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

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Abstract

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

Keywords: *Aggregatibacter actinomycetemcomitans*; Integration host factor; Bacterial gene expression regulation; Leukotoxin

1. Introduction

Periodontal disease, which can lead to soft tissue degeneration and significant bone loss, results from the inflammatory response to bacteria in the subgingival crevice. The development of periodontitis is associated with a shift in the subgingival flora; there is a dramatic increase in the levels of several species of gram-negative bacteria. Because the periodontal pocket is a relatively anaerobic environment, most of the bacteria found in deep pockets are anaerobes (Haffajee and Socransky, 1994; Slots and Ting, 1999), but at least one periodontal pathogen, *Aggregatibacter actinomycetemcomitans,* is a facultative organism. This gram-negative bacterium is the major pathogen in the etiology of localized aggressive periodontitis (LAP) (Fine et al., 2006, 2007; Slots and Ting, 1999), although it can also be involved in cases of rapidly progressive and adult periodontitis (Slots and Ting, 1999). Understanding the mechanisms by which *A. actinomycetemcomitans* establishes a subgingival infection requires the identification and characterization of potential virulence factors. A number of virulence components have been described in *A. actinomycetemcomitans* (Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003), including leukotoxin, a 116 kDa protein that kills human neutrophils, monocytes, natural killer cells (Fine et al., 2006; Henderson et al., 2003; Taichman and Wilton, 1981) and erythrocytes (Balashova et al., 2006). At lower doses, leukotoxin can also induce apoptosis in other cell types, including lymphocytes (Fine et al., 2006; Korostoff et al., 1998). Thus, this protein is

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thought to be critical in helping *A. actinomycetemcomitans* evade the host innate immune response (Bueno et al., 1998; Fine et al., 2006; Haubek et al., 1997; Taichman and Wilton, 1981).

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

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

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

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. actinomycetemcomitans cells were grown anaerobically (5% CO_2, 10% H_2, 85% N_2) in a Coy chamber (Coy Laboratory Products, Ann Arbor, MI) at 37 °C or aerobically (5% CO_2, 95% air) in a CO_2 incubator with shaking at 37 °C. The growth media was TSBYE (3% tryptic soy broth plus 0.6% yeast extract) containing, where indicated, spectinomycin to a final concentration of 50 μg/ml or ampicillin at a final concentrations of 10 μg/ml.

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

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

2.2. Purification and identification of the ORE binding protein

JP2 cells (2 L) were grown anaerobically overnight, harvested and disrupted on a French press. After centrifugation (15 min, 11,000×*g*), the soluble material was made 4 M in NaCl and 1:3 w:w polymer mix (6.4% Dextran 500 K, 25.6% PEG 8000, 4 M NaCl) was added slowly and stirred on ice for 60 min to remove nucleic acid (Schleif and Wensink, 1987).