Induction of *Aggregatibacter actinomycetemcomitans* leukotoxin expression by IS1301 and *orfA*

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Most *Aggregatibacter actinomycetemcomitans* strains express relatively low levels of leukotoxin, encoded by the *orfA-ltxCABD* operon. However, several strains isolated from patients with localized aggressive periodontitis are hyperleukotoxic and transcribe the *ltx* operon at high levels. These strains possess a copy of IS1301 in the *ltx* promoter and previous studies have suggested that the presence of the insertion sequence increases *ltx* transcription by uncoupling a *cis*-acting negative regulator of *ltx* expression from the basal elements of the *ltx* promoter. However, we now report that replacing IS1301 with an equal length of random sequence has little effect on transcriptional activity of the *ltx* promoter, suggesting that the physical displacement of the negative regulatory element does not contribute to the hyperleukotoxic phenotype of IS1301-containing strains. Instead, we show that a –10-like element upstream of the transposase ORF of IS1301 is required for increased transcriptional activity of the *ltx* promoter. Site-specific mutation of the –10 sequence, or reversing the orientation of IS1301 relative to the basal *ltx* promoter elements, reduced transcriptional activity to levels exhibited by the native *ltx* promoter. However, no increase in transcription was observed when IS1301 was recombinantly inserted into a *ltx* promoter that contained a truncated copy of *orfA*, suggesting that an intact *orfA* may also be required for IS1301-mediated induction of *ltxCABD*. Therefore, to determine if *orfA* functions as a regulator of *ltx* expression, three independent *ltx*-promoter–*lacZ*-reporter constructs containing frameshift mutations in *orfA* were analysed. Each exhibited significantly lower expression of β -galactosidase than the control reporter with intact *orfA*. In addition, OrfA protein was shown, by mobility shift electrophoresis, to interact with the *ltx* promoter at or downstream of the –35 sequence. These results suggest that a potential transposase promoter and the OrfA polypeptide may modulate leukotoxin expression in hyperleukotoxic *A. actinomycetemcomitans* strains containing IS1301.

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INTRODUCTION

Aggregatibacter (Actinobacillus) actinomycetemcomitans is a capnophilic, Gram-negative bacterium that is associated with localized aggressive periodontitis (Haraszthy *et al.*, 2000; Slots *et al.*, 1980; Zambon, 1985; Zambon *et al.*, 1983, 1996), endocarditis (Block *et al.*, 1973; Page & King, 1966) and subcutaneous abscesses (Page & King, 1966). *A. actinomycetemcomitans* expresses several virulence factors that may contribute to pathogenesis, including two toxins that may play a role in suppressing the host response to infection by targeting various components of the immune system. One of these, a cytolethal distending toxin (Cdt) similar to the Cdt of *Haemophilus ducreyi*, induces G2 arrest of proliferating human lymphocytes (Shenker *et al.*, 1999, 2000). *A. actinomycetemcomitans* also expresses a

leukotoxin that is a member of the repeats in toxin (RTX) family of host-specific cytolysins (Welch, 1991). The leukotoxin targets cells of the lymphocytic and monomyelocytic lineages expressing the β 2-integrin LFA-1 (CD11a/CD18) (Karakelian *et al.*, 1998; Lally *et al.*, 1997, 1999) and a recent report suggests that it may also have haemolytic activity (Balashova *et al.*, 2006). At high doses *in vitro*, intoxication of target cells by the leukotoxin leads to cell lysis via the development of membrane pores (Iwase *et al.*, 1990; Korostoff *et al.*, 1998, 2000), whereas lower doses of toxin perturb mitochondrial functions, leading to apoptosis of host cells (Korostoff *et al.*, 1998, 2000).

Most *A. actinomycetemcomitans* strains express relatively low levels of leukotoxin and have been designated minimally leukotoxic (Brogan *et al.*, 1994; Hritz *et al.*, 1996). However, several *A. actinomycetemcomitans* strains have been isolated that exhibit a hyperleukotoxic phenotype. These strains exhibit significantly increased transcription of the *ltx* operon

Abbreviations: EMSA, electrophoretic mobility shift assay; RTX, repeats in toxin.

and express elevated levels of the leukotoxin (Brogan *et al.*, 1994; He *et al.*, 1999; Kolodrubetz *et al.*, 1996). Hyperleukotoxic strains are also isolated at higher frequency from patients with localized aggressive periodontitis (Bueno *et al.*, 1998; Haraszthy *et al.*, 2000; Haubek *et al.*, 1997, 2001), suggesting that the level of leukotoxin production may in part determine whether *A. actinomycetemcomitans* contributes to the development of periodontitis or is tolerated as a commensal in the oral flora.

The leukotoxin operon consists of four genes, *ltxCABD*, and has an arrangement typical of other RTX family toxins (Kraig *et al.*, 1990; Lally *et al.*, 1989). However, immediately upstream of *ltxCABD* is a co-transcribed ORF of ~450 bp designated *orfA* (or *orfX*) (Brogan *et al.*, 1994; Kolodrubetz *et al.*, 1996; Mitchell *et al.*, 2003) that is capable of encoding a polypeptide of approximately 15 kDa of unknown function. Interestingly, the hyperleukotoxic phenotype of *A. actinomycetemcomitans* is correlated with insertions or deletions that do not affect the major structural genes of the *ltx* operon (*ltxCABD*) but occur within or upstream of *orfA* (Brogan *et al.* 1994; He *et al.*, 1999). Other than the inserted or deleted segments, all of the remaining sequences in the *ltx* promoter and in *orfA* are virtually identical in minimally leukotoxic and hyperleukotoxic strains (Brogan *et al.*, 1994; He *et al.*, 1999). For example, the hyperleukotoxic JP2 strain expresses approximately 10-fold higher levels of cytotoxic activity than minimally leukotoxic strains and has been found extensively in localized aggressive periodontitis patients in United States, Northern African and European populations (Cortelli *et al.*, 2005; Haraszthy *et al.*, 2000; Haubek *et al.*, 1997, 2001, 2004, 2007). This strain possesses a 530 bp deletion that truncates *orfA* (Brogan *et al.*, 1994), suggesting either that *orfA* plays a role in regulating *ltx* expression or that the truncation of *orfA* in *A. actinomycetemcomitans* JP2 increases transcription simply by bringing the *ltx* promoter into closer proximity to *ltxCABD* (Kolodrubetz *et al.*, 1996).

