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Characterization of the Hemolytic Activity of *Haemophilus ducreyi*

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***H. ducreyi* is the causative agent of chancroid, a genital ulcer disease most prevalent in developing countries. Chancroid enhances the heterosexual transmission of human immunodeficiency virus and is identified in focal outbreaks in the United States, but little is known about its pathogenesis. We studied the hemolysin produced by *H. ducreyi* because this molecule might be an important virulence factor in the pathogenesis of chancroid. Ten strains of *H. ducreyi* were tested on newly devised blood agar plates and were found to have hemolytic activity. We examined the hemolytic activity of *H. ducreyi* 35000 further and found that it was heat labile, cell associated, greatest at pH 7.0, and produced in logarithmic- but not stationary-phase cultures. Using transposons Tn*916* and Tn*1545*-Δ*3*, we have isolated three classes of transposon mutants of strain 35000: those with no detectable hemolytic activity, those with reduced hemolytic activity, and those with enhanced hemolytic activity. Transposon insertions in the nonhemolytic mutants were located in a DNA sequence which hybridized to the *Proteus mirabilis* hemolysin gene. Analysis of clones containing overlapping sections of this region served to further localize the *H. ducreyi* hemolysin gene and allow its expression in *Escherichia coli* and complementation of the nonhemolytic defect in an *H. ducreyi* mutant. These experiments indicate that *H. ducreyi* 35000 produces a hemolysin that is related to the calcium-independent hemolysin produced by *P. mirabilis*. Further experiments are needed to define the similarity of the *H. ducreyi* hemolysin to other calcium-independent hemolysins and to determine its role in the pathogenesis of chancroid.**

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease prevalent in Africa, where it is associated with the heterosexual transmission of human immunodeficiency virus (5, 26, 50). In the United States, periodic outbreaks of chancroid and the association of chancroid with human immunodeficiency virus transmission (44) make this disease an important public health concern. Currently, diagnosis of chancroid depends upon the isolation of *H. ducreyi* from the lesion. However, even under optimal conditions, *H. ducreyi* is isolated from only 50 to 80% of chancroidal lesions (36). In addition, only 14% of sexually transmitted disease clinics in the United States have the appropriate media for isolation of this organism (37). Thus, even with the increasing recognition of chancroidal disease in the United States, the disease is undoubtedly underreported.

Histologically, chancroidal lesions are characterized by a necrotic ulcer which contains disintegrating epithelial cells, inflammatory cells, and viable bacteria (1, 18). These histological findings are compatible with the production of cytotoxins, and indeed, *H. ducreyi* cytotoxin(s) appears to act on cultured fibroblasts and epithelial cells (2, 30). The *H. ducreyi* lipooligosaccharide may also be required for ulcer formation, perhaps by enhancing migration of inflammatory cells to the lesion site and increasing the resistance of *H. ducreyi* to phagocytosis (6, 22, 23).

Whether clinical isolates of *H. ducreyi* produce a hemolysin has been controversial. The organism has been variously reported as either being nonhemolytic (13) or having weak (21) or variable (42) hemolytic activity or alpha-hemolytic activity (1, 39) on different formulations of blood agar plates. Recently, Palmer et al. (25) have described the ability of *H. ducreyi* 35000 to lyse horse blood in a liquid-phase assay. Many hemolysins, originally identified by their ability to hemolyze erythrocytes (RBCs), are also able to lyse other cell types or affect their cellular functions (51). Thus, an *H. ducreyi* hemolysin which acts upon epithelial and inflammatory cells could be an important virulence factor in the pathogenesis of chancroidal disease.

We utilized newly devised blood agar plates and liquid-phase assays to assess the abilities of 10 *H. ducreyi* strains to lyse RBCs and characterized factors modifying the expression and detection of hemolysin. In addition, we analyzed the gene disrupted in nonhemolytic transposon mutants, isolated clones that expressed hemolytic activity in *Escherichia coli*, and constructed a derivative of these clones that restored hemolytic activity to the nonhemolytic mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. *H. ducreyi* strains were obtained from diverse sources and have been previously described (48). Other *Haemophilus* species used in this report, *H. influenzae* ATCC 33391, *H. aphrophilus* ATCC 33389, *H. segnis* ATCC 33393, and *H. parainfluenzae* ATCC 33392, were kindly provided by Arnold Smith (University of Washington, Seattle). *H. influenzae* Rd (32) was obtained from M. Roberts (University of Washington).

