Regulation of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* Leukotoxin Secretion by Iron[▽]

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The gram-negative oral and systemic pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans* produces a leukotoxin (LtxA) that is a member of the RTX (repeats in toxin) family of secreted bacterial toxins. We have recently shown that LtxA has the ability to lyse erythrocytes, which results in a beta-hemolytic phenotype on Columbia blood agar. To determine if LtxA is regulated by iron, we examined beta-hemolysis under iron-rich and iron-limiting conditions. Beta-hemolysis was suppressed in the presence of FeCl₃. In contrast, strong beta-hemolysis occurred in the presence of the iron chelator deferoxamine. We found that secretion of LtxA was completely inhibited by free iron, but expression of *ltxA* was not regulated by iron. Free chromium, cobalt, and magnesium did not affect LtxA secretion. Other LtxA-associated genes were not regulated by iron. Thus, iron appears to play an important role in the regulation of LtxA secretion in *A. actinomycetemcomitans* in a manner independent of gene regulation.

Aggregatibacter (formerly *Actinobacillus*) *actinomycetemcomitans* (30) is a highly adherent (10, 20) gram-negative pathogen that colonizes the oral cavity of humans and Old World primates. In humans, *A. actinomycetemcomitans* is the etiologic agent of localized aggressive periodontitis, a rapidly progressing oral disease that occurs primarily in adolescents (11, 35). *A. actinomycetemcomitans* is also a member of the HACEK group of bacteria, which is implicated in causing subacute infective endocarditis (5).

A. actinomycetemcomitans produces the RTX toxin leukotoxin (LtxA) as part of its array of virulence factors (23, 26, 27). LtxA is a secreted ~114-kDa protein that is homologous to *Escherichia coli* α -hemolysin (42, 43), *Bordetella pertussis* adenylate cyclase (12), and *Mannheimia haemolytica* leukotoxin (6, 13). RTX toxins are soluble proteins that destroy target cells by disrupting cell membranes (4, 32).

LtxA appears to act as an important virulence factor for *A. actinomycetemcomitans* by helping the bacterium evade the host immune response. The toxin has been reported to be highly specific for human and primate leukocytes (38, 40, 41). In addition, we recently showed that LtxA is able to lyse both human and nonprimate erythrocytes (3). As a result, LtxA-mediated erythrocyte lysis confers beta-hemolytic activity to *A. actinomycetemcomitans* (3). Erythrocyte lysis is one mechanism by which a bacterial pathogen could acquire iron.

Previous studies showed that LtxA production is regulated by oxygen (16, 24, 31, 37), but little is known about other factors that might regulate production or secretion of LtxA in *A. actinomycetemcomitans*. Because of our recent discovery that LtxA is able to lyse erythrocytes (3), we wondered whether

leukotoxin also plays a role in iron acquisition. Proteins that are involved in iron uptake and metabolism are often regulated by iron. We show here that LtxA secretion is strongly affected by the presence of free iron. This represents the first report of iron-mediated regulation of LtxA in *A. actinomycetemcomitans*.

Beta-hemolysis under different iron conditions. To determine if iron plays a role in LtxA regulation, we assayed beta-hemolysis under excess-iron and iron-limiting conditions on Columbia agar with 5% sheep blood (3). We found that, compared to results obtained with media with no added iron, beta-hemolysis was suppressed when *A. actinomycetemcomitans* was grown on Columbia agar containing 300 μ M FeCl₃ (Fig. 1). This difference was less pronounced with the highly leukotoxic strain JP2 (8, 36, 41) than with the minimally leukotoxic strain DF2200 (21), but it was highly reproducible and could be due to the greater total amount of LtxA produced by strain JP2. In contrast to the results obtained under high-iron conditions, beta-hemolysis was increased when an iron chelator, deferoxamine (DFO; 100 μ M), was included in the medium (Fig. 1). Both strains JP2 and DF2200 showed strongly hemolytic phenotypes on the blood agar with DFO. We observed similar effects with another iron chelator, 2,2'-dipyridyl, but found that DFO was more effective (data not shown). Because LtxA is required for beta-hemolysis (3), these results suggest that LtxA production is regulated by iron.

