Mlc Is a Transcriptional Activator with a Key Role in Integrating Cyclic AMP Receptor Protein and Integration Host Factor Regulation of Leukotoxin RNA Synthesis in *Aggregatibacter actinomycetemcomitans*

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Aggregatibacter actinomycetemcomitans, a periodontal pathogen, synthesizes leukotoxin (LtxA), a protein that helps the bacterium evade the host immune response. Transcription of the *ltxA* operon is induced during anaerobic growth. The cyclic AMP (cAMP) receptor protein (CRP) indirectly increases *ltxA* expression, but the intermediary regulator is unknown. Integration host factor (IHF) binds to and represses the leukotoxin promoter, but neither CRP nor IHF is responsible for the anaerobic induction of *ltxA* RNA synthesis. Thus, we have undertaken studies to identify other regulators of leukotoxin transcription and to demonstrate how these proteins work together to modulate leukotoxin synthesis. First, analyses of *ltxA* RNA expression from defined leukotoxin promoter mutations in the chromosome identify positions –69 to –35 as the key control region and indicate that an activator protein modulates leukotoxin transcription. We show that Mlc, which is a repressor in *Escherichia coli*, functions as a direct transcriptional activator in *A. actinomycetemcomitans*; an *mlc* deletion mutant reduces leukotoxin RNA synthesis, and recombinant Mlc protein binds specifically at the –68 to –40 region of the leukotoxin promoter. Furthermore, we show that CRP activates *ltxA* expression indirectly by increasing the levels of Mlc. Analyses of Δmlc , Δihf , and $\Delta ihf \Delta mlc$ strains demonstrate that Mlc can increase RNA polymerase (RNAP) activity directly and that IHF represses *ltxA* RNA synthesis mainly by blocking Mlc binding. Finally, a $\Delta ihf \Delta mlc$ mutant still induces *ltxA* during anaerobic growth, indicating that there are additional factors involved in leukotoxin transcriptional regulation. A model for the coordinated regulation of leukotoxin transcription is presented.

Periodontitis is an inflammatory disease of the gums, which, if left unchecked, can lead to connective tissue degradation and, ultimately, to tooth loss. In addition, periodontal infections can lead to serious nonoral systemic problems (1, 2). Compared to the chronic periodontal disease typically seen in adult patients, localized aggressive periodontitis (LAP) causes more rapid bone and tissue destruction, affects mostly nonadults, and is localized mainly to the first molars and central incisors (3, 4). *Aggregatibacter actinomycetemcomitans* is a Gram-negative facultative bacterium found in the oral cavity as part of the subgingival microbiota, and an increase in the proportion of this bacterium is strongly associated with LAP (5, 6, 7). *A. actinomycetemcomitans* expresses a number of virulence factors (5, 8, 9), including a 116-kDa leukotoxin protein (LtxA). This protein lyses lymphoid and myeloid cells, including neutrophils, monocytes, and natural killer cells (5, 8, 10, 11), but only if the cells are from human or from certain nonhuman primates (12). At high concentrations, leukotoxin can destroy human leukocytes by forming pores in host cell membranes and by disruption of mitochondrion function, leading to apoptosis (5, 8, 11, 12, 13). At low concentrations of leukotoxin, there is apoptosis and neutrophil degranulation, including the release of the collagenolytic matrix metalloproteinase MMP-8 (14), and phagocytosis can be inhibited (15). All of these results are consistent with a role for leukotoxin as a key player in modulating the host immune response to *A. actinomycetemcomitans* and possibly other bacteria found at the same sites.

Leukotoxin (LtxA) is encoded by the second gene in a four-gene operon, *ltxCABD* (16), whose transcription start site is 337 bp before the first open reading frame in strain JP2 (17). The *ltxC*

gene is required for leukotoxin acylation and function (18), whereas *ltxB* and *ltxD* are required for LtxA secretion (19). Not surprisingly, given the importance of leukotoxin as a virulence factor, transcription of its operon is regulated by various changes in its growth environment. For example, leukotoxin expression is subject to quorum sensing (20). The autoinducer-2 receptors LsrB and RbsB may both play a role in this regulation (21), but the protein that binds the leukotoxin promoter to modulate this regulation is unknown. We, along with others, have shown that leukotoxin levels increase when *A. actinomycetemcomitans* is grown anaerobically (17, 22, 23). Interestingly, leukotoxin synthesis in *A. actinomycetemcomitans* mutants in *fir* and *arcA* is still induced anaerobically (24). This indicates that leukotoxin expression is not modulated by FNR or ArcA/ArcB, which control the synthesis of most of the oxygen-regulated genes in *Escherichia coli* and other Gram-negative bacteria (25, 26, 27, 28, 29). Thus, the response of leukotoxin to aerobic versus anaerobic conditions appears to use a

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TABLE 1 Oligonucleotides in the leukotoxin region used to construct and analyze promoter mutations

Primer name	Sequence	Position in the <i>ltx</i> operon (nt) ^a	Orientation ^b	Restriction site ^c
LKT467	5'-GAGCTCTTACAGATCAAAACCT-3'	-163	For	SacI
LKT483	5'-CTGCAGTATTAACCCTAAGAAAAAGG-3'	+47	For	PstI
LKT484	5'-CTGCAGCCTTAGCTCTATCTGCAAC-3'	+931	RC	PstI
LKT485b	5'-AAAGATAACGAAGCGGAAGTGAT-3'	-319	For	None
LKT486c	5'-CAATCCCTAACTTCTGTGCTGCT-3'	+1081	RC	None
LKT526	5'-GAGCTCCGGTAATGAAAATTGCC-3'	-210	For	SacI
LKT527	5'-GAGCTCTACAATACGGGATTGCG-3'	-108	For	SacI
LKT528	5'-GAGCTCATCAAAAACTAATAAT-3'	-78	For	SacI
LKT529	5'-GAGCTCTTCTATTGACTATTAAAG-3'	-40	For	SacI
LKT530	5'-CCCGGGGCCATAATCTATTCTCC-3'	+860	RC	XmaI
LKT531	5'-TCCTTAGCTCTATCTGC-3'	+932	RC	None
LKT548	5'-GAGCTCTAATAATTTTATGAAAT-3'	-68	For	SacI
LKT549	5'-GAGCTCATGAAATTAATAATTT-3'	-58	For	SacI
LKT550	5'-GAGCTCATAATTTTCTATTGAC-3'	-48	For	SacI
LKT768	5'-CTGCAGATAACCTTTGACCGGA-3'	+44	RC	PstI

^a The nucleotide (nt) position indicated is for the 5' end of each primer. The leukotoxin transcription start site (17) is defined as +1. The 5' noncoding region goes from +1 to +336. Bases +337 to +840 encode the LtxC protein, and the open reading frame for LtxA goes from +856 to +4020.

^b Orientation of the oligonucleotide, where a forward (For) sequence is in the same orientation as the genes in the operon, and a sequence that is in the reverse complement orientation is indicated by RC.

^c Added to the 5' end.

novel, unidentified regulatory pathway in *A. actinomycetemcomitans*. Finally, carbon source is a regulator of leukotoxin synthesis. In *A. actinomycetemcomitans*, leukotoxin protein and RNA levels decrease when cells are grown in high fructose (30, 31), with a corresponding drop in the levels of cyclic AMP (cAMP) (31). This led Inoue et al. (30) to speculate that the classic carbon catabolite repression system, including the global regulatory protein cAMP receptor protein (CRP) (32), is involved in regulating leukotoxin production in *A. actinomycetemcomitans*. Indeed, deletion of the *A. actinomycetemcomitans* *crp* gene results in a significant reduction in leukotoxin RNA expression, indicating that CRP is a transcriptional activator of leukotoxin (33). However, *in silico* analysis indicates that CRP must be acting through another protein to modulate leukotoxin transcription; the leukotoxin promoter does not have a good match (30, 33) to the consensus DNA sequence for CRP-specific binding, as defined in *E. coli* (34, 35, 36), even though 65 other CRP-regulated operons in *A. actinomycetemcomitans* are predicted to have CRP binding sites (33). Thus, the identity of the intermediary between CRP and the leukotoxin promoter is unknown. Only one transcription factor has been shown to bind to the leukotoxin promoter, integration host factor (IHF). This small heterodimeric protein, which is a global regulator in *E. coli* (37), binds to the -50 to -77 region of the *A. actinomycetemcomitans* leukotoxin promoter. The phenotype of an *ihf* deletion mutant in *A. actinomycetemcomitans* showed that IHF is a repressor of leukotoxin expression although leukotoxin synthesis is still modulated by aerobic versus anaerobic growth (38).

