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Repression of aerobic leukotoxin transcription by integration host factor in *A. actinomycetemcomitans*

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Abstract

Aggregatibacter actinomycetemcomitans has been implicated as the primary etiologic agent in localized aggressive periodontitis. This bacterium produces a leukotoxin which may help the bacterium evade the host immune response. Leukotoxin transcription is induced when *A. actinomycetemcomitans* is grown anaerobically, as in the periodontal pocket. Previously, a 35 bp oxygen-response-element (ORE) was shown to be responsible for oxygen regulation at the leukotoxin promoter. However, the gene's transcription is not controlled by Fnr or ArcA, the major oxygen regulators in other bacteria. To identify the potentially novel protein(s) that regulate leukotoxin transcription, protein extracts of *A. actinomycetemcomitans* were tested for ORE binding by mobility shift assays; one ORE-specific binding complex was found. Standard fractionation protocols and protein sequencing identified the ORE binding protein as integration host factor (IHF). DNaseI protection assays showed that the IHF binding site overlaps the first half of the ORE. To assess the effect of IHF on leukotoxin synthesis, an *A. actinomycetemcomitans* deletion mutant in *ihfB* was constructed and characterized. Interestingly, leukotoxin RNA and protein synthesis was de-repressed in the *ihf* mutant, although leukotoxin synthesis is still oxygen-regulated in the mutant cells. Thus, IHF plays a direct role in repressing leukotoxin transcription, but another protein is also involved in regulating leukotoxin expression in response to oxygen.

Keywords

Aggregatibacter actinomycetemcomitans; Integration host factor; Bacterial gene expression regulation; Leukotoxin

1. Introduction

Periodontal disease, which can lead to soft tissue degeneration and significant bone loss, results from the inflammatory response to bacteria in the subgingival crevice. The development of periodontitis is associated with a shift in the subgingival flora; there is a dramatic increase in the levels of several species of gram-negative bacteria. Because the periodontal pocket is a relatively anaerobic environment, most of the bacteria found in deep pockets are anaerobes (Haffajee and Socransky, 1994; Slots and Ting, 1999), but at least one periodontal pathogen,

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Aggregatibacter actinomycetemcomitans, is a facultative organism. This gram-negative bacterium is the major pathogen in the etiology of localized aggressive periodontitis (LAP) (Fine et al., 2006; Fine et al., 2007; Slots and Ting, 1999), although it can also be involved in cases of rapidly progressive and adult periodontitis (Slots and Ting, 1999). Understanding the mechanisms by which *A. actinomycetemcomitans* establishes a subgingival infection requires the identification and characterization of potential virulence factors. A number of virulence components have been described in *A. actinomycetemcomitans* (Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003), including leukotoxin, a 116 kDa protein that kills human neutrophils, monocytes, natural killer cells (Fine et al., 2006; Henderson et al., 2003; Taichman and Wilton, 1981) and erythrocytes (Balashova et al., 2006). At lower doses, leukotoxin can also induce apoptosis in other cell types, including lymphocytes (Fine et al., 2006; Korostoff et al., 1998). Thus, this protein is thought to be critical in helping *A. actinomycetemcomitans* evade the host innate immune response (Bueno et al., 1998; Fine et al., 2006; Haubek et al., 1997; Taichman and Wilton, 1981).

In addition to identifying the bacteria and virulence factors involved in an infection, it is critical to understand their regulation by the host milieu. The periodontal pocket is a unique microenvironment that can change dramatically. For example, during disease, the oxygen tension of the subgingival crevice decreases dramatically and is lowest, and essentially anaerobic, in the deepest periodontal pockets. Thus, a periodontal pathogen such as *A. actinomycetemcomitans* is likely to regulate some of its virulence factors in response to relatively aerobic versus anaerobic conditions. In fact, a shift of *A. actinomycetemcomitans* from aerobic to anaerobic growth has been shown to increase the bacterium's interaction with host cells (Meyer and Fives-Taylor, 1994), but to decrease its binding to host molecules such as collagen (Mintz and Fives-Taylor, 1999). In addition, aerobic versus anaerobic growth results in altered levels of numerous proteins (Fletcher et al., 2001; Scannapieco et al., 1987), including leukotoxin (Kolodrubetz et al., 2003; Spitznagel et al., 1995). The leukotoxin regulation is occurring at the RNA level, since anaerobiosis has been shown to induce transcription from the leukotoxin promoter (Spitznagel et al., 1995).

