HK1651R (MasterPure™ DNA Purification Kit, Epicentre Biotechnologies, Madison, WI) was amplified by polymerase chain reaction (PCR) with primers as listed in Table 2. Reactions for JA01, JA02 and JA03 were: 2 min at 95°C (one cycle), followed by 30 cycles of 30 s at 95°C, 1 min at 51°C, 2 min at 72°C and then 10 min at 72°C (one cycle). An annealing temperature of 56°C was used with JA04 primers. The PCR products were column-purified (QIAquick, Qiagen) and quantified using the Nanodrop spectrophotometer. DNA was labeled with biotin using the BrightStar®–Psoralen-Biotin kit (Ambion, Applied Biosystems), and assessed for sensitivity according to the manufacturer's protocol. The dsDNA probes were used at 1 pM and denatured before use. After hybridization, blots were washed with low-stringency and high-stringency buffers (NorthernMax®) followed by detection of the biotinylated probes using the BrightStar®–Biodetect™ Kit (Ambion, Applied Biosystems) according to the manufacturer's protocols. Signals were detected by exposure to autoradiography Hyperfilm ECL (Amersham, GE Healthcare).

Reverse transcription PCR

To synthesize cDNA, total RNA (5 μg) was pretreated with 1 μl Turbo DNase (Ambion) and RNA was re-quantified using the NanoDrop spectrophotometer. A 2-μg aliquot of DNase-treated RNA was used as template to synthesize first-strand cDNA using random primers as per the manufacturer's protocol (Invitrogen). Superscript II reverse transcriptase (200 U; Invitrogen) was added to the reaction tubes only. As a negative control for each RNA template, DEPC-treated water was added in place of Superscript II. For reverse transcription-PCR experiments, 5 μl cDNA was used for each reaction. The primers used are indicated in Table 2. Reactions were run as follows: one cycle of 2 min at 95°C, followed by 25–28 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C followed by a final cycle of 72°C for 5 min. Samples were resolved on a 1% agarose gel and stained with ethidium bromide. Genomic DNA was used as template for the positive control and no template was used for the negative control.

Real-time quantitative PCR

Complementary DNA was synthesized from total RNA, as described above. TaqMan primers and probes used for real-time quantitative PCR (qPCR; Primer Express, Version. 2.0; Applied Biosystems) are listed in Table 2. All reactions were run in triplicate according to the manufacturer's protocol, and repeated from three independent biological replicates. Controls included: (i) amplification with a primer/probe set for 16S rRNA, (ii) amplification with a reaction mixture without a template, and (iii) amplification without the reverse transcriptase. Reaction plates were processed on an Applied Biosystems 7500 real-time PCR system as follows: hot-start AmpliTaq Gold polymerase was activated at 95°C for 10 min followed by 40 cycles of denaturation for 15 s at 95°C with annealing and extension for 60 s at 60°C. The non-iron-regulated gene *glyA* was used as a control, and 16S rRNA was used as an endogenous control.

For real-time qPCR analysis using SYBR green dye, specific primers were designed for selected genes, as indicated in Table 2. Each primer set was pretested by PCR using genomic DNA as template to ensure that a single amplicon was produced. For real-time qPCR, each reaction mixture (total volume, 25 μl) contained 5 μl template, 3.5 μl DEPC water, 12.5 μl Power SYBR green PCR master mixture (Applied Biosystems) and 2 μl each of the forward and reverse primers (100 nM). Real-time qPCR was performed using an Applied Biosystems 7500

Table 2 List of oligonucleotides used in this study

Primer name	Primer sequence (5′–3′)[1,2,3]
Plasmid verification	
T7-F (pGEMT vectors)	TAATACGACTCACTATAGGG
pTAC-F	TTGACAATTAATCATCGGCTCGTATAAT
pJAK16-R	CACACTACCATCGGCGCTAC
Northern blot probes	
JA01-F	ACCGCTAAATAATAGCCAACTGGC
JA01-R	CAGTGCACTTGTGCCGATAACCT
JA02-F	GCACAAGATAATGTGTTGGATGTGA
JA02-R	AATGCGATTTTTACCACGCA
JA03-F	AGCCACGTAATAACTGCTGA
JA03-R	TGTTATGCGAAATTAGTGACAAAGT
JA04-F	ATTCTTGCGAACTTTTTCCGTT
JA04-F	TCTCACGAAGAGAGGCAAATAACC
Small RNA and Fur overexpression	
JA03-KpnI-F	CGGGGTACCCCGTAATGCGAAACGTGCTTGAA; *Kpn*I
JA03-BamHI-R	CGCGGATCCGCGCCAACTCCTTTCCTTAATTTGTT; *Bam*HI
Fur-KpnI-F	CGGGGTACCCCGGCTTAATAGCAAAAAGGAGCTG; *Kpn*I
Fur-BamHI-R	CGCGGATCCGCGAGACCGCCTTTTGCATGGT; *Bam*HI
ActF-194-F	TGACACAGGCAATACGGAAA
FURTA and EMSA	
JA01-pro-F	TTCTACGCAAGGTTGGTTTATTT
JA01-pro-R	TGTTATCTACGCACTATATCAAATTCA
JA02-pro-F	ATTTTGCACAAGATAATGTGTTGG
JA02-pro-R	AAATTTGTCCCTTTACACAAATTTTA
JA03-pro-F	AAAGCCACGTAATAACTGCTGA
JA03-pro-R	GAAAACACAGAGTTATTCAAAGGTCA
JA04-pro-F	TTTTGATTAAAAATTAAGCGTTCG
JA04-pro-R	AAGCCTGCGATTTTCCTGTA
Antibiotic cassette	
Km-R	GCAATGTAACATCAGAGATTTTGAG
TaqMan probes/primers for real-time qPCR	
tadV-F	GCGGCCAAGTTTTTTCTTTTTCTT
tadV-R	GCAATCCGTTTTCTTTAATTGATTTACG
tadV-MGB probe	CCGGATTGGGACTAATT
pgaC[3]-F	TGGTTCAAGCCTTAGAGCAAGATC
pgaC[3]-R	CGGTTACGTACACGCGGATTA
pgaC[3]- MGB probe	CCTGTGGTAGCAGCATAT
rmlB-F	AACGTATTAGAAGATTGGTGCCTGTT
rmlB-R	CCTCCCATGACCCAATCGAAA
rmlB-MGB probe	AAACCACGCCTTATTC
JA01-F	TGACCGCTAAATAATAGCCAACTG
JA01-R	TGTGCCGATAACCTTAAAATCATTTT
JA01-Probe	FAM-CGATTTCTTTGGATGGTTTTCAAGGCACTATAAC-NFQ
JA03-F	CGTGCTTGAATTTATGCTTACACTTC
JA03-R	AACACAGAGTTATTCAAAGGTCACTATTACTAG
JA03-Probe	FAM-TGTTACAAGGCTTTATTTCTTCAGTTTTCATCACGTT-NFQ
JA04-F	TCTTGCGAACTTTTTCCGTTATACT
JA04-R	CCTAAGGAACCAATTTTATTAAAACAACTG
JA04-Probe	FAM-TGTCATAAATTACGAGCAACT-NFQ
16S rRNA-F	ACGCGAAGAACCTTACCTACTCT
16S rRNA-R	CCTAAGGCACAAACCACATCTCT
16S rRNA-MGB probe	CATCCGAAGAAGAACTC
tadG-F	ATCCCTTCCTTGCCGAGTGT

Table 2 (Continued)

Primer name	Primer sequence (5′–3′)[1,2,3]
tadG-R	CAATAGATTTTGCTGTGCCATTTT
tadG-Probe	FAM-AAAACACAGCCTAAGAAT-NFQ
ltxD-F	TGTTAAAACTGACCGCACTTGGT
ltxD-R	CTTGAGAAAGCGATGTCTTCGTT
ltxD-Probe	FAM-CCGAAGCGGATACGTTA-NFQ
fur-F	TGAAGGCAATAAATCAGTTTTCGA
fur-R	CGGTGCAGATAATGTGGTCATG
fur-Probe	FAM-TGGCGCCAACCC-NFQ
Primers for RT-PCR	
JA01-F	TTCTACGCAAGGTTGGTTTATTT
JA01-R	TGTTATCTACGCACTATATCAAATTCA
JA02-F	ATTTTGCACAAGATAATGTGTTGG
JA02-R	AAATTTGTCCCTTTACACAAATTTTA
JA03-F	AAAGCCACGTAATAACTGCTGA
JA03-R	GAAAACACAGAGTTATTCAAAGGTCA
JA04-F	TTTTGATTAAAAATTAAGCGTTCG
JA04-R	AAGCCTGCGATTTTCCTGTA
SYBR GREEN Primers for real-time qPCR	
hemU-F	CGTTATTGATGATGCGTTGG
hemU-R	GGAATAATCAAGCCGATCCA
murD-F	CCTTAACCGGTTTGCAGGTA
murD-R	CAATGGCGTGTTCTATGGTG
rsgA-F	CACACCAATTCGCTTCGTTA
rsgA-R	CTTCGTGCTGTTCTTCAACG
hitC-F	CTTGGGTAACGGCAAAGGTA
hitC-R	TTCATCCAGGGCACTAAAGG
galE-F	CCGTGTTCGGTAGCGACTAT
galE-R	TGTCATTTGCCTGCTCAAAG
glyA-F	GGATTCTCCGCGTATTCTCA
glyA-R	CGCCTAAGGTTTTGTGGGTA
16S rRNA-F	ACGCGAAGAACCTTACCTACTCT
16S rRNA-R	CCTAAGGCACAAACCACATCTCT

[1]Underline denotes restriction sites of enzyme listed to the right side of the sequence.