Although two transcription factors, IHF and CRP, have been shown to play a role in regulating leukotoxin RNA synthesis in *A. actinomycetemcomitans*, there are still a lot of unanswered questions about the mechanisms of this regulation. What protein interacts with the leukotoxin promoter to function as the intermediary for CRP modulation of leukotoxin synthesis? What is the mechanism of aerobic/anaerobic regulation of leukotoxin transcription? How do the IHF and CRP regulatory pathways interact with each other to accurately control leukotoxin RNA levels? Ad-

ditional studies into the mechanisms of transcriptional regulation of the leukotoxin (*ltxA*) promoter are reported here. Examination of *ltxA* RNA expression in defined promoter mutants identifies the region between -69 and -35 as an important control region in the leukotoxin promoter and indicates that an activator protein plays a major role in modulating leukotoxin transcription. We show genetically and biochemically that this direct activator of leukotoxin transcription is Mlc and that CRP works through Mlc to modulate leukotoxin synthesis. Our results demonstrate that IHF, which represses *ltxA* transcription, functions mainly by blocking the binding of Mlc to the leukotoxin promoter. Finally, we show that a $\Delta ihf \Delta mlc$ mutant still has higher *ltxA* RNA levels in anaerobic than aerobic cells, indicating that additional factors also play a role in the regulation of leukotoxin transcription. All of these regulatory mechanisms are combined into a model for the coordinated regulation of leukotoxin transcription.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. actinomycetemcomitans* clone JP2, which was originally isolated from a young patient with periodontitis and which expresses high levels of LtxA because it contains a 528-bp deletion in the 5' noncoding region of the leukotoxin operon (17), was the parental strain in this study. The cells were grown anaerobically (in 5% CO₂, 10% H₂, and 85% N₂) in a Coy chamber (Coy Laboratory Products, Ann Arbor, MI) at 37°C or aerobically (in 5% CO₂ and 95% air) in a CO₂ incubator at 37°C. The growth medium was TSBYE (3% tryptic soy broth plus 0.6% yeast extract) containing, when needed to select for transformants, spectinomycin (Spec) to a final concentration of 100 µg/ml or ampicillin to a final concentration of 10 µg/µl. In experiments where leukotoxin protein levels were evaluated, 300 µM FeCl₃ was included in the medium to suppress leukotoxin secretion (39).

Constructing strains with leukotoxin promoter deletions in the chromosome. To introduce leukotoxin promoter deletions into the *A. actinomycetemcomitans* chromosome, seven PCR products, containing differing lengths of the leukotoxin promoter (primers LKT526 to LKT529 and LKT548 to LKT550) (Table 1) and the first 860 bp of the leukotoxin operon (primer LKT530) were synthesized. These PCR products were each cloned into the SacI/XmaI sites of pDK919, which is pUC19 (40)

TABLE 2 Vector oligonucleotides used to analyze promoter mutations

Name	Sequence	Description
LKT66	5'-GGGTTTCCCAGTCACGAC-3'	Forward sequencing primer for pUC-based vectors
LKT197a	5'-CAGGAAACAGCTATGAC-3'	Reverse sequencing primer for pUC-based vectors
Spc3	5'-CTTGACTTTTATGTCGTCGTATCTG-3'	These two primers are in opposite orientations, 140 bp apart in the <i>Spec^r</i> gene
Spc4	5'-ACGAATCCATAATGGCTCTTCTC-3'	

with a spectinomycin resistance gene in the BamHI site. The deletion plasmid DNAs were electroporated individually into JP2 to produce stable spectinomycin-resistant (*Spec^r*) transformants. In each transformant, the plasmid should have recombined into the leukotoxin locus such that the promoter deletion was directing transcription of the leukotoxin operon. This recombination event was confirmed by PCR using primers LKT66 and LKT531 (Tables 1 and 2).

Constructing strains with BamHI linker mutations in the chromosomal leukotoxin promoter. DNAs from plasmids pDK759 to pDK766, which contain BamHI linker mutations at sequential positions in the leukotoxin promoter (24), were used as DNA templates in PCRs with primers LKT467 and LKT768. Each of the 205-bp PCR products, which extend from -163 to +44 in the leukotoxin promoter region, was cloned into the SacI/PstI sites of pDK810. The pDK810 vector consists of the spectinomycin-resistant plasmid pUS19 (41) with the counterselectable marker *SacB*

(42) cloned into the vector *NdeI* site. An 884-bp PCR product, generated from primers LKT483 and LKT484 and containing the region from +47 to +931 of the leukotoxin operon, was then cloned in the unique PstI site in each of these intermediate plasmids. Plasmids with the correct orientation of the 884-bp insert, relative to the BamHI linker regions, were identified by diagnostic PCR with primers LKT467 and LKT484. Eight plasmids, named pCC759 to pCC766, were generated. Each plasmid (Fig. 1A) contains 1,089 bp from the leukotoxin operon (-163 to +931), including a different BamHI linker mutation in each region from -20 to -85 and a TA-to-GC change at positions +46/+47 in the 5' noncoding region of the leukotoxin locus (from the PstI ends used for cloning).

Plasmids pCC759 to pCC766 were introduced into *A. actinomycetemcomitans* strain JP2 by electroporation (43), and stable spectinomycin-resistant transformants were selected. To check that the recombination occurred correctly, PCR analysis with two sets of primers (Fig. 1B) was

FIG 1 Constructing strains with BamHI linker mutations in the leukotoxin promoter. (A) Schematic of a typical plasmid with a BamHI mutation in the leukotoxin promoter and the possible recombination events when it is transformed into *A. actinomycetemcomitans*. *ltxC* is the first gene in the four gene leukotoxin operon; *ltxA'* indicates a partial *ltxA* coding region. (B) Possible recombination results when a BamHI linker mutation plasmid recombines into the JP2 chromosome. In one case (1) the normal parental promoter is still driving synthesis of the full leukotoxin operon, but in the other recombinant (2) the expression of the leukotoxin operon is being controlled by the BamHI mutant promoter. The dashed lines indicate the leukotoxin region DNA introduced by the plasmid recombination events. The positions of the primers used in PCR to confirm the accuracy of the constructs are marked by arrows below recombination event 2. (C) PCR analysis was done on DNA from transformants of JP2 with the various BamHI linker plasmids to identify those in which the BamHI mutation was controlling the leukotoxin operon. Two sets of primers were used. LKT486C and LKT66 amplify a 1.3-kb region from within the vector outward into the *ltxA* gene. The second set of primers, LKT485B and LKT197a, amplify a region from the gene (*glyA*) just upstream of leukotoxin into the plasmid vector (panel B). These PCR products were then digested with BamHI. A typical result, with DNA from recombinant AAM766 is shown. Lanes 1 and 2 were amplified with primers LKT66 and LKT486C. Lanes 3 and 4 were amplified with primers LKT485B and LKT197a. After amplification, half of each sample was digested with BamHI as indicated.

done on chromosomal DNA from each transformant. The correct recombinants, in which different BamHI mutant promoters are driving leukotoxin synthesis, were designated AAM759 to AAM766.