To determine the mechanism by which leukotoxin transcription responds to aerobic versus anaerobic growth, we characterized a series of leukotoxin promoter mutants in *A. actinomycetemcomitans* (Kolodrubetz et al., 2003) and found that a 35 bp oxygen response element (ORE) upstream of the leukotoxin mRNA start site is responsible for the repression of transcription in aerobically grown cells. The sequence of the ORE differs from the consensus elements for transcription factors from other bacteria. In addition, mutants in the *A. actinomycetemcomitans* homologues of Fnr and ArcA, which play major roles in oxygen regulation in other bacteria, still repress leukotoxin synthesis in aerobic conditions (Kolodrubetz et al., 2003). These results suggest that oxygen regulation of leukotoxin expression involves an uncharacterized regulatory pathway.

In this report, gel mobility shift assays with *A. actinomycetemcomitans* extracts and ORE DNA were used to try to identify the potentially novel transcription factor(s) that regulates the leukotoxin promoter in response to aerobic versus anaerobic growth. The *A. actinomycetemcomitans* homologue of integration host factor (IHF) (Bewley et al., 1998; Rice et al., 1996; Swinger and Rice, 2004), a small heterodimeric protein, was found to bind specifically to the leukotoxin promoter region. DNaseI protection experiments showed that the IHF binding site overlapped with the first half of the ORE. Analysis of an *ihfB* deletion strain demonstrated that IHF represses leukotoxin protein and RNA synthesis, but that IHF is not responsible for oxygen regulation; leukotoxin in the mutant is still oxygen-regulated. Overall, the results show that IHF is involved in the regulation of *A. actinomycetemcomitans*' leukotoxin transcription, but that another protein is a key player in the response of leukotoxin to aerobic versus anaerobic conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. actinomycetemcomitans cells were grown anaerobically (5% CO₂, 10% H₂, 85% N₂) in a Coy chamber (Coy Laboratory Products, Ann Arbor, MI) at 37°C or aerobically (5% CO₂, 95% air) in a CO₂ incubator with shaking at 37°C. The growth media was TSBYE (3% tryptic soy broth plus 0.6% yeast extract) containing, where indicated, spectinomycin to a final concentration of 50 µg/ml or ampicillin at a final concentrations of 10 µg/ml.

A. actinomycetemcomitans strain JP2 (Tsai et al., 1984) was the parental wild-type strain used in these studies. To construct an *ihfB* deletion mutant in *A. actinomycetemcomitans*, standard recombinant DNA techniques and PCR were used to construct plasmid pDK795, in which 0.91 kb of the DNA immediately upstream of *ihfB* and 0.82 of the DNA immediately downstream of *ihfB* flank a spectinomycin resistance gene in place of the *ihfB* coding region. The plasmid is a pUC-based replicon that does not replicate in *A. actinomycetemcomitans* and it also contains the counterselectable marker *sacB* (Reyrat et al., 1998). Plasmid pDK795 was electroporated into *A. actinomycetemcomitans* strain JP2 and single crossover events at the *ihfB* locus were selected as spectinomycin-resistant colonies. After passaging these strains several times, cells in which a subsequent second recombination event had occurred, removing the vector and also possibly removing the wild type *ihfB* gene from the chromosome, were selected by plating the cells on TSBYE with 7.5% sucrose. The sucrose-resistant, spectinomycin-resistant colonies should be those in which the *ihfB* deletion allele had replaced the wild type gene. Mutation of the *ihfB* locus was confirmed by PCR and by Southern blot analysis (Spitznagel et al., 1991) using a 143 bp probe internal to *ihfB*. One correct isolate, AAM109, was used in subsequent experiments.