[2]Primer sequences based on HK1651 sequence unless otherwise noted.

[3]TaqMan probe and primer sequences based on D7 sequence.

MGB, minor groove binder; FAM, fluorophore; NFQ, non-fluorescent quencher.

FURTA, ferric uptake regulator titration assay; EMSA, electrophoretic mobility shift assay; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription PCR.

Real-time PCR System with the following thermal cycle recommended for the Power SYBR green PCR master mixture: 95°C for 10 min and then 40 cycles of 30 s at 95°C and 1 min at 56°C. Dissociation curves were generated by incubating reaction products at 95°C for 1 min and at 56°C for 30 s and then incrementally increasing the temperature to 95°C. Fluorescence data were collected at the end of the 56°C primer annealing step for 40 amplification cycles and throughout the dissociation curve analysis. Analysis of the melting curves with both primer sets revealed a single sharp peak. The non-iron-regulated gene *glyA* was used as a control, and 16S rRNA was used as an endogenous control.

Amplification data were analysed using ABI PRIMER DESIGN SDS 2.1 software (Applied Biosystems). Relative quantification of gene expression was performed by the C_t method, with 16S rRNA expression serving as an endogenous control to normalize target expression within each sample (Winer *et al.*, 1999). By comparing the threshold cycle, the relative expression of a given gene was determined. Relative quantification of gene transcription from JA03 overexpression strain HKR(pJAK16-JA03) was calibrated to HKR.Nal

levels. Statistical significance was determined by Student's *t*-test.

Ferric uptake regulator titration assay (FURTA)

Ferric uptake regulator titration assay, performed as previously described, employs a Fur-regulated *lacZ* fusion as a reporter gene that allows the detection of transformants carrying multicopy Fur-binding sites as Lac^+ colonies on MacConkey agar plates (Stojiljkovic *et al.*, 1994). In brief, potential sRNA promoter sequences were amplified by PCR using the primers listed in Table 2 with genomic DNA (MasterPure DNA Purification kit, Epicentre Biotechnologies) from HK1651R as template. Amplicons were cloned into the pGEM-T cloning vector (Promega, Madison, WI) according to the manufacturer's instructions, and subsequently transformed into *E. coli* TOP10 cells. Transformants were selected on LB agar containing ampicillin, and confirmed by colony PCR using the T7 forward primer (T7-F) and the appropriate sRNA-specific reverse primer (Table 2). Plasmid DNA was isolated (Qiagen) and transformed into *E. coli* H1717, a Fur^+ strain containing the Fur-regulated *fhuF* gene fused to *lacZ.* To perform the FURTA, transformants were screened for the Lac^+ phenotype on Difco™ MacConkey lactose agar plates (Becton Dickinson and Co.) containing 100 mg ml^{-1} ampicillin in the presence or absence of 50 μM $FeSO_4$ supplementation after 30 h of growth at 37°C. In the Fur^+ H1717 in the presence of iron, Fur binds to the *fhuF* promoter region preventing *lacZ* reporter gene expression that results in white colonies on MacConkey lactose agar plates. However, Fur will not bind to the *fhuF* promoter region in the absence of iron or in the presence of both iron and Fur-binding sequences carried on a multicopy plasmid that will titrate out the Fur protein from the binding site within the reporter construct. In both of these latter cases, the *lacZ* reporter gene is expressed and the colonies appear red on MacConkey lactose plates.

β-galactosidase assay

For quantitative analysis of FURTA experiments, *E. coli* H1717 cells containing the sRNA promoter constructs with putative Fur-box sequences grown on MacConkey plates supplemented with 50 μM $FeSO_4$ were suspended in LB medium (iron-rich, 10–30 μM Fe^{3+}) containing 100 mg ml^{-1} ampicillin and grown overnight. The cells were diluted 1 : 20 in fresh LB–ampicillin medium and grown to stationary phase. Cells were permeabilized and the β-galactosidase activity was measured as previously described (Miller, 1992). Each sample was analysed in triplicate from two independent assays, and the data were pooled for statistical analysis.

Overproduction and purification of Fur

For use in subsequent Fur-binding assays recombinant Fur protein was overproduced using the T7 RNA polymerase/promoter system. The corresponding coding sequence of the *fur* gene from *A. actinomycemcomitans* database strain HK1651 was PCR amplified and cloned into the *Nco*I/*Not*I sites of a pET-30b expression vector (Novagen, EMD Biosciences, San Diego, CA). Novasingles competent *E. coli* cells (Novagen) were transformed with the *fur* construct (pAaFur). Transformants were selected on kanamycin-containing LB agar plates and the sequence of the selected clone was verified by nucleotide sequencing. The plasmid (pAaFur) was isolated using QIAprep miniprep spin columns (Qiagen) and transformed into *E. coli* expression host BL21(DE3) strain. Transformants were selected on LB agar containing kanamycin and grown at 37°C. Transformants containing pAaFur were confirmed by PCR using primers (Fur-*Kpn*I-F and Fur-*Bam*HI-R, Table 2) and by nucleic acid sequencing. To induce Fur expression, the selected transformant [BL21(DE3)(pAaFur)] was grown in LB broth containing kanamycin to OD_{600} 0.7–0.8, isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5–1 mM) was added, and the culture was shaken for 3 h at 37°C or 16 h at 25°C. After induction, the cell lysate was produced by sonication and unbroken cells were removed by centrifugation. The overexpressed recombinant protein was identified by electrophoresis on Coomassie-stained 14% SDS–polyacryamide gels in comparison with whole cell lysates from uninduced cells. Results were confirmed on Western blot using an *E. coli* anti-Fur antibody obtained from Dr Michael Vasil (University of Colorado).

Electrophoretic mobility shift assay

Non-radioactive electrophoretic mobility shift assay (EMSA) was performed using the digoxigenin (DIG)

gel shift kit (Roche Applied Science, Indianapolis, IN) as described by the manufacturer's instructions. DNA probes specific to the promoter of each sRNA were amplified from chromosomal DNA template by PCR using the primers listed in Table 2. End-labeling of the probes was carried out with digoxigenin-11-ddUTP (DIG-ddUTP) using terminal transferase in 20 μl reaction buffer containing 0.2 M potassium cacodylate, 0.25 M Tris–HCl, pH 6.6, 0.25 mg bovine serum albumin ml^{-1} and 5 mM $CoCl_2$ incubated at 37°C for 15 min, and then 2 μl 0.2 M EDTA (pH 8.0) was added to terminate the reaction. Binding reactions were carried out with 10 nM DIG-labeled probe and cell lysate from pAaFur plasmid (6 μM) in 20 μl reaction buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, Tween-20 (0.2%, weight/volume), 30 mM KCl, 1 μg poly(dI-dC), and 0.1 μg poly-L-lysine incubated at 37°C for 15 min. For the competition reaction, cell lysate was first incubated in the presence of 100-fold molar excess of competitor DNA (unlabeled specific DNA) followed by incubation with the labeled probe (Vogel *et al.*, 2009). The *E. coli* anti-Fur antibody (obtained from Dr Michael Vasil), diluted 1 : 50 or 1 : 250 in phosphate-buffered saline, was preincubated with cell lysate before incubation with labeled probe for supershift experiments. The bound product was resolved on a 5% polyacrylamide gel in 0.25 × TBE buffer at 4°C. The gel was electroblotted onto a nylon membrane and treated with an anti-DIG–alkaline phosphatase conjugate. DNA was detected by exposure to autoradiography Hyperfilm ECL.

Engineering the *fur* mutant

A mutation in the *fur* gene was constructed by transposon mutagenesis using the suicide vector pMB78 (Bhattacharjee *et al.*, 2007). The *fur* gene was PCR amplified with primers engineered with *Kpn*I and *Bam*HI restriction enzyme sites (Table 2). The *fur* gene was cloned into the TA-cloning vector pGEM-T (Promega), transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) and plasmid was isolated (Qiagen) according to the manufacturers' instructions. Following digestion of plasmid pGEMT-*fur* with the appropriate restriction enzymes, gel-purified fragments (Qiagen) were ligated into pMB78 to generate pMfur, which was then transformed into *E. coli* TOP10 cells. Transformants were selected on LB agar supplemented with ampicillin and confirmed by colony PCR using primers specific for the *fur* gene (Fur-*Kpn*I-F and Fur-*Bam*HI-R, Table 2). *In vitro* transposon mutagenesis (EZ-Tn5 < KAN-2 > ; Epicentre Biotechnologies) was performed according to the manufacturer's instructions on isolated plasmid to insert a kanamycin resistance (Km^r) cassette into the *fur* gene. After transformation of *E. coli* TOP10 with the intact plasmid, transformants were selected on LB agar supplemented with kanamycin and verified by colony PCR using the appropriate primers listed in Table 2. Plasmid was isolated from colonies containing the Km^r cassette in the proper orientation in the *fur* gene. Plasmid pM*fur*.KanEZ linearized by digestion with *Xma*I enzyme was either electroporated or transformed into chemically competent *A. actinomycetemcomitans* HKR.NaI to generate a *fur::kan* insertion mutant (Sreenivasan *et al.*, 1991; Bhattacharjee *et al.*, 2007). Transformants were selected on TSBY agar plate containing kanamycin (40 μg ml^{-1}). Correct incorporation of the *fur::kan* insertion mutation sequences into chromosomal DNA was verified by PCR using *fur* primers Fur-*Kpn*I-F and Fur-*Bam*HI-R, as well as forward primer (ActF-194-F) from the upstream gene *actF* and reverse primer Km-R from the Km^r cassette, and nucleotide sequencing (Biopolymer Facility of Roswell Park Cancer Institute, Buffalo, NY).

