cis Elements and *trans* Factors Are Both Important in Strain-Specific Regulation of the Leukotoxin Gene in *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans, the etiologic agent of localized juvenile periodontitis, produces a potent leukotoxin that kills human neutrophils. The production of leukotoxin RNA can vary more than 50-fold among isolates of *A. actinomycetemcomitans*, and strains expressing high levels of leukotoxin RNA are most often found at sites of periodontal disease. To assess the relative contributions of transcription factors and promoter sequences in setting the disparate levels of leukotoxin RNA found, we have undertaken classical *cis/trans* analyses. First, the leukotoxin promoter regions from moderately leukotoxic (Y4) and minimally leukotoxic (ATCC 33384) strains of *A. actinomycetemcomitans* were cloned, sequenced, and compared with the previously sequenced leukotoxin promoter region of the high-producer strain JP2. The Y4 and ATCC 33384 promoter regions each contain a 528-bp segment that is absent from JP2. Interestingly, the analysis of various deletion constructs in *A. actinomycetemcomitans* indicated that Y4, despite the large insertion, initiates leukotoxin RNA synthesis at the same promoter as JP2 does. To perform *cis/trans* analyses, these three leukotoxin promoter regions were cloned into a plasmid upstream of the reporter gene β -galactosidase. Each plasmid was transformed into JP2, Y4, and ATCC 33384, and the β -galactosidase levels were determined. The results indicated that the sequences responsible for down-regulating leukotoxin RNA levels in Y4 relative to JP2 are found within the transcribed region of the Y4 leukotoxin operon. Importantly, in ATCC 33384, strain-specific *trans* factors and promoter sequence differences are equally significant in determining the lower levels of leukotoxin RNA. We hypothesize that either strain ATCC 33384 has a negative regulatory protein (which is missing or mutated in JP2/Y4) or that JP2 and Y4 carry an activator that is missing or mutated in ATCC 33384.

The gram-negative bacterium *Actinobacillus actinomycetemcomitans* is considered to be a primary pathogenic agent in various periodontal diseases, especially localized juvenile periodontitis (24, 32). Although *A. actinomycetemcomitans* has been purported to have numerous virulence factors, the one that has garnered the most attention is a 116-kDa protein called leukotoxin (28). This toxin specifically lyses human and primate polymorphonuclear leukocytes (29, 34) and thus is assumed to help the bacterium evade the innate host immune defense system. The contention that leukotoxin is a virulence factor is supported by the finding that it is produced in most strains of *A. actinomycetemcomitans* isolated from patients with periodontitis but is not usually produced in the few isolates that can be cultured from healthy individuals (33). The lack of leukotoxin production in some strains of *A. actinomycetemcomitans* is not due to a deletion of the leukotoxin structural gene, since the operon is present in all strains examined to date (7, 11, 25). Instead, the strain-specific differences in leukotoxin levels correlate with the amount of leukotoxin RNA produced (25). The mechanisms responsible for the interstrain regulation of leukotoxin RNA are unknown.

To allow a molecular analysis of leukotoxin function and

regulation, the gene for this toxin has been cloned and sequenced (16–18). The protein encoded by the leukotoxin structural gene (*lktA*) is a member of the RTX family of toxins, whose prototypical member is the α -hemolysin from *Escherichia coli*. Like many of the genes encoding RTX proteins, the *lktA* gene is found within an operon that contains four genes (3, 30). The other three genes, *lktC*, *lktB*, and *lktD*, encode proteins that are presumably involved in the activation and localization of the leukotoxin; this has been shown for their homologs in the α -hemolysin operon of *E. coli* (4, 10, 12, 13, 30). Two transcripts derive from the leukotoxin operon: a minor ~9.5-kb species, which contains the sequences of all four genes, and a predominant ~4.3-kb RNA, which contains only the *lktC* and *lktA* sequences (25).

Brogan et al. (5) recently presented an analysis of the leukotoxin promoter regions from *A. actinomycetemcomitans* JP2, a high-level leukotoxin expresser, and from strain 652, which produces about 5% of the JP2 levels of LktA. They identified two leukotoxin RNA start sites in JP2 by primer extension; both of these have potential –10 and –35 promoter sequences associated with them. One promoter, called P₁, is 350 bp upstream of *lktC*, and the second, called P₂, is only 50 bp in front of *lktC*. Interestingly, the sequence of the region upstream of *lktC* from strain 652 revealed an insertion of 528 bp between the P₁ and P₂ promoters. This additional sequence has an open reading frame (which we will refer to as *orfX*) that has the potential to encode a 150-amino-acid protein in strain 652, whereas JP2 could produce only a truncated version of this protein. The position of the leukotoxin promoter in strain 652

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was not determined experimentally, nor did the sequence comparison of Brogan et al. (5) provide any strong clues to the mechanism by which these two operons express different levels of leukotoxin RNA.

None of the previous studies investigating the mechanisms of interstrain differences in leukotoxin levels have examined the possibility that strain-specific transcription factors play a role. To assess the relative contributions of transcription factors and leukotoxin promoter sequences (*cis* elements) in setting the disparate levels of leukotoxin RNA in different strains of *A. actinomycetemcomitans*, we have undertaken classical *cis/trans* analyses. As a first step, we cloned and sequenced the leukotoxin promoter regions from three strains of *A. actinomycetemcomitans*: JP2, a high-level leukotoxin producer; Y4, a moderate-level producer of LktA (10% of the level produced by JP2); and ATCC 33384, which produces only 2% as much leukotoxin as JP2 (25). RNA end mapping and other strategies have been used with JP2 and Y4 to show that, unlike the results reported by Brogan et al. (5), leukotoxin RNA is transcribed only from the P₁ promoter. Sequence comparisons and classic *cis/trans* tests with leukotoxin promoter reporter gene plasmids in *A. actinomycetemcomitans* were used to demonstrate that the extra 528 bp in Y4 relative to JP2 contain the sequences responsible for determining the lower level of *lkt* RNA in Y4. Interestingly, similar analyses with the ATCC 33384 promoter region indicated that DNA element and transcription factor differences are equally important in determining the lower levels of *lkt* RNA found in strain ATCC 33384.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Three strains of *A. actinomycetemcomitans* were used: JP2 (29), Y4 (28), and ATCC 33384 (NCTC 9710) (35). *A. actinomycetemcomitans* cells were grown in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) (5% CO₂, 10% H₂, 85% N₂) at 37°C in TSBYE (3% tryptic soy broth plus 0.6% yeast extract). The recombinant constructs were propagated in *E. coli* TB-1 at 37°C in Luria broth (1). Where indicated, spectinomycin was added to a final concentration of 50 µg/ml. Ampicillin was used at final concentrations of 50 µg/ml for *E. coli* and 10 µg/ml for *A. actinomycetemcomitans*.