Strain AAM111 was constructed by electroporating an *ihfB*⁺ plasmid into AAM109. Briefly, a 0.47 kb *HindIII/BamHI* DNA fragment containing the 287 bp *ihfB*⁺ gene and 90 bp each of its upstream and downstream DNA, was cloned into the broad-host-range plasmid pMMB67HE (Goncharoff et al., 1993). This *ihfB*⁺ plasmid was electroporated into AAM109 and transformants were selected on TSBYE with ampicillin.

2.2. Purification and identification of the ORE binding protein

JP2 cells (2 liters) were grown anaerobically overnight, harvested and disrupted on a French press. After centrifugation (15 min, 11,000 × g), the soluble material was made 4M in NaCl and 1:3 w:w polymer mix (6.4% Dextran 500K, 25.6% PEG 8000, 4M NaCl) was added slowly and stirred on ice for 60 min to remove nucleic acid (Schleif and Wensink, 1987). The supernatant, after a 15 min centrifugation at 4500 × g, was dialyzed overnight at 4°C against dialysis buffer (10 mM Tris (pH7.5), 50 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF). The PEG was removed by a 30% ammonium sulfate precipitation step and the resulting pellet was enriched for ORE binding activity by stepwise 40%, 50% and 70% ammonium sulfate precipitations. The material precipitated by 70% (NH₄)₂SO₄ was dialyzed overnight at 4°C against the same buffer as above and then applied to an Affi-Gel Heparin (Bio-Rad Laboratories) column which had been equilibrated with dialysis buffer. Proteins were eluted with a linear (50 mM to 2M) NaCl gradient in buffer and dialyzed at 4°C against dialysis buffer which included 4% glycerol. ORE binding activity was measured for each fraction and the proteins present were assessed by SDS polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. For the gel mobility shift experiment with the *ihf* deletion mutant, AAM109 cells were grown and ammonium sulfate extracts were prepared exactly as for wild type cells.

To identify the two proteins whose levels correlated with ORE binding activity, material from the most active Affi-Gel heparin column fraction was separated by SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories). The membrane was stained with 0.2% ponceau S, 1% acetone and after destaining with water, the two appropriate protein bands were cut out of the filter and submitted to the UTHSCSA Mass Spectrometry Core Facility for amino terminal sequence analysis.

2.3. Gel mobility shift and DNaseI protection assays

The template for the gel mobility shift assays was a 68 bp PCR product containing the -33 to -87 region of the leukotoxin promoter () and primer-encoded *EcoRI* ends. After digestion with *EcoRI*, the DNA was labeled by end-filling with Klenow polymerase and α -³²P-dATP/dTTP. The probe was gel-purified before use. For the competition binding experiment, the specific competitor DNA was simply unlabeled ORE DNA (Fig. 1, Specific Probe "A") and the non-specific competitor DNA was a gel-purified 71 bp PCR product with the -5 to +50 region of the leukotoxin gene. Both competitor DNAs had primer-encoded *EcoRI* ends. Gel mobility shift assays were done in 20 μ l (final volume) of binding buffer (10 mM Tris, pH7.5, 75 mM KCl or 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 50 μ g/ml BSA, 10 μ g/ml poly-dI:dC). Labeled Probe "A" DNA and protein, which had been dialyzed, were added. For the competition binding experiment, unlabelled specific or non-specific competitor DNA was also included in the reaction. After 20 min incubation at 25°C, the reaction products were electrophoresed on a low ionic strength (45 mM Tris, pH8.0, 45 mM boric acid, 2 mM EDTA) polyacrylamide gel and the gel was exposed to film.