To generate revertants of the BamHI promoter mutants, strains AAM759 to AAM766 were passaged in TSBYE broth cultures without spectinomycin, and then dilutions were plated onto 10% sucrose-TSBYE plates. Individual sucrose-resistant colonies were spotted onto plates with and without spectinomycin. To confirm that spectinomycin-sensitive cells had lost the plasmid, PCR using primers Spc3 and Spc4 (Table 2) internal to the vector was done. To determine whether a given BamHI linker mutation or the parental sequence remained at the leukotoxin promoter after a plasmid recombined out, additional PCR analyses were done with primers LKT485B and LKT486C, which flank the leukotoxin promoter. Half of each PCR product was then incubated with BamHI; PCR products from strains in which the parental-type promoter was left behind were BamHI insensitive, whereas BamHI-sensitive PCR products were those from cells that had the BamHI mutation in the leukotoxin promoter. The former types of cells were named AAM759R to AAM766R, and the latter were named AAM759B to AAM766B.

Single deletion strains of IHF, CRP, and Mlc. In order to construct various double mutants we first had to make Δihf and Δcrp deletion strains that were not spectinomycin resistant so that a *Spec^r* gene could be used to introduce the Δmlc mutation. To construct an unmarked Δcrp strain, standard recombinant DNA techniques and PCR were used to construct plasmid pDK945 in which 0.98 kb and 0.94 kb of DNA from the regions immediately upstream and downstream of the *crp* gene are cloned together in their relative chromosomal orientations into the multiple cloning site of vector pDK810 (carrying the *Spec^r* gene and *SacB*, described above). This construct could synthesize, at most, the first three and last four amino acids of the 206-amino-acid (aa) Crp protein. The Δcrp plasmid was electroporated into *A. actinomycetemcomitans* strain JP2, and a two-step allelic replacement strategy (24) was used to generate strains in which the *crp* deletion, instead of the wild-type *crp* gene, was present in the chromosome with no vector sequences. PCR with primers flanking the *crp* gene was used to confirm the genotype, and isolate AAM945 was used in subsequent experiments.

To construct an unmarked Δihf mutant in *A. actinomycetemcomitans*, the same two-step allelic replacement strategy was followed with plasmid pDK946. In this plasmid, the upstream (0.81 kb) and downstream (0.9 kb) regions flanking the *ihf* β gene were cloned together into the multiple cloning site of vector pDK810. This construct could synthesize, at most, the first three and last nine amino acids of the 94-aa IHF β protein. PCR confirmed that the *ihf* β gene was deleted in the *Spec^r* isolate AAM946.

The *Spec^r* Δmlc mutant in *A. actinomycetemcomitans* was made with plasmid pDK940, in which 0.98 kb of the DNA immediately upstream of *mlc* and 0.83 kb of the DNA immediately downstream of *mlc* flank a 0.98-kb spectinomycin gene (44) in place of amino acids 9 through 392 of the *mlc* coding region. After pDK940 was used in the two-step allelic replacement protocol, PCR was used to confirm that the genomic *mlc⁺* gene had been replaced by the *Spec^r* gene deletion allele in isolate AAM155.

Construction of the double knockout Δcrp Δmlc and Δihf Δmlc strains. To construct the Δihf Δmlc double mutant, the *Spec^r* Δihf β mutant AAM946 was electroporated with the *Spec^r* Δmlc plasmid pDK940. This plasmid was also electroporated into the *Spec^r* Δcrp strain AAM945 to construct a Δcrp Δmlc mutant. In both cases, the two-step allelic replacement strategy (24) was used to isolate strains in which a *Spec^r* gene had replaced the *mlc* gene. The genotype of each recombinant was confirmed by PCR with primers flanking the gene of interest. The strains used in subsequent studies were named AAM948 (Δihf Δmlc) and AAM949 (Δcrp Δmlc).

Complementation of deletion mutants with Mlc⁺ on a plasmid. To construct the Mlc⁺ complementation plasmid pDK941, a DNA segment that contained a 326-bp region immediately upstream of the *mlc* gene, the

entire *mlc* gene, and 31 bp of DNA from immediately downstream of the *mlc* gene was cloned into the EcoRI and SalI sites of the shuttle vector pMMB67EH (45). Plasmid pDK941 DNA was electroporated into the Δmlc strain AAM155 and into AAM167, a Δcrp deletion mutant (33), and ampicillin-resistant colonies were selected. One of each of the resulting ampicillin-resistant isolates was used in subsequent experiments.

Making recombinant *A. actinomycetemcomitans* Mlc protein and using it in gel mobility shift assays. Standard cloning techniques and PCR were used to construct an Mlc expression plasmid where the entire *mlc* gene, except for the last 9 bp, was cloned into the pET-30a(+) vector (Novagen, Madison, WI). This plasmid produces a recombinant version of Mlc in which its three carboxy-terminal amino acids are replaced with 22 vector-encoded amino acids including a six-histidine (His) tail. The insert was confirmed by sequencing. It contains one amino acid change relative to the sequence from *A. actinomycetemcomitans* strain HK1651: a phenylalanine-to-leucine change at amino acid 229 which is distant from the helix-turn-helix DNA binding domain (46). The pET-30a Mlc expression plasmid, as well as vector alone as a control, were individually transformed into *E. coli* strain BL21(DE3), and protein overexpression was induced in the resulting transformants by growing cells overnight at 30°C in LB medium plus kanamycin (final concentration of 30 μ g/ml) and isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM). His-tagged Mlc protein was then purified from the *E. coli* extracts using a Ni-NTA Fast Start kit (Qiagen, Valencia, CA) following the manufacturer's nondenaturing protocol. Protein that bound to the Ni-nitrilotriacetic acid (NTA) column was eluted with native elution buffer (Qiagen, Valencia, CA), diluted 1:5 into buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 250 mM KCl, 5 mM EDTA, 40% glycerol) containing bovine serum albumin (BSA; 50 μ g/ml), and frozen in aliquots at -20°C . The presence of recombinant Mlc was assessed in various protein samples by SDS-gel electrophoresis and staining with Coomassie brilliant blue and/or by Western blotting with anti-His tag antibody. Protein concentrations were measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA).

A gel purified 240-bp PCR product, spanning the region from -164 to $+67$ of the leukotoxin promoter with primer-encoded KpnI and EcoRI ends, was used as the binding substrate in the gel mobility shift assays. The DNA was labeled by end-filling with Klenow polymerase and [α - ^{32}P]dATP as previously described (38). For the competition binding experiment, the specific competitor DNAs were unlabeled, gel-purified PCR products containing various segments of the leukotoxin promoter. Gel mobility shift assays were done in 20 μ l (final volume) of binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 8% glycerol) containing labeled probe DNA at a final concentration of 1.2 nM. Unlabeled competitor DNAs were also included in the competition binding experiment reaction mixtures. The reaction was started by the addition of protein, incubated for 30 min at 25°C, and electrophoresed on a low-ionic strength (45 mM Tris, pH 8.0, 45 mM boric acid, 2 mM EDTA) polyacrylamide gel.

Evaluating levels of leukotoxin protein. Bacterial samples were grown to log phase (optical density at 600 nm [OD₆₀₀] of ~ 0.4) or overnight to stationary phase, as indicated in each figure legend, and 1-ml aliquots were taken for total protein analysis. The cell cultures were centrifuged to pellet the cells, and the supernatant was removed. Each cell pellet was resuspended with 1 $\times$ SDS-PAGE loading buffer, boiled for 3 to 4 min, and then centrifuged again (4 min) to pellet cellular debris. Aliquots from each sample were electrophoresed on SDS-polyacrylamide gels, and the proteins were then visualized by staining with Coomassie brilliant blue. Western blot analysis of leukotoxin expression was done with rabbit polyclonal antileukotoxin antibody as described previously (33) except that LumiGLO reagent (Cell Signaling Technologies, Danvers, MA) and autoradiography were sometimes used to detect the horseradish peroxidase on the secondary antibody.