Cloning and sequencing leukotoxin promoter regions from strains Y4 and ATCC 33384. A 7-kb *Pst*I DNA fragment containing the leukotoxin promoter region from *A. actinomycetemcomitans* ATCC 33384 was cloned from a limited genomic DNA library (16) with an *Eco*RI fragment internal to the JP2 *lktC* gene (17) as a hybridization probe; the resulting clone was called pEK30. The same hybridization probe was used to clone a 3-kb *Pst*I DNA fragment containing the leukotoxin promoter region from a limited genomic library (16) of strain Y4 DNA; the resulting clones are called pCJ2 and pCJ6. The DNA sequence of part of each clone was determined by the dideoxy-chain termination method with a Sequenase version 2.0 sequencing kit (U.S. Biochemicals, Cleveland, Ohio). All reported sequences were determined from both strands and were analyzed with the Pustell DNA/protein sequence analysis system (International Biotechnologies, Inc.).

RNA isolation and analysis. *A. actinomycetemcomitans* RNA was prepared by a sodium dodecyl sulfate (SDS) lysis/CsCl cushion procedure modified from that of Deretic and Konyecsni (6). A 30- to 50-ml sample of JP2 cells was harvested in the mid-logarithmic phase of growth by centrifugation, washed with cold lysis buffer (50 mM Tris-HCl [pH 7.5], and brought up in 5 ml of lysis buffer to which 1 ml of 20% SDS had been added. After incubation at 37°C for 1 to 2 min, 4 g of CsCl was added and mixed in by gentle rocking. An additional 5 ml of lysis buffer was mixed in by gentle inversion, and the precipitate that formed was removed by centrifugation at 11,000 × g for 10 min. The resulting supernatant was removed, and aliquots were layered onto 5.7 M CsCl cushions of equal volume. The RNA was pelleted overnight in an SW50.1 rotor at 150,000 × g for 15°C. The tubes were emptied by inversion, and each clear RNA pellet was dissolved in 200 µl of water and stored as a precipitate in 67% ethanol in a microcentrifuge tube. All solutions were prepared with diethylpyrocarbonate-treated water, and diethylpyrocarbonate-treated plasticware and baked glassware were used.

Primer extension assays followed the protocol of Welch and Pellet (31) with some minor modifications. Synthetic oligonucleotide primers were radiolabeled with T4 polynucleotide kinase and [γ -³²P]ATP (1). Between 0.01 and 0.03 pmol of labeled probe was combined with 30 µg of *A. actinomycetemcomitans* RNA in 10 µl of reverse transcriptase buffer, and the mixture was heated to 80°C for 3 min and then allowed to anneal at 37°C for 90 min. Moloney murine leukemia

virus (MMLV) reverse transcriptase (200 U; Life Technologies) was added to the reaction mixture along with each deoxynucleoside triphosphate to 0.63 mM, and the mixture incubated for 60 min at 37°C. Each sample was then precipitated with ethanol, dried, and dissolved in sequencing sample buffer (1) for electrophoresis on 6% polyacrylamide-urea gels.

S1 nuclease analyses were adapted from the protocol of Deretic and Konyecsni (6). The DNA probes were end labeled with T4 polynucleotide kinase, and 25,000 to 75,000 cpm of each probe was mixed with 20 to 25 µg of RNA in 15 µl of hybridization buffer [0.83 M NaCl, 1.7 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.5)]. After being boiled for 3 min, the samples were incubated at 67°C for 60 min, and then 300 µl of iced S1 nuclease buffer and 880 to 1,200 units of S1 nuclease were added. The samples were incubated for 30 min at 37°C and then extracted with phenol, precipitated with ethanol, and electrophoresed as described above.

Reverse transcriptase-coupled PCR. The procedure for reverse transcriptase-coupled PCR was modified from that of Pierce et al. (22). RNA from *A. actinomycetemcomitans* Y4 was first treated with RNase-free RQ1 DNase (Promega Corp., Madison, Wis.) at 0.25 U/µg for 15 min at 37°C. After extraction with phenol and chloroform-isoamyl alcohol, the RNA sample was ethanol precipitated and suspended in water to 1 mg/ml. RNA (10 µg) was incubated in Moloney murine leukemia virus reverse transcriptase buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂) containing 0.12 U of human placental RNase inhibitor (Life Technologies, Gaithersburg, Md.) per µl, 0.5 mM each deoxynucleoside triphosphate, 20 ng of primer LKT57 per µl and 2 U of MMLV reverse transcriptase (Life Technologies) per µl. cDNA synthesis was allowed to proceed for 2 h at 37°C, at which point the reaction was stopped by placing the reaction mixture at -20°C. The sample was extracted with phenol and chloroform, ethanol precipitated, and suspended in Tris-EDTA to 0.2 µg/µl. For PCR amplification, 0.8 µg of the cDNA sample was used as a template with 0.5 µg of each primer and amplified for 30 cycles (1). The resulting PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

Construction of a strain deleted of the chromosomal copy of *orfX*. Standard recombinant DNA techniques and PCR were used to construct plasmid pDK533, in which a spectinomycin resistance gene has replaced the coding region of gene *orfX*. Briefly, a 1.48-kb *Ngo*MI-*Eco*RI DNA fragment containing the end of *glyA*, all of *orfX*, and most of *lktC* from strain Y4 was cloned between the *Xma*I and *Eco*RI sites of the vector pBluescript KS (Stratagene Cloning Systems, La Jolla, Calif.) to generate an intermediate plasmid called pDK527. This plasmid was linearized with *Afl*II, which cuts just upstream of *orfX*, and *Syl*I, which has a site at the termination codon of *orfX*; a 1.1-kb PCR product encoding spectinomycin resistance was cloned into this DNA to generate pDK533. In this plasmid, *orfX* has been replaced with the spectinomycin resistance gene and its promoter and transcription termination sequences. The 1.1-kb PCR product used in this protocol was generated with a primer pair that flanked the spectinomycin adenyltransferase gene of *Enterococcus faecalis* (19). The primers were synthesized to contain a *Syl*I site at the end of the sense primer (5'-CCCCATGGCGTGAA TACATGTTATAATAAC-3') and an *Afl*II site on the end of the antisense primer (5'-CGCCTTAAGGTTTCTAAATCTGATTACC-3'). The orientation of the spectinomycin resistance gene in pDK533 is opposite to the orientation of *orfX*.

