INFECTION AND IMMUNITY, Apr. 2006, p. 2015–2021 Vol. 74, No. 4
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.4.2015–2021.2006

Leukotoxin Confers Beta-Hemolytic Activity to *Actinobacillus actinomycetemcomitans*

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Received 11 October 2005/Returned for modification 6 December 2005/Accepted 8 January 2006

Actinobacillus actinomycetemcomitans **is the etiologic agent of localized aggressive periodontitis, a rapidly progressing oral disease that occurs in adolescents.** ***A. actinomycetemcomitans*** **can also cause systemic disease, including infective endocarditis. In early work on** ***A. actinomycetemcomitans*** **workers concluded that this bacterium is not beta-hemolytic. More recent reports have suggested that** ***A. actinomycetemcomitans*** **does have the potential to be beta-hemolytic. While growing** ***A. actinomycetemcomitans*** **on several types of growth media, we noticed a beta-hemolytic reaction on media from one manufacturer. Beta-hemolysis occurred on Columbia agar from Accumedia with either sheep or horse blood, but not on similar media from other manufacturers. A surprising result was that mutants of** ***A. actinomycetemcomitans*** **defective for production of leukotoxin, a toxin that is reportedly highly specific for only human and primate white blood cells, are not beta-hemolytic. Purified leukotoxin was able to lyse sheep and human erythrocytes in vitro. This work showed that in contrast to the accepted view,** ***A. actinomycetemcomitans*** **leukotoxin can indeed destroy erythrocytes and that the production of this toxin results in beta-hemolytic colonies on solid medium. In light of these results, the diagnostic criteria for clinical identification of** ***A. actinomycetemcomitans*** **and potentially related bacteria should be reevaluated. Furthermore, in studies on** ***A. actinomycetemcomitans*** **leukotoxin workers should now consider this toxin's ability to destroy red blood cells.**

Actinobacillus actinomycetemcomitans is a gram-negative bacterium that inhabits the oral cavity of a large percentage of the population (16, 35, 46). Under certain conditions, *A. actinomycetemcomitans* is able to cause disease, notably localized aggressive periodontitis (LAP) (35, 45, 46). LAP is a rapidly progressing and destructive disease of the mouth that affects approximately 0.5% of the adolescent population of the United States (29). LAP is irreversible, and when untreated, it may result in loss of teeth. *A. actinomycetemcomitans* is also a systemic pathogen and is a HACEK organism (3, 8, 30). The HACEK bacteria (*Haemophilus aphrophilus*, *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) are responsible for a large fraction of the infective endocarditis cases caused by gram-negative organisms (3, 8). Interestingly, all the HACEK bacteria are also part of the human normal oral flora. Indeed, the link between oral bacteria and systemic disease is well established (2, 12, 31).

An important virulence trait exhibited by many pathogens is hemolysis. Hemolysins play a role in disease by releasing iron from erythrocytes and making it available for the invading pathogen (7, 17, 28). Three types of hemolysis have been described (11). When grown on solid medium supplemented with blood, bacteria are alpha-hemolytic, beta-hemolytic, or gamma-hemolytic. In alpha-hemolysis, bacteria do not completely lyse erythrocytes, and the area surrounding colonies becomes yellowish green due to the action of peroxide. During beta-hemolysis, bacteria completely lyse erythrocytes, which results in clear zones around colonies. The lack of any type of hemolysis is given the unfortunate designation gamma-hemolysis (or the preferred designation nonhemolysis) (11).

In general, *A. actinomycetemcomitans* is described as an organism that is not beta-hemolytic (4). In one study, a survey of 69 strains of *A. actinomycetemcomitans* showed that only alpha-hemolysis was observed on blood agar (1). However, in a few reports workers have suggested that a hemolysin may be present in *A. actinomycetemcomitans*. In 1996, Kimizuka et al. (24) reported that approximately 60% of the *A. actinomycetemcomitans* strains that they tested formed beta-hemolytic colonies on blood agar. A year later, Haubek et al. (14) noted that only serotype b strains of *A. actinomycetemcomitans* formed beta-hemolytic colonies on blood agar after prolonged incubation. Thus, reports on the hemolytic properties of *A. actinomycetemcomitans* appear to be inconsistent, and the identity of a potential hemolysin in *A. actinomycetemcomitans* remains elusive.