The functionality of the *fur::kan* mutation was determined using a Fur-deficient *E. coli* H1780 strain by testing its capacity to complement the Fur deficiency of *E. coli* H1780. The Fur-deficient *E. coli* H1780 strain contains a Fur-regulated *fiu* gene fused to a *lacZ* reporter gene. The *fiu* gene is constitutively expressed regardless of the iron conditions because of an engineered *fur* mutation in this strain. Consequently, β-galactosidase is also constitutively expressed. The *A. actinomycetemcomitans fur* gene was PCR amplified using specific primers from the parental and HKR.*fur::kan* strains, and cloned into pGEM-T plasmid generating pGEMT-*fur* and pGEMT-*fur::kan*, respectively. These plasmids, as well as the vector only (pGEM-T), were transformed into *E. coli* H1780. The H1780 strain with pGEM-T alone, H1780 with *A. actinomycetemcomitans* with wild-type *fur*, and H1780 with mutated *fur* were grown in iron-replete LB broth. β-Galactosidase activity was determined from stationary-phase cells, as described previously (Miller, 1992), but without IPTG induction.

Genetic complementation of *fur* mutation

The broad-host-range plasmid pJAK16 was used for *fur* complementation. The pJAK16 plasmid is an IncQ expression vector that contains a chloramphenicol-resistance gene and an IPTG-inducible P_{tac} promoter located upstream from a multiple cloning site (Thomson *et al.*, 1999). Plasmid pJAK-*fur* was constructed by restriction enzyme digestion of pMfur (Table 1) with *Kpn*I/*Bam*HI, yielding a 506-base pairs (bp) *fur* fragment which was gel purified and ligated into the *Kpn*I/*Bam*HI sites of pJAK16. The pJAK-*fur* plasmid and pJAK16 vector alone (negative control) were each transformed into chemically competent *E. coli* SK140, a TOP10 strain carrying RK2 *oriT*-defective mobilization plasmid pRK21761 (Table 1), as previously described (Thomson *et al.*, 1999), resulting in SK140(pJAK-*fur*) and SK140(pJAK16), respectively. Each *E. coli* strain was then used as the donor for conjugation with the HKR. *fur::kan* mutant strain, using a protocol previously described for rough phenotype strains of *A. actinomycetemcomitans* (Thomson *et al.*, 1999). Transconjugants were selected on TSBY agar containing chloramphenicol (2 μg ml^{-1}) and nalidixic acid (20 μg ml^{-1}). Uptake of pJAK-*fur* was confirmed by colony PCR using specific primers for the *fur* gene (Fur-*Kpn*I-F and Fur-*Bam*HI-R) and the pJAK-16 plasmid (pTac-F and pJAK16-R), and by nucleotide sequencing. Plasmid-harboring strains were grown in TSBY containing chloramphenicol (2 μg ml^{-1}); IPTG induction was not required for gene expression, because the P_{tac} promoter is leaky.

Overexpression of JA03 sRNA

The JA03 gene was PCR amplified with primers engineered with *Kpn*I and *Bam*HI sites (Table 2), cloned into pGEM-T, and transformed into *E. coli* TOP10. Following isolation of plasmid pGEMT-JA03 (Qiagen), the JA03 fragment (650 bp) produced by digestion with *Kpn*I/*Bam*HI was gel purified and cloned into pMB78 to yield pMJA03 (Table 1). The sRNA overexpression plasmid (pJAK-JA03) was constructed by enzyme restriction digestion of plasmid pMJA03 with *Kpn*I/*Bam*HI. The resulting JA03 fragment (650 bp) was gel purified and ligated into the *Kpn*I/*Bam*HI sites of the broad-host-range plasmid pJAK16 donated to us by Dr Jeffrey Kaplan at University of Columbia, NY, which placed JA03 under the control of an IPTG-inducible P_{tac} promoter (Fine *et al.*, 2005). The resulting plasmid, pJAK-JA03, and the pJAK16 vector alone (negative control) were transformed chemically into *E. coli* SK140. Each *E. coli* strain, SK140(pJAK16-JA03) and SK140(pJAK16), was used as the donor for conjugation with HKR.Nal, as previously described (Thomson *et al.*, 1999; Kachlany *et al.*, 2000). Transconjugants were selected on TSBY agar containing chloramphenicol and nalidixic acid. Uptake of extrachromosomal pJAK-JA03 generating SK140(pJAK16-JA03) was confirmed by colony PCR using specific primers for the gene encoding JA03 (JA03-*Kpn*I-F and JA03-*Bam*HI-R, Table 2), as well as by nucleic acid sequencing. Similarly uptake of the pJAK16 plasmid generating SK140(pJAK16) was confirmed by colony PCR using primers pTac-F and pJAK16-R (Table 2). Plasmid-harboring strains were grown in TSBY containing chloramphenicol (2 μg ml^{-1}); IPTG induction was not required for gene expression because the P_{tac} promoter is leaky.

Microtiter plate biofilm assay

Biofilm growth and quantification were determined using a modification of a standard microtiter plate biofilm assay, as described previously (Haase *et al.*, 2006). Briefly, frozen stock cultures were plated onto TSBY agar and incubated in a candle jar for 72 h at 37°C. One to five isolated colonies were transferred to TSBY broth and grown overnight. Cultures were standardized to OD_{600} of 0.050 and cells were harvested by centrifugation and resuspended in TSBY, and applied to 96-well, flat-bottomed, untreated polystyrene microtiter plates (Nalge Nunc International, Rochester, NY). Plates were covered and incubated for 21 h or 48 h in 5% CO_2 at 37°C. Growth was monitored at OD_{595} using a microplate reader (Beckman Coulter AD340; Brea, CA). Culture supernatants were decanted and unbound bacteria were removed by washing with phosphate buffered saline, pH 7.2. Biofilm cells were fixed with methanol and air-dried before staining with 0.1% (weight/volume) crystal violet (Sigma) for 5 min. Dye was decanted and wells were washed with distilled water until negative control wells were clear. After air-drying the plates, ethanol was used to solubilize the bound crystal violet. Plates were mixed briefly and the absorbance of the dye was quantified at 595 nm using a microplate reader. Each sample was tested in three biological replicates

with six replicates per assay. Statistical significance was determined by the Student's *t*-test.

RESULTS

Identification of sRNA in *Aggregatibacter actinomycetemcomitans*

We identified several Fur-regulated sRNA molecules in *A. actinomycetemcomitans* HK1651 using a bioinformatics approach based on criteria previously reported to identify sRNA sequences in other bacterial species. The sRNA target prediction tool TargetRNA was then used to identify potential mRNA targets of the sRNA molecules. The region proximal to the ribosome-binding site of each message in the *A. actinomycetemcomitans* genome was evaluated for a likelihood of interacting with sRNAs by homologous base pairing. TargetRNA predicted statistically significant interactions of four sRNAs (JA01, JA02, JA03 and JA04) with biofilm-associated genes, including the *flp* fimbrial operon and genes associated with EPS, LPS and several known iron-regulated proteins (see Table S1) (Chen *et al.*, 2002; Carrondo, 2003; Grifantini *et al.*, 2003; Balashova *et al.*, 2006b). Predicted base-pairing regions of each sRNA with putative targets are shown in Table S2. The putative nucleotide coordinates of each sRNA within the IGRs annotated from the online *A. actinomycetemcomitans* HK1651 sequence (Roe *et al.*, 2006) are as follows: JA01 (1366985–1367137 within IGR1299), JA02 (1109159–1109274 within IGR1069), JA03 (1446694–1446967 within IGR1375), and JA04 (356690–356770 within IGR0328). Computer modeling using Mfold predicted that each of these sRNAs had a stable RNA secondary structure (see Fig. S1).