Secretion of LtxA is regulated by iron. To determine which step of LtxA production is regulated by iron, the highly leukotoxic *A. actinomycetemcomitans* strain JP2 was grown in AAGM broth (10) and Columbia broth (Accumedia, Baltimore, MD) in the presence or absence of excess FeCl₃ (300 μ M). After approximately 16 h of growth, bacterial cells were separated from the supernatant by centrifugation. Equal amounts of cell-associated and secreted protein (supernatant) were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

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FIG. 1. Beta-hemolysis by *A. actinomycetemcomitans* on Columbia agar with 5% sheep blood. Strains JP2 and DF2200 were streaked onto medium containing 300 μ M FeCl_3 , no additional components (0), or 100 μ M DFO. Plates were incubated for 3 days at 37°C in 10% CO_2 before imaging.

analysis using antileukotoxin antibody (9). Figure 2 shows that without added FeCl_3 , LtxA is predominantly secreted, while a small amount remains associated with cells. In contrast, under conditions of excess iron, LtxA was not secreted but was instead associated with cells (Fig. 2). The results were identical for both AAGM and Columbia broth. Further, chromium (300 μ M $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), cobalt (300 μ M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), and magnesium (300 μ M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) had no effect on LtxA secretion (Fig. 2).

The above results suggest that LtxA secretion, rather than production per se, is regulated by iron. To confirm that *ltxA* synthesis is not affected by iron, we determined the levels of *ltxA* mRNA produced under normal and excess-iron growth conditions by quantitative real-time PCR (qRT-PCR) using a quantitative RT-PCR ReadyMix kit according to the manufacturer's protocol (Sigma, St. Louis, MO) (Table 1). Highly leukotoxic strains JP2 (36, 41) and NJ4500, a fresh clinical isolate (9, 21), were grown in AAGM broth with and without 300 μ M FeCl_3 . Relative to the expression of housekeeping genes *glyA* (29) and *gapdh* (34) (Table 1), we found that the ratio of *ltxA* mRNA levels under excess-iron conditions to those under normal growth conditions was approximately 1 (Table 2). This result indicates that expression of *ltxA* is not affected by iron.

We offer two models to explain how iron might regulate LtxA secretion in *A. actinomycetemcomitans*. First, modulation

FIG. 2. Western blot analysis of secreted (super) and cell-associated (pellet) LtxA. Strain JP2 was grown in AAGM with or without the metal indicated at the left. Supernatant and cell pellet protein were resolved through an SDS-PAGE gel, transferred to a nitrocellulose membrane, processed for Western blot analysis using anti-LtxA antibody.

TABLE 1. Primers and molecular beacons used for qRT-PCR analysis^a

Gene and primer or beacon	Sequence
Primers	
<i>glyA</i>	
glyA-s	CCCAATTCACCAACAAATATGC
glyA-as	ATTCTTTCGCACGCTCAATAG
<i>gapdh</i>	
gapdh-s	CCCAAAACATCATCCCATCTTC
gapdh-as	GGAACACGGAACGCCATAC
<i>ltxA</i>	
ltxA-s	GTGCTAGGTAACATCGGTAAAG
ltxA-as	GACCACAGAGGCAATTAACC
<i>ltxD</i>	
ltxD-s	CCAGCAAGTCTCTGAAATTCTG
ltxD-as	CTTCTTCGGGCACAACCTACC
<i>tdeA</i>	
tdeA-s	TCCACAAGCTCTGACTCAAG
tdeA-as	AGCAAATAGTTTACGGGAATGG
Molecular beacons	
<i>glyA</i>	
glyA-mb	CGCGATCTTCACAACCGCCATA ATAACGCTTGGATCGCG
<i>gapdh</i>	
gapdh-mb	CGCGATCGCAGGAATAACTTTA CCTACGGCTTTGATCGCG
<i>ltxA</i>	
ltxA-mb	CGCGATCTTGATTGCTCAACGT GCTGCAATCGCG
<i>ltxD</i>	
ltxD-mb	CGCGATCCCGTAGTAACAACGC CATCTATTGTGATCGCG
<i>tdeA</i>	
tdeA-mb	CGCGATAAGCATCGGCACTCAC TACTGTAATCGCG

^a Primers and beacons specific for *A. actinomycetemcomitans* were designed using Beacon Designer version 5.1 software (Premier Biosoft Int., Palo Alto, CA).