During growth of *A. actinomycetemcomitans* on different types of media, we noticed various hemolytic reactions. We report here that all *A. actinomycetemcomitans* strains tested showed beta-hemolysis on blood agar and that this phenotype is dependent on the type of growth medium used for culturing. Significantly, we found that the hemolysin is in fact the widely studied leukotoxin (LtxA) of *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strain JP2 (43), strains DF2200, IDH781, and NJ4500 (22), and strain CU1000 (9, 10) have been described previously. Strain JP2 is a nonadherent, smooth laboratory isolate, while strains DF2200, NJ4500, and CU1000 are all adherent, rough clinical isolates obtained from patients with LAP. Strain IDH781 is an adherent, rough isolate that was isolated from the mouth of an individual who, at the time of isolation, did not show symptoms of LAP. *A. actinomycetemcomitans* growth medium (AAGM) has been described previously (9, 20). The following growth media were prepared according the manufacturers' instructions: Trypticase soy agar from BBL-Becton

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Dickinson, Columbia agar from BBL-Becton Dickinson, and Columbia agar from Accumedia. When indicated below, blood was added to the media after they were allowed to cool to 48°C. Sheep blood (final concentration, 5%; PML Microbiologicals, Inc., Wilsonville, OR, or HemoStat Laboratories, Dixon, CA) was used for all blood agar plates. After bacteria were streaked on solid media, plates were incubated at 37°C in the presence of 10% CO_2 for 2 to 3 days. Colonies were inoculated into AAGM broth (9, 10) and incubated for 24 h unless indicated otherwise.

Isolation of *ltxA* mutants. The mutagenesis method used for isolation of an *ltxA* mutant of strain JP2N (a nalidixic acid-resistant variant of JP2) has been described previously (20, 21). Briefly, bacteria harboring the transposon mutagenesis plasmid pVJT128 (20, 41) were maintained in AAGM with chloramphenicol (2.0 μg/ml). The nonpolar transposon IS*903*φ*kan* was induced by growing cells in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Transposon mutants were selected by plating cells on AAGM with kanamycin (40 μg/ml). We screened for leukotoxin mutants by inoculating independent transposon mutants into wells of 96-well dishes, incubating the dishes (as described above) for 24 h, and then centrifuging the dishes and collecting supernatants. Several microliters of each supernatant were spotted onto nitrocellulose, and the membrane was then probed with anti-leukotoxin antibody (7a). The membrane was processed for Western blot analysis as previously described (6). Supernatants that did not yield a dark spot after exposure of the blot to X-ray film were considered mutants.

Allelic exchange of a transposon containing a kanamycin resistance cassette into *ltxA* in DF2200N (a nalidixic acid-resistant variant of DF2200) was carried out using a recently developed method (M. K. Bhattacharjee, B. A. Perez, S. C. Kachlany, and D. H. Figurski, unpublished data). This technique has recently been used by Rhodes et al. (33). Briefly, strain DF2200N expressing a competence-inducing gene was transformed with a suicide vector containing *ltxA* insertionally inactivated with EZ-Tn<KAN-2> (Epicenter Biotechnologies, Madison, WI). The EZ-Tn<KAN-2> transposon contains a cassette encoding kanamycin resistance. Allelic exchange mutants were selected on AAGM with kanamycin (40 μg/ml). Thus, EZ-Tn< KAN-2> transposition was carried out in vitro, followed by recombination of the cassette into *ltxA* in vivo.

Complementation of *ltxA* mutation. For complementation studies, we first amplified *ltxA* from *A. actinomycetemcomitans* using high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The ~3.0-kb product was cloned into pCR-XL-TOPO according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The *ltxA* gene was then subcloned into pJAK16 as described previously (20). The new plasmid containing *ltxA* (pSK248) or the empty vector (pJAK16) was mobilized into *A. actinomycetemcomitans* as previously described (20). Expression of LtxA was carried out on blood medium containing 0.5 mM IPTG. Plates were incubated as described above until strong beta-hemolysis was observed, usually 7 days after inoculation.