RNA isolation and real-time reverse transcriptase PCR (RT-PCR). RNA was isolated from log-phase *A. actinomycetemcomitans* cells using an RNeasy Mini Kit (Qiagen). The manufacturer's instructions were em-

FIG 2 Chromosomal deletions define the region between -69 and -35 as an important control region in the leukotoxin promoter. Plasmids with the deletions indicated in the region upstream of the leukotoxin operon (diagrammed at the top of the figure) were transformed into *A. actinomycetemcomitans* strain JP2. Recombination into the host chromosome resulted in a set of strains ($\Delta 526$ to $\Delta 529$) with different segments deleted (missing line) immediately upstream of the leukotoxin operon. The transcription start site for the leukotoxin operon is shown as are key promoter elements (17). For data in the graph shown, each sample measured was grown anaerobically to log phase, RNA was isolated, and leukotoxin RNA levels were measured by quantitative RT-PCR. The amount of *ltxA* mRNA in each sample was normalized to the level of *pdxY* mRNA in the same sample, and then the *ltxA* mRNA level in each mutant was compared to that of the parental JP2 cells.

played with the following notes: 2.5 ml of cells was lysed, in the presence of RNAProtect Bacteria Reagent (Qiagen), with 10 to 15 mg/ml lysozyme (Sigma) for 10 to 15 min at room temperature. DNA was removed with an RNase-free DNase I set from Qiagen, except that twice the recommended amount of DNase was used. Each RNA sample (400 ng) was made into cDNA using random hexamer primers (Qiagen) and an Omniscript Reverse Transcriptase kit (Qiagen). RNase inhibitor (10 units; Applied Biosystems) was included in each reaction mixture.

Real-time PCR was performed on dilutions (1/1,600) of the cDNA samples as detailed in Kolodrubetz et al. (38). Duplicate sample sets were used, on the same PCR plate, with primers LKT516 (5'-CGAGGGAAGGTTACCGATCTAC-3') and LKT517 (5'-TGCTGCCGATAATTTCTCTAC-3'), which amplify a 119-bp region in the middle of the *ltxA* gene (not included in any plasmid constructs), and primers LKT518 (5'-CGTTGGCGTGGGTGATTAAAC-3') and LKT519 (5'-CAAAAGCCTCCACATCGGAT-3'), which amplify a 77-bp region of the *pdxY* gene. We have found the latter gene to be constitutively expressed at the same level in multiple microarrays and real-time PCR assays from cells grown under a variety of conditions. All reactions were performed in duplicate on a Chromo4 Real Time PCR Detection System (Bio-Rad). A dilution series of the control reaction (JP2 without O_2) was done on each plate to assess the efficiency of the reaction with each primer pair. For each sample, the levels of *ltxA* mRNA were normalized to the levels of *pdxY* mRNA by calculating the difference in the averaged *ltxA* and *pdxY* threshold cycles (C_T) for that sample.

Statistical analyses. In experiments with only two samples, a Student *t* test was used to evaluate statistical significance. In the real-time RT-PCR experiments with multiple samples, the ratios of *ltxA* to *pdxY* mRNA levels from individual samples were first log 2 transformed (47), and then multiple pairwise comparisons were done with a mixed-model analysis of variance (ANOVA) (PROC MIXED in SAS, version 9.3) using the Tukey-Kramer method for determination of statistical significance.

RESULTS

Chromosomal deletions define the region between -69 and -35 as an important control region in the leukotoxin promoter. Previous studies of leukotoxin promoter mutations from two labs

reached somewhat different conclusions about the regulatory elements involved in controlling transcription of the leukotoxin operon (24, 48). This disparity could be due to the use, in both cases, of reporter genes on plasmids, which might not accurately reflect the chromosomal environment of the leukotoxin operon. To eliminate this as a possible confounding factor, a series of strains with various lengths of deletions in the chromosome upstream of the leukotoxin operon were constructed (Fig. 2). Analysis of leukotoxin (*ltxA*) RNA levels in these strains using quantitative reverse transcriptase PCR (RT-PCR) showed that there were no regulatory elements upstream of -69 (Fig. 2). Importantly, the levels of *ltxA* mRNA in deletion strains $\Delta 526$, $\Delta 527$, $\Delta 528$, and $\Delta 548$ were the same as those in the parental strain, JP2, which proves that the presence of the vector DNA in the chromosome does not alter leukotoxin expression. However, deletion strains $\Delta 549$, $\Delta 550$, and $\Delta 529$ all had dramatically lower levels of *ltxA* mRNA, indicating that there is a positive-control element in, at a minimum, the -69 to -58 segment of the leukotoxin operon regulatory region although this positive-control element is likely to extend further toward the -35 as well.

BamHI linker mutations define key regulatory element(s) within the -69 to -35 region of leukotoxin promoter. To identify additional leukotoxin promoter regulatory elements that may have been missed because of the nature of promoter deletion experiments, a BamHI linker scanning analysis was done in the chromosome. As an initial screen of the BamHI linker mutants' phenotypes, the level of leukotoxin protein production in each mutant was evaluated by SDS-PAGE. Although samples AAM759 and AAM760 had LtxA levels similar to those in the parental strain (JP2), the other six strains that contained mutations within the -71 to -30 region, AAM761 through AAM766, all showed decreased amounts of leukotoxin protein (Fig. 3A and data not shown). This is consistent with the chromosomal deletion results, which showed reduced leukotoxin synthesis only in deletions

FIG 3 The phenotype of the BamHI linker mutations is not altered by vector DNA in the chromosome. (A) Cells from the parent strain (JP2) or the mutants indicated were grown anaerobically, and protein from log phase cells was analyzed by SDS-PAGE and Coomassie blue staining. Strains AAM760, AAM761, and AAM764 have a BamHI mutation driving synthesis of leukotoxin and also contain vector sequences upstream of the leukotoxin operon. Strains AAM760B, AAM761B, and AAM764B also have a BamHI promoter controlling leukotoxin synthesis but without any vector DNA in the chromosome. Strains AAM760R, AAM761R, and AAM764R are revertants which have lost their BamHI promoter mutant as a consequence of the plasmid recombining out of AAM760, AAM761, and AAM764, respectively. (B) Position and base changes in the indicated BamHI promoter mutants. The numbers in parenthesis indicate the proportion of bases changed in a given mutant.

closer to the transcription start site than -69 . To eliminate the possibility that the vector sequences in the chromosome upstream of the leukotoxin promoter were somehow influencing the BamHI linker phenotype, strains were isolated in which the vector recombined out of the chromosome, leaving only the BamHI mutation in the leukotoxin promoter (strains designated in the form AAMxxxB). Leukotoxin protein expression in these strains was similar to that seen in the corresponding strains with the vector next to the BamHI linker mutations (Fig. 3A, strains AAM760 versus AAM760B, AAM761 versus AAM761B, and AAM764 versus AAM764B; also data not shown), proving that the presence of the vector in the chromosome upstream of the leukotoxin promoter has no effect on leukotoxin expression. Finally, on occasion, JP2 can spontaneously lose leukotoxin expression when the strain is passaged continuously *in vitro* (A. Burgum and D. Koldrubetz, unpublished observation). To rule out this possibility for mutants AAM761 to AAM766, revertants were isolated. These are strains in which the vector used to construct strains AAM761 to AAM766 was allowed to recombine out of the chromosome, leaving behind a parental-type promoter. In all of the revertants, leukotoxin protein synthesis was recovered (Fig. 3A, strains AAM760R, AAM761R, and AAM764R; also data not shown), indicating that the leukotoxin phenotypes observed were due to the BamHI mutations and not due to the spontaneous loss of leukotoxin synthesis. Thus, the BamHI mutations in the -71 and -35 parts of the leukotoxin promoter all appear to alter a positive regulatory element, presumably involved in transcriptional regulation.

To show that leukotoxin mRNA levels are affected, quantitative RT-PCR was done with RNA from the BamHI mutation strains grown anaerobically and aerobically (Fig. 4). The changes in leukotoxin mRNA levels seen in the BamHI mutants mirrored the changes seen at the protein level, indicating that the BamHI promoter mutants affected leukotoxin transcription. As seen in

the protein gels, mutants AAM759 and AAM760 had, on average (Fig. 4A and B), essentially the same amount of leukotoxin mRNA as parental strain JP2. Mutant AAM761, centered at -67 , has a drastic (>8 -fold) and significant ($P < 0.04$ relative to JP2) reduction of the *ltxA* transcript, establishing the segment from -71 to -65 as a key positive regulatory element. Strains AAM762 through AAM765 also appear to have decreased *ltxA* mRNA levels, relative to JP2, but the changes are more moderate than those seen with AAM761, and they do not reach the threshold of statistical significance (all P values are >0.05). However, in combination with the protein results, we conclude that strains AAM762 to AAM765 with mutations in the -64 to -38 region also alter a positive-control element in the leukotoxin promoter. Finally, the AAM766 mutant had very low levels of *ltxA* mRNA, as expected for a strain which changes the promoter's -35 sequence.