Transcription of sRNAs

To verify that each candidate sRNA is indeed transcribed as a relatively small transcript and is not part of the downstream transcript, Northern blot analysis was performed. Total RNA was isolated from early stationary phase, separated on denaturing acrylamide gels, transferred to Hybond N+ membranes, and probed with biotinylated DNA probes corresponding to the complementary DNA sequence of each sRNA. The PCR amplicon used to generate the dsDNA probe was included on the blot as a positive control (data not shown). Transcript sizes were estimated from the RNA Century Plus Ladder (Ambion); the 500, 400 and 100 base markers consistently were detected on the blot using the Bright-Star Psoralen-Biotin kit, regardless of the probe. Bands visible for JA01 (*c.*155 nt), JA02 (*c.*105 nt) and JA04 (*c.*76 nt), as shown in Fig. 1, correlated well with the predicted size for JA01 (153 nt), JA02 (116 nt) and JA04 (81 nt) (see Fig. S1). Several attempts were made to obtain a transcript for JA03 on Northern blot using both agarose gels and different percentage acrylamide gels. Also, dsDNA probes from different regions in the putative sRNA region were used. The dsDNA probe could detect the probe loaded onto the gel, but no transcripts could be detected for JA03. This could be the result of secondary structure problems with the RNA on the gel or insufficient sensitivity of DNA probes (Vogel *et al.*, 2009).

Potential coding capacities of the sRNAs

The nucleotide sequence of each sRNA was examined for potential open reading frames in all three reading frames using DNASIS (ver. 3.0). There were no potential peptides greater than 2668 Da for JA01,

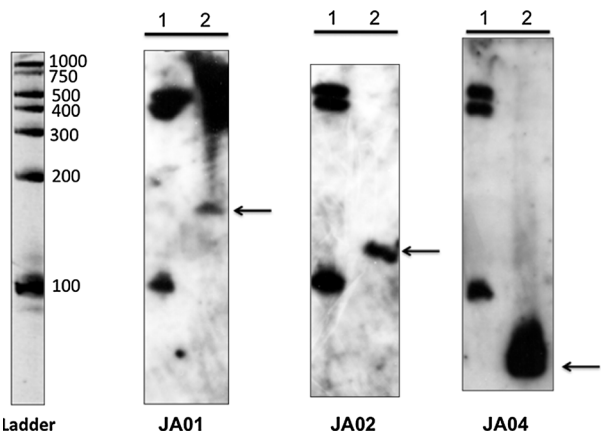

Figure 1 Northern blot analysis of small RNA (sRNA). The RNA Century Plus size ladder was loaded in lane 1 of each gel. The ladder was visualized by ethidium bromide staining as shown (inverted photograph). Total RNA was extracted from parental strain HKR.Nal grown in Bacto™ tryptic soy broth containing 0.6% yeast extract with 300 μM dipyridyl chelator. About 10 μg RNA was loaded in lane 2. After electrophoresis, each gel was electroblotted onto positively charged nylon membranes and hybridized with biotinylated dsDNA probes specific to JA01, JA02 and JA04, as indicated. Hybridization was detected with the BrightStar Psoralen-Biotin kit.

1303 Da for JA02 and 3096 Da for JA04. The size of the transcript for JA03 has yet to be determined so the entire IGR potentially encodes peptides no greater than 1809 Da, whereas the putative JA03 associated with a promoter containing a Fur box encodes for peptides no greater than 1614 Da.

Iron-responsive transcription of sRNAs

Initially, to determine if iron limitation had any effect on sRNA transcription, reverse transcription-PCR was performed to compare cells grown in iron-sufficient conditions (TSBY) with iron-chelated TSBY. Each of the sRNA molecules (JA01–JA04) was expressed to a greater extent under iron-limited (chelated) growth conditions (Fig. 2A). Then, to more accurately quantify the difference between these conditions, and to determine if additional iron had any effect on transcription, two-step real-time qPCR was performed with the addition of iron-supplementation of TSBY to the assay. Transcription of JA01, JA03 and JA04 increased by 12-fold, ~ 12-fold and 19-fold, respectively, in iron-chelated conditions when compared with growth in TSBY (Fig. 2B). Iron-supplemented TSBY suppressed sRNA transcription relative to TSBY. As a consequence of the short length and the nucleotide sequence of the IGR (211 bp) containing JA02, a reliable TaqMan probe could not be designed for the real-time qPCR uniform cycling conditions.

Fur regulation of sRNAs

In many cases, genes that respond to iron are commonly regulated by Fur, a global iron-dependent transcriptional regulator that binds to operators (Fur boxes) located proximally to the promoters (Kirby *et al.*, 2001; Bosch *et al.*, 2002; Osorio *et al.*, 2004; Najimi *et al.*, 2008). *In silico* analysis determined that each sRNA possessed a Fur box-like motif proximal to the putative −35 and −10 promoter sequences. These Fur box-like motifs were 63–74% (12–14 of the 19 bp) identical to the consensus Fur box of *E. coli* (see Table S3). Previously, a Fur box-like sequence with 68% identity to the consensus Fur box of *E. coli* was located downstream of a −35 and −10 sequence in the *fur* gene of *A. actinomycetemcomitans* (Haraszthy *et al.*, 2002). The Fur box of the *fur* gene of *A. actinomycetemcomitans* is 52–65% identical to the putative sRNA Fur box motifs reported in this study.

Although a consensus 19-bp nucleotide sequence for the Fur box has been determined for *E. coli*, it is clear that Fur binds to Fur boxes with only partial homology to that consensus sequence (Kirby *et al.*,

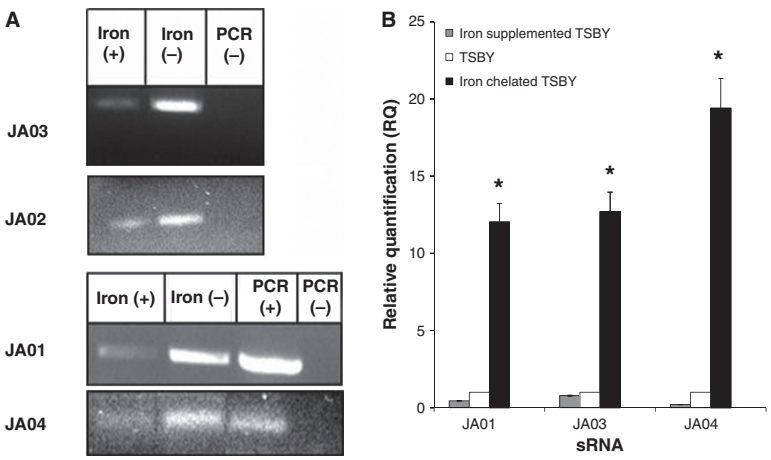

Figure 2 Iron regulation of small RNA (sRNA) candidates. (A) Reverse transcription-polymerase chain reaction (PCR) analysis: comparison of sRNA transcript expression in HKR.Nal grown in Bacto™ tryptic soy broth containing 0.6% yeast extract (TSBY; iron-sufficient) and iron-chelated TSBY, genomic DNA (PCR + control), and no template (PCR – control). (B) Real-time quantitative PCR analysis using TaqMan primers and probes: quantitative expression sRNA molecules JA01, JA03 and JA04 under iron-supplemented TSBY, TSBY (iron-sufficient), and iron-chelated TSBY growth conditions normalized to 16S rRNA gene expression. Expression level of each transcript under the iron-supplemented or iron-chelated conditions was relative to the expression in TSBY. *A significant difference ($P < 0.01$) of the test condition from the control.

2001; Bosch *et al.*, 2002; Osorio *et al.*, 2004; Najimi *et al.*, 2008). FURTA, a genetic assay for detecting Fur boxes, is routinely used to evaluate the Fur-binding potential of DNA sequences from various bacterial species that respond to iron (Kirby *et al.*, 2001; Bosch *et al.*, 2002; Osorio *et al.*, 2004; Najimi *et al.*, 2008). Genes from other bacteria possessing putative Fur boxes that differ from the consensus sequence [e.g. *Bordetella avium* (13/19 bp), *Photobacterium damselae* ssp (14/19 bp), *Aeromonas salmonicida* (13/19 bp) and *Pasteurella multocida*) are strongly positive in FURTA (Kirby *et al.*, 2001; Bosch *et al.*, 2002; Osorio *et al.*, 2004; Najimi *et al.*, 2008). Hence, FURTA was used to determine whether the putative Fur-box nucleotide sequences of each sRNA had the capacity to bind Fur.

Colonies of *E. coli* strain H1717 transformed with the control plasmid pGEM-T alone exhibited only a minimal LacZ expression phenotype (pale pink) when plated on iron-supplemented MacConkey agar. In contrast, colonies of strain H1717 transformed with plasmids encoding each of the four sRNA promoter constructs (pGEMT-JA01pro, pGEMT-JA02pro, pGEMT-JA03pro and pGEMT-JA04pro) exhibited dark red colony morphologies (Table 3). Quantification of β-galactosidase activity by the transformants confirmed the FURTA results (Table 3). These data strongly indicated that each of the DNA fragments encoding the sRNA putative promoter constructs was capable of titrating Fur in an iron-replete environment.

To better define the specificity of Fur binding to the predicted Fur box sequence of the four sRNA molecules, EMSA were performed using cell lysates from *E. coli* overexpressing the *A. actinomycetemcomitans* Fur protein (AaFur) (Fig. 3). As expected, no band shift in mobility was observed for each DIG-labeled sRNA promoter DNA in the absence of cell lysate (Fig. 3, lane 1). Preincubation of each DIG-labeled sRNA promoter DNA with 0.4 μg of cell lysate of *E. coli* overexpressing the AaFur, however, was associated with a shift in fragment mobility resulting in a band shift (Fig. 3, lane 2).