To generate leukotoxin promoter DNA labeled at one end for the DNaseI protection experiments, a 260 bp product containing 225 bp from the leukotoxin promoter region, as well as an *EcoRI* site at one end and an *SphI* site near the other end, was generated by PCR. After digestion with *EcoRI*, the DNA was labeled by end-filling with Klenow polymerase and α -³²P-dATP/dTTP. The labeled DNA was digested with *SphI* and the resulting singly-end-labeled 230 bp fragment was purified by gel electrophoresis. Varying amounts of an IHF-enriched fraction from an Affi-Gel heparin column were incubated for 20 min (25°C) with the end-labeled leukotoxin-promoter DNA in 50 μ l (final volume) of 50 mM NaCl, 5 mM Tris (pH 7.5), 5 mM MgCl₂, 1 mM CaCl₂, 2 μ g/ml poly-dI:dC. DNaseI (0.2 units) was then added for 30 s at which time the reactions were quenched with 100 μ l of 1% SDS, 0.2M NaCl, 20 mM EDTA. After phenol/ChCl₃ extraction and ethanol precipitation, the samples were dissolved in sequencing sample buffer (Ausubel et al., 1984) for electrophoresis on 6% polyacrylamide-urea gels. For sizing, a Sanger sequencing reaction was performed with ddATP on a piece of leukotoxin promoter DNA with a primer that starts at the same position as the *EcoRI* site in the labeled DNA used for footprinting.

2.5. Analysis of leukotoxin protein and RNA expression

Cells from cultures of various strains of *A. actinomycetemcomitans* were pelleted by centrifugation and then suspended in 10 mM Tris (pH 7.5), 50 mM NaCl to 1/50th of the original volume. Total protein was prepared from these cells by heating a 50 μ l aliquot with 30 μ l of 2 $\times$ loading buffer (Kolodrubetz et al., 2003) to 100°C for 2 min. After pelleting debris for 5 min at 13,000 $\times$ g, equal volumes of the soluble material from each sample were electrophoresed on an SDS-polyacrylamide gel (Kolodrubetz et al., 2003). Proteins were visualized by staining with Coomassie brilliant blue.

RNA was prepared from *A. actinomycetemcomitans* using the RNeasy Mini Kit (Qiagen). The manufacturer's instructions were followed with the following notes: 3 ml of cells were used and they were lysed with 10 mg/ml lysozyme for 15 min. After DNase treatment, an Omniscript Reverse Transcriptase kit (Qiagen) was used to convert each RNA sample (400 ng) into cDNA

using random hexamers as primers. Real-time PCR was performed on dilutions (1/1600 or 1/400) of the cDNAs. GoTaq® qPCR Master Mix (Promega), cDNA and appropriate primers (300 nM for *ltx516*, *ltx517* and *pdx519*; 900 nM for *pdx518*) were gently mixed together in a final volume of 50 µl in 96-well PCR plates. PCR was done on a Chromo4 Real-Time PCR Detection System (BioRad) using an initial denaturation step for 2 min at 95°C followed by 39 cycles of amplification (15 s at 95°C, 1 min at 63°C, read plate). At the end of each reaction, a melting curve of the reaction products was generated to show that the primers had specifically amplified the single PCR product expected. A dilution series of control reactions was run to show that the primer pairs worked with >97% efficiency. All reactions were performed in triplicate. For each sample, the levels of *ltx* mRNA were normalized to the levels of *pdxY* mRNA, by calculating the difference in the averaged *ltx* and *pdxY* threshold cycles (C_t) for that sample. Primers *ltx516* (5'-CGAGGGAAGGTTACCGATCTAC-3') and *ltx517* (5'-TGCTGCCGATAATTCCTCTAC-3') amplify a 119 bp fragment from the middle of the leukotoxin gene. Primers *pdx518* (5'-CGTTGGCGTGGGTGATTAAAC-3') and *pdx519* (5'-CAAAAGCCTCCACATCGGAT-3') make a 77 bp fragment from the *pdxY* gene which we have found to be non-regulated in multiple microarrays (data not shown).