In contrast, analysis of clinical isolates from periodontitis patients in a Japanese population failed to detect the JP2 clone. Instead, hyperleukotoxic *A. actinomycetemcomitans* strains were identified that exhibited increased transcription of the *ltx* operon and possessed an intact copy of *orfA*. He *et al.* (1999) showed that these strains contained an insertion sequence related to IS1301 of *Neisseria meningitidis* in the *ltx* promoter upstream of *orfA*. Spontaneous loss of the IS element resulted in reduced expression of leukotoxin, similar to that of minimally leukotoxic strains (He *et al.*, 1999), indicating that the hyperleukotoxic phenotype was dependent on the presence of IS1301. Integration of IS1301 occurred at the 5'-end of an AT-rich positive regulatory sequence and downstream from a *cis*-acting negative regulatory sequence in the *ltx* promoter (Mitchell *et al.*, 2003). In addition, the IS element itself possesses an outwardly directed -35 sequence. However, Mitchell *et al.* (2003) showed that IS1301 does not functionally disrupt the AT-rich regulatory sequence and

furthermore, that the outwardly directed -35 element does not influence transcription of *ltx* genes. These results led the authors to suggest that the hyperleukotoxic phenotype may arise from the uncoupling of the *cis*-acting negative regulator from the basal elements of the *ltx* promoter upon the acquisition of IS1301.

In this report, we show that replacing IS1301 with an equal length of random sequence does not affect the transcriptional activity of the *ltx* promoter, suggesting that physical displacement of the *cis*-acting negative regulator does not contribute to the hyperleukotoxic phenotype of IS1301 containing strains. Instead, we show that a -10-like sequence upstream of the transposase gene in IS1301 is necessary and sufficient for increased transcription of the *ltx* operon. In addition, reporter constructs containing frameshift mutations in *orfA* exhibited significantly reduced *ltx* promoter activity, suggesting that the OrfA polypeptide itself modulates expression of *ltxCABD*. Consistent with this, purified OrfA was shown to interact with the *ltx* promoter. Together, these results suggest that both IS1301 and *orfA* influence leukotoxin expression and may contribute to the hyperleukotoxic phenotype exhibited by some *A. actinomycetemcomitans* strains.

METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (Becton Dickinson) at 37 °C with aeration. *A. actinomycetemcomitans* strains were grown in brain-heart infusion (BHI) broth (Becton Dickinson) that was supplemented with 40 µg NaHCO₃ ml⁻¹ at 37 °C in a standing culture. Where indicated, media were supplemented with the appropriate antibiotics at the following concentrations: ampicillin, 100 µg ml⁻¹, kanamycin, 25 µg ml⁻¹, spectinomycin 80 µg ml⁻¹. Plates containing 15% (w/v) granulated agar (Becton Dickinson) were used where indicated. Plasmids were isolated from *A. actinomycetemcomitans* strains using the Wizard Plus Mini-Prep DNA purification system (Promega), following the protocol supplied by the manufacturer.

Construction of *lacZ* reporters for examination of the IS1301 insertion. Fragments for construction of the *lacZ* reporters were generated by PCR using primers listed in Table 2. To facilitate directional cloning of the PCR amplicons, restriction sites (underlined in Table 2) were incorporated into all primers. The PCR products were initially cloned into pGEM-T Easy vector (Promega) using the TA sites provided. The resulting plasmids were transformed into *E. coli* DH5α and confirmed both by blue/white colour selection on LB agar containing IPTG and X-Gal, and by restriction digestion with *EcoRI* to release the insert fragment. Plasmid purifications were carried out using the Wizard Plus Miniprep DNA purification system (Promega). PCR products were subsequently excised using *Clal* and *XmaI* sites and ligated into the acceptor plasmid pBluescript-*orfA*. pBluescript-*orfA* contains the 1000 bp *ltx* promoter of *A. actinomycetemcomitans* 652 (Brogan *et al.*, 1994) with *Clal* and *XmaI* restriction sites engineered in at the point of IS1301 insertion (Mitchell *et al.*, 2003). Since the *ltx* promoter of strain JP2 is identical in sequence to that of strain 652, with the exception of the 530 bp deletion, a similar approach was used to produce the truncated *ltx* promoter of strain JP2 containing the *Clal* and *XmaI* restriction sites. Thus, each of the PCR fragments that were generated was inserted