Plasmids used in hybridization experiments included pWPM177, provided by Rodney Welch (University of Wisconsin, Madison); pUC3-7, obtained from Irene Kieba (University of Pennsylvania, Philadelphia); and pBS+, obtained from Stratagene Cloning Systems (La Jolla, Calif.). Other plasmids, used for cloning experiments with *E. coli*, included pUC19 (GIBCO BRL, Gaithersburg, Md.) and pTZ18R and pTZ19R (Pharmacia LKB Biotechnology, Piscataway, N.J.). Plasmid pLS88, an *H. ducreyi* shuttle vector (52), was kindly provided by William Albritton (University of Alberta, Edmonton, Alberta, Canada). Plasmid pAM120, which contains the transposon Tn*916* (12), was provided by Craig Rubens (University of Washington). Plasmid pMGC20.1 was obtained from Maggie So (University of Oregon, Portland). This plasmid contains transposon Tn*1545*-Δ*3* and is a derivative of pMGC20 (20) from which the erythromycin gene has been deleted (20a). These two transposons have been previously used to mutagenize *H. ducreyi* (25, 41). All plasmids were maintained in *E. coli* DH5α (GIBCO BRL) unless otherwise indicated. C2110, a *polA* strain of *E. coli* (28), was obtained from S. Jin (University of Washington).

Media and general methods. Charcoal medium (CM) plates and Hd broth, used for culturing *H. ducreyi*, have been described elsewhere (48). *H. influenzae* was cultured on chocolate agar (GC agar base, 1% hemoglobin, and 1% XV factor enrichment added after autoclaving) or in Hd broth without fetal bovine

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serum. All plates were used for growth of *H. ducreyi* incubated in candle jars at 35°C unless otherwise indicated. Hd broth was incubated at 35°C with shaking as described previously (48). All strains were maintained at −70°C in L broth (17) with 25% glycerol and recovered from these stock cultures as described elsewhere (48).

Horse blood agar plates (HBAPs) were bilayer plates consisting of GC agar base (Difco), 1% XV factor supplement, and 5% horse blood. These plates were prepared as follows: 13 ml of the base medium (GC agar base with 1% XV factor supplement) was dispensed into the petri plates and allowed to solidify, and then 8 ml of the same medium containing 7.5% horse blood was overlaid on top of the solidified base medium. Sheep blood agar plates (SBAPs) were prepared in a similar manner except sheep, rather than horse, blood was used.

E. coli strains were cultured in L broth and L agar (17). When appropriate, antibiotics were incorporated in these media at the following concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 30; and tetracycline, 20.

Detection of hemolysis on plates. The abilities of different *H. ducreyi* strains to lyse RBCs on agar plates were observed after incubation for 2 to 3 days at 35°C in a candle jar. Hemolysis after growth in other microaerophilic conditions was tested by inoculation of strain 35000 onto HBAPs and incubation for 2 days in an anaerobic jar with a CampyPak Microaerophilic System (BBL Microbiology Systems, Cockeysville, Md.) with a catalyst and in an anaerobic jar with a GasPak Anaerobic System (BBL) with the catalyst removed (38). Strain 35000 grew very poorly under the anaerobic conditions generated in the GasPak Anaerobic System with a catalyst, so its ability to produce a hemolysin under these conditions could not be evaluated.

Liquid hemolysis assays. To prepare *H. ducreyi* for liquid hemolysis assays, a culture of the appropriate strain was grown overnight with shaking at 35°C in Hd broth. A portion of this culture was then used to inoculate fresh Hd broth at a 1:10 or 1:25 dilution and incubated for the periods indicated below.