3. Results

3.1. A. actinomycetemcomitans has a protein(s) that binds preferentially to the ORE of the leukotoxin promoter

Previous mutational analysis of the leukotoxin promoter revealed that a 35 bp segment (−70 to −36), called the oxygen response element (ORE) (Fig. 1), was involved in the aerobic/ anaerobic regulation of leukotoxin transcription (Kolodrubetz et al., 2003), so gel mobility shift assays were used to identify *trans* factors that bind to this sequence. Extracts from strain JP2 grown aerobically or anaerobically were tested for binding activity. The same two shifted bands were seen in extracts from both aerobic cells (data not shown) and anaerobic cells (Fig. 2A, lane 1). To determine which, if either, of these bands was specific to the leukotoxin regulatory region, a competitive binding assay was done with increasing concentrations of either "specific" ORE-containing DNA (Fig. 1, Specific Probe "A") or a "non-specific", non-promoter AT-rich DNA fragment from the 5' non-coding region of leukotoxin. The intensity of one of the shifted bands, the smaller one, was reduced more by the ORE-containing competitor DNA ("A") (Fig. 2A, lanes 2–3) than by the non-specific competitor DNA ("B") (Fig. 2A, lanes 4–5), indicating that the lower band shift was due to a protein that bound preferentially to the leukotoxin regulatory region. The upper band appeared to bind equivalently to both promoter and non-promoter DNA and thus was not pursued further. The ORE-specific band was also found with extracts from Y4, another strain of *A. actinomycetemcomitans*, and its abundance, as measured by binding activity, appeared to be the same in extracts from JP2 cells grown aerobically or anaerobically (data not shown).

Since the ORE was somewhat large for a transcriptional regulatory sequence, it seemed possible that there could be more than one ORE-specific binding protein. Thus, numerous experimental parameters were altered to see if another ORE-specific binding protein could be found in *A. actinomycetemcomitans*' extracts. We varied the methods of extract preparation (sonication, French press), binding conditions (salt, different cations, adding polydI-dC or single-stranded salmon sperm DNA) and gel buffers. None of these conditions yielded additional specific binding activities. Since the binding of an oxygen-responsive transcription factor can be sensitive to the oxidation/ reduction status of the sample (Bauer et al., 1999), the extractions and binding assays were also performed with and without DTT or β-mercaptoethanol. These agents did not alter any of the binding activities, including that of the protein initially found. Finally, since transcription factors can be present in low abundance,

ORE multimers were used as templates in order to increase the assay sensitivity (Singh et al., 1989); no additional ORE-specific bands were seen.

3.2. IHF is the protein complex that binds to the leukotoxin ORE

To identify the protein causing the specific shift of the leukotoxin -regulatory region, protein fractionations were performed. Briefly, JP2 cells were grown anaerobically, harvested and disrupted on a French press. PEG/dextran was used to remove nucleic acid and then stepwise (30%, 40%, 50%, 70%) ammonium sulfate precipitations were used to remove the PEG and the majority of the proteins. The material precipitated by 70% (NH₄)₂SO₄ was separated on an Affi-Gel heparin column. Mobility shift assays were used to monitor ORE binding activity throughout the purification protocol. Two small (~11 kD) proteins were the only proteins that cofractionated with the binding activity in the final chromatography step (Fig. 3, lanes 18 and 19). The two proteins were subjected to amino terminal sequence analysis and were identified as the two subunits (α and β) of the sequence-specific DNA binding protein IHF (Bewley et al., 1998; Rice et al., 1996; Swinger and Rice, 2004). The *A. actinomycetemcomitans* proteins share 69% and 63% amino acid sequence identity with the well characterized IHF α and IHF β proteins from *E. coli*.

To demonstrate that the specific binding activity we identified in *A. actinomycetemcomitans* extracts was the *A. actinomycetemcomitans* homologue of IHF, a two-step allelic replacement procedure was used to make a strain in which the *ihfB* gene had been replaced by a spectinomycin resistance gene. Extracts from this *ihf* mutant strain of *A. actinomycetemcomitans* do not have the ORE-specific binding activity found in wild type cells (Fig. 2B), proving that IHF is the ORE binding protein.

3.3 IHF binds to sequences that overlap the first half of the ORE

To define the IHF binding site in the leukotoxin ORE more precisely, the Affi-Gel heparin-purified IHF protein complex was used in DNaseI footprinting experiments. The results indicate that IHF protects a region from -50 to -77 in the leukotoxin promoter (Fig. 4). This is consistent with the fact that there is a sequence element, from -53 to -64, that is a good match (11/13) to the consensus IHF binding site in *E. coli* (Goodrich et al., 1990). Interestingly, although the IHF footprint overlaps the ORE (Fig. 1), it does not cover the entire ORE. This suggests that IHF should impact transcription and/or oxygen regulation at the leukotoxin promoter, but that there is likely to be at least one other protein involved.