To identify the DNA sequences involved in the anaerobic/aerobic regulation of leukotoxin expression, the ratio of leukotoxin RNA in anaerobic versus aerobic cells for each mutant was calculated (Fig. 4C). Surprisingly, all of the mutants, except the AAM766 mutant at -35 , still had at least 5-fold higher levels of *ltxA* mRNA when grown anaerobically than when grown aerobically. These results suggest either that the -70 to -38 region of the leukotoxin promoter is not involved in controlling the anaerobic induction response or that the BamHI mutants in that region could be decreasing, but not totally inhibiting, the binding of an oxygen-responsive activator protein to the positive-control element in the leukotoxin promoter. These possibilities, among others, might be resolved by identifying and characterizing this activator protein.

Mlc is an activator of leukotoxin transcription that binds to the -68 to -40 region of the leukotoxin promoter. Two transcription factors that regulate leukotoxin synthesis have been identified previously; IHF represses leukotoxin transcription by binding to the leukotoxin promoter (38), and CRP activates *ltxA*

A. Anaerobic **B. Aerobic** **C. Anaerobic/Aerobic Ratio**

FIG 4 Quantitative RT-PCR of BamHI mutations define key regulatory elements within the leukotoxin promoter. Each of the indicated strains was grown anaerobically (A) or aerobically (B) to log phase, total RNA was isolated, and cDNA was prepared with random hexamer primers and then used in quantitative RT-PCR with *ltxA*- and *pdxY*-specific primers. The levels of *ltxA* mRNA in a single sample were normalized to *pdxY* (internal control) levels in the same sample. The value for a given strain is the average of the ratios of the *ltxA* mRNA to *pdxY* mRNA levels from three biological replicates (samples) of that strain. The error bars are the sample standard deviation. A single asterisk indicates a sample for which the ratio of *ltxA* mRNA to *pdxY* mRNA is statistically significantly ($P < 0.04$) lower than the value in anaerobically grown JP2 cells. The double asterisks mark samples for which the ratio of *ltxA* mRNA to *pdxY* mRNA is statistically significantly ($P < 0.04$) lower than the value from aerobically grown cells of JP2, AAM759, AAM760, AAM763, AAM764, and AAM765. (C) The anaerobic ($-O_2$) values for at least three biological replicates of a given strain from panel A were averaged and then expressed as a ratio relative to the averaged aerobic ($+O_2$) values from at least three biological replicates of the same strain from panel B.

transcription (33). However, the effect of CRP appears to be indirect since there is no apparent CRP binding site in the leukotoxin promoter. This suggests that a CRP-regulated transcription factor may exist that is an intermediate in the regulation of leukotoxin mRNA synthesis. In fact, whole-genome microarray analyses using RNA from parental JP2 and *crp* mutant cells show that 11 putative transcription factors are CRP regulated in *A. actinomycetemcomitans* (33). To investigate the possibility that one of these CRP-regulated transcription factors is part of the *ltxA* transcriptional activation pathway identified by the BamHI linker analysis

above, 11 deletion mutants in *A. actinomycetemcomitans* strain JP2 or ATCC 29523L were screened for possible effects on leukotoxin expression. These mutants are part of a genomic deletion collection our lab has generated (L. Phillips, S. Bryant, A. Burgum, and D. Kolodrubetz, unpublished data) in which a number (five, on average) of adjacent genes are deleted in each strain. Wild-type and mutant cells were grown anaerobically and aerobically, and whole-cell lysates were analyzed by SDS-PAGE for leukotoxin production. The results showed that only mutant AAL284 significantly altered leukotoxin expression relative to wild-type cells (data not shown); the AAL284 mutant has dramatically reduced levels of leukotoxin protein. This deletion mutant is missing four genes, *ydeW*, *xylB*, *bioD1*, and *mlc*, but only the *mlc* gene is CRP regulated and encodes a homologue of a known *E. coli* transcriptional regulator (49). Overall, the *E. coli* and *A. actinomycetemcomitans* Mlc protein sequences are 26% identical.

To prove that *mlc*, and not one of the other three genes deleted in strain AAL284, plays a role in leukotoxin regulation, the phenotype of the *mlc* deletion mutant AAM155 was examined. Analysis of protein expression in anaerobically grown cells of AAM155 demonstrates that *mlc* is required for maximal expression of leukotoxin (Fig. 5). Importantly, this effect is due to deletion of *mlc* and not to a polar effect on the *bioD1* gene downstream of *mlc* since a plasmid containing only the wild-type *mlc* gene complements the AAM155 mutant (Fig. 5). Finally, to see if activation of leukotoxin expression by Mlc was occurring at the level of transcription, RNA was isolated from JP2 and *mlc* mutant cells grown anaerobically and analyzed by quantitative RT-PCR. Consistent with the protein results, the *mlc* mutant had a significant reduction (~5-fold) in *ltxA* RNA levels (Fig. 5C). This indicates that Mlc is an activator of leukotoxin RNA expression, consistent with the possibility that it is the protein that interacts with the positive regulatory element identified by the BamHI mutations in the leukotoxin promoter region.

If Mlc enhances leukotoxin transcription directly, there should be an Mlc binding sequence in the *ltx* operon's promoter. The *A. actinomycetemcomitans* Mlc protein shares 56% amino acid sequence identity with the helix-turn-helix and linker domains, including key arginine and proline residues (50) that determine the DNA binding specificity of the *E. coli* Mlc protein. This suggests that the *A. actinomycetemcomitans* Mlc could bind to the same DNA sequence to which the *E. coli* protein binds. Thus, the matrix-scan software at the Regulatory Sequence Analysis Tools (RSAT) web site (51) was used to look for matches to the *E. coli* Mlc consensus binding site ($W_5TTN_9AAW_5$) (52) in the *A. actinomycetemcomitans* leukotoxin promoter region. A significant match, which included the absolutely conserved TT/AA bases, was found at position -65 to -43 , which is within the -69 to -38 region identified as a positive-control element of leukotoxin transcription in the BamHI promoter mutation experiments. To prove that Mlc binds specifically to this DNA segment, gel mobility shift assays were performed. *A. actinomycetemcomitans* Mlc, with a carboxy terminus polyhistidine tag, was expressed in *E. coli* and partially purified by nickel column chromatography (Fig. 6A). As little as $0.06 \mu\text{g}$ (62 nM final concentration) of this protein fraction bound to a DNA fragment containing 232 bp of the leukotoxin promoter region, whereas $1.0 \mu\text{g}$ of protein purified from *E. coli* cells with the expression vector alone did not show a similarly sized protein-DNA complex (Fig. 6B). These data show that we are observing binding by the *A. actinomycetemcomitans*

FIG 5 An *mlc* mutant makes less leukotoxin protein and RNA. Samples were taken at log phase from aerobically (+) and anaerobically (–) grown cultures of the parent strain (JP2) and Δ*mlc* (AAM155) cells as well as the deletion strain with a plasmid carrying *mlc*⁺ (the Δ*mlc*/*mlc*⁺ strain). Cells were harvested by centrifugation, resuspended in SDS-PAGE loading buffer, boiled, and centrifuged, and the soluble material was electrophoresed on duplicate gels. (A) Gel stained with Coomassie brilliant blue. (B) Western blot analysis of total cell protein from the indicated strains using rabbit antileukotoxin antibody as the primary antibody. The arrows mark the position of leukotoxin. The numbers show the positions of molecular size markers (not shown) in kDa. (C) For each sample, total cell RNA was prepared from anaerobically grown log phase cells, and cDNA was synthesized with random hexamer primers and then used in quantitative RT-PCR. The level of *ltxA* mRNA in each sample is normalized to the level of *pdxY* mRNA in the same sample. The data are the average of the expression ratios from three biological replicates. The error bars are standard error, and the difference between the two samples is statistically significant ($P \leq 0.05$, by Student's *t* test).