To ensure that AaFur and not EcoliFur caused the decreased mobility of the promoter sequence, *E. coli* lysate without AaFur failed to show a shift (data not shown). Unlike the FURTA, in which the titration of relatively small amounts *E. coli* Fur by the AaFur boxes transiently freed enough of the reporter gene of Fur repression to produce indicator enzyme, the gel shift assay requires relatively high levels of the Fur repressor to bind tightly and constantly to produce a shift in mobility. Only *E. coli* overexpressing AaFur was able to cause a gel shift. To confirm the association between AaFur and the AaFur box, a

Table 3 Ferric uptake regulator titration assay (FURTA) analysis of the small RNA promoters

sRNA promoter plasmid	Lac-FURTA[1]	Mean β-galactosidase activity (± SD)[2]
pGEMT-JA01pro	+	603 (± 85)
pGEMT-JA02pro	+	533 (± 22)
pGEMT-JA03pro	+	426 (± 46)
pGEMT-JA04pro	+	681 (± 18)
pGEMT-alone	–	176 (± 26)

[1]Lac phenotype of the *Escherichia coli* H1717 (Fur^+) containing the indicated plasmid after 30 h of incubation at 37°C on MacConkey agar supplemented with 50 μM $FeSO_4$. Lac^+ (red), lac^- (pale pink).
[2]β-Galactosidase activity, in Miller units, of *E. coli* H1717 containing the indicated plasmid grown to stationary phase in LB medium. SD, standard deviation.

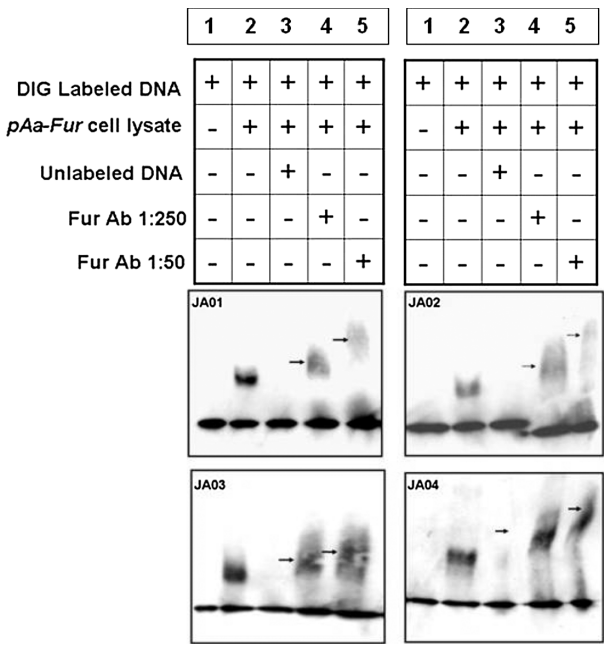

Figure 3 Electrophoretic mobility shift assay (EMSA). To demonstrate the binding of Fur to predicted small RNA (sRNA) promoters, digoxigenin-labeled DNA probes corresponding to JA01, JA02, JA03, and JA04 sRNA promoter regions were used in EMSAs with cell lysates from a plasmid overexpressing AaFur protein (pAaFur). Mouse anti-Fur antibody (diluted 1 : 50 and 1 : 250) was used to show the supershift of DNA–protein–antibody complex (arrows).

competition reaction was performed in which a 100-fold molar excess of unlabeled, sRNA-specific promoter DNA was employed. Shifting of mobility of the sRNA fragments in EMSA was inhibited by preincubation with this excess of unlabeled probe (Fig. 3, lane 3).

When anti-*E. coli* Fur antibody was added to the reaction, a supershifted band appeared in the gels (Fig. 3, arrows, lanes 4, 5). The mobility shift correlated with antibody concentration, i.e. more antibody (1 : 50 dilution) produced a greater shift than less antibody (1 : 250 dilution). The binding of anti-Fur antibody to the DNA : Fur complex further retarded the mobility, and the more antibody bound the greater the retardation of mobility. These data strongly indicate that the mobility shifts observed in the EMSA were the result of the direct interaction between AaFur and the Fur boxes located in the DNA fragments encoding the sRNA promoters.

Characterization of a *fur* mutant

To further investigate the role of the *fur* gene in regulating sRNAs in *A. actinomycetemcomitans*, a *fur* mutant strain was constructed by inserting a Km^r cassette into the *fur*-encoding region of the parental strain via transposon mutagenesis. The lack of functionality of the *fur::kan* mutation was verified by testing its capacity to complement the Fur deficiency of *E. coli* strain H1780 relative to HK1651R Fur. As expected, introduction of functional *A. actinomycetemcomitans* Fur on plasmid pGEMT.*fur* into *E. coli* H1780 was able to restore the defect of the *E. coli* H1780 strain and shut down β-galactosidase production. The mutated *fur* gene carried on plasmid pGEMT.*fur::kan* was unable to provide a functional Fur, and unable to complement the Fur defect of the H1780 strain; hence, constitutive β-galactosidase activity was observed in H1780, similar to pGEM-T vector alone (Fig. 4A). These data confirm that the Km^r cassette successfully disrupted the *fur* gene in the parental strain, rendering it non-functional.

The transcript levels of *fur* in HKR.*fur::kan* and in the parental strain HKR.Nal were compared by real-time qPCR. As expected, the *fur* gene was transcribed ~ 15-fold ($P = 0.02$) more in the parental strain than in the *fur::kan* mutant strain (Fig. 4B). Introducing a functional copy of *fur* carried on the pJAK-*fur* expression plasmid restored the *fur* deficiency of HKR.*fur*::*kan*. The complemented *fur::kan* strain, HKR(*fur::kan*-pJAK*fur*), had a 21.6-fold ($P = 0.008$) increase in *fur* transcription compared

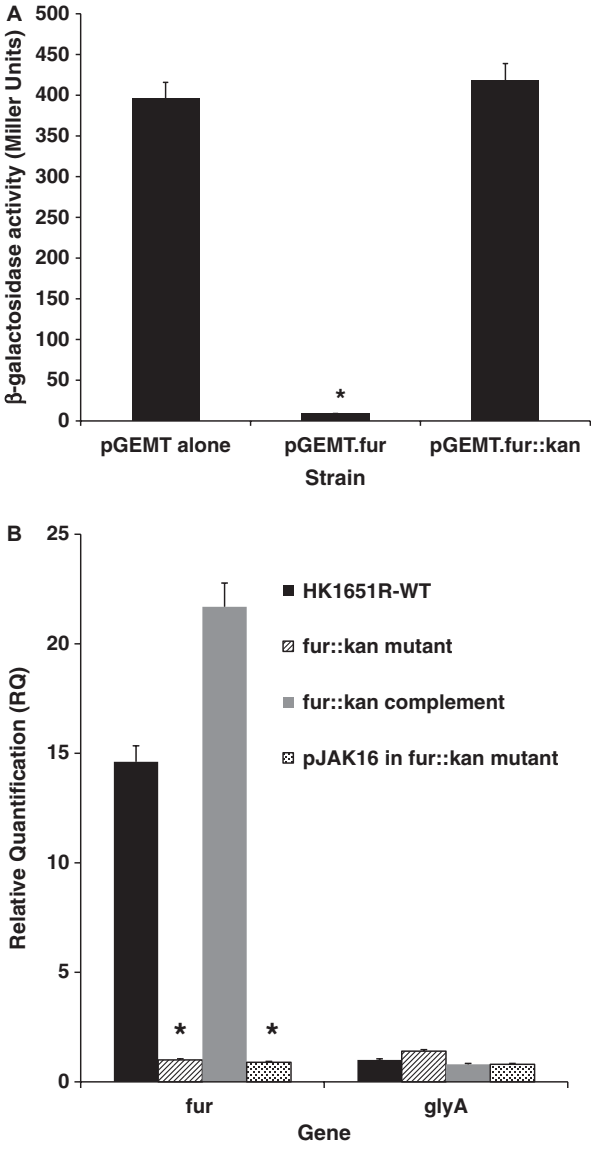

Figure 4 (A) Fur-complementation assay. Fur-deficient *Escherichia coli* H1780 was used to test for the ability of plasmids to complement the Fur deficit. The promoter of the Fur-regulated *fiu* gene is fused to a promoterless *lac*Z. β-Galactosidase is constitutively expressed in H1780. Samples: pGEMT-(vector alone); pGEMT.*fur* (strain complemented with Aa*fur*); pGEMT.*fur::kan* (Aa*fur* disrupted with Km^r cassette). (B) Effect of the *fur::kan* mutation and *fur* mutation complementation on the expression of *fur*. Real-time quantitative polymerase chain reaction was used to quantify *fur* expression in parental HKR.Nal and *fur::kan* mutant strains. The non-iron-regulated *gly*A gene was used as a control. 16S rRNA was used as the endogenous control. *A significant difference ($P < 0.05$) of the test condition from the control.

with mutant strain HKR.*fur::kan*, whereas *fur* expression in the *fur::kan* mutant strain with pJAK16 vector alone (HKR.*fur::kan*-pJAK16) was similar to that of HKR.*fur::kan*. Real-time qPCR data, together with quantitative FURTA experiments, confirm that *fur* gene expression was successfully interrupted by insertion of the Km^r cassette and that the *fur::kan* strain probably produced a non-functional or partially functional Fur.