RT-PCR of *ltxB*. Strains JP2N and 1704 were grown overnight in AAGM broth. Cells from 1 ml of medium were pelleted by centrifugation at 12,000 × *g* for 3 min. Total RNA was isolated from the cell pellet using TRIzol reagent (Roche, Nutley, NJ) according to the manufacturer's protocol. The reverse transcription (RT)-PCR was carried out using a OneStep RT-PCR kit (QIAGEN, Valencia, CA). Reverse transcription was performed at 50°C for 30 min, and this was followed by initial PCR activation at 94°C for 15 min. PCR amplification was performed for 35 cycles using an annealing temperature of 60°C for 1 min and primer extension at 72°C for 2 min. The primers used were LtxBUP (5′-CGCAAATTCGTTAGCAACTACTGC-3′) and LtxBDOWN (5′-CAAGTTTTCATTATCGTTCGTTCC-3′).

Examination of secretion of LtxA. To confirm that AA1704 and AA1700 were defective in LtxA production, we isolated total protein from supernatants of cultures as previously described (19). Briefly, after cells were grown overnight in AAGM, cultures were centrifuged, and 500 μl of supernatant was precipitated with 1 ml ice-cold ethanol. For JP2 and AA1704, the pelleted protein was resuspended in sodium dodecyl sulfate (SDS) loading dye and electrophoresed on an SDS-polyacrylamide gel electrophoresis (PAGE) gel. The gel was stained with Coomassie blue and visualized. For DF2200N and AA1700, the pellet was resuspended in phosphate-buffered saline, spotted onto a nitrocellulose membrane, and probed with anti-leukotoxin antibody as described above. Because DF2200N is a minimally leukotoxic strain, less LtxA is present in the supernatant of this strain than in the supernatant of JP2. Thus, a dot blot format provides greater sensitivity than SDS-PAGE.

Purification of LtxA from *A. actinomycetemcomitans*. LtxA was isolated from JP2 as previously described (18), with some modifications (7a). Briefly, cells were grown in 5 ml AAGM broth for 7 to 9 h and then diluted into 400 ml fresh AAGM broth. The new cultures were then grown for 13 to 17 h before supernatant was harvested. To obtain supernatant, cultures were centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was filtered through a 0.22-μm low-protein-binding membrane filter. For every 100 ml of filtered supernatant, 32.5 g $(NH_4)_2SO_4$ was added. The mixture was gently rocked at 4°C for 1 h. The precipitated protein was collected by centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet from 400 ml supernatant was then resuspended in 2 ml LtxA buffer (20 mM Tris-HCl [pH 6.8], 250 mM NaCl, 0.2 mM $CaCl_2$). The resuspended pellet was loaded onto a column packed with 40 ml of Sephadex G-100 (Sigma, St. Louis, MO). The protein was eluted in 1-ml fractions with LtxA buffer. The protein content in each fraction was determined with the Bradford reagent. The three fractions with the highest protein contents were combined, aliquoted, and stored at −80°C. The purity of LtxA was determined on a 4 to 20% SDS–PAGE gel, and the concentration was determined by the bicinchoninic acid assay performed according to the manufacturer's protocol (Pierce, Rockford, IL).

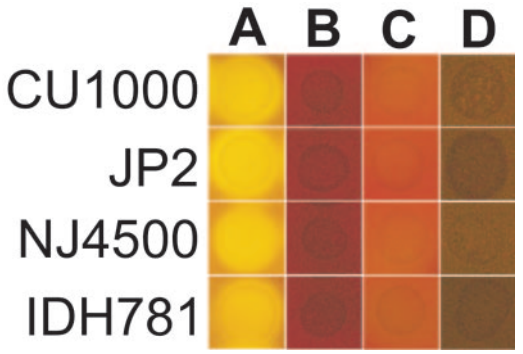

FIG. 1. Hemolytic reactions of *A. actinomycetemcomitans* strains on different growth media. Liquid cultures of strains CU1000, JP2, NJ4500, and IDH781 were spotted on growth media containing 5% sheep blood. Lane A, Columbia agar from Accumedia; lane B, Columbia agar from BBL-Becton Dickinson; lane C, Trypticase soy agar from BBL-Becton Dickinson; lane D, AAGM.