Mlc protein. To delimit the sequence to which the *A. actinomycetemcomitans* Mlc binds, four DNAs containing different segments of the leukotoxin promoter were used as binding competitors in a gel shift assay. The two DNAs containing the –68 and –40 regions of the leukotoxin promoter competed significantly better for Mlc binding than the two DNAs lacking the –68 to –40 sequences (Fig. 7). Thus, *A. actinomycetemcomitans* Mlc is binding specifically to the leukotoxin promoter in the region containing the match, at –65 to –43, to the consensus sequence for *E. coli* Mlc binding. Taken together, all of these results indicate that Mlc binds to the leukotoxin promoter upstream of the –35/–10 promoter element to activate *ltxA* transcription.

Maximal leukotoxin transcription occurs mainly through an Mlc-dependent CRP activation pathway. The facts that (i) there is a 9/10-bp match to the consensus CRP binding site 96 bp upstream of the *A. actinomycetemcomitans* *mlc* gene (33), (ii) *mlc* mRNA levels are lower in a Δ*crp* mutant in microarray analyses (33) and in real-time RT-PCR ($P < 0.005$) (L. Phillips and D. Kolodrubetz, unpublished data), and (iii) there is no match to the

FIG 6 The *A. actinomycetemcomitans* Mlc protein binds to DNA containing the leukotoxin promoter region. (A) *E. coli* cells containing an expression plasmid with a His-tagged Mlc (Mlc-His plasmid) were grown at 30°C to mid-log phase, IPTG was added overnight to induce recombinant protein expression, and then His-tagged Mlc was purified from lysed cells on a nickel column. A transformant with the empty vector plasmid (Empty Vector) was grown, induced, and processed in parallel. Protein samples from various stages of purification were analyzed by SDS-gel electrophoresis and staining with Coomassie brilliant blue. Lanes 1 and 4, uninduced whole-cell lysates; lanes 2 and 5, IPTG-induced whole-cell lysates; lanes 3 and 6, protein eluted from a Ni-NTA column, used in gel shift experiments. The asterisk marks the His-tagged Mlc protein. The numbers on the left show the positions of molecular size markers (not shown) in kDa. (B) Increasing amounts of His-tagged Mlc (Mlc-His Plasmid) protein purified on a Ni-NTA column or of protein similarly purified from cells with the empty vector plasmid (Empty Vector) were used in a gel mobility shift assay. A reaction mixture with 0.25 μg of purified His-tagged Mlc protein corresponds to a final concentration of 250 nM Mlc. The labeled DNA (1.2 nM final concentration) in the reaction mixtures was a 240-bp PCR product, spanning –164 to +67 of the leukotoxin promoter. After a 30-min incubation at 25°C, the reaction products were electrophoresed on a low-ionic strength polyacrylamide gel, and the gel was then exposed to film.

consensus CRP binding site in the *ltx* promoter region (30, 33) suggest that CRP activates leukotoxin transcription by increasing Mlc levels. If this is so, then having an extra copy of a wild-type *mlc* gene in a Δ*crp* strain should lead to increased leukotoxin expression. To test this, a plasmid containing an *mlc*⁺ gene was transformed into a *crp* deletion mutant, and leukotoxin synthesis was measured. An extra *mlc*⁺ gene copy restored leukotoxin expression in a *crp* deletion mutant (Fig. 8), indicating that Mlc can indeed work downstream of *crp* to activate leukotoxin production. Although this result is consistent with the hypothesis that CRP acts through Mlc to regulate leukotoxin production, it does not exclude the possibility that CRP could also alter *ltxA* transcription through a separate, Mlc-independent pathway. The latter possibility was tested genetically by comparing *ltxA* mRNA levels in a Δ*crp* Δ*mlc* double mutant to those in a Δ*mlc* mutant. As expected, anaerobically grown Δ*crp* and Δ*crp* Δ*mlc* mutants each displayed a significant ($P \leq 0.007$) ~5-fold reduction in leukotoxin RNA compared to the JP2 parent (Fig. 9A). Importantly, the amount of *ltxA* message in the Δ*mlc* strain is not significantly higher than that in the Δ*crp* Δ*mlc* cells, indicating that CRP uses an Mlc-dependent pathway to activate leukotoxin synthesis under these anaerobic growth conditions. However, the results with the

FIG 7 The *A. actinomycetemcomitans* Mlc protein binds specifically to the -68 to -40 region of the leukotoxin promoter. (A) The positions of the competitor DNAs used in the competition gel shift assay are shown relative to key sequences in the leukotoxin promoter, including the match (boxed sequence) to the *E. coli* Mlc consensus binding site. (B) His-tagged Mlc protein (192 ng; which is 192 nM in the reaction mixture) purified on a Ni-NTA column was incubated with a 32 P-labeled 240-bp DNA fragment (1.2 nM final concentration) spanning -164 to $+67$ of the leukotoxin promoter and then subjected to nondenaturing gel electrophoresis. A set of parallel reactions was run using mixtures containing a 1-, 3-, 10-, or 30-fold molar excess of the unlabeled competitor DNAs indicated. No protein lane, no protein or unlabeled DNAs were added to the reaction mixture.

Δmlc mutant grown aerobically are somewhat different. Aerobically, the amount of *ltxA* mRNA in the Δmlc mutant is ~ 3 -fold higher than that seen in the $\Delta crp \Delta mlc$ strain. The difference between the two strains may not be statistically significant ($P \leq 0.08$), but when the *ltxA* mRNA values from another $\Delta crp \Delta mlc$ isolate grown and assayed at the same time (data not shown) are included, the difference does become statistically significant ($P \leq 0.03$). Thus, the CRP present in the Δmlc mutant appears to be

FIG 8 Mlc enhances leukotoxin production in a *crp* mutant. Samples were taken from overnight cultures (stationary phase) of anaerobically grown cells of the indicated strains. JP2/*mlc*⁺ is the parental strain with a plasmid carrying *mlc*⁺. The $\Delta crp/mlc$ ⁺ strain is the *crp* deletion strain with a plasmid carrying *mlc*⁺. The strains with empty vector are controls for possible indirect plasmid effects. Cells were harvested by centrifugation, resuspended in loading buffer, boiled, and centrifuged, and the soluble material was electrophoresed on duplicate gels. (A) Gel stained with Coomassie brilliant blue. (B) Western blot analysis of total cell protein from the indicated strains using rabbit antileukotoxin antibody as the primary antibody. The arrow marks the position of leukotoxin. The numbers show the positions of molecular size markers (not shown) in kDa.

FIG 9 The genetic interactions of IHF, CRP, and Mlc in altering leukotoxin transcription. After each sample was grown to log phase, total cell RNA was isolated, and cDNA was prepared with random hexamer primers and then used in quantitative RT-PCR. The level of *ltxA* mRNA in each sample is normalized to the level of *pdxY* mRNA in the same sample. The data are the average of the expression ratios from three biological replicates. The error bars are standard error. A single asterisk indicates samples for which the ratio of *ltxA* mRNA to *pdxY* mRNA is statistically significantly ($P < 0.007$) lower than the value in anaerobically grown JP2 cells. The double asterisks mark samples for which the ratio of *ltxA* mRNA to *pdxY* mRNA is statistically significantly ($P < 0.05$) lower than the value from the Δihf strain grown under the same conditions. The strains used are as follows: JP2, parental strain, Δcrp , strain AAM945; Δmlc , strain AAM155; $\Delta crp \Delta mlc$, strain AAM949; Δihf , strain AAM946; $\Delta ihf \Delta mlc$, strain AAM948.

able to partially activate leukotoxin RNA synthesis via an Mlc-independent pathway in aerobic cells but not in anaerobic cells.