Plasmid pDK533 was electroporated into *A. actinomycetemcomitans* Y4 to produce ampicillin-resistant, spectinomycin-resistant transformants (e.g., AAM52 [see Fig. 6A]) in which the plasmid had recombined into the host chromosome. These constructions were verified by Southern blot analysis. AAM52 and three other similar transformants were then passaged six to eight times in TSBYE without antibiotics and plated onto TSBYE for isolation. A total of 140 individual colonies were tested for their resistance to spectinomycin and ampicillin; 4 were now ampicillin sensitive but still spectinomycin resistant. These colonies should be cells in which pDK533 had recombined out of the chromosome in such a way that it left the Δ Gene X::*spec^{res}* gene in the chromosome (AAM54-57). This was confirmed by Southern blot analysis.

***lktA::lacZ* reporter gene plasmids: construction and isolation for three different promoters.** To construct the JP2 reporter gene plasmid pDK445, a 1.65-kb *Pst*I-*Ssp*I fragment of JP2 DNA containing 1 kb of the leukotoxin promoter region, the 500-bp *lktC* gene, and the DNA coding for the first 26 amino acids of LktA (Fig. 1) was cloned into *Pst*I-*Sma*I-digested pUC18. This allowed the same region of DNA to be isolated as a 1.65-kb *Pst*I-*Kpn*I DNA fragment by using the *Kpn*I site provided by the vector. This DNA fragment was cloned into plasmid YEp358R (21) to create an in-frame fusion of the first 26 amino acids of LktA to β -galactosidase. The fusion junction was confirmed by DNA sequence analysis. The entire *lktA::lacZ* fusion gene with the JP2 promoter region was then isolated as a 4.5-kb *Pst*I-*Nsi*I DNA fragment and cloned into the *Pst*I site of the broad-host-range vector pMMB67 (8, 9) to generate plasmid pDK445.

A 1.05-kb *Ssp*I fragment containing 450 bp of the Y4 leukotoxin promoter region, all of *lktC*, and the DNA coding for the first 26 amino acids of LktA (see Fig. 1) was cloned into the *Sma*I site of pUC18 as a first step in making a Y4 *lktA::lacZ* reporter gene fusion plasmid. The same DNA segment was then isolated as a *Pst*I-*Kpn*I fragment (with *Kpn*I at the *lktA* end) by using the restriction endonuclease recognition sites in the multiple-cloning site of the vector and cloned between the *Pst*I and *Kpn*I sites of YEp356R (21). DNA sequence analysis was used to confirm that this plasmid had an in-frame fusion

of *LktA*, at amino acid 26, to β -galactosidase. The entire *lktA::lacZ* fusion gene with the Y4 promoter region was then isolated as a 4.2-kb *PstI*-*NsiI* DNA fragment and cloned into the *PstI* site of pMMB67 (9) to generate plasmids pDK493 and pDK494; these plasmids have the insert present in opposite orientations. To put additional Y4 leukotoxin promoter region DNA onto this plasmid, a 1.0-kb *NaeI*-*HindIII* DNA fragment was isolated, *HindIII* linkers were added to the *NaeI* end, and the resulting DNA was cloned between the two *HindIII* sites of pDK493 to generate plasmid pDK541. The cloning junctions and insert orientation of this plasmid were confirmed by DNA sequence analysis. In plasmid pDK541, the *lktA::lacZ* fusion is flanked by the *lktC* gene and 1.2 kb of additional upstream DNA starting at the *NaeI* site in *glyA*.

The same recombinant DNA strategy used for Y4 was used to construct the ATCC 33384 reporter gene plasmid with two minor differences: the starting DNA fragment was a 1.05-kb *SspI* DNA fragment from the leukotoxin promoter region of strain ATCC 33384, and the final 1.0-kb *HindIII* fragment added to the clone was generated from a *Ngo*MI segment to which *HindIII* linkers were added.

Generation of promoter deletions in the Y4 *lktA::lacZ* reporter gene plasmid.

A series of deletions in the Y4 leukotoxin promoter region was generated by cloning various PCR products into the Y4 *lktA::lacZ* reporter plasmid pDK494. One primer, LKT66 (5'-GGGTTTCCAGTCACGAC-3'), was present in all of the PCR amplifications; it is the reverse complement of a sequence in *lacZ* so that the resulting PCR product will include the *KpnI* restriction endonuclease site at the *lktA-lacZ* junction. The other primers used in individual PCR amplifications (LKT68 to LKT76) annealed to sites at approximately 100-bp intervals within the Y4 leukotoxin promoter region (see Fig. 2). The LKT68-76 primers had a non-template encoded *KpnI* site at their 5' ends.

LKT66 was used in combination with each of the primers, LKT68 to LKT76, in PCR amplifications (30 cycles) on a PTC-100 programmable thermal controller (M. J. Research, Inc.) with plasmid pDK541 as a template. The nine resultant PCR products were made blunt ended by reaction with the Klenow fragment of DNA polymerase I, phosphorylated with T4 polynucleotide kinase and ATP, and then isolated by gel purification (2). Each PCR fragment was cloned into the *SmaI* site of pUC18, isolated from those intermediate plasmids as *KpnI* fragments, and cloned in between the *KpnI* sites of plasmid pDK494. This resulted in the replacement of the full-length Y4 promoter with truncated versions of the promoter in *lktA::lacZ* reporter plasmids pDK563 to pDK571. Plasmid DNAs were isolated from *E. coli* (1, 15), and large-scale isolations were done by a previously described modification of an alkali lysis procedure (14). The junctions and orientations of each insert were confirmed by DNA sequence analysis.