For the liquid hemolysis assay, 1-ml aliquots of the bacterial suspensions were centrifuged for 1 min in a microcentrifuge (model MCF-100; Fotodyne, New Berlin, Wis.) and the supernatant was separated from the cell pellet. After the remaining liquid was removed from the tube, the cell pellet was suspended in assay buffer and serial twofold dilutions were prepared in the microtiter plates so that a 200-μl suspension remained in each well. The RBCs used in these assays were extensively washed in 0.85% NaCl, an equal volume (10 to 20 μl) of this suspension was then added to each well in the microtiter plate, and the contents of each well were mixed by repeated pipetting. After 1 h of incubation at 35°C, the microtiter plates were centrifuged (162 × *g*, 10 min) in a Sorval RT 6000 refrigerated centrifuge (DuPont Co., Newton, Conn.) to pellet the unlysed RBCs, 100 μl of the liquid from each well was transferred to a clean microtiter plate, and the optical density at 540 nm (OD_{540}) was analyzed with a microtiter plate reader (Titertek Multiskan MC; Flow Laboratories, Inc., McClean, Va.).

The percent hemolysis in each assay was calculated as a fraction of the value for complete hemolysis (determined by suspending RBCs in distilled water) for each experiment. The volume of the RBC suspension added in each experiment was calibrated to give an OD_{540} of 1.0 to 2.0 when completely lysed. All assays were performed in duplicate or triplicate and were repeated in a separate experiment at least once. Background hemolysis in these assays, determined by incubating buffer with RBCs, was always <5%.

DNA manipulations. Plasmid DNA was extracted by small-scale preparations by the alkaline lysis method or on a preparative scale by using alkaline lysis followed by cesium chloride-ethidium bromide density gradients as described previously (33). Whole-cell DNA was purified as described elsewhere (33). Restriction enzyme digestions and ligations were performed according to the directions of the manufacturer (GIBCO BRL). *E. coli* was made competent for the uptake of DNA by the rubidium chloride method (9). Colony and Southern blots were performed as described previously (3).

Hybridizations were performed either at high (50% formamide) or low (35% formamide) stringency (19) as described previously except 5× rather than 1× Denhardt's solution was added to the hybridization buffers. Fragments used as probes in these experiments were purified from agarose gels by using the Gene Clean II kit (Bio 101, Inc., La Jolla, Calif.) according to the manufacturer's directions and then radiolabelled with [α-^{32}P]dATP (New England Nuclear, Boston, Mass.) by using the Random Primers DNA Labeling System as recommended by the manufacturer (GIBCO BRL).

DNAs containing hemolysin genes from other organisms were used as probes in hybridization experiments and were derived from the following sources. The *Proteus mirabilis* hemolysin probe (Hpm probe) was prepared from a 7-kb *Hin*dIII fragment from pWPM177. This plasmid contains the *Hin*dIII fragment from pWPM140 cloned into pBS+ (48a, 49) and contains the *P. mirabilis* calcium-independent hemolysin HpmA and HpmB genes. The *Actinobacillus actinomycetemcomitans* leukotoxin gene probe (AaLt probe) was the 3.5-kb fragment from pUC3-7 derived from *Sal*I and *Hin*dIII digestion. This fragment contains 2.1 kb of the B gene, 1.0 kb of the D gene, and 0.35 kb of the A gene from this operon (15, 16). Plasmid pBS+ was used as a control in homology experiments to detect hybridization of vector sequences. The probe used to detect insertion of the Tn*1545*-Δ*3* and Tn*916* transposons in *H. ducreyi* DNA Tn*1545*-Δ*3* probe) was prepared from the 5-kb *Hin*dIII fragment from pMGC20.1 and designated the transposon probe. This piece contains all of the transposon and a portion of the vector. The 3-kb *Hin*dIII fragment derived from this plasmid was used as a probe to detect the insertion of plasmid sequences into the *H. ducreyi* chromosome, always with negative results.

Plasmid DNA was sequenced as described previously (3).

Construction of a DNA library of *H. ducreyi* whole-cell DNA. A *Sau*3A library of *H. ducreyi* DNA in pUC19 was prepared as follows. *H. ducreyi* whole-cell DNA was subjected to limited digestion with *Sau*3A to maximize the amount of 10-kb fragments and then subjected to centrifugation in a 10 to 40% sucrose gradient as described elsewhere (3). After centrifugation, the bottom of the tube was punctured and 500-μl fractions were collected. Fractions containing fragments of approximately 10 to 20 kb were collected and ligated to pUC19 which had been digested with *Bam*HI and then treated with calf intestinal phosphatase according to the directions of the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). This ligation mixture was then transformed into *E. coli* DH5α. The gene library resulting from this procedure contained 14,000 transformants with an average insert size of 7 kb.