Erythrocyte lysis assay. Hemolytic activity was measured by detection of released hemoglobin from sheep and human erythrocytes. Sheep blood was obtained from PML Microbiologicals, Inc. (Wilsonville, OR) and HemoStat Laboratories (Dixon, CA). For routine culturing and hemolytic determination, the blood from PML Microbiologicals was used. Human blood from a healthy volunteer was collected in a tube containing heparin sulfate. Prior to the assay, both sheep blood and human blood were centrifuged to collect erythrocytes. The erythrocytes were centrifuged at 600 × *g* for 10 min and washed in 0.85% NaCl–20 mM $CaCl_2$–10 mM Tris-HCl (pH 7.4) until the supernatant was clear (five times). Reactions were performed in 1-ml reaction mixtures containing 1% erythrocytes and 10 μg of leukotoxin, heat-inactivated leukotoxin (65°C for 20 min), or an equivalent volume of LtxA resuspension buffer. Sonic extract from JP2N was added to 1% sheep erythrocytes at a final concentration of 35 μg/ml. The reaction mixtures were incubated at 37°C for the times indicated below. At each time, erythrocytes were removed by centrifugation, and the optical density at 450 nm of the supernatant was determined with a Synergy HT plate reader (BioTek Instruments, Winooski, VT). One hundred percent cell lysis was determined by resuspending erythrocytes in distilled water.

RESULTS

Beta-hemolysis of *A. actinomycetemcomitans* is dependent on the growth medium. While growing *A. actinomycetemcomitans* on different growth media supplemented with 5% sheep blood, we noticed various beta-hemolytic reactions. The results were consistent regardless of the strain used. Figure 1 shows that strains CU1000, JP2, NJ4500, and IDH781 all exhibited beta-hemolysis on Columbia agar from Accumedia (Fig. 1, lane A) but not on Columbia agar from BBL-Becton Dickinson (lane B), Trypticase soy agar (lane C), or AAGM (lane D). The effect was independent of the blood since sheep blood from two different suppliers and horse blood all yielded identical results (data not shown). Hemolysis was apparent on solid media after 2 to 3 days of growth.

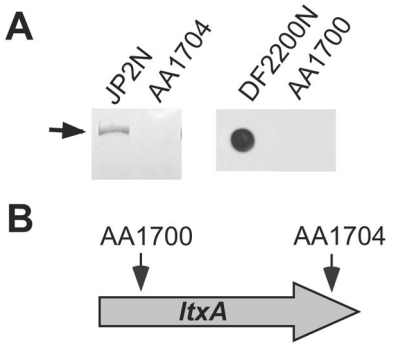

FIG. 2. Construction of *ltxA* mutants of *A. actinomycetemcomitans*. (A) Coomassie blue-stained SDS-PAGE gel of supernatants from JP2N and AA1704 (left panel) and dot blot of supernatants from DF2200N and AA1700 probed with anti-LtxA antibody (right panel). The arrow indicates the band representing LtxA. (B) Schematic diagram of *ltxA* and the insertion sites of mutations in AA1700 and AA1704.

Construction of *ltxA* mutants. *A. actinomycetemcomitans* produces leukotoxin, a toxin that is in the same RTX family of proteins as *Escherichia coli* alpha-hemolysin. While alpha-hemolysin is active against erythrocytes, *A. actinomycetemcomitans* LtxA is reported to have very high specificity for human and primate white blood cells. Because LtxA is similar to alpha-hemolysin, we examined whether LtxA could contribute to the hemolytic reaction that we observed for *A. actinomycetemcomitans*. To do this, we generated insertion mutations in the gene that encodes leukotoxin, *ltxA*. We used two strains, the highly leukotoxic strain JP2 (36) and the minimally leukotoxic strain DF2200 (22). For isolation of an *ltxA* mutant of strain JP2, supernatants from random transposon mutants were screened for the presence of LtxA using anti-LtxA antibody as described in Materials and Methods. For generation of an *ltxA* mutant of DF2200, a kanamycin resistance cassette was inserted into *ltxA* using an allelic exchange technique, as described in Materials and Methods. Figure 2A shows that supernatant from either wild-type strain JP2N or wild-type strain DF2200N (spontaneous nalidixic acid-resistant variants of JP2 and DF2200, respectively) contained LtxA, as expected. In contrast, the culture supernatants of the JP2N and DF2200N *ltxA* mutants (AA1704 and AA1700, respectively) both lacked LtxA protein. To identify the sites of insertion in *ltxA*, we amplified *ltxA* from the mutants and sequenced the products. Figure 2B shows that AA1700 had an insertion at the 5′ end of the gene, while AA1704 contained an insertion at the 3′ end.