Assay of β -galactosidase from *lktA::lacZ* reporter plasmids in *A. actinomycetemcomitans*. The leukotoxin promoter-*lacZ* fusion constructs (plasmids pDK445, pDK541, pDK543, and pDK563 to pDK571) were introduced into various strains of *A. actinomycetemcomitans* by electroporation (12, 27). To measure *lktA::lacZ* activity, cells from a given transformant were grown overnight in 5 ml of TSBYE plus ampicillin broth in a Coy anaerobic chamber with shaking, diluted 1:25 into a flask with 25 ml of fresh medium, and grown overnight once again. The overnight culture was then diluted to an optical density at 600 nm (OD_{600}) of 0.1 in 25 ml of TSBYE. When the culture density had doubled ($OD_{600} \approx 0.2$), a 1-ml aliquot was removed and assayed for β -galactosidase activity. Each sample was measured in triplicate as described (20) with cells that had been permeabilized with chloroform-SDS. β -Galactosidase units were calculated as $(1,000 \times OD_{420}) \div [\text{time of assay (minutes)} \times \text{volume of cells (milliliters)} \times OD_{600} \text{ of cells}] (20)$.

Nucleotide sequence accession numbers. The GenBank accession numbers for the Y4 and ATCC 33384 sequences are U51861 and U51862, respectively.

RESULTS

Strains producing different levels of leukotoxin have distinct sequences upstream of the leukotoxin coding region. To see if leukotoxin promoter sequence differences could readily explain the variability in *lkt* RNA levels seen in different strains of *A. actinomycetemcomitans*, the leukotoxin promoter regions from strains Y4 (leukotoxin moderate; 10% of JP2) and ATCC 33384 (leukotoxin low; 2% of JP2) were cloned, sequenced, and compared with the previously analyzed sequence of the corresponding region from strain JP2 (17). All three sequences were identical in the *lktC* coding region and in the adjacent 27 bases; the upstream sequences diverged. The most dramatic variation is a 528-bp deletion in the JP2 leukotoxin promoter relative to Y4 and ATCC 33384 (Fig. 1). The same deletion was also found by Brogan et al. (5) when comparing the leukotoxin promoter regions from strains JP2 and 652. The 528-bp deletion removes a DNA segment starting in the middle of *orfX*, a 450-bp open reading frame found in Y4 and ATCC 33384, and continuing to a position 54 bp 5' of the *lktC* start codon (Fig. 2). Except for the deletion, strains Y4 and JP2

FIG. 1. Gene organization of the leukotoxin loci in strains of *A. actinomycetemcomitans*. Protein-coding regions are denoted by boxes; an open-ended box indicates that only part of a gene is shown. The position of the leukotoxin P_1 promoter is marked by an arrow, as is the position of the putative P_2 promoter in JP2 (5). The nucleic acid probe used in the S1 analysis of JP2 *lkt* RNA is indicated by the line above the JP2 map. The positions and orientations of the oligonucleotides used in JP2 primer extension experiments (SPITZ8) and Y4 reverse transcriptase-coupled PCR assays (LKT16, LKT56, and LKT57) are designated by small arrows. The labeled ends of the primers and probes are indicated by asterisks. The oligonucleotide sequences are as follows: SPITZ8, GGGAACTGAATAAAACGGAGATAAAATGGAGCATATTCGTACTG GAC; LKT16, CCGGTCAAAGGTTAT; LKT56, GGTT(A/G)TAAATAAC GG; LKT57, AATTATTATTTTCTCCATATAAATCTCC. Relevant restriction endonuclease sites are marked as follows: A, *Afl*II; E, *Eco*RI; H, *Hind*III; Na, *Nae*I; Ng, *Ngo*MI; P, *Pst*I; Ss, *Ssp*I; St, *Sty*I.

are identical in the region sequenced. This includes the end of the *glyA* gene which encodes serine hydroxymethyltransferase (5, 23). On the other hand, the sequences in the *glyA*-to-*lktC* regions from Y4 and ATCC 33384 diverged from each other by 8% on average (Fig. 2). Interestingly, the least conserved region (~89% sequence identity) was in the *orfX* coding region.

Brogan et al. (5) previously reported that the leukotoxin operon in strain JP2 has two promoters, P_1 and P_2 , which are located 336 and 38 bp, respectively, in front of *lktC*. From sequence analysis, it is clear that differences in the "strengths" of the P_1 promoters from the various strains cannot be responsible for the interstrain variation in leukotoxin RNA levels; the -10 and -35 sequences of the P_1 promoter are identical in Y4, ATCC 33384 (Fig. 2), 652 (5), and JP2. Thus, the most straightforward hypothesis would be that leukotoxin RNA synthesis in strains Y4, ATCC 33384, and 652 does not start at P_1 but instead initiates from a "weaker" promoter between the end of *orfX* and the beginning of *lktC*. Consistent with this possibility, there are a number of base pair differences among the strains in the vicinity of P_2 , any of which may lead to a weaker leukotoxin promoter in that region. In fact, the -35 sequence of P_2 in JP2 is not present in Y4 or ATCC 33384 because of the 528-bp insertion. However, this simple hypothesis appears incorrect. We will present data below indicating that there is no P_2 promoter for leukotoxin RNA synthesis in strains JP2 and Y4. This leads to the alternate hypothesis that leukotoxin RNA synthesis initiates at P_1 in all strains of *A. actinomycetemcomitans* but transcription through to *lktC* is inefficient in strains Y4, ATCC 33384, and 652 because they have an extra 528 bp DNA relative to JP2.

Leukotoxin RNA synthesis initiates at P_1 in strain JP2. To help distinguish among the possible mechanisms involved in producing lower leukotoxin RNA levels in some strains of *A. actinomycetemcomitans*, the *lkt* RNA initiation site(s) must be mapped. The leukotoxin RNA initiation site(s) was first mapped in JP2, since this strain produces the most leukotoxin RNA (25) and since we wanted to confirm the results of Brogan et al. (5). RNA was isolated from *A. actinomycetemcomitans* JP2 grown to different cell densities, and S1 nuclease protection mapping was done with an end-labeled DNA fragment encompassing the region from *glyA* to *lktC* as a probe (Fig. 1). The one RNA-dependent band was 590 bp (Fig. 3A)