Table 1. Bacterial strains used in this study

Strain (plasmid)	Description
652	Wild-type minimally leukotoxic strain of <i>A. actinomycetemcomitans</i>
JP2	Hyperleukotoxic strain of <i>A. actinomycetemcomitans</i>
IS	Hyperleukotoxic strain of <i>A. actinomycetemcomitans</i> ; contains IS1301 in the <i>orfA</i> promoter
652(pYGK: <i>lacZ</i>)	652 with a promoterless <i>lacZ</i> on plasmid pYGK
652(pYGK:652: <i>lacZ</i>)	652 containing pYGK with <i>lacZ</i> driven by the 652 <i>ltx</i> promoter
652(pYGK:JP2: <i>lacZ</i>)	652 containing pYGK with <i>lacZ</i> under the control of the JP2 <i>ltx</i> promoter
JP2(pYGK:IS: <i>lacZ</i>)	JP2 containing pYGK with <i>lacZ</i> under the control of the IS <i>ltx</i> promoter
652(pYGK:IS: <i>lacZ</i>)	652 containing pYGK with <i>lacZ</i> under the control of the IS <i>ltx</i> promoter
652(pYGK: <i>ltxA</i> 300: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with 300 bases of <i>ltxA</i> inserted at the site of IS1301 insertion
652(pYGK: <i>ltxA</i> 600: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with 600 bases of <i>ltxA</i> inserted at the site of IS1301 insertion
652(pYGK: <i>ltxA</i> 900: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with 900 bases of <i>ltxA</i> inserted at the site of IS1301 insertion
652(pYGK:IS100: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but lacking 800 bp from the 5' end of IS1301
652(pYGK:IS400: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but lacking 500 bp from the 5' end of IS1301
652(pYGK:IS550: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but lacking 350 bp from the 5' end of IS1301
652(pYGK:IS700: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but lacking 200 bp from the 5' end of IS1301
652(pYGK:IS800: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but lacking 100 bp from the 5' end of IS1301
652(pYGK:ISmut: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> with a 3-base mutation in the putative transposase –10 site
652(pYGK:ISinverted: <i>lacZ</i>)	652 containing pYGK:IS: <i>lacZ</i> but with IS1301 reversed in orientation relative to <i>orfA</i>
652Δ <i>orfA</i> (pYGK:Xba270: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with a frameshift mutation at nucleotide 270 of <i>orfA</i>
652Δ <i>orfA</i> (pYGK:Sph400: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with a frameshift mutation at nucleotide 400 of <i>orfA</i>
652Δ <i>orfA</i> (pYGK:Xba500: <i>lacZ</i>)	652 containing pYGK:652: <i>lacZ</i> with a frameshift mutation at nucleotide 500 of <i>orfA</i>

Table 2. Primers used in the generation of *lacZ* reporter constructs

Primers F1–F3 were used with reverse primer R1. Primers F4–F9 were used with reverse primer R2. Underlining indicates added restriction sites.

Construct	Primers (5'–3')
<i>ltxA</i> 300	F1 <u>GCCATCGATAGATCCAATCAAAGG</u> R1 <u>GGCCCGGGCATAATCATCTTTAGCAC</u>
<i>ltxA</i> 600	F2 <u>GCCATCGATGCTCGCCGATAAAATTAAAGC</u>
<i>ltxA</i> 900	F3 <u>GCATCGATGCGAAACAATTTGATCG</u>
IS100	F4 <u>GGCTATCGATTGCGCAAGGAATTATGC</u> R2 <u>GCCTCCCGGGGGGGCTGTTGATCTTTCGAG</u>
IS400	F5 <u>GGCTATCGATTAAACGGGAGGCGAAGTTC</u>
IS550	F6 <u>GCGATCGATGTGTATTCTGAATGGAT</u>
IS700	F7 <u>GGCTATCGATAATACCGTCGAAGGTATTCTG</u>
IS800	F8 <u>GCGATCGATGGGATCGCCTGGCTTATTAC</u>
IS900	F9 <u>GGCTATCGATGGGCGTGTGATCTTTCC</u>
ISinverted	F <u>GCCCCGGGGGGCGTGTGATCTTTCCCGGTATAAAAA</u> R <u>GCATCGATGGGGCTGTTGATCTTTCGAGTACA</u>
FS270(1)	F <u>GCGGTACCTTTTTCGCTATTAATACAATACGG</u> R <u>GCTCTAGAGTAAACTTCATTACTTGGG</u>
FS270(2)	F <u>GCTCTAGATGAATACTGGAACTTGTTT</u> R <u>CCGGATCCATATTAATCTCCTTCT</u>
FS500(1)	F <u>GCGGTACCTTTTTCGCTATTAATACAATACGG</u> R <u>GCTCTAGATTTAAAGTGGATAACTATGGC</u>
FS500(2)	F <u>GGTCTAGATTTTACCAACCTTCTTATACTG</u> R <u>CCGGATCCATATTAATCTCCTTCT</u>

into the appropriate *ltx* promoter at the site of IS1301 integration. The entire *orfA/ltx* promoter region containing the desired PCR fragments was then released by digestion with *KpnI* and *BamHI* and ligated into the *E. coli/A. actinomycetemcomitans* shuttle vector

pYGK*lacZ* (Brogan *et al.*, 1996). Recombinant reporter plasmids were transformed into *E. coli* DH5 α and verified by restriction analysis. Reporter constructs were subsequently electroporated into competent *A. actinomycetemcomitans* strain 652 by pulsing for 2.5 ms at 2.5 kV.

Plasmid uptake was confirmed by the presence of blue colonies on BHI agar containing X-Gal and by recovery of the appropriate plasmid from the recombinant strains. Promoter activity was assessed by determining β -galactosidase activity using the colorimetric substrate ONPG assays as described below. A similar approach was used to insert *IS1301* into the *ltx* promoter of strain JP2.

Site-specific mutation of *IS1301*. Site-specific mutation of *IS1301* was carried out by introducing a 3-base alteration into the -10 -like element (TATAAT to GGGAAAT; see underlined region in the primer below). Mutagenesis reactions were carried out using the primer 5'-CGACATAGAAGTTTGGGGAATTGAGTCCGCAAAAACC-3' with the Quick-Change Multi Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The template for mutagenesis was the intact *ltx* promoter region from *A. actinomycetemcomitans* strain IS contained in pBluescript. Sequence alteration was confirmed by nucleotide sequencing of the recombinant plasmids. The intact promoter containing the mutated *IS1301* sequence was then excised from pBluescript by digestion with *KpnI* and *BamHI*, cloned into pYGKlacZ and introduced into *A. actinomycetemcomitans* strain 652 by electroporation as described above.