IHF works mainly by blocking Mlc activation of leukotoxin transcription. IHF binds the leukotoxin promoter at -63 to -56 to repress *ltxA* transcription (38), and the results above show that Mlc activates leukotoxin synthesis by binding to the promoter at a site (-65 to -43) that overlaps the IHF binding site. Thus, there are several mechanisms by which IHF repression and Mlc activation could occur at the same promoter, including interfering with each other's binding and/or directly affecting RNA polymerase (RNAP) binding or activity (open complex formation or promoter clearance) at the leukotoxin promoter. To determine which of these possibilities is most likely occurring *in vivo*, a $\Delta ihf \Delta mlc$ double mutant was constructed and compared to the corresponding single mutant strains for the effects on *ltxA* mRNA levels. In anaerobic and aerobic cells, Mlc increases leukotoxin mRNA expression ~ 3 -fold ($P < 0.05$) in the absence of IHF (Fig. 9B, compare the $\Delta ihf \Delta mlc$ and Δihf strains with and without oxygen). This indicates that Mlc can directly stimulate RNAP binding or activity at this promoter. On the other hand, IHF does not inter-

fer directly with RNAP binding or activity at the *ltxA* promoter in aerobic cells since *ltxA* mRNA levels are the same in the $\Delta ihf \Delta mlc$ and Δmlc strains (Fig. 9B, with oxygen); however, IHF may interfere to some extent in cells grown anaerobically since the *ltxA* mRNA level in the $\Delta ihf \Delta mlc$ strain is 1.7-fold higher ($P > 0.1$) than that in the Δmlc strain (Fig. 9B, without oxygen). Mlc must compete effectively with IHF for binding to the leukotoxin promoter in anaerobic cells since Mlc increases *ltxA* transcription at least 3-fold ($P < 0.01$) even when IHF is present (Fig. 9B, Δmlc strain versus JP2, without oxygen). However, in aerobic cells, IHF must bind better than Mlc to the *ltxA* promoter since Mlc does not increase leukotoxin transcription when IHF is present (Fig. 9B, Δmlc strain versus JP2, with oxygen). Thus, the major function of IHF at the leukotoxin promoter under aerobic growth conditions, and to some extent in anaerobic cells, is to reduce transcriptional activation by precluding Mlc binding to the promoter DNA.

DISCUSSION

In order to define the mechanism of transcriptional regulation of leukotoxin, *ltxA* promoter mutations were introduced into the *A. actinomycetemcomitans* chromosome. Quantitative RT-PCR analysis of these deletion and substitution mutations showed that the -69 to -38 region of the promoter was required for maximal transcription of the leukotoxin operon. These results indicate that a transcriptional activator is involved in *ltxA* regulation. We have shown here that Mlc binds specifically to the -68 to -40 region of the leukotoxin promoter sequence and directly increases *ltxA* transcription. In a previous study, CRP was also shown to be an activator of leukotoxin transcription (33), but this protein was thought to be unlikely to be acting directly at the leukotoxin promoter since it does not contain a CRP binding site consensus sequence. However, the *mlc* promoter does have a CRP binding site, and *mlc* transcription is induced by CRP (33). Thus, we tested the possibility that Mlc is the intermediary between CRP and the leukotoxin promoter. Indeed, this appears to be the case since we show here that leukotoxin expression increases when a plasmid containing the *mlc*⁺ gene is transformed into a *crp* deletion mutant and since the Δcrp and $\Delta crp \Delta mlc$ mutants have the same low levels of *ltxA* mRNA. The five Mlc-regulated operons in *E. coli* are also modulated by CRP (53), but, in those cases, CRP binds to their promoters to directly activate their transcription. When glucose is being metabolized in *E. coli*, the transcription of the genes encoding the glucose phosphotransferase system (PTS) proteins increases due to direct activation by CRP and derepression of Mlc. It will be interesting to see if a similar type of direct coregulation by Mlc and CRP occurs at nonleukotoxin promoters in *A. actinomycetemcomitans*.

IHF and Mlc both bind to the *ltxA* promoter but with opposite effects on transcription; IHF represses leukotoxin synthesis (38) while Mlc is an activator. To dissect the regulatory hierarchy of IHF and Mlc at the leukotoxin promoter, a $\Delta ihf \Delta mlc$ mutant was made and assessed for its effect on leukotoxin transcription relative to the Δihf and Δmlc strains. Mlc appears to be able to enhance RNA polymerase (RNAP) activity directly since Mlc increases *ltxA* transcription at least 3-fold in the absence of IHF (the Δmlc strain versus the $\Delta ihf \Delta mlc$ strain). The step at which Mlc will activate RNAP (possibly at promoter binding, open complex formation, or promoter clearance) is unknown. What about IHF? In *E. coli*, IHF can directly repress RNAP function by several different mechanisms at a number of different promoters (54, 55, 56). However,

a direct effect on RNAP is not the major function of IHF at the *A. actinomycetemcomitans* leukotoxin promoter since removing IHF by deletion has minimal or no effect on *ltxA* RNA levels in the absence of Mlc. Instead, IHF most likely works mainly by interfering with Mlc's binding to the *ltxA* promoter since their binding sites overlap (Fig. 10A). Such a mechanism has been shown to be true, by *in vitro* binding experiments, for NarL/IHF and for OmpR/IHF at the *nrf* and *ompF* promoters in *E. coli* (57, 58). However, our data cannot rule out a mechanism in which IHF binds to the promoter at the same time as Mlc, presumably on different faces of the DNA helix, and interferes with Mlc's activation of RNAP.

Another goal of these studies was to identify transcription factors that modulate leukotoxin RNA levels in response to aerobic versus anaerobic growth. Thus, the observation that leukotoxin RNA expression was still repressed when cells deleted of both IHF and Mlc were grown aerobically was unexpected since they are the only two proteins that have been shown to bind directly to the leukotoxin promoter. This result indicates that the aerobic/anaerobic response at the *ltxA* promoter is not simply a matter of increasing or decreasing the levels or activity of IHF or Mlc in response to oxygen. Instead, either a third unidentified transcription factor or a DNA topology change must be involved in the aerobic/anaerobic modulation of leukotoxin transcription. The former possibility seems unlikely since we have demonstrated that the classical oxygen-regulatory proteins FNR and ArcA/B do not regulate *ltxA* transcription (24) and since all of the BamHI mutants are still oxygen regulated; if a third transcription factor were the oxygen response regulator, we would have expected at least one of the base substitution mutants to have mutated the binding site for a putative third transcription factor resulting in nonregulated *ltxA* transcription. This was not observed (Fig. 4C). Thus, we favor the hypothesis that a change in DNA conformation/structure is involved in regulating leukotoxin transcription in response to anaerobic versus aerobic growth of *A. actinomycetemcomitans*. The best candidate for the DNA conformational change that might be involved in the *ltxA* aerobic/anaerobic response is superhelical density. Supercoiling increases in *E. coli* cells grown without oxygen, and this increase in negative supercoils enhances transcription at a number of promoters because the DNA is more "open" for RNAP activity (59, 60, 61, 62, 63). Given this background, will the *A. actinomycetemcomitans* *ltxA* promoter be among the promoters which are particularly sensitive to changes in superhelical density? One way to predict this is to look for stress-induced DNA duplex destabilized (SIDD) sequences in the DNA. SIDD sequences are DNA elements identified and characterized in *E. coli* as being more likely than other sequences to change conformation (unwind the DNA duplex) when the overall superhelical density is increased (64). In fact, the SIDD site prediction program at <http://orange.genomecenter.ucdavis.edu/benham/sidd/> (65) indicates that the leukotoxin promoter has a SIDD element. This observation is consistent with our hypothesis that supercoiling is involved in the response of the *ltxA* operon to aerobic versus anaerobic growth.