FIG. 3. Mapping the leukotoxin RNA start site in *A. actinomycetemcomitans* JP2. (A) S1 nuclease protection experiment with an end-labeled *NaeI-EcoRI* probe (Fig. 1) from JP2 DNA that covered the entire region between *ghyA* and *lktC*. RNA was harvested from JP2 cells at different cell densities (as measured by OD₆₀₀): lane a, 0.1; lane b, 0.18; lane c, 0.28; lane d, 0.4. Each RNA sample was hybridized to the end-labeled probe and then subjected to S1 nuclease treatment as described in Materials and Methods. The size of the band expected for initiation at promoter P₁ is marked, as is the expected position of a band from the P₂ promoter identified by Brogan et al. (5). The lane marked stds contains radiolabeled 123-bp ladder DNA (Life Technologies, Gaithersburg, Md.). (B) Primer extension reaction products obtained with the 5'-end-labeled primer SPITZ8 (Fig. 1) and JP2 RNA. The sample (lane a) was subjected to electrophoresis on a high-resolution sequencing gel. The positions of the two major primer extension products at -336/-337 relative to the start codon of *lktC* are indicated, as are the positions of the -35 and -10 consensus sequences predicted by Kraig et al. (17). The DNA-sequencing reactions of JP2 DNA with primer SPITZ8 are shown in the first four lanes and are labeled as the complements of the actual sequencing reactions. (C) Lack of major primer extension product at P₂, using the 5'-end-labeled primer LKT77 (5'-TCCATATTAAAT CTCCTTGT-3'), which is identical to the one used by Brogan et al. (5), and JP2 RNA (lane marked primer + RNA). The lane marked primer contains the reaction products from a primer extension reaction without JP2 RNA. The lane marked stds contains radiolabeled 123-bp ladder DNA (Life Technologies).

before the *lktC* coding region (data not shown but summarized in Fig. 2). The apparent discrepancy between the S1 nuclease and primer extension mapping results could be a consequence of interference from secondary structures in the leukotoxin RNA or could be an artifact due to difficulties in controlling the extent of RNA degradation or processing among samples of *A. actinomycetemcomitans* RNA. However, a more interesting possibility is that leukotoxin RNA synthesis initiates at P₁ in strain Y4 but that the RNA, containing the *orfX* and *lktC* genes, is rapidly cleaved at a site (or sites) between *orfX* and *lktC*. This could easily lead to a mixture of RNA ends between *orfX* and *lktC* which would be found in RNA-mapping experiments.

If this final possibility were correct, then *orfX*, *lktC*, and *lktA* should be cotranscribed as part of a ~4.6-kb RNA in strain Y4 (25). To test for the existence of this transcript, Northern (RNA) blot analysis was done with Y4 RNA and an *orfX*-specific hybridization probe. Surprisingly, the only signal seen was from hybridization to a somewhat broad band of approximately 750 bases (Fig. 4A, lane a). The lack of hybridization to a larger band did not appear to be due to general RNA degradation during sample preparation, since a ~4.3-kb leukotoxin RNA band was seen when the Y4 RNA was hybridized with an *lktA*-specific probe (Fig. 4A, lane b). We therefore hypothesized that leukotoxin RNA synthesis initiates at the P₁ promoter to produce a primary 4.6-kb transcript containing *orfX*, *lktC*, and *lktA*, which is rapidly processed or degraded into two RNAs, one encoding *orfX* and the other encoding *lktCA*. The levels of this initial 4.6-kb transcript could be too low for detection by Northern blot analysis.

Therefore, to provide evidence for this proposed unstable, low-abundance 4.6-kb primary RNA containing both *orfX* and *lktC* sequences, a more sensitive technique, reverse transcrip-

FIG. 4. Analysis of *orfX* RNA from *A. actinomycetemcomitans* Y4. (A) Autoradiograph of RNA from Y4 that was subjected to Northern blot analysis with *orfX*- and *lktA*-specific hybridization probes. The *orfX* hybridization probe (lane a) was a labeled 400-bp PCR product which had been amplified from the *orfX* gene of Y4. The *lktA* hybridization probe (lane b) was a ³²P-labeled 600-bp *PstI-HindIII* DNA fragment from the beginning of the *lktA* gene. The positions of RNA size standards (in kilobases) are marked on the left, as is the position of the major transcript containing *orfX*. (B) Ethidium bromide-stained gel of PCR products after amplification of *lktC/orfX* cDNA with various primers. Y4 RNA was made into cDNA with reverse transcriptase and primer LKT57. This sample was then used as a template in PCR amplifications with various primer pairs (lanes a, c, and e). As controls to show that the PCR products are not being amplified from contaminating genomic DNA, lanes b, d, and f contain samples which were not treated with reverse transcriptase. The primer pairs, whose positions are shown in Fig. 1, were LKT68/LKT57 (lanes a and b), LKT16/LKT57 (lanes c and d), and LKT56/LKT57 (lanes e and f). The positions of the DNA size standards (in bases) are marked on the left.

FIG. 5. Structure and activity of β -galactosidase reporter gene plasmids with Y4 promoter region deletions. The diagonal lines represent the junctions between *A. actinomycetemcomitans* DNA (thick lines) cloned from strain Y4 and vector sequences (thin lines), which are not all shown for these circular plasmids. The boxes indicate genes (or parts of genes) including the leukotoxin- β -galactosidase fusion gene (*lktA::lacZ*). The position of the leukotoxin P_1 promoter is marked. The β -galactosidase values were derived from Y4 cells containing each plasmid and are the averages of results from two experiments.

tase-coupled PCR, was used. DNase-treated RNA from *A. actinomycetemcomitans* Y4 was used in a reverse transcriptase reaction with the *lktC*-specific oligonucleotide LKT57 as a primer. This cDNA sample was then used as the PCR substrate for several *orfX*-specific (LKT16 and LKT56) and *lktC*-specific (LKT57) oligonucleotide primer pairs (Fig. 1). Each primer pair generated a reverse transcriptase-dependent band of the size expected, 856 bp with primers LKT16/LKT57 and 699 bp with primers LKT56/LKT57 (Fig. 4B, lanes c and e), indicating that *orfX* and *lktC* are cotranscribed. The unexpected ~ 1.4 -kb PCR product in both lanes is from an irrelevant RNA, since it failed to hybridize to an oligonucleotide specific for the region between *lktC* and *orfX* (data not shown). Thus, all of the results lead us to conclude that the *lktC* and *lktA* genes are cotranscribed with *orfX* from P_1 in strain Y4 and that this 4.6-kb RNA is rapidly processed into two smaller RNAs, one containing *orfX* and a second containing *lktC* and *lktA*.