Construction of lacZ reporter constructs containing frameshift mutations of *orfA*. Three frameshift mutants of *orfA* were constructed and were designated FS270, FS400 and FS500. FS400 was constructed by cleaving *orfA* at its unique *SphI* site and filling the overhanging nucleotides with T4 DNA polymerase in the presence of all four dNTPs for 30 min at 37 °C. The blunted ends were then religated with T4 DNA ligase. This mutant was capable of translating

the first 80 amino acids of *OrfA* before prematurely terminating (full-length *OrfA* contains 150 residues). FS270 and FS500 were each constructed in pBluescript by assembling two promoter fragments that were amplified from strain 652 genomic DNA. The primers for these reactions are shown in Table 2. Fragments FS270(1) and FS500(1) encoded 24 and 98 residues of *OrfA* respectively and were ligated to pBluescript cleaved with *KpnI* and *XbaI*. Inclusion of the *XbaI* site in these fragments generated the frameshift in *orfA*. Subsequently, amplified fragments FS270(2) and FS500(2) were ligated into the constructs described above cleaved with *XbaI* and *BamHI* to regenerate the full-length *orfA* containing the frameshift mutation. Clone FS270 was capable of expressing only the first 24 residues of *OrfA*. Clone FS500 possessed a frameshift mutation at the codon encoding amino acid 98, but encoded an additional 38 residues from an alternative reading frame before terminating. FS500 was therefore capable of producing a polypeptide of 136 amino acids. All three frameshift mutants were excised from pBluescript by cleavage with *KpnI* and *BamHI* and cloned into pYGKlacZ as described above. The resulting reporter constructs were introduced into an *A. actinomycetemcomitans* background that was deficient in *OrfA* (see below).

Insertional inactivation of *orfA* in *A. actinomycetemcomitans*.

The *orfA* gene was inactivated by insertion of a spectinomycin resistance marker obtained from pVT1461 (kindly provided by Dr K. Mintz, University of Vermont) by cleavage with *SphI*. This fragment was ligated into the unique *SphI* site of *orfA* in the *ltx* promoter region of *A. actinomycetemcomitans* 652 contained in pBluescript. The *SphI* site is located at nucleotide 396 in the 1000 bp *ltx* promoter region of this strain and is shown in Fig. 1. After selection for spectinomycin-resistant

Fig. 1. (a) Schematic representation of the leukotoxin operons from *A. actinomycetemcomitans* strains JP2, IS and 652. *A. actinomycetemcomitans* 652 is minimally leukotoxic. The acquisition of *IS1301* in the *ltx* promoter upstream from *orfA*, or the deletion of 530 bp encompassing the 3'-end of *orfA* and a portion of the *orfA*–*ltxC* intergenic region, results in the hyperleukotoxic phenotypes exhibited by strains IS and JP2, respectively. (b) An expanded comparison of the *ltx* promoter upstream of *orfA* in *A. actinomycetemcomitans* 652 and IS. Regulatory sequences in the *ltx* promoter identified by Mitchell *et al.* (2003) are labelled; a *cis*-acting negative regulator (CNR), an AT-rich inverted repeat (IR), and the -35 and -10 sequences are shown. The transcriptional start site is numbered as $+1$ and the translation start codon of *orfA* is indicated by ATG. The site of *IS1301* insertion in *A. actinomycetemcomitans* IS is near the 5' end of the IR (Mitchell *et al.*, 2003).

clones, the promoter fragment was removed by cleavage with *KpnI* and *BamHI* and cloned into pGEM-T containing the *sacB* gene under the control of the inducible *tac* promoter. The resulting plasmid was introduced into *A. actinomycetemcomitans* 652 by electroporation and recombinants were selected on agar plates containing 50 µg spectinomycin ml⁻¹. Resistant clones were then grown in broth culture (BHI containing 50 µg spectinomycin ml⁻¹) to mid-exponential phase and were induced for 2 h with 1 mM IPTG before plating on BHI agar plates containing 50 µg spectinomycin ml⁻¹, 5% sucrose and 1 mM IPTG. Spec^R Suc^R recombinants were selected and confirmed by two additional passages on the above selective agar medium. Integration of *orfA* containing the Spec^R determinant was then confirmed by PCR amplification using primers FS270(1)F and FS270(2)R (see Table 2). Loss of *orfA* transcripts in the recombinant organisms was then confirmed by RT-PCR using primers 16 (5'-TAGGAATTTA-TCCGGTCAAAG-3') and 20 (5'-TTTATTAGGTTTAGGGCGATG-3') of Mitchell *et al.* (2003), which flank the *SphI* site used to insert the spectinomycin-resistance marker. The resulting strain was used for analysing the *orfA* frameshift reporter plasmids described above.

ONPG assays. β-Galactosidase activity was measured using the colorimetric substrate ONPG (Sigma Aldrich). Reporter strains were grown to mid-exponential phase and centrifuged to pellet bacteria. Cells were then resuspended in PBS, pH 7.4 to OD₆₀₀ ~0.5. Enzymic activity was determined in the following reaction: 5 µl bacteria were suspended in 226 µl 0.1 M sodium phosphate buffer, pH 7.5, containing 0.0003% SDS and 10 µl chloroform. Samples were incubated at room temperature for 10 min and then 3 µl magnesium buffer (0.1 M MgCl₂, 4.5 M β-mercaptoethanol) and 66 µl ONPG (4 mg ml⁻¹) were added. Samples were incubated for 10 min at 37 °C and reactions were stopped by the addition of 500 µl 1 M Na₂CO₃. Units of β-galactosidase activity were calculated using the method of Miller (1972).