of secretion might occur through regulated expression of *ltx* secretion genes, such as *ltxB*, *ltxD*, or *tdeA*. LtxB and LtxD are part of the type I secretion system for LtxA (1, 14, 25). TdeA is a TolC-like protein we recently identified in *A. actinomycetemcomitans* as the putative outer membrane channel-form-

TABLE 2. Expression level ratios of genes in *A. actinomycetemcomitans* grown under excess-iron and normal-iron conditions^a

Control gene	Strain	Expression level ratio with test gene		
		<i>ltxA</i>	<i>ltxD</i>	<i>tdeA</i>
<i>gapdh</i>	JP2	0.87 $\pm$ 0.49	0.64 $\pm$ 0.05	0.52 $\pm$ 0.05
	NJ4500	0.77 $\pm$ 0.12	0.39 $\pm$ 0.04	0.57 $\pm$ 0.20
<i>glyA</i>	JP2	1.69 $\pm$ 0.09	0.58 $\pm$ 0.01	0.38 $\pm$ 0.01
	NJ4500	0.91 $\pm$ 0.50	0.99 $\pm$ 0.17	0.85 $\pm$ 0.14

^a Ratio of expression during growth under excess-iron conditions to expression during growth under normal-iron growth conditions.

ing component required for secretion of LtxA and drug efflux (8a). To test if the levels of *ltxD* and *tdeA* mRNA are affected by iron, we performed RT-PCR (Table 1) as described for *ltxA*. The levels of these genes were very similar under excess-iron and normal growth conditions (Table 2), indicating that differences in expression do not account for the complete lack of LtxA secretion under iron-rich conditions.

Second, iron may regulate the activity of a protein required for LtxA secretion. For example, binding of iron to TdeA may result in a structural change in the protein, preventing transport of LtxA through the pore. In support of this hypothesis, Andersen et al. found that the function of TolC was severely inhibited by divalent and trivalent cations (2). They noted that trivalent cations, such as Cr^{3+} , were more potent because of irreversible binding of the metals to an aspartate ring that lines the periplasmic entrance of the assembled protein (2). Interestingly, a comparison of *A. actinomycetemcomitans* TdeA with *E. coli* TolC reveals that TdeA contains two glutamate residues with a relative location (in the last α -helix) and configuration (separated by two amino acid residues) similar to those of the two aspartate residues in TolC. Thus, in our model, iron might bind the negatively charged glutamate residues of TdeA, causing constriction of the pore and preventing secretion of LtxA.

Other RTX toxins have been shown to be regulated by iron. Interestingly, iron regulates expression of these other RTX toxins at the transcriptional level, which represents a mechanism different from what we have observed for *A. actinomycetemcomitans*. Marciel and Highlander (28) showed that transcription of *Mannheimia haemolytica* leukotoxin (Lkt) is increased in the presence of an iron chelator. Similarly, the RTX toxin of *Neisseria meningitidis* (FrpC) is up-regulated under iron-limiting conditions (39). Thus, whatever the mechanism, iron appears to play a negative regulatory role in RTX toxin production.

A. actinomycetemcomitans LtxA is both secreted (18, 19) and associated with the outer membrane of the cell (7, 9, 17, 22). We show here that secretion of LtxA is required for hemolysis, since iron affects secretion but not *ltxA* synthesis. Thus, it is tempting to speculate that the cell-associated and secreted forms of the toxin may play distinct functional roles (9).

Conclusions. Suggesting a role for LtxA in iron acquisition may also shed light on the evolution of high leukotoxicity in *A. actinomycetemcomitans*. High leukotoxicity results from increased *ltx* promoter activity due to a 530-bp deletion in the promoter region (8). Interestingly, Hayashida et al. (15) found that none of the highly leukotoxic strains (JP2 type) of *A. actinomycetemcomitans* they examined was able to utilize hemoglobin as an iron source due to a nonfunctional hemoglobin binding protein A (HgpA). Because *A. actinomycetemcomitans* is unable to obtain iron from human transferrin or lactoferrin (15, 44) and does not produce siderophores (33, 44), hemoglobin may be an important physiological source of iron for *A. actinomycetemcomitans*. It is tempting to speculate that the 530-bp deletion was selected for in strains that contained a nonfunctional HgpA because the increased production of LtxA may have allowed these bacteria to lyse more erythrocytes and release a greater amount of other usable forms of iron.

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