To confirm that P_1 is the primary leukotoxin promoter in Y4, a series of deletions in the region upstream of the Y4 *lktC* gene were constructed and their effects on leukotoxin levels were assayed. These deletions were generated in a broad-host-range plasmid in which the leukotoxin structural gene (*lktA*) was fused, in frame, at amino acid 26 to *lacZ* (*lktA::lacZ*). Thus, the effects of the Y4 leukotoxin promoter deletions on leukotoxin synthesis could be assessed by measuring the levels of β -galactosidase. The plasmids with the promoter deletions were electroporated into *A. actinomycetemcomitans* Y4, and several transformants of each were assayed for the level of LktA- β -galactosidase fusion protein (Fig. 5). Deletion of the P_1 sequences (plasmids 564, 565, and 566) led to the loss of β -galactosidase synthesis; this suggests very strongly that leukotoxin synthesis in Y4 initiates at the P_1 promoter (as it does in strain JP2).

As expected if P_1 were the major leukotoxin promoter, the three smallest deletions led to the loss of LktA::LacZ synthesis. Surprisingly, however, the other larger deletions showed progressive increases in the level of β -galactosidase (Fig. 5, plasmids pDK567 to pDK571). There are two possible explanations for these data: (i) Y4 has a second promoter near *lktC* that is repressed by sequences within *orfX*, or (ii) the isopropyl-

β -D-thiogalactopyranoside (IPTG)-inducible *tac* promoter (8) in the vector may drive the synthesis of LktA::LacZ in plasmids pDK567 to pDK571. We favor the latter explanation since the levels of β -galactosidase can be induced fourfold by IPTG in cells containing pDK569, one of the larger deletions, but only twofold in cells with pDK565, a small deletion.

To eliminate definitively the possibility that there is a promoter for leukotoxin in the region between *orfX* and *lktC* in strain Y4, an alternate molecular genetic approach was used. We generated strains of *A. actinomycetemcomitans* in which a spectinomycin resistance gene, with its transcription termination sequences, has replaced the normal genomic copy of *orfX* (Fig. 6A, AAM57 for example). In these mutants, the P_1 promoter cannot be used to synthesize *lkt* RNA. If there were a second promoter immediately upstream of *lktC*, a leukotoxin RNA would be synthesized in these mutant strains even when the transcript from P_1 has been terminated. RNA was prepared from several *orfX* deletion strains (AAM54 to AAM57) and examined by Northern blot analysis with a leukotoxin-specific probe; no *lkt* RNA was detected (Fig. 6B, top panel). Signals for leukotoxin RNA were found in the samples from the leukotoxin-positive strains AAM50 and AAM60 (Fig. 6B, top panel), indicating that the hybridization probe was working. As a positive control to show that the RNA samples were intact, the same blot was rehybridized with a *glaA* probe which gave appropriately sized signals (Fig. 6B, bottom panel). Finally, the lack of leukotoxin RNA in the Δ *orfX* strains was confirmed by checking extracts from several of the deletion mutant strains for leukotoxic activity by chromium release assays (16); no activity was found (data not shown). These results prove that a second leukotoxin promoter is not present within the 330 bases of Y4 DNA immediately upstream of *lktC*. This provides further evidence that the P_1 promoter must be the only one used for leukotoxin RNA synthesis in strain Y4.

However, these experiments left one, albeit remote, possibility: perhaps there is a promoter between *orfX* and *lktC* whose activation requires the presence of the protein product of *orfX*. To eliminate this possibility, a Northern blot analysis was performed with RNA from AAM52, an intermediate strain made in the construction of the *orfX* deletion mutants (Fig. 6A). AAM52 has a complete, transcribed (data not shown) copy of *orfX* near but separated from the 330-bp segment upstream of *lktC* (Fig. 6A), yet it synthesizes no detectable leukotoxin RNA (Fig. 6B). Taken together, the RNA-mapping results, the plasmid deletion experiments, and the *orfX* deletions lead us to conclude that leukotoxin RNA synthesis initiates from the same promoter, P_1 , in strains Y4 and JP2; there is no second leukotoxin promoter in strain Y4 between *orfX* and *lktC*.

Promoter differences and strain-specific *trans* factors are both important in determining the level of leukotoxin produced by different strains of *A. actinomycetemcomitans*. Having demonstrated that the JP2 and Y4 leukotoxin operons use the same promoter, P_1 , we now could begin to examine the mechanism(s) responsible for the different levels of leukotoxin expressed in the two strains and in the low-leukotoxin-expressing strain ATCC 33384. Therefore, classical *cis/trans* experiments were done to assess the relative contributions of promoter differences (*cis* elements) and possible strain-specific transcription proteins (*trans* factors) in determining different leukotoxin levels. To facilitate the analysis, the JP2 leukotoxin gene (*lktA*) and its promoter region, including P_1 and 700 bp of additional upstream DNA, were fused in frame at amino acid codon 26 to the reporter gene *lacZ* (26). This fusion construct was then cloned into the broad-host-range plasmid vector pMMB67 (9) to generate plasmid pDK445. Similar reporter gene plasmids

FIG. 6. Scheme used to make a strain deleted of *orfX* and assay for leukotoxin RNA in such deletion strains. (A) Schematic of the genetic structures expected at the leukotoxin locus during the construction of *orfX* deletion derivatives (strains AAM54 to AAM57) of *A. actinomycetemcomitans* Y4. Strain AAM52 is an intermediate in the construction of several *orfX* deletion strains in which plasmid pDK533 had recombined with the 0.6-kb region of homology downstream of *orfX*. The *P₁* promoters in AAM52 are separated from the *lktCA* genes. Abbreviations: *P₁*, leukotoxin promoter; *amp*, gene encoding ampicillin resistance; *spec*, gene encoding spectinomycin resistance, including the promoter and transcription termination site. Key restriction endonuclease sites are indicated as follows: E, *EcoRI*; H, *HindIII*; N, *NaeI*. The physical maps are not drawn to scale. (B) RNA isolated from the indicated strains of *A. actinomycetemcomitans* was subjected to Northern blot analysis with a labeled 600-bp *PstI-HindIII* DNA fragment from the beginning of the *lktA* gene as the hybridization probe (upper panel). After exposure to X-ray film to generate the autoradiogram in the upper panel, the same filter was hybridized with a labeled 376-bp *PstI-NaeI* DNA fragment containing the end of the *glyA* gene (lower panel). The positions of RNA size standards (in kilobases) are marked on the right. Strain AAM50 is an intermediate in the construction of several *orfX* deletion strains in which plasmid pDK533 had recombined with the 0.4-kb region of homology upstream of *orfX*. AAM50 has a normal *P₁* promoter region in front of *lktCA*, so it should (and does) express the *lktCA* RNA. In strain AAM60, the plasmid pDK533 has recombined out of the intermediate transformant AAM50 to give back a wild-type, Y4-like leukotoxin locus.

were generated for the leukotoxin operons from strains Y4 and ATCC 33384. These two constructs, pDK541 and pDK543, have the same *lktA::lacZ* fusion but contain only 350 bp of DNA upstream of *P₁*, so that the overall sizes of the three plasmids differ by less than 2%. This should ensure that there are no significant differences in plasmid copy number when they are introduced into *A. actinomycetemcomitans*.