Real-time PCR. Real-time PCRs were performed using Ready-to-Go reverse transcriptase beads (Amersham Biosciences) in a reaction volume of 25 µl. Reverse transcription was carried out according to the manufacturer's instructions. Each reaction mixture contained 7.5 pmol of the appropriate antisense primer and 50 ng bacterial RNA. After completion of cDNA synthesis, serial dilutions of cDNA were amplified by addition of 7.5 pmol sense primer and 0.5 × SYBR-Green dye (Roche Applied Science). Amplifications were carried out using the Smart Cycler system (Cepheid). The amplification conditions for real-time PCR were as follows: denaturation at 95 °C for 270 s for a single cycle, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 60 s. The threshold cycle for each reaction was determined from a second-derivative plot of total fluorescence as a function of cycle number using the software package supplied with the Smart Cycler system. All reactions were normalized against real-time PCR reactions using primers specific for the *A. actinomycetemcomitans* 5S RNA gene. The threshold cycle for each reaction was determined from a second-derivative plot of total fluorescence as a function of cycle number using the software package supplied with the Smart Cycler system. All reactions were carried out at least twice using independently obtained template samples with consistent results.

Expression of His₆-tagged OrfA. The *orfA* open reading frame was amplified by PCR using the RTS *E. coli* Linear Template Generation set for His₆ tags (Roche Applied Sciences). In this two-step protocol, the initial PCR was performed using *A. actinomycetemcomitans* 652 genomic DNA as the template. This reaction used the forward primer 5'-CTTTAAGAAGGAGATATACCATGTCCGGTACAGAATATGC-TCC-3', which contains an *orfA*-specific sequence (underlined) and additional nucleotides that are required for priming of the secondary PCR reaction. The reverse primer (5'-TGATGATGAGAACCCCC-CCCCATGGCAACGGTAGAAGG-3') also possesses an *orfA*-specific

region (underlined) along with additional sequence encoding a portion of the His₆ tail and necessary for priming the secondary PCR reaction. The PCR reaction profile was 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, for 30 cycles. The secondary PCR was performed with the *orfA* amplicon as the template, using primers provided by the manufacturer. This reaction added a ribosome-binding sequence and a T7 polymerase promoter to the 5' end of the *orfA* amplicon, and the hexahistidine tag and a translational termination sequence to the 3' end of the fragment. The product of the secondary PCR was cloned directly into the pGEM-T Easy vector using the TA cloning sites provided (Promega). Ligated plasmids were transformed into competent *E. coli* DH5α and the appropriate plasmid construct was confirmed by digestion of purified plasmids with *EcoRI*. Plasmid purification was carried out using the Wizard Plus Miniprep DNA purification system (Promega). For expression of OrfA, 15 µg purified plasmid DNA was used in the RTS500 *in vitro* coupled transcription-translation system (Roche) according to the manufacturer's instructions. Expression of OrfA was confirmed by PAGE analysis and Western blotting using monoclonal anti-polyhistidine antibodies and goat anti-mouse peroxidase-conjugated antibody.

His₆-tagged OrfA was purified from the *in vitro* expression mixture by immunoprecipitation. Monoclonal anti-polyhistidine antibody, clone His-1 (Sigma), was added to samples to a final concentration of 10 µg ml⁻¹ and incubated at 4 °C for 2 h. Following antibody binding, 100 µl of a 50% (w/v) slurry of Protein A-Sepharose 4 Fast Flow beads (GE Healthcare) were added and samples were incubated for an additional 2 h at 4 °C with gentle rotation. Beads were then pelleted by centrifugation for 5 min at 300 g and washed three times in 500 µl 50 mM Tris, pH 7.6 and bound OrfA was eluted by five 1 min incubations in 200 µl each of 0.1 M glycine, pH 2.0 followed by centrifugation as above. Samples were neutralized by the addition of an equivalent volume of 1 M Tris, pH 8.0 and then lyophilized. Dried fractions were suspended in 200 µl sterile MilliQ H₂O and 20 µl volumes were analysed by PAGE followed by Coomassie staining and Western blotting. The sample was subjected to two additional rounds of immunoprecipitation and the final OrfA protein concentration was determined using a BCA microplate assay (Pierce Biotechnology). Samples were then separated into 50 ng aliquots, lyophilized and stored at -80 °C until used.

Electrophoretic mobility shift assays (EMSAs). Probes corresponding to various segments of the *lyx* promoter of strain 652 (Fig. 1) were generated by PCR using the biotinylated primers (Biosynthesis) shown in Table 3. PCRs were carried out in a volume of 100 µl for 40 cycles using an annealing temperature of 60 °C for 1 min and a 2 min extension reaction at 72 °C followed by melting at 94 °C for 1 min. The resulting products were precipitated with 2-propanol, dried and resuspended in 50 µl TE buffer. All products were confirmed by agarose gel electrophoresis. Biotinylated probes were diluted 1:100 or 1:50 as needed in MilliQ H₂O and 20 µl binding reactions were set up containing 4 µl 5× binding buffer (50 mM Tris/HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton X-100, 62.5% v/v, glycerol and 1 mM DTT) and 1 µg poly(dI.dC). Where indicated, 2 µl diluted biotinylated probe and 5 ng His₆-OrfA were added to the reactions. For competition reactions, 2 µl undiluted and unlabelled probe was added. All binding reactions were incubated for 20 min at room temperature. After the addition of 5 µl loading dye (15% Ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue), samples were separated by electrophoresis on 10% TBE gels (Invitrogen) and transferred to Hybond Immobilon N+ nylon membranes (GE Healthcare) at a constant current of 380 mA for 1.5 h. Membranes were immediately UV cross-linked at 1200 µJ cm⁻² and then probed using the LightShift nucleic acid detection kit (Pierce), following the manufacturer's instructions. Developed membranes were visualized on a Kodak 1D ImageStation.