3.4. IHF is required for normal regulation of leukotoxin synthesis, but it is not the only protein involved

To elucidate the role of IHF in leukotoxin transcription, the effect of the *ihfB* mutant on leukotoxin synthesis was assessed. First, the levels of leukotoxin protein were examined at different growth phases in cells grown aerobically or anaerobically. Wild type and *ihf* mutant cells, which have the same growth rates, appeared to have approximately equal levels of leukotoxin in anaerobic growth conditions (Fig. 5). However, when cells were grown aerobically, the *ihf* mutant cells had more leukotoxin than wild type cells (Fig. 5). The de-repression of aerobic leukotoxin synthesis in *ihf*⁻ cells was more obvious in stationary phase cells than in log phase cells (Fig. 5B); this would appear to be due to a higher starting level of leukotoxin in aerobic, stationary phase wild type cells. Although leukotoxin appeared to be absent in some aerobic samples in Fig. 5, the protein was indeed expressed aerobically, as has been reported previously (Fong et al., 2001; Hritz et al., 1996). The apparent lack of leukotoxin protein in some aerobic samples was due to the fact that staining to detect protein levels is not as sensitive as using western blots with anti-leukotoxin antisera, as was done by others (Fong et al., 2001; Hritz et al., 1996). To see whether IHF affected leukotoxin transcription, quantitative reverse-transcriptase PCR was done on RNA from wild type and mutant cells. As

expected, when transcriptional regulation was involved, the pattern of the relative leukotoxin RNA levels (Fig. 6) correlated well with the pattern of leukotoxin protein expression (Fig. 5). The increase in leukotoxin RNA seen in mutant cells grown anaerobically was not obvious on the protein gels because the levels of leukotoxin protein were close to saturation on the stained gels. On other gels with less protein loaded, increased leukotoxin was seen in the *ihf*⁻ cells (data not shown), but then leukotoxin protein levels in the aerobic samples were too low to be seen. Overall, the levels of leukotoxin RNA in *ihf*⁻ cells were approximately twofold higher than in wild type cells irrespective of growth phase or oxygen concentration. Thus, these results show that IHF acted as a repressor of leukotoxin transcription. Importantly, the IHF mutant and wild type cells grew with the same doubling times and to the same final cell densities. This ruled out indirect effects of the loss of IHF on leukotoxin synthesis due to altered cellular physiology. Finally, the *ihf* mutant phenotype was reversed when a wild type *ihfB* gene was put into the mutant strain on a plasmid (Fig. 5B). This proved that the effects on leukotoxin synthesis were due to the deletion of *ihfB* and not to a fortuitous mutation in another gene or to a polar effect on a gene downstream of *ihfB*. Thus, IHF plays a role in repressing leukotoxin synthesis. However, the data also indicate that another protein must be involved in the aerobic/anaerobic regulation of leukotoxin synthesis, since leukotoxin RNA and protein expression was still oxygen-regulated in cells lacking IHF activity.

4. Discussion

In an attempt to identify protein(s) involved in regulating *A. actinomycetemcomitans* leukotoxin transcription in response to changes in oxygen tension, we found that IHF binds to a segment of DNA overlapping the first half of the ORE, a 35 bp DNA element involved in regulating leukotoxin transcription (Kolodrubetz et al., 2003). However, analysis of an IHF mutant shows that it is not the protein that senses oxygen; anaerobically grown mutant cells still have higher levels of leukotoxin than aerobic IHF⁻ cells. The inability of oxygen to modulate IHF activity at the leukotoxin promoter is not surprising, since IHF is a heterodimer that basically consists of a DNA binding motif (Rice et al., 1996; Swinger and Rice, 2004); IHF does not have an additional domain that might be involved in sensing environmental cues.