The three plasmids were individually electroporated into the set of three *A. actinomycetemcomitans* strains, and the β -galactosidase levels were determined (Table 1). As expected, the *LktA::LacZ* levels were highest for the JP2 promoter plasmid in strain JP2, intermediate for the Y4 promoter plasmid in strain Y4, and low for the ATCC 33384 promoter plasmid in strain ATCC 33384 (Table 1, top left to bottom right diagonal). The ratios of these three values, 100:22:3, are very close to the ratios 100:10:2, which we have measured directly for leukotoxin activity in JP2, Y4, and ATCC 33384 (25). These results indicate that the reporter gene system accurately reproduces the regulation of leukotoxin in *A. actinomycetemcomitans*.

The data show that the JP2 promoter plasmid pDK445 gives the highest level of expression in all of the strains (Table 1). Similarly, the ATCC 33384 promoter has the lowest levels of

expression in all strains and the Y4 promoter plasmids always give intermediate levels of expression (Table 1). Thus, we conclude that differences in the promoters play a critical role in determining the different levels of leukotoxin RNA in JP2, Y4, and ATCC 33384. In fact, the 10-fold disparity in leukotoxin levels between strains JP2 and Y4 (25) appears to be due mainly to the differences in their leukotoxin promoter regions; the β -galactosidase values for a given plasmid in JP2 are al-

TABLE 1. β -Galactosidase levels of *lktA::lacZ* fusion gene plasmids containing leukotoxin promoters from different strains of *A. actinomycetemcomitans*

Plasmid	Promoter origin	β -Galactosidase level (U/cell) in <i>A. actinomycetemcomitans</i> strain ^a :		
		JP2	Y4	ATCC 33384
pDK445	JP2	28,127 $\pm$ 14%	25,521 $\pm$ 13%	10,312 $\pm$ 8%
pDK541	Y4	9,147 $\pm$ 17%	6,294 $\pm$ 17%	1,936 $\pm$ 17%
pDK543	ATCC 33384	6,426 $\pm$ 21%	4,095 $\pm$ 25%	960 $\pm$ 14%

^a The results are the means of at least three independent experiments.

most the same as the values for the same plasmid in Y4. On the other hand, strain-specific *trans* factors must play a significant role in determining leukotoxin level differences between ATCC 33384 and the other two strains, since the levels of β -galactosidase from a given promoter construct are always lower in strain ATCC 33384 than in strains Y4 or JP2 (Table 1). On the basis of these results, it is clear that differences in leukotoxin promoter sequences and in strain-specific *trans* factors are both important in determining the level of leukotoxin produced by different strains of *A. actinomycetemcomitans*.

DISCUSSION

Most strains of *A. actinomycetemcomitans* isolated from individuals with periodontal disease produce high levels of the virulence factor leukotoxin, whereas most strains isolated from healthy individuals produce significantly lower levels of leukotoxin (32). We had previously shown by Northern blot analysis that the difference in the levels of leukotoxin in the assorted strains was a direct reflection of the levels of leukotoxin RNA (25). In this study, classical *cis/trans* experiments were used to show that both *cis* regulatory elements and *trans*-acting transcription factors are involved in determining the different levels of leukotoxin produced among the three strains tested. Sequence comparisons and promoter mapping indicate that some of the *cis* elements involved in the interstrain regulation are found downstream of the leukotoxin promoter, within the transcribed region of the operon.

The leukotoxin promoter regions have been cloned and sequenced from four strains: JP2 (5, 17), a high-level leukotoxin producer; Y4 and 652 (5), which produce moderate levels of leukotoxin; and ATCC 33384, a low-level leukotoxin producer. The major difference among the sequences is that JP2 has a deletion of 528 bp that moves the first half of an open reading frame of unknown function (*orfX*) to a position 53 bp upstream of *lktC*. This is identical to the result reported by Brogan et al. (5). This group found three additional base pair changes between the leukotoxin promoter regions of JP2 and 652, a moderate-level leukotoxin producer. These three changes are probably not important in determining the relative levels of leukotoxin produced, since we have shown that the sequence of another moderate-level producer of leukotoxin, strain Y4, is identical in sequence to JP2, except for the 528-bp deletion. Importantly, the -10 and -35 sequences that constitute the P_1 leukotoxin promoter are identical in all four strains sequenced. This indicates that the "strength" of the P_1 promoter is not the only factor in determining the different levels of *lkt* RNA produced by the four strains of *A. actinomycetemcomitans*. Finally, the sequence comparisons suggest that strains ATCC 33384 and Y4 diverged from each other before JP2 arose from Y4.

Strains Y4 and ATCC 33384 have a 450-bp open reading frame (*orfX*) which is truncated in JP2. Brogan et al. (5) found the same open reading frame in strain 652. This gene is transcribed (and presumably translated) in strain Y4. The predicted sequences of the OrfX proteins from the three strains are different. The six base pair changes between Y4 and 652 result in five amino acid changes, three of which are conservative. Strains ATCC 33384 and Y4 have 29 amino acid differences between their OrfX proteins, whereas the 652 and ATCC 33384 OrfX proteins differ at 31 positions. In both cases, about half of the amino acid changes are conservative and half are nonconservative. The possible effects of these amino acid changes on *orfX* function are unclear, since its activity is undefined; there are no close homologs in the protein databases. However, we have shown that *orfX* is not an

essential gene under standard laboratory growth conditions, since the *orfX* deletion strains created for the studies reported here grow as well as do cells of the parental Y4 strain (data not shown).