Table 3. Primers used in the generation of EMSA probes

Forward primers F2 to F4 were used with reverse primer R1. Reverse primer R2 was used with forward primer F1.

Probe	Primers (5'–3')
Full-length	F1 GCGGTACCTTCTCAGTCTAATTTTTCG R1 AGGGTTAATACTTAAGATAACCTTTGACC
CNR	R2 GATAATACTTTATCTATGCAATTCGGTATT
ΔCNR	F2 GGAATTGCATAGATAAAGTATTATCAAAAACT
IRprom	F3 GTATTATCAAAAACTAATAATTTTATCAAATTAAT
ΔIRprom	F4 TATTGACTATTAAGAATCCGGAGTAAATTAGTCTCCAAAATTA

Statistical analysis. Samples were analysed for significant differences using an unpaired Student's *t*-test assuming unequal variances. Analyses were performed using Microsoft Excel.

RESULTS

Physical displacement of regulatory sequences by IS1301 does not increase transcriptional activity of the *ltx* promoter

Mitchell *et al.* (2003) previously suggested that upstream displacement of a *cis*-acting negative regulator in the *ltx* promoter (CNR in Fig. 1b) by the integration of IS1301 may contribute to increased expression of the leukotoxin operon. To test this hypothesis, we generated *lacZ* reporter constructs in which progressively larger fragments of random sequence derived from the coding region of *ltxA* were inserted into the native *ltx* promoter at the site of integration of IS1301. As shown in Fig. 2, the transcriptional activity of these constructs was similar to that of the control reporter in which *lacZ* was driven by the *ltx* promoter from the minimally leukotoxic parent strain *A. actinomycetemcomitans* 652, even when a fragment of approximately the

same size (~900 bp) as IS1301 was inserted. This suggests that displacement of CNR does not lead to increased transcriptional activity of the *ltx* promoter.

IS1301 sequences direct elevated leukotoxin expression in *A. actinomycetemcomitans*

To determine how the presence of IS1301 contributes to increased transcription of the *ltx* operon, additional reporter plasmids were constructed that contained a nested series of 5' deletions in IS1301 (see Fig. 3a). As shown in Fig. 3(b), deleting 100 bp from the 5' end of IS1301 (construct IS800) reduced promoter activity by 5.7×10^4 Miller units, which represents 60 % of the difference in expression between the IS promoter and the parent 652 promoter constructs. Further deletions in IS1301 resulted in slight additional reductions in β -galactosidase activity. This suggests that sequences within 100 bp of the 5'-end of IS1301 are primarily responsible for elevated transcriptional activity of the *ltx* promoter of strain IS. The sequence of this region of IS1301 is shown in Fig. 3(c); it contains the 5' imperfect terminal repeat (underlined) and a consensus *E. coli* –10 element (in bold) upstream of the start codon

Fig. 2. β -Galactosidase activity of reporter constructs containing increasing lengths of *ltxA* sequence inserted at the site of IS1301 integration. (a) Schematic diagrams showing the promoter fragments cloned into the *lacZ* reporter plasmid. In each of these constructs, *lacZ* replaces the *ltx* operon (*ltxCABD*). (b) β -Galactosidase activity determined from each reporter construct is indicated in Miller units $\pm$ SE. The parental strain 652 serves as a basis for comparison. *, $P < 0.0001$.

Fig. 3. β -Galactosidase activity of reporter constructs containing a nested series of 5'-end deletions of IS1301. A schematic representation of the individual constructs is shown in (a) and β -galactosidase activity determined from each reporter construct is indicated in Miller units $\pm$ SE in (b). *, $P < 0.0001$ in pairwise comparison with the construct containing the intact IS1301 sequence. (c) Schematic representation of the *ltx* promoter of strain IS and the sequence of the 5' end of the IS1301 insertion element. The 5' imperfect direct repeat of IS1301 is underlined and the putative -10 sequence is indicated in bold type in the nucleotide sequence. Nucleotides of the putative transposase -10 element that were changed by site-specific mutagenesis are indicated above the sequence.

of the putative transposase gene. To determine if the -10 element contributes to increased promoter activity, a *lacZ* reporter was constructed in which the TATAAT sequence was altered to GGGAAT by site-specific mutagenesis. As shown in Fig. 4(a, b), this construct showed β -galactosidase activity similar to the parent *A. actinomycetemcomitans* 652 reporter construct. A second *lacZ* reporter in which the unaltered IS1301 sequence was inverted in orientation relative to *orfA* and *lacZ* also exhibited β -galactosidase activity similar to the parent strain promoter construct. Finally, real-time RT-PCRs using primers complementary to the transposase ORF of IS1301 showed a 5–6-fold decrease in the appropriate transcripts in the strain containing the site-specific mutation (see Fig. 4c). However, a transcript spanning the transposase ORF and *orfA* was not detected, consistent with the results of Mitchell *et al.* (2003). Together, these results suggest that the -10 element and its orientation relative to *orfA* contribute either directly or indirectly to increased transcription of the *ltx* operon.