Leukotoxin is required for beta-hemolysis. Generation of *ltxA* mutants in two different strains of *A. actinomycetemcomitans* allowed us to ask whether LtxA might act as a hemolysin. To our surprise, mutations in *ltxA* (strains AA1704 and AA1700) completely abolished the beta-hemolytic activity of *A. actinomycetemcomitans* (Fig. 3A). The results were identical for both highly a leukotoxic strain (JP2N) and a minimally leukotoxic strain (DF2200N). To confirm that our mutation did not have polar effects on downstream genes, we performed complementation experiments with AA1700. We found that beta-hemolysis was restored when wild-type *ltxA* was expressed on a plasmid in *trans* (pSK248) (Fig. 3B). In contrast, the mutant harboring the vector control (pJAK16) was unable to carry out beta-hemolysis (Fig. 3B). We further confirmed that our mutations were not polar by performing RT-PCR with AA1704. We found that *ltxB*, the gene immediately downstream from *ltxA*, was transcribed at the same level in both JP2N and AA1704 (Fig. 3C). Thus, we obtained genetic evidence for a new activity of LtxA.

Purified leukotoxin destroys erythrocytes. Previous results indicated that LtxA is at least necessary for beta-hemolysis of *A. actinomycetemcomitans*. To determine if the toxin is indeed responsible for the hemolytic reaction, we tested purified LtxA with erythrocytes (Fig. 4A). We found that LtxA was able to lyse sheep erythrocytes (Fig. 4B) and human erythrocytes (Fig. 4C) in a time-dependent manner. Heat-inactivated LtxA or buffer alone caused insignificant lysis of either sheep or human erythrocytes (Fig. 4B and 4C). Additionally, cell extract from *A. actinomycetemcomitans* strain JP2 caused no sheep erythrocyte lysis (Fig. 4B). We also demonstrated that lysis of sheep erythrocytes is strictly dependent on the dose of LtxA used (Fig. 4D). Our results indicated that in contrast to the findings of previous studies, LtxA is capable of lysing erythrocytes.

DISCUSSION

In this work we found that the oral bacterium *A. actinomycetemcomitans* possesses beta-hemolytic activity and that leukotoxin is necessary and sufficient for this property. Previously, *A. actinomycetemcomitans* has usually been reported to be an organism that is not beta-hemolytic (1, 4); however, in two previous reports workers claimed that certain strains of *A. actinomycetemcomitans* can be beta-hemolytic. Kimizuka et al. (24) showed that approximately 60% of the strains that they tested were beta-hemolytic on horse blood agar. In 1997, Haubek et al. (14) reported that only highly leukotoxic strains showed beta-hemolysis on blood agar. In contrast, we found that all strains tested, both highly and minimally leukotoxic strains, exhibited beta-hemolysis. We suggest that the discrepancy between our results and the results of other workers may be due to differences in the type of growth media used for culturing *A. actinomycetemcomitans*. For example, Avila-Campos (1) concluded that *A. actinomycetemcomitans* exhibited only alpha-hemolysis based on growth on Trypticase soy agar with blood, a growth medium that we demonstrate here does not reveal beta-hemolysis (Fig. 1, lane C).

We found that *A. actinomycetemcomitans* is beta-hemolytic on Columbia agar from Accumedia but not on other types of growth media. The reasons for the various hemolytic reactions on the different media are currently being investigated, but other workers have found that growth media and environmental factors can affect hemolytic activity of bacteria (34). It is possible that the Accumedia formula contains something that enhances or activates hemolysis in *A. actinomycetemcomitans* or that the BBL-Becton Dickinson formula and other growth media used in this study (e.g., AAGM) contain a reagent that suppresses hemolysis. In this regard, the possibility of identifying a compound that might interfere with the hemolytic activity of *A. actinomycetemcomitans* is very attractive from a clinical perspective.