Although IHF is not the factor responsible for leukotoxin's oxygen regulation in *A. actinomycetemcomitans*, it does repress leukotoxin transcription. Since we do not yet know any other transcription factors that bind to the leukotoxin promoter, the exact mechanism by which IHF alters leukotoxin transcription is a matter of speculation. IHF is a conserved DNA binding protein that bends the DNA to which it binds by >160° (Rice et al., 1996) and it can affect most DNA transactions in the cell (Friedman, 1988), including transcription. Although IHF was first described as an activator of transcription at several promoters (Bertoni et al., 1998; Goosen and van de Putte, 1995; Mettert and Kiley, 2007; Sheridan et al., 1998; Stonehouse et al., 2008), it is clearly acting as a repressor of *A. actinomycetemcomitans* leukotoxin synthesis. This is not unprecedented, since IHF has been shown to repress transcription at a number of promoters in other bacteria using several different mechanisms. Thus, how might IHF be repressing leukotoxin transcription? It is unlikely simply to be directly blocking RNA polymerase binding, as is seen at several *E. coli* promoters (Goosen and van de Putte, 1995; Tsui et al., 1991), since the IHF binding site does not overlap the -10 or -35 sequences in the leukotoxin promoter. In *E. coli*, IHF binding at -88 reduces RNA polymerase occupancy at the *nir* operon promoter, possibly by interfering with the polymerase's interaction with an UP element in the *nir* promoter (Browning et al., 2008). In *A. actinomycetemcomitans*, Mitchell et al. (2003) identified an activator element at position -40 to -71 in the leukotoxin promoter and called it an UP element since it was a good match to the consensus UP sequence (Estrem et al., 1998). Maybe IHF binding to -50 to -77 in the leukotoxin promoter interferes with the activity of this putative UP element. However, we think this model is unlikely to be correct since the putative UP element in the leukotoxin promoter

is centered at -60 as opposed to the ideal -50. Tagami et al. (1999) showed that an UP element becomes inactive and even repressive when it is moved 5 to 10 bp upstream from its normal position (centered at -50). However, the results of Mitchell et al. (2003) are also consistent with an unidentified activator protein binding to the positive *cis*-element they identified. Thus, IHF could be repressing leukotoxin transcription because its binding site overlaps the binding site for an unidentified activator protein. In fact, this has been shown to be the mechanism involved in the repression of two *E. coli* promoters (Browning et al., 2006; Ramani et al., 1992). Conversely, our previous analysis of leukotoxin promoter mutants showed that a repressor, not an activator, is involved in regulating leukotoxin transcription (Kolodrubetz et al., 2003). If, as our analysis indicated, a repressor is involved in leukotoxin's transcriptional regulation, the best model for IHF's role at the leukotoxin promoter is that IHF binding enhances the binding of a transcriptional repressor. This type of mechanism has been described for the *ompF* gene in *E. coli* and the *farAB* operon in *Neisseria gonorrhoeae* (Lee et al., 2006; Ramani et al., 1992; Slauch and Silhavy, 1991). The reason for the discrepancy between the two analyses (Kolodrubetz et al., 2003; Mitchell et al., 2003) of what appears to be the same *cis*-element in the leukotoxin promoter is not clear, so additional speculation on how IHF alters the binding of a repressor or activator to the -37 to -71 region of the *A. actinomycetemcomitans* leukotoxin promoter is premature until this unknown regulatory protein is identified and its DNA binding properties are characterized. The identification of the other protein(s) involved in leukotoxin regulation would also allow us to do experiments to rule out the formal, but unlikely, possibility that IHF affects leukotoxin transcription indirectly by regulating synthesis of this other transcription factor. We are currently using genetic approaches to identify this other protein(s), which may well be the transcription factor that responds to changes in oxygen concentration.

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Fig. 1.

Location of key elements in the leukotoxin promoter region sequence. The transcription start site for leukotoxin RNA (Kolodrubetz et al., 1996) is indicated as position 0. The position of the putative RNA polymerase binding site is marked as -10 and -35. The positions of the ORE (Kolodrubetz et al., 2003) and the specific probe ("A") used in the gel mobility shift assays are shown by the lines below the sequence. Probe "A" has primer-encoded *Eco*RI (RI) sequences at its ends. The extent of the region protected by IHF in the DNaseI protection experiment (Fig. 4, footprint) is indicated by the line above the sequence. The "core IHF consensus" is a close match to the consensus binding site for the *E. coli* IHF protein; YAANNNTTGGATW, Y = T or C, W = A or T (Goodrich et al., 1990), and includes the absolutely conserved dinucleotide TG.