Transposon mutagenesis and electroporation of *H. ducreyi*. Electroporation of *H. ducreyi* 35000 was performed by a modification of the procedure of Hansen et al. (10). Briefly, strain 35000 was cultured on five CM plates overnight, scraped off these plates with a cotton swab, and then suspended in 3 ml of cold 10% glycerol. These bacterial suspensions were washed twice in 10% cold glycerol by centrifugation (1 min, Fotodyne microcentrifuge) and suspension of the resulting bacterial pellet in 10% glycerol by repeated pipetting. After the last centrifugation, the bacterial cells were suspended in 10% glycerol with a volume equal to the volume of the bacterial pellet (approximately 200 μl) and maintained on ice. For electroporation, 40 μl of this bacterial suspension and 2 to 20 μl of DNA (containing approximately 1 μg) were mixed in a microcentrifuge tube, transferred to a Pulser cuvette with a 0.1-cm electrode gap, and then exposed to a current of 2.5 V, 200 Ω, and 3.0 capacitance with a Gene Pulser System (Bio-Rad Laboratories, Hercules, Calif.). The bacterial suspension was then transferred to 12- by 75-mm tubes containing 1 ml of Hd broth and incubated without shaking for 6 h at 35°C to allow for expression of the antibiotic resistance. Finally, the bacteria in each 1-ml suspension were collected by centrifugation (1 min, Fotodyne microcentrifuge), spread on CM plates containing the appropriate antibiotic, and incubated for 2 days at 35°C in a candle jar.

Two independent transposon systems were used to mutagenize *H. ducreyi*: transposon Tn*916*, present on plasmid pAM120, and Tn*1545*-Δ*3*, encoded on plasmid pMGC20.1. We obtained approximately 100 tetracycline-resistant transformants after electroporation of 10^8 CFU of bacteria with 1 μg of pAM120 DNA. In contrast, we obtained 100,000 kanamycin-resistant transformants in similar experiments using pMGC20.1 DNA. These transformants were frozen in aliquots at −70°C. The aliquots were used to screen individual colonies for their hemolytic phenotype on HBAPs containing kanamycin (20 μg/ml). Transposon mutants derived from electroporation with pAM120 were screened for hemolytic activity on HBAPs containing tetracycline (2 μg/ml).

Cloning into pLS88, a shuttle vector for *H. ducreyi*. The pUC derivatives described in Results were cloned into pLS88 by digesting the appropriate clones with *Sst*I and then ligating them into pLS88 which had been similarly digested. Next, *E. coli* C2110 was transformed with the ligation mixtures and the resulting recombinants were selected on L agar containing ampicillin and kanamycin. Because C2110 has a mutation in *polA* and thus will not support the replication of pUC plasmids, recombinants expressing ampicillin and kanamycin resistance must have the pUC recombinant plasmids ligated to pLS88. *H. ducreyi* was transformed with the resulting constructs by electroporation and selection on CM plates containing kanamycin.

RESULTS

Detection of hemolytic activity of *H. ducreyi* on HBAPs and SBAPs. In initial studies, we found that plates with a thin top layer of medium containing 5% horse blood enhanced the detection of hemolysis produced by *H. ducreyi* (data not shown). On these bilayer plates (HBAPs), clear zones of hemolysis were seen with *H. ducreyi* 35000 which had been incubated for 3 days at 35°C in a candle jar (Fig. 1A and B). Hemolysis was not enhanced by incubation under the different atmospheric conditions described in Materials and Methods. Less hemolysis was seen after strain 35000 was incubated on similar plates containing sheep blood (SBAPs).

Ten strains of *H. ducreyi* were tested for hemolysis on HBAPs and SBAPs incubated for 3 days in a candle jar. While the 10 strains of *H. ducreyi* differed in the amount of hemolysis produced on HBAPs, all were hemolytic after 3 days of incubation. Strains V149/91, 35000, CF101, and CIP 542 were the most hemolytic; strains V-180, V-1168, and CH2 were moderately hemolytic; and strains LA228R, CIP A75, and CIP A77 were the least hemolytic. All strains of *H. ducreyi* were more hemolytic on HBAPs than on SBAPs. *H. influenzae* Rd and