A. actinomycetemcomitans produces leukotoxin (LtxA) (25, 27), an RTX toxin belonging to the same family of proteins as *E. coli* alpha-hemolysin, *Bordetella pertussis* adenylate cyclase, and *Mannheimia hemolytica* leukotoxin (37, 44). It is likely that

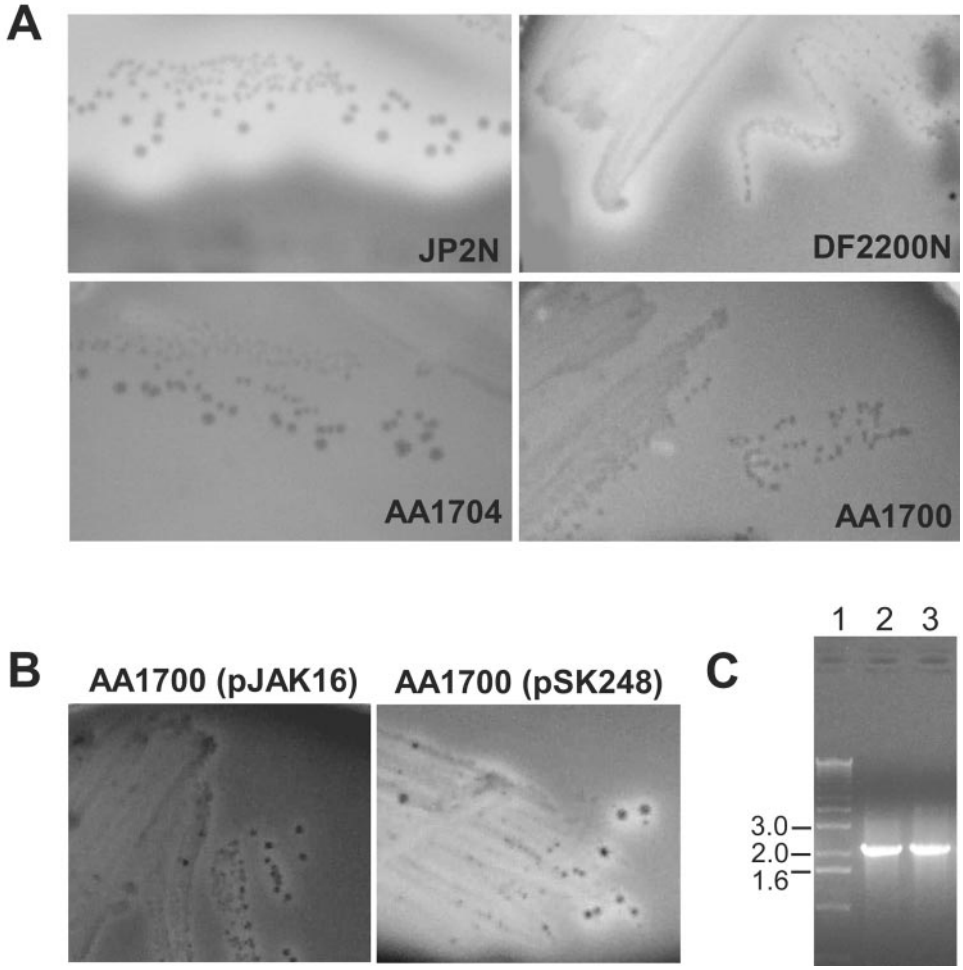

FIG. 3. Beta-hemolysis of the wild type and *ltxA* mutants. (A) Strains were streaked onto Columbia agar (Accumedia) with 5% sheep blood and incubated for 3 days as described in Materials and Methods. AA1704 is an isogenic *ltxA* mutant of JP2N, and AA1700 is an isogenic *ltxA* mutant of DF2200N. (B) Beta-hemolysis complementation of AA1700 with wild-type *ltxA* expressed in *trans*. Bacteria were plated on medium containing 0.5 mM IPTG. (Left panel) AA1700 harboring the vector control; (right panel) AA1700 harboring wild-type *ltxA* in *trans*. (C) Reverse transcription-PCR with *ltxB*. Lane 1, molecular weight ladder (sizes [in kb] are indicated on the left); lane 2, strain AA1704; lane 3, strain JP2N.