Interestingly, recombinant insertion of IS1301 into the AT-rich sequence in the *ltx* promoter of *A. actinomycetemcomitans* JP2

(which contains a deletion that truncates *orfA*) had little effect on transcriptional activity (see Fig. 4a). This suggests that an intact *orfA* gene may be required for IS1301 to exert its effect. To address this, the activity of a JP2 promoter-*lacZ* reporter was examined in both 652 and JP2 backgrounds. As shown in Fig. 4(b), *lacZ* expression was significantly higher in the 652 background. This suggests that OrfA may be a positive regulator of *ltx* expression.

Frameshift mutations in *orfA* alter *ltx* promoter activity

To determine if *orfA* regulates *ltx* promoter activity, three independent reporter plasmids containing frameshift mutations in *orfA* were analysed in an OrfA-deficient *A. actinomycetemcomitans* strain that was constructed by insertional inactivation of *orfA* (see Methods). As shown in Fig. 5, each of the frameshift mutations resulted in significantly lower β -galactosidase activity than the parental construct. Frameshift mutations at nucleotides 270 and 400 of the *orfA* sequence lead to 93% and 96% reductions of *ltx* promoter activity respectively. These

Fig. 4. (a) Schematic diagrams of the promoter fragments containing the site-specific mutation of the putative transposase -10 element and inversion in IS1301. The sequence of the putative -10 region is shown for each construct and its orientation relative to *orfA* is indicated by arrows. The promoter region of strain JP2 contains a deletion that truncates *orfA* and removes much of the *orfA-ltxC* intergenic region (see Fig. 1a). The site of recombinant insertion of IS1301 into the *ltx* promoter of *A. actinomycetemcomitans* JP2 is identical to the naturally occurring site in strain IS. (b) β -Galactosidase activity of reporter constructs containing altered IS1301 sequences is indicated in Miller units $\pm$ SE. The left axis indicates Miller units for the 652 and IS reporters and the right axis indicates Miller units for the JP2 reporter constructs. The ISJP2:*lacZ* construct was expressed in a 652 background. The JP2:*lacZ* construct was expressed in both 652 and JP2 backgrounds. *, $P < 0.0001$. (c) Real-time RT-PCR analysis of RNA from strain IS and the isogenic -10 region site-specific mutant using primers specific for the transposase and *orfA* ORFs.

constructs are capable of expressing OrfA polypeptides of 24 and 80 residues whereas the full-length ORF encodes a putative protein of 150 residues. The frameshift mutation at nucleotide 500 directed higher levels of β -galactosidase expression than the reporters containing mutations at nucleotides 270 and 400 but still had only 30 % of the β -galactosidase activity expressed by the control reporter plasmid. Thus, the introduction of premature translational stop codons into *orfA* dramatically affected *ltx* promoter activity.

OrfA interacts with the *ltx* promoter

To determine if the OrfA polypeptide interacts with the *ltx* promoter *in trans*, OrfA protein was expressed with a C-terminal His₆ tag using an *in vitro* coupled transcription-translation system. The affinity-purified polypeptide was

then used as a probe with various fragments derived from the *ltx* promoter region in EMSAs (see Fig. 6a). As shown in Fig. 6(b), a biotinylated fragment corresponding to the intergenic region between *orfA* and the upstream *glyA* gene exhibited reduced mobility when incubated with His₆-OrfA and the interaction of OrfA with this fragment was inhibited by the addition of excess unlabelled promoter DNA. To localize the putative OrfA binding site, a series of nested 5' deletions of the target DNA fragment were amplified and analysed. As shown in Fig. 6(c, d), OrfA retarded the mobility of all of the promoter fragments that contained the region between the -35 element and the start codon of *orfA*. The mobility of fragments lacking this region, such as probe CNR (see Fig. 6e), was unaffected by OrfA. These results suggest that OrfA interacts with sequences residing downstream from the -35 element in the *ltx* promoter.

Fig. 5. Activity of reporter constructs containing frameshift mutations in *orfA*. The sites of the frameshift mutations in *orfA* and the resulting truncated peptides that are potentially expressed by these constructs are described in Methods. All the mutations result in a statistically significant ($P \leq 0.0001$) decrease in activity relative to the parental strain, 652.

DISCUSSION

Both positive and negative *cis*-acting regulatory sequences have been identified in the *ltx* promoter of *A. actinomycetemcomitans* (Kolodrubetz *et al.*, 1996; Mitchell *et al.*, 2003), and hyperleukotoxic strains of the organism are known to possess genetic alterations in the promoter region that lead to increased transcription of the *ltx* operon. Indeed, He *et al.* (1999) showed that integration of IS1301 occurred near the 5'-end of an AT-rich regulatory sequence that resembles an UP element (Estrem *et al.*, 1999; Gourse *et al.*, 2000; Ross *et al.*, 1993) in the hyperleukotoxic strain IS, but IS1301 does not appear to functionally disrupt the putative UP element (Mitchell *et al.*, 2003). Instead, Mitchell *et al.* (2003) suggested that the acquisition of IS1301 may uncouple negative regulation of the *ltx* operon by displacing a *cis*-acting negative regulatory sequence ~800 bp upstream. Our current results, however, show that displacement of this negative regulator by random sequences derived from *ltxA* did not result in increased transcription of downstream genes, suggesting that specific sequences within IS1301 are required to stimulate toxin expression.