Both S1 nuclease analysis and primer extension experiments clearly showed that leukotoxin RNA in strain JP2 initiates at two sites 336 and 337 bp before the *lktC* gene. These sites are immediately downstream of the -10 and -35 promoter consensus sequences identified by Kraig et al. (17) at positions -344 and -366 by sequence analysis. Although Brogan et al. (5) also found that the leukotoxin RNA started at -336 in JP2, they did not find the -337 start site. The reason for the discrepancy between the two sets of data is not clear, but they both lead to the conclusion that the consensus sequences at $-344/-366$ constitute the leukotoxin promoter called P_1 .

Unlike previous reports (5), we found no evidence for a second leukotoxin initiation site near *lktC* in strain JP2 even if we used the same primer as they did in a primer extension experiment. As was suggested by Brogan et al., this additional site, called P_2 , could result from an RNA degradation intermediate as opposed to being a consequence of a true initiation event. Consistent with this, we did observe a faint band at the position expected for a " P_2 " RNA on long exposures of some S1 nuclease analysis gels. In addition, Brogan et al. found 4.2- and 3.8-kb leukotoxin transcripts on Northern blots of JP2 RNA (5). The sizes and hybridization patterns of these RNAs were consistent with initiation of the 4.2-kb RNA at P_1 and initiation of the 3.8-kb RNA at P_2 , the interpretation favored by Brogan et al. (5). Alternatively, we suggest that the 3.8-kb RNA is a relatively stable intermediate in the breakdown of the 4.2-kb *lkt* RNA. Our conclusion is supported by the fact that the major leukotoxin transcript we found on Northern blots of JP2 RNA was 4.3 kb (25) and by the fact that we did not see a start site at P_2 by S1 nuclease analysis or primer extension experiments. Finally, Brogan et al. (5) reported that deletion of the P_2 sequences on a leukotoxin promoter reporter gene plasmid led to a decrease in leukotoxin levels (as measured from a β -galactosidase fusion in *E. coli*). We believe that their experiments do not provide strong evidence for the existence of a P_2 promoter in JP2, for two reasons. First, there was no significant difference between the levels of LacZ expressed from the plasmid without P_2 (plasmid pLTX63) and those made from the plasmid containing an extra 70 bp of DNA, including P_2 (plasmid pLTX135) (5). Second, Brogan et al. (5) assayed their promoter constructs in *E. coli*, which may not reflect accurately what occurs in *A. actinomycetemcomitans*. Indeed, we have shown that the Y4 promoter deletion plasmids gave different absolute and relative values when the levels of LktA::LacZ were measured in *E. coli* and *A. actinomycetemcomitans* (data not shown). Therefore, we conclude that there is no second leukotoxin promoter just upstream of *lktC* in strain JP2.

Leukotoxin RNA synthesis in strain Y4 also appears to initiate at P_1 . This would generate an RNA transcript that should be 550 bases longer than the P_1 -initiated transcript from strain JP2. However, our previous results indicated that the major *lkt* transcript in strain Y4 was, if anything, slightly smaller than the major *lkt* RNA in JP2 (25). This apparent discrepancy can be explained by our results with Y4, indicating that the leukotoxin RNA that initiates at P_1 is rapidly degraded or processed into an *orfX*-containing RNA about 750 bp in size and a 3.85-kb RNA containing *lktCA*. It is this latter RNA species that appears as the major leukotoxin transcript on Northern blots of Y4 RNA. From the DNA sequences, these *lktCA* RNAs from Y4 should be about 100 to 200 bases shorter than the major JP2 *lkt* transcript; this is what was observed on Northern blots

(25). The Y4 leukotoxin RNA end-mapping data are also consistent with this interpretation. Both S1 and primer extension analyses indicate that there is a leukotoxin RNA end in the region between *orfX* and *lktC*, but the results are often different between experiments. This could arise from the distinct possibility that the leukotoxin RNA is degraded or processed to somewhat different extents in different experiments. It is not clear whether this processing occurs *in vivo* or during sample preparation.

Why are leukotoxin levels lower in strain Y4 than in JP2? The *cis/trans* experiments indicate that the different leukotoxin levels are due mainly, if not solely, to differences in the sequences in the leukotoxin regions of these two strains. Since both JP2 and Y4 *lkt* RNA initiate at the P₁ promoter and since the only sequence difference between the two leukotoxin regions is the extra 528 bp between *orfX* and *lktC* in Y4, there must be a sequence in the 528-bp element that is involved in down-regulating Y4 leukotoxin RNA relative to JP2 *lkt* RNA. Several mechanisms are possible for this down-regulation, including specific RNA turnover and termination models; these are being investigated.

Why are leukotoxin levels lower in ATCC 33384 than in JP2 (and Y4)? In this case, the *cis/trans* experiments indicate that the 30-fold difference in *lkt* RNA levels between strain JP2 and strain ATCC 33384 is due equally to *cis* (promoter sequence) and *trans* (strain-specific DNA binding factor) effects. Specifically, the extent of the *trans* effect is demonstrated by the fact that the level of LktA::LacZ from an ATCC 33384 promoter construct (pDK543) is six- to sevenfold lower in strain ATCC 33384 than in strain JP2 (Table 1). One possibility is that the levels are lower in strain ATCC 33384 because the plasmid copy number is lower in that specific strain. We do not think that this is true, for two reasons. First, the vector used encodes its own replication initiation proteins. Second, if the lower LktA::LacZ levels in ATCC 33384 were due to a lower plasmid copy number, the ratio of β -galactosidase between ATCC 33384 and Y4 (or ATCC 33384 and JP2) for a given plasmid should be the same for all three plasmids. This was not the case (Table 1). However, even if the plasmids had lower copy numbers in ATCC 33384, the data would still lead to the conclusion that there are *trans* factor differences among the strains, although their contribution to the different leukotoxin levels would not be as great. Thus, we conclude that there must be a regulatory protein that controls a portion of the interstrain difference in expression. We hypothesize that either strain ATCC 33384 has a negative regulatory protein (that is missing or mutated in Y4 and JP2) or that JP2 and Y4 carry an activator that is missing or mutated in ATCC 33384. This factor (positive or negative) is presumed to work in regulating the initiation of transcription from the leukotoxin P₁ promoter. Studies are under way to identify and characterize the protein involved in the interstrain regulation of leukotoxin RNA synthesis.

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