leukotoxin plays an important role in *A. actinomycetemcomitans* pathogenesis by helping the bacterium evade host immune responses. Indeed, there is evidence that highly leukotoxic strains are correlated with disease in humans more often than minimally leukotoxic variants are (13, 15). Unlike many of the other RTX toxins, LtxA is not supposed to be active against erythrocytes or cells of nonprimate origin. LtxA has been reported to be specific for human and Old World primate white blood cells (38–40). We were therefore surprised to find that *ltxA* mutants of two different strains of *A. actinomycetemcomitans* were not beta-hemolytic. The fact that a single nonpolar transposon insertion in *ltxA* resulted in a complete defect in beta-hemolysis indicates that LtxA is the only hemolysin in *A. actinomycetemcomitans* expressed under our experimental conditions.

The find that *ltxA* mutants are not beta-hemolytic offers the exciting possibility of using solid media to efficiently screen for leukotoxin mutants of *A. actinomycetemcomitans*. Indeed, we have already begun screening random mutants on Columbia agar with blood (Accumedia) and have identified many mutants with defects in the different stages of LtxA production. This work should reveal a great deal about the genetics of LtxA production and will be the subject of another report (M. P. Palacio, M. S. Duncan, and S. C. Kachlany, unpublished data).

To exclude the possibility that LtxA acts with another protein or factor in vivo to cause hemolysis, we tested the effects of purified LtxA with erythrocytes. Until recently, it was believed that *A. actinomycetemcomitans* does not actively secrete its leukotoxin (5, 26, 32, 43). However, we showed that LtxA is indeed secreted under normal growth conditions, and we developed an efficient protocol to purify a large amount of active LtxA from culture supernatants (18, 19). We report here that purified LtxA indeed lyses both sheep and human erythrocytes in a dose-dependent manner. LtxA appeared to be more active against human erythrocytes than against sheep erythrocytes; however, differences in blood sample preparation, age of the sample, and erythrocyte concentration may account for differences in lysis activity.

In their original study (42), Tsai et al. concluded that LtxA has no lysis activity against erythrocytes. A likely explanation for this conclusion, which conflicts with the work that we de-