Fig. 2.

A. actinomycetemcomitans has a protein, IHF, that binds specifically to the ORE in the leukotoxin promoter. (A) A dialyzed 40% ammonium sulfate extract from JP2 was incubated with a ^{32}P -labeled DNA fragment (Fig. 1, specific probe "A") containing the ORE element from the leukotoxin promoter, and subjected to non-denaturing gel electrophoresis (lane 1). A set of parallel reactions was run which contained a 100- or 300-fold molar excess of unlabelled specific (ORE-containing, "A") competitor DNA (lanes 2, 3), unlabeled non-specific (without ORE, "B") competitor DNA (lanes 4, 5), or a 100-fold molar excess of unlabelled poly dA:dT (lane 6). No protein or unlabeled DNAs were added to the reaction in lane 7. (B) Equal amounts of dialyzed ammonium sulfate extracts from JP2 or from AAM109 (*ihf* β deletion mutant) were incubated with a ^{32}P -labeled DNA fragment containing the ORE element from the leukotoxin promoter and subjected to non-denaturing gel electrophoresis.

Fig. 3.

The levels of two small proteins correlate with ORE binding activity. Proteins from the indicated fractions of an Affi-Gel heparin column were used in a gel mobility shift assay (A) with a ^{32}P -labeled DNA fragment (Fig. 1, specific probe "A") containing the ORE element from the leukotoxin promoter or were electrophoresed on an SDS-polyacrylamide gel (B). The asterisks mark the two protein bands that were subjected to amino-terminal sequencing and shown to be IHF α and IHF β . The positions at which molecular size standards migrated on the SDS gel are also indicated.

Fig. 4.

IHF binds to a sequence that overlaps the ORE in the leukotoxin promoter. Increasing amounts of IHF protein from an Affi-Gel heparin column fraction that had the most ORE binding activity in a gel mobility shift assay were used in a DNaseI protection assay. The labeled DNA in the reactions was 230 bp and included the ORE as well as 190 bp of additional sequence from the leukotoxin promoter and 5' non-coding region. To localize the protected region, a Sanger sequencing reaction was performed with ddATP (ddA Seq.) on a piece of leukotoxin promoter DNA with a primer whose 5'-end was at the same position as the position of the ^{32}P -label in the DNA used for footprinting. All of the samples were dissolved in sequencing sample buffer (Ausubel et al., 1984), electrophoresed on 6% polyacrylamide-urea gels and exposed to film.

Fig. 5.

Leukotoxin synthesis is de-repressed in *IHF* β mutant. For each sample, total cell protein was prepared by boiling cell pellets in SDS gel-loading buffer. The proteins were then analyzed by SDS-PAGE and Coomassie blue staining. (A) Overnight cultures of JP2 (the parental wild type strain) and AAM109 (*IHF* $^{-}$) were diluted into fresh media and then grown aerobically or anaerobically, as indicated. Samples were prepared from these cells 8 h later when they were in the log phase of growth. (B) Samples were prepared from anaerobic or aerobic overnight cultures of JP2, AAM109 (*IHF* $^{-}$) and AAM111, which is AAM109 with a wild type *ihfB* gene on a plasmid (*IHF* $^{-}$ /pIHF $^{+}$). Ltx indicates the position of leukotoxin.

Fig. 6.

Leukotoxin RNA levels increase in the *IHF* β mutant. (A) For each sample, total cell RNA was prepared from log phase cells, cDNA was prepared with random hexamer primers and then used in real-time PCR. The levels of *ltx* mRNA in the indicated samples, after normalization to the *pdxY* mRNA (internal control) levels, are expressed as a ratio relative to the level of *ltx* mRNA in the JP2 anaerobic sample. As a control, a parallel set of samples were run without adding reverse transcriptase (RT); in each case the no-RT threshold cycle appeared at least nine cycles later than in the C_t for the corresponding sample with RT (data not shown). (B) Total cell RNA was prepared from stationary phase cells of the samples indicated, cDNA was made for and used in real-time PCR and analyzed as in (A).