Effect of Fur on transcription of sRNAs

To determine the role of Fur in the expression of selected sRNA molecules (JA01, JA03 and JA04), the transcript levels of these genes were examined using real-time qPCR comparing the *fur::kan* mutant strain and parental strain grown in TSBY (iron-sufficient) medium. Interestingly, the expression of all three sRNA molecules was significantly increased in the *fur::kan* mutant strain compared with the parental strain (Fig. 5A,B); notably, expression increased 4.9-fold for JA01 ($P = 0.032$), 9.7-fold for JA03 ($P = 0.039$) and 53.9 fold for JA04 ($P = 0.007$). When the *fur::kan* mutant strain was complemented by introduction of *fur* on an over-expressing plasmid, transcription of these sRNA molecules was reduced to the level in the parental strain. The sRNA expression level in the pJAK16 vector control strain was similar to the *fur::kan* mutant strain (Fig. 5A,B). These data indicate that these sRNA molecules are under direct control from the Fur master regulator.

The possibility of polar effects of *fur::kan* mutation on upstream and downstream genes was evaluated using real-time qPCR. Transcription of the flavodoxin-encoding gene, *fldA*, located upstream of *fur*, and AA02518 a gene directly downstream of *fur* were increased 3.0-fold ($P = 0.03$) and 2.7-fold ($P = 0.09$), respectively, in the *fur::kan* mutant strain compared with the parental strain. Both *fldA* and AA02518 are transcribed in the same direction as *fur*. The *fldA* gene is known to be iron repressible, and a similar increase in *fldA* gene transcription in *fur* mutant strains has been observed in other bacteria (Ghassemian & Straus, 1996; Achenbach & Genova, 1997; Bender *et al.*, 2007). Therefore, it is possible that the expression of *fldA* and AA02518 of *A. actinomycetemcomitans* are also repressed by Fur, as in other bacteria, which accounts for the increase of transcription of these genes observed in the *fur::kan* mutant strain.

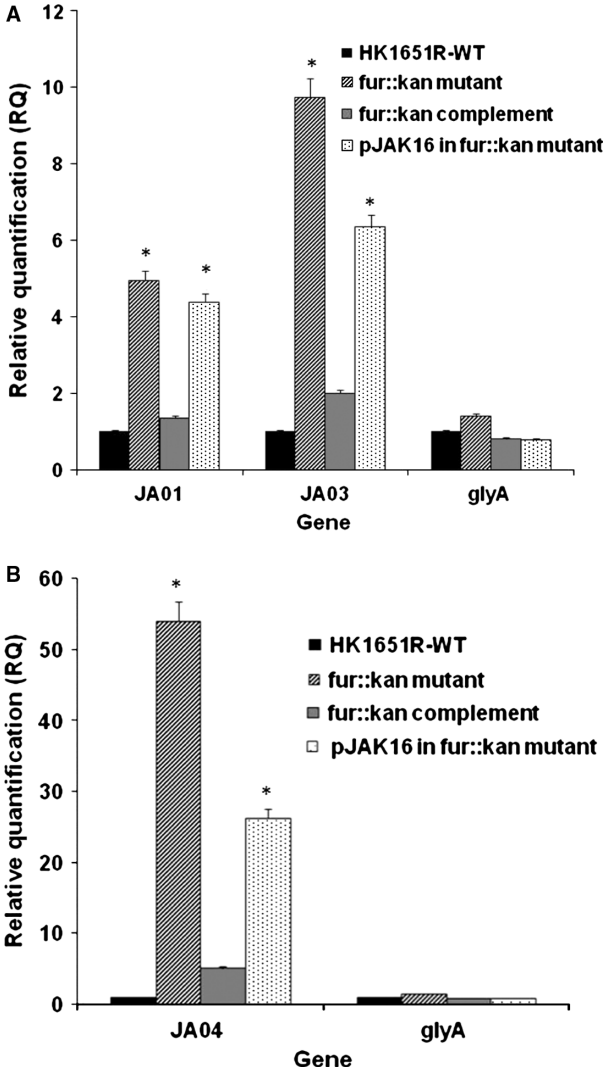

Figure 5 Effect of *fur::kan* mutation on expression of small RNAs (sRNAs). Expression of sRNA molecules JA01, JA03 and JA04 in parental HKR.Nal (indicated as HK1651R) and *fur::kan* mutant strains in real-time quantitative polymerase chain reactions. The non-iron regulated *gly*A gene was used as control. 16S rRNA was used as an endogenous control. *A significant difference ($P < 0.05$) of the test condition from the control.

Effect of Fur on biofilm

To determine whether biofilm formation is affected by *fur* mutation, a quantitative biofilm assay was performed in TSBY broth on stationary-phase (21 h) cells of the parental, *fur::kan* mutant, and *fur*.:*kan* complement strains, as well as the pJAK16 vector alone in the *fur::kan* mutant strain. The growth and quantity of biofilm mass was not significantly different for any of the strains tested (Fig. 6A,B). When biofilm

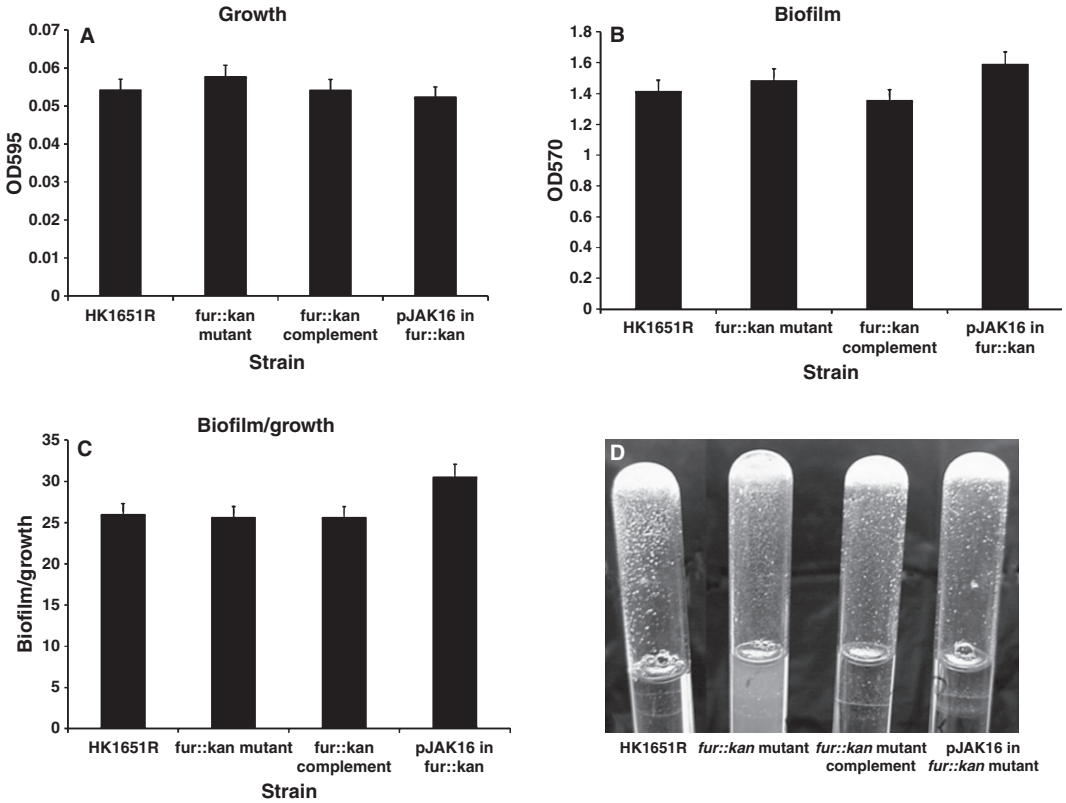

Figure 6 Effect of Fur on biofilm formation in Bacto™ tryptic soy broth containing 0.6% yeast extract (TSBY). Microtiter plate biofilm assay; (A) growth of cells as measured by optical density at 595 nm, (B) biofilm mass as measured by absorbance at 570 nm of crystal violet eluted from biofilm cells, and (C) biofilm mass normalized to cell density, i.e. biofilm : growth ratio. (D) Cultures grown for 24 h in glass tubes; biofilm formation on surface of the tube and supernatant turbidity. Antibiotic-resistant HKR.Nal (designated here as HK1651R) was used as wild-type (WT).

was standardized to growth, no significant differences were noted among the strains (Fig. 6C). However, when the strains were grown in glass tubes in TSBY broth the *fur::kan* mutant produced a biofilm with more slime and also produced a slightly turbid broth compared with other strains, as shown in Fig. 6D, suggesting a change in expression of some biofilm determinant genes when grown under these conditions.