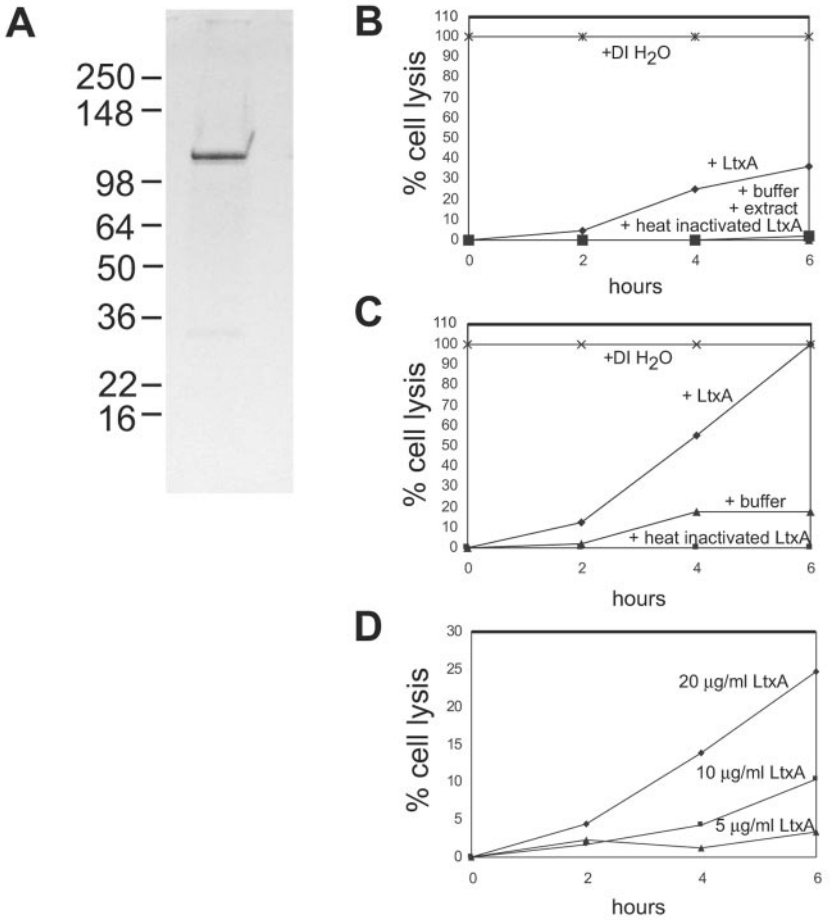

FIG. 4. Erythrocyte lysis by LtxA. (A) Coomassie blue-stained gel of purified LtxA from strain JP2. Approximately 4 μg of protein was loaded on the gel. (B) LtxA activity against sheep erythrocytes. Erythrocytes were washed and resuspended to a concentration of 1% in Tris-HCl buffer. Where indicated, LtxA was added to a final concentration of 10 μg/ml and JP2N cell extract was added to a concentration of 35 μg/ml. The buffer was the buffer in which purified LtxA was resuspended. The results for incubation of erythrocytes in distilled H_2O (DI H_2O) were defined as 100% cell lysis. The lines for erythrocyte incubation with buffer, with JP2N cell extract, and with heat-inactivated LtxA are nearly superimposable. (C) LtxA activity against human erythrocytes. The experimental details are the same as those described above for sheep erythrocytes (excluding extract). The data are representative of the results of triplicate experiments. (D) Dose-dependent lysis of sheep erythrocytes by LtxA.

scribe here, is that Tsai et al. used cell extracts instead of purified LtxA protein. In support of this theory, we found that cell extract from a highly leukotoxic strain of *A. actinomycetemcomitans* (JP2) showed no activity against erythrocytes. Perhaps there was a relatively low level of LtxA in the extract compared to the level of another cellular protein or some factor in the extract inhibited anti-LtxA activity. In subsequent work performed with LtxA purified from whole bacterial cells with polymyxin B Tsai et al. reported that the LtxA was not active against erythrocytes (43). It is possible that the cell-associated form of LtxA has a different specificity than the secreted form that we used. Alternatively, other components (such as lipopolysaccharide) in the polymyxin B preparation may inhibit the hemolytic effects of LtxA.

Kimizuka et al. previously reported isolation of a putative hemolysin from *A. actinomycetemcomitans* (24). However, they found that their hemolysin had a molecular mass of 12 kDa, which is much less than the molecular mass of LtxA (~114 kDa). These workers noted that in their purification scheme most of the hemolytic activity was present in the cell-associated lipid vesicle fraction. Interestingly, it has been shown that lipid vesicles of *A. actinomycetemcomitans* are enriched in LtxA (23). We have also shown previously that breakdown of LtxA is common in older cultures of *A. actinomycetemcomitans* (19). Thus, we suggest that Kimizuka et al. (24) may have indeed isolated LtxA as the hemolysin but that the 12-kDa protein represented one of leukotoxin's major breakdown products (19).

Our work which demonstrated that *A. actinomycetemcomitans* is beta-hemolytic on certain growth media has important diagnostic significance. One of the properties that clinical microbiologists use to identify pathogens in diagnostic laboratories is the hemolysis reaction on blood agar plates. Because microbiology manuals indicate that *A. actinomycetemcomitans* is not beta-hemolytic, cases of systemic *A. actinomycetemcomitans* may be mis- or underidentified. Furthermore, based on the new information presented here, *A. actinomycetemcomitans* pathogenesis should be viewed in a new light. *A. actinomycetemcomitans* can become

systemic and infect several other organs of the body (16). Most significantly, the pathogen can cause infective endocarditis, a serious disease of the heart valves (8). When it becomes systemic and infects heart tissue, *A. actinomycetemcomitans* may use its leukotoxin to destroy erythrocytes. We propose that *A. actinomycetemcomitans* hemolysis may indeed be an important property for the bacterium to cause or sustain disease. The contribution of LtxA-induced hemolysis to pathogenesis should become clear as we continue to study the mechanisms by which LtxA acts.

ACKNOWLEDGMENTS

We thank Paul Planet, Maria Palacio, Laurie Bluth, Angela Jackson-Lee, John Foote, and Christine Cooper for thoughtful comments and suggestions throughout this project.

This work was generously supported by grant R01 DE16133 from the National Institute of Dental and Craniofacial Research.

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Editor: J. T. Barbieri