Fig. 6. Interaction of OrfA with the *ltx* promoter region. (a) Schematic diagram of the *ltx* promoter region and the DNA fragments used as probes in EMSAs. For clarity, the drawing is not to scale; the sizes of the probes are listed on the right. ATG represents the start codon of *orfA*. Biotinylated DNA probes were incubated with His₆-OrfA and electrophoresed in a 10 % TBE gel, transferred to a nylon membrane and detected using streptavidin-horseradish peroxidase and a chemiluminescent substrate as described in Methods. (b) Lanes: 1, full-length *ltx* promoter probe alone; 2, probe + His₆-OrfA; 3, probe + His₆-OrfA + unlabelled probe. (c) Lanes: 1, ΔCNR alone; 2, ΔCNR + His₆-OrfA; 3, ΔCNR + His₆-OrfA + unlabelled ΔCNR; 4, IRprom alone; 5, IRprom + His₆-OrfA; 6, IRprom + His₆-OrfA + unlabelled IRprom. (d) Lanes: 1, ΔIRprom alone; 2, ΔIRprom + His₆-OrfA; 3, ΔIRprom + His₆-OrfA + unlabelled ΔIRprom. (e) Lanes: 1, CNR alone; 2, CNR + His₆-OrfA.

Analysis of *lacZ* reporters containing serial deletions in IS1301 indicated that a 100 bp region at the 5' end of IS1301 was required for increased transcriptional activity. This region contains a consensus -10 element upstream from the transposase gene, and several lines of evidence suggest that this putative -10 sequence functions to modulate the activity of the *ltx* promoter. First, inverting the orientation of the intact IS1301 relative to *orfA* and second, site-specific mutagenesis of the putative -10 sequence both reduced *ltx* promoter activity to that of the parental minimally leukotoxic strain which lacks IS1301. In addition, real-time PCR indicated a 5–6-fold reduction in the steady-state levels of transposase transcripts in the strain possessing the -10-site-specific mutation. However, consistent with Mitchell *et al.* (2003), no transcript was detected that spanned the transposase and *orfA* ORFs. Thus, alteration of the putative transposase -10 sequence may have an indirect effect on *orfA* expression or, alternatively, the primary transcript encompassing both genes may undergo rapid post-transcriptional processing. Indeed, the primary transcript from the *ltx* promoter in both minimally leukotoxic and hyperleukotoxic *A. actinomycetemcomitans* strains is quickly processed to generate independent *orfA* and *ltxCA* (or *ltxCABD*) transcripts (Kolodrubetz *et al.*, 1996). Together, these data suggest that the TATAAT sequence upstream of the IS1301 transposase gene contributes to increased expression of leukotoxin.

Interestingly, recombinant insertion of IS1301 into a *lacZ* reporter plasmid containing the *ltx* promoter of *A. actinomycetemcomitans* JP2 did not increase β -galactosidase expression. *A. actinomycetemcomitans* JP2 contains a 530 bp deletion that truncates *orfA* and removes much of the *orfA*–*ltxC* intergenic region (see Fig. 1a). This led us to examine a potential regulatory role for the putative OrfA polypeptide. Reporter plasmids containing three independent frameshift mutations in *orfA* resulting in premature termination of translation each produced significantly decreased β -galactosidase activity compared to the unaltered ORF. Furthermore, recombinantly expressed OrfA protein bound to a region of the *ltx* promoter at or near the -35 sequence, suggesting that OrfA may function to regulate *ltx* promoter activity. Thus, it is possible that increased transcription of *orfA* mediated by the -10 element in IS1301 results in overproduction of the OrfA protein, which in turn stimulates *ltx* operon expression leading to the hyperleukotoxic phenotype. However, the OrfA binding site near the -35 sequence suggests that it may not function as a typical *trans*-regulatory protein. One possibility is that OrfA is a DNA-binding protein that alters the structure of the *ltx* promoter, which in turn facilitates increased transcription. Interestingly, the C-terminus of OrfA is highly basic (11 of 35 residues are positively charged), consistent with a potential DNA-binding activity, and this basic region is encoded by a sequence that is deleted in strain JP2. OrfA does not possess a predicted signal sequence or transmembrane domain, suggesting that

it is a cytoplasmic protein. However, it exhibits no significant similarity to other polypeptides in the protein database and does not possess any conserved structural domains when analysed by PFAM. Thus, OrfA may represent a novel regulatory protein that controls the expression of the leukotoxin in *A. actinomycetemcomitans*; studies are currently under way to further examine its DNA-binding activity.

While our results suggest a potential role for OrfA in stimulating *ltx* expression in *A. actinomycetemcomitans* 652, they do not fully explain the hyperleukotoxic phenotype of strain JP2, where truncation of *orfA* is associated with increased *ltx* expression. The expression of *lacZ* from the JP2 promoter in a 652 background was significantly greater than that in a JP2 background. This is consistent with a role for OrfA as a positive regulator of *ltx* expression. However, the JP2 promoter still directed higher overall expression of *lacZ* in the 652 background than did the 652 promoter, suggesting that other factors may also contribute to the elevated expression observed from the JP2 promoter. One possible explanation is that the closer proximity of the *ltx* promoter to *ltxCABD* in strain JP2 (as a result of the 530 bp deletion) partially compensates for the loss of OrfA induction in JP2. In addition, the primary *ltx* transcript appears to be rapidly cleaved between *orfA* and *ltxC* (Kolodrubetz *et al.*, 1996; D. R. Demuth, unpublished). Thus, it is also possible that the 530 bp deletion spanning the *orfA*–*ltxC* intergenic region alters processing of the *ltx* primary transcript in JP2, resulting in increased expression of the *ltx* operon.

In summary, hyperleukotoxic strains of *A. actinomycetemcomitans* are associated with localized aggressive periodontitis but the molecular mechanisms leading to the hyperleukotoxic phenotype are poorly understood. Our results suggest that the acquisition of IS1301 upstream of *orfA* introduces a new promoter-like sequence that may increase transcription of *orfA* and the downstream *ltx* genes. We also show that the OrfA protein acts as a positive regulator of *ltx* expression, which may further amplify toxin expression in strain IS. However, the mechanism of OrfA *trans*-activation may be unique and will require further study to define.

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