However, IHF or Mlc must also play some role in the maximal induction of *ltxA* transcription in anaerobic cells since the magnitude of the change (10-fold) in leukotoxin expression in wild-type cells grown with and without oxygen is larger than the extent of the change (3-fold) seen in $\Delta ihf \Delta mlc$ double mutant cells (Fig. 9B). The possibility that aerobic cells either have less Mlc or more IHF than anaerobic cells is unlikely since the levels of their

FIG 10 Model for transcriptional regulation at the leukotoxin promoter. (A) Two DNA transcription factors compete for binding to the leukotoxin promoter at the positions indicated by lines above the sequence; final levels of transcript are determined by which one, the repressor (IHF) or the activator (MLC), binds more frequently. (B) In the presence of oxygen the conformation of the DNA favors IHF binding more frequently, thus reducing the amount of leukotoxin RNA levels present. (C) In the absence of oxygen, a change of DNA conformation occurs allowing Mlc to bind more frequently than IHF, thus resulting in an increase of leukotoxin RNA levels.

mRNAs do not change in aerobic versus anaerobic growth (based upon eight microarray experiments, the average anaerobic/aerobic ratio is 0.77 ± 0.3 for the *mlc* gene and 1.1 ± 0.3 for the *ihf* β gene [A. Burgum and D. Kolodrubetz, unpublished data]). Perhaps Mlc activity increases in anaerobic *A. actinomycetemcomitans* cells by an unknown mechanism. This possibility is precluded by the fact that, in the absence of IHF as a confounder, Mlc activates leukotoxin transcription ~ 3 -fold both aerobically and anaerobically. On the other hand, our analysis of the Δmlc strain data (Fig. 9B) indicates that Mlc competes more effectively with IHF for binding to the leukotoxin promoter in anaerobic cells than in aerobic cells. Thus, we propose the following model to explain the roles of Mlc and IHF in aerobic/anaerobic regulation of the *ltx* promoter. In aerobic cells (Fig. 10B), where the DNA has a certain superhelical density, IHF binds better than Mlc to the leukotoxin promoter, so the cumulative level of leukotoxin transcription is low. In anaerobic cells (Fig. 10C), which have a higher superhelical density, we propose that the altered DNA conformation at the leukotoxin promoter allows Mlc to bind better than IHF. This would lead to enhanced transcription both from the inherent increase in RNAP activity at a more supercoiled promoter and from the increased occupancy of the *ltxA* promoter by Mlc and the subsequent Mlc activation of RNAP activity. Although we do not have direct evidence that Mlc and IHF binding changes in response to the superhelical density of the leukotoxin promoter, it is

well known that the binding properties of some DNA binding proteins can be altered by the degree of supercoiling (64).

The linker scanning mutant promoter data are mostly consistent with the proposed model, with the possible exception of strain AAM761 which will be discussed below. The model predicts, as seen, that the levels of *ltxA* RNA in AAM759 and AAM760 would be similar to those in parental type cells since these two mutants do not alter the Mlc or IHF binding sites. On the other hand, mutants AAM762 through AAM765, which do alter the sequence of the presumed Mlc binding site in the leukotoxin promoter, all have lower leukotoxin RNA levels, as expected. However, the levels of leukotoxin transcription seen with these four mutants cannot be explained solely by their effects on Mlc binding since the mutants also alter the IHF binding site sequence in the leukotoxin promoter. Thus, the final level of leukotoxin expression in strains AAM762 to AAM765 is assumed to reflect a balance in each mutant between its effect on activator (Mlc) versus repressor (IHF) binding.

The significantly lower levels of leukotoxin RNA seen in BamHI promoter mutant AAM761 ($\sim 10\%$ of JP2) are harder to explain solely in terms of our proposed model. The sequence changes in AAM761 do not alter the predicted Mlc binding site in the leukotoxin promoter, so a loss of Mlc binding cannot explain the reduced *ltxA* transcription. Could the 761 mutation be reducing SIDD function at the leukotoxin promoter? Once again, this

explanation seems unlikely since both the AAM761 and normal leukotoxin promoters are predicted to have SIDD sites with approximately equal “strengths.” Another formal possibility to explain the low levels of leukotoxin in AAM761 is that its sequence changes allow IHF to bind with higher affinity to the leukotoxin promoter and block transcription. This possibility also seems highly improbable since the sequence changes in AAM761 alter the IHF consensus binding site and thus would be expected to decrease IHF binding. Could there be an unidentified transcriptional activator that normally binds to the promoter sequence changed in AAM761? If this were the case, deletion mutant AAM548, which removes two of the six bases mutated by AAM761, would be expected to have some reduction in leukotoxin expression; instead, AAM548 has the same levels of *ltxA* RNA as JP2 cells. Therefore, we favor the hypothesis that the region mutated in AAM761 contains an UP element, a DNA sequence that interacts with the alpha subunit of the C-terminal domain (α CTD) of RNAP to increase polymerase binding (66). Indeed, there is a reasonable match to the consensus sequence for a distal UP half-site at -66 to -56 . In strain AAM761, the match to this distal UP half-site, which is basically an AT-rich sequence, is much poorer because 761 introduces four new G/C bases in place of four A/T bases.

Mitchell et al. (48) also concluded that the leukotoxin promoter, in strain 652, has an UP element. They used mutational analysis of reporter gene plasmids to identify two *cis*-acting regions that influence the expression of leukotoxin in *A. actinomycetemcomitans*. Their first DNA element, encompassing sequences -68 to -35 , contained an activation sequence, which is essentially the same results we have reported here. However, Mitchell et al. concluded that the activation function of the -68 to -35 region was due solely to the presence of an UP element because they did not find any proteins that bound to this sequence in gel mobility shift assays. We can now conclude that an UP sequence is not the sole explanation for their positive-control element since we have shown that this region of the *ltx* promoter, whose sequence is identical in strains 652 (their strain) and JP2 (our strain) (17), contains a binding site for the transcriptional activator Mlc.

The second *cis*-acting regulatory region identified by Mitchell et al. (48) was further upstream, somewhere between -262 and -75 . When this region was deleted, there was a 4-fold increase in transcription of *ltxA*, indicating that this second region contains a negative *cis*-acting element. They demonstrated that an unknown *A. actinomycetemcomitans* protein, possibly a transcriptional repressor, binds somewhere in the region of -111 to -87 . However, there have been no further reports identifying this protein and proving that it is involved in controlling leukotoxin synthesis in *A. actinomycetemcomitans*. The validity of this negative-control element is somewhat questionable since our experiments with chromosomal promoter deletion mutants across the same region did not show any evidence for a negative-control element upstream of -64 . Of course, the discrepancies in these results can also be attributed to (i) different growth conditions (anaerobic versus aerobic), (ii) the use of different strains (we have shown that differences in *trans*-acting factors contribute to the differences in leukotoxin expression among strains of *A. actinomycetemcomitans* [17]), and/or (iii) the fact that our promoter deletions were in the chromosome as opposed to being on a plasmid.

Previously, we reported that the BamHI linker mutants, when driving the synthesis of a reporter gene on a plasmid in *A. actino-*

mycetemcomitans, altered a repressor binding site, as opposed to a transcriptional activator binding site, in the leukotoxin promoter (24). One possible explanation for the different effects of these mutants in the previous versus current experiments is that the superhelical density of the promoter mutants on the plasmid differs from their superhelical density in the chromosome, as is known to occur in *E. coli*. However, this explanation is not compelling because the extent, but not the direction (activation versus repression), of regulation would be expected to change in response to differences in superhelical density. Thus, we do not have a satisfactory explanation for the discrepancy in the results with the plasmid versus chromosomal promoter mutants AAM762, AAM763, and AAM764, but we believe that the chromosomal mutant data are correct because they more accurately reflect the normal chromosomal “environment.”

Overall, our results with the various promoter and transcription factor mutants indicate that leukotoxin transcription can be affected by four control elements in its promoter: an Mlc binding site centered at -55 that can enhance RNA synthesis when Mlc is bound, an IHF binding site also centered at -55 that reduces *ltxA* RNA when IHF is bound, a putative UP element near position -65 , and a putative SIDD sequence, encompassing most of the -69 to -35 region.

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