Effect of JA03 sRNA overexpression on its mRNA targets and biofilm determinant genes

A strain overexpressing JA03 sRNA, HKR(pJAK-JA03), was engineered to determine the regulatory role exerted by JA03 sRNA on cellular functions. The JA03 sRNA insert in the expression plasmid was derived using primers encompassing nearly the entire IGR. In these experiments, JA03 was expressed from a plasmid under the control of the IPTG-inducible P_{tac} promoter in the adherent variant nalidixic-acid-resistant clinical isolate HKR.Nal. Hence, JA03 sRNA expression was independent of the Fur repressor, allowing analysis of target behavior independent of growth limitations resulting from iron starvation stress or iron toxicity, and in the presence of a functional Fur repressor protein. The positive effect of JA03 overexpression on JA03 sRNA transcription was clearly evident as indicated by real-time qPCR where expression of JA03 sRNA increased $\sim$ 70-fold ($P < 0.01$) compared with the parental strain or with HKR(pJAK16) harboring the pJAK16 vector alone. Expression of a control gene, *glyA* (serine hydroxymethyltransferase), a housekeeping gene with no iron-binding function, was similarly expressed in both JA03 sRNA overexpression and parental strains.

To examine the effect of JA03 sRNA overexpression on cellular functions, six targets of JA03 sRNA (*murD*, *rsgA*, *galE*, *ltxD*, *tadG* and *hitC*) predicted by TargetRNA and three biofilm determinant genes (*tadV*, *rmlB*, *pgaC*) that had been previously characterized (Amarasinghe *et al.*, 2009) were selected for evaluation by real-time qPCR. The expression of each potential target in HKR(pJAK16-JA03) was compared with the parental strain (Table 4). Interestingly, expression of JA03 from a heterologous promoter resulted in significant repression of transcription of *hitC*, an ATPase and a member of the *hitABC* gene cluster coding for a known periplasmic iron-binding transport system in *A. actinomycetemcomitans* (Rhodes *et al.*, 2007). JA03 sRNA overexpression correlated with repression of several other genes with non-iron functions, e.g. *murD*, a gene that encodes UDP-*N*-acetylmuramoylalanine-D-glutamate ligase, which is involved in the peptidoglycan-based cell wall biogenesis pathway, and *ltxD*, a gene encoding a protein involved in leukotoxin secretion. The *galE* gene that encodes a UDP-glucose-4-epimerase involved in the nucleotide sugar metabolic pathway was nominally increased, suggesting that JA03 sRNA probably does not control its expression. Biofilm-associated genes, *tadV* and *tadG* genes of the *flp* fimbrial operon, LPS-associated *rmlB* and EPS-associated *pgaC*, were repressed approximately 1.7-fold to 2.1-fold (Table 4), suggesting that JA03 sRNA may have a role in the regulation of biofilm formation/adherence in this organism. The non-heme ferritin gene *rsgA*, another putative target, was also slightly upregulated. Other potential targets (see Table S1) involved in biofilm formation, such as autoaggregation and type IV secretion genes, have yet to be tested by real-time qPCR. Transcriptome analysis of the overexpression strain will give a more global view of genes affected by this sRNA.

Effect of JA03 sRNA overexpression strain on biofilm formation

To determine if JA03 sRNA overexpression regulates *in vitro* biofilm formation by *A. actinomycetemcomitans*, the ability to form biofilm by HKR(pJAK16-JA03) was compared with HKR.Nal. Biofilm growth and quantification were determined using a modification of a standard microtiter plate biofilm plate assay, as described previously. All assays were performed in iron-sufficient TSBY broth. Although there were no significant differences in growth between the strains after 48 h as measured by OD_{600}, the HKR(pJAK16-JA03) overexpression strain produced less biofilm in comparison with the amount of biofilm produced by either HKR.Nal or HKR(pJAK16) carrying the pJAK16 expression vector alone (Fig. 7A–C). HKR.Nal formed

Table 4 Effect of JA03 overexpression on selected genes in rough variant HK1651R by real-time quantitative polymerase chain reaction

Gene accession number	Gene	Gene Function/Category	Expression of HKR(pJAK16-JA03) relative to HKR.Nal[1] Fold change (± SD)	Expression of HKR(pJAK16) relative to HKR.Nal[1]
Upregulated genes				
AA02121	*rsgA*	non-heme ferritin	1.65 ± 0.39	1.21 ± 0.30
AA01886	*galE*	UDP-glucose-4-epimerase	1.31 ± 0.1	1.09 ± 1.23
Downregulated genes				
AA00869	*tadV*	tight adherence protein V; prepilin peptidase	1.75 ± 1.1	ND
AA00880	*tadG*	tight adherence protein G	1.71 ± 0.19	1.34[2]
AA00492	*pgaC*	*N*-glycosyltransferase	1.79 ± 0.8	ND
AA02623	*rmlB*	dTDP-D-glucose 4,6-dehydratase	2.06 ± 0.1*	ND
AA00838	*murD*	UDP-*N*-acetylmuramate-alanine ligase	3.58 ± 1.1*	1.01[2]
AA02803	*ltxD*	leukotoxin secretion protein D	3.13 ± 1.2*	ND
AA01051	*hitC*	Fe(III) ABC transporter, ATP-binding protein	196 ± 232*	0.96 ± 0.26

ND, not done.

[1]HKR.Nal (nalidixic-acid-resistant HK1651R, parental strain).

[2]Sample assayed once.

*Significant threshold: At least twofold change in expression with $P \leq 0.05$.

approximately 24-fold ($P < 0.05$) more biofilm in an untreated, polystyrene microtiter plate compared with the HKR(pJAK16-JA03) overexpressing strain (Fig. 7D). When standardized cultures were grown in glass test tubes for 48 h at 37°C in a candle jar, the JA03 overexpression strain formed large flocs (microcolonies) that clearly did not adhere to the glass surface, unlike the HKR.Nal and vector control strains (Fig. 7E,F). These data demonstrate that JA03 sRNA overexpression did not interrupt

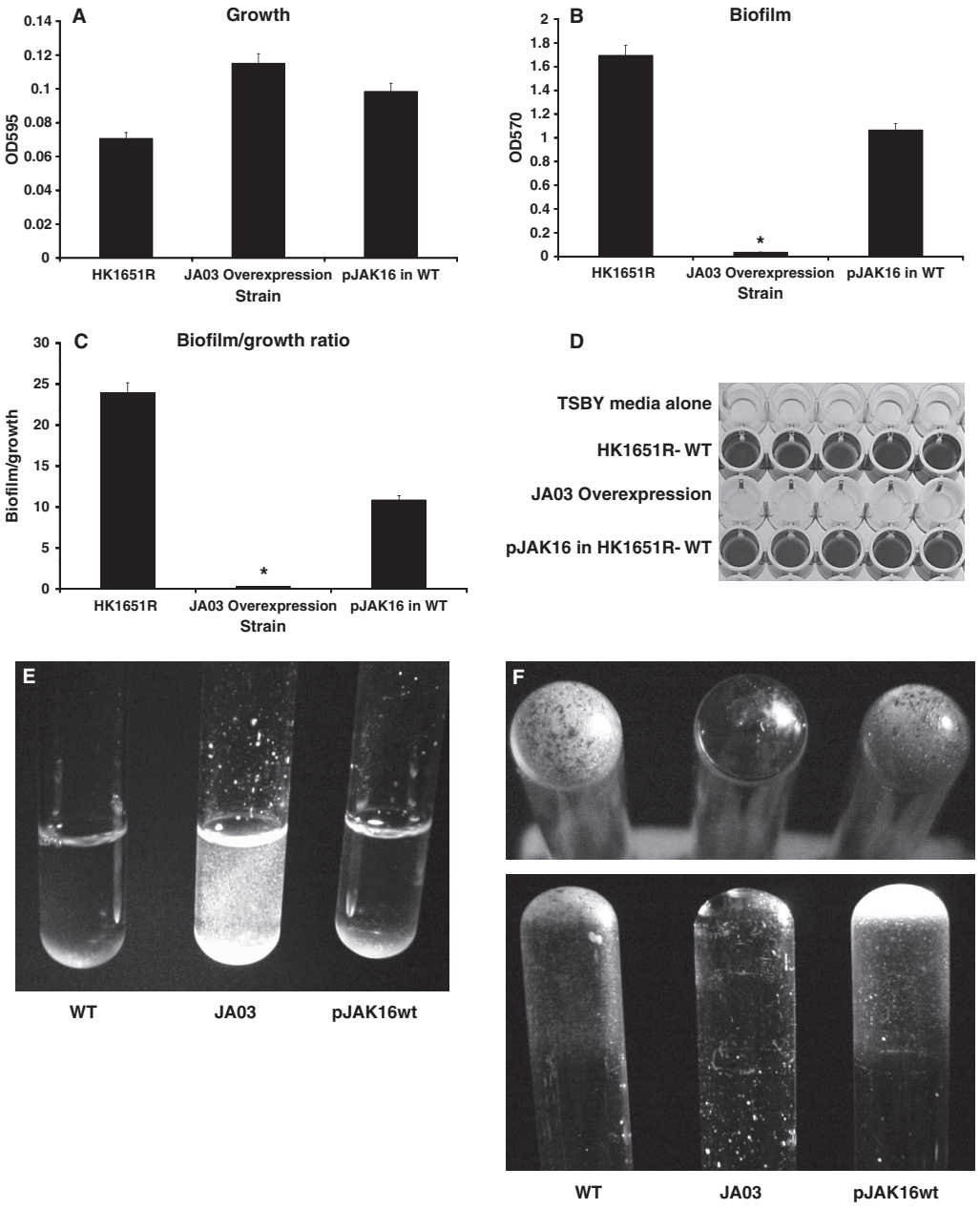

Figure 7 Effect JA03 small RNA overexpression on biofilm formation in Bacto™ tryptic soy broth containing 0.6% yeast extract (TSBY). Microtiter plate biofilm assay: (A) growth of cells as measured by turbidity at optical density 595 nm, (B) biofilm mass as measured by absorbance at 570 nm, (C) biofilm mass normalized to cell density, i.e. ratio: optical density (OD) at 570 nm of biofilm/OD at 595 nm for growth, and (D) microtiter plate biofilm stained with crystal violet. Cultures grown 24 h in glass tubes: (E) supernatant after vortexing, and (F) biofilm formation on surface of the tube. *A significant difference ($P < 0.05$) of the test condition from the control. Antibiotic-resistant HKR.Nal (designated here as HK1651R) was used as wild-type (WT).

intercellular adhesion, but significantly reduced cell adhesion to an abiotic surface.

DISCUSSION

A bioinformatics screen of the *A. actinomycetemcomitans* genome identified several potentially Fur-regulated sRNA molecules within intergenic regions. Each of the four sRNA molecules selected for further study, JA01–JA04, possessed a Fur box-like sequence within a probable −35 and −10 promoter sequence that was able to titrate *E. coli* Fur in a FURTA. This was confirmed by EMSA using *E. coli* cell lysates overexpressing AaFur, which clearly demonstrated that Fur is able to bind to each sRNA promoter. The transcription of each sRNA molecule in *A. actinomycetemcomitans* was decreased by iron supplementation and increased by limiting iron by chelation, demonstrating the influence of environmental iron availability on gene expression.

This study provides the first evidence for iron-regulated, Fur-responsive sRNAs in *A. actinomycetemcomitans*. There are several advantages for bacteria to use sRNA to regulate gene expression. First, their rapid synthesis requires considerably less energy compared with the synthesis of protein regulators. Therefore, the use of sRNA may be more economical to the bacteria under stressful environments (Guillier *et al.*, 2006). Second, sRNA provides bacteria with a means to fine-tune the expression of genes, stabilize mRNA, and initiate the expression of colonization factors under iron-limited conditions. Small RNA also provides a rapid means to turn off particular genes when the environmental stress signal disappears (Guillier *et al.*, 2006).

Based on *in silico* analysis using TargetRNA, it is interesting to note that the mRNA of several biofilm determinant genes recently found to be iron-regulated (fimbriae, EPS, LPS and *lux*S; Fong *et al.*, 2003; Amarasinghe *et al.*, 2009), potentially base pair with the four Fur-regulated sRNA molecules identified in this study. Genes associated with these biofilm determinants apparently do not possess Fur-binding boxes in their promoter regions, suggesting a possible indirect Fur regulatory mechanism.

Regulation of biofilm formation in *A. actinomycetemcomitans* could be accomplished by several sRNA molecules, as in other bacteria where multiple redundant sRNAs act together in response to environmental stress signals to better adapt to their environment (Tu & Bassler, 2007; Svenningsen *et al.*, 2009; Tu *et al.*, 2010). Expression of sRNA molecules under stress conditions and their repression upon disappearance of the particular stress signal is precisely controlled by negative feedback loops that help to fine-tune sRNA-mediated expression of genes in response to the environment (Tu *et al.*, 2010). This type of precise control could also be present in *A. actinomycetemcomitans* controlling the sRNA molecules.

When JA03 sRNA was overexpressed, multiple iron-regulated and Fur-regulated genes were repressed, including the *hitC* gene involved in *hitABC* iron-uptake system in *A. actinomycetemcomitans.* In the mRNA target predictions of JA03 sRNA, it was observed that significant base pairing occurred between JA03 sRNA and *hitC* mRNA. Previous studies have shown that isogenic mutants of *A. actinomycetemcomitans* with inactivation of *hitA*, *hitB* and *hitC* were unable to grow in iron-limited growth medium compared with the parental strain, indicating an essential role of *hitABC* in iron acquisition under iron-stressed conditions (Rhodes *et al.*, 2005). There are two possibilities for sRNA-mediated repression of the *hitC* gene. First, it is possible that JA03 sRNA binding to the 5′ untranslated region of *hitC* gene masks the ribosome-binding site to inhibit ribosome entry onto mRNA, as has been described for other bacteria (Storz *et al.*, 2004; Sharma *et al.*, 2007). This blockage of ribosomal entry onto mRNA by sRNA–mRNA pairing at 5′ untranslated regions will lead to rapid translational inhibition of these mRNA targets by accelerating the RNaseE-mediated mRNA degradation (Masse *et al.*, 2003; Deana & Belasco, 2005; Morita *et al.*, 2005). In *A. actinomycetemcomitans*, the *hitA* (*afuA*) gene is known to be iron-regulated and Fur-regulated (Willemsen *et al.*, 1997; Rhodes *et al.*, 2007). Hence, as part of the *hitABC* locus, repression of *hitC* transcription probably occurs under iron-sufficient conditions when activated Fur binds to iron-regulated gene promoters. Therefore, the second reason why *hitC* may be repressed could be that JA03 sRNA overexpression, as it occurs in an iron-limited environment, may lead to the degradation of iron-containing proteins (i.e. iron storage proteins such as bacterioferritins). This phenomenon, known as the iron-sparing effect (Masse & Gottesman, 2002; Gaballa *et al.*, 2008), causes an increase in

intracellular iron concentration, which consequently activates Fur and represses Fur-regulated genes. Similarly, JA03 sRNA overexpression in this study led to the downregulation of multiple iron-regulated and Fur-regulated genes, suggesting an occurrence of an iron-sparing effect.

Another target of JA03 sRNA was *ltxD*, part of the *ltxCABD* operon that is responsible for production of LtxA (leukotoxin). The *ltxC* gene encodes for a protein responsible for acylation of the toxin, *ltxA* encodes the toxin, and *ltxB* and *ltxD* encode for predicted components of the membrane transport system. LtxA expression is partly regulated by LuxS (also regulated by iron), and levels of LtxA are affected by cAMP concentration (Fong *et al.*, 2001; Inoue *et al.*, 2001). The *A. actinomycetemcomitans* leukotoxin can also act as a hemolysin that has the ability to lyse erythrocytes resulting in β-hemolysis of blood agar. This leukotoxin-dependent hemolysis is iron dependent (Balashova *et al.*, 2006a,b); hemolysis is suppressed in the presence of $FeCl_3$, whereas strong hemolysis was observed upon iron chelation. Erythrocyte lysis is one mechanism by which a bacterial pathogen acquires iron, it is suggested that leukotoxin may play a role in iron acquisition (Balashova *et al.*, 2006a,b).

Downregulation of the *ltxD* gene upon JA03 overexpression could be either a direct or an indirect effect. JA03 sRNA could directly regulate the *ltxD* gene at the post-transcriptional level to achieve the maximum level of efficiency in iron utilization in a low-iron environment. One possibility is that JA03 sRNA is degrading the transcripts of non-essential iron-utilizing proteins (i.e. LtxD) to spare available iron for essential proteins. Alternatively, our studies indicated that expression of *ltxD* in the *fur* mutant was upregulated 2.2-fold compared with the wild-type strain, indicating that Fur protein is negatively regulating the expression of the *ltxD* gene in wild-type strain in iron-replete conditions. JA03 overexpression may indirectly downregulate several iron-regulated genes via an iron sparing effect thereby activating the Fur cascade and downregulating the *ltxD* gene.

Expression of biofilm determinant genes *tadV* (fimbriae), *pgaC* (EPS) and *rmlB* (LPS) were only modestly changed by the overexpression of JA03 sRNA, though bacterial cells produced non-adherent flocculation in broth, and little or no biofilm on untreated polystyrene and glass surfaces. This is characteristic of an intermediate phenotype where intercellular adhesion is maintained permitting flocculation, but adhesion to surfaces is abolished. These data also suggest that biofilm regulation goes beyond the regulation of the standard biofilm determinants, yet to be explored through transcriptional profiling of this overexpression strain.

In summary, we have so far identified and characterized at least one *A. actinomycetemcomitans* sRNA that is iron-responsive, regulated by Fur, and has an effect on biofilm formation. Although these sRNAs appear to base pair with the mRNA of previously identified biofilm determinant genes, their effect on global transcription needs to be further investigated. As a result of the multifactorial nature of biofilm formation and the multiple potential targets of sRNA molecules, other approaches are required to more accurately determine the pathways and genes affected. Current studies are underway to more precisely map each sRNA by determining the transcriptional start and stop sites, and to verify putative mRNA targets. The exact role of these sRNA in the regulation of biofilm formation in *A. actinomycetemcomitans* will be explored through further mutant analysis and transcriptional profiling.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Putative small RNAs (sRNAs). Highlighted areas submitted to MFOLD structure analysis. Red (−35, −10 promoter regions), boxed nucleotides (Fur box), underline (stem loop at 3′ end of the sRNA).

Table S1. Relevant potential small RNA target messenger RNA.

Table S2. Base-pairing of small RNA (sRNA) with potential target messenger RNA using TARGETRNA; predicted targets for JA01 to JA04 sRNAs.

Table S3. Putative Fur boxes in small RNA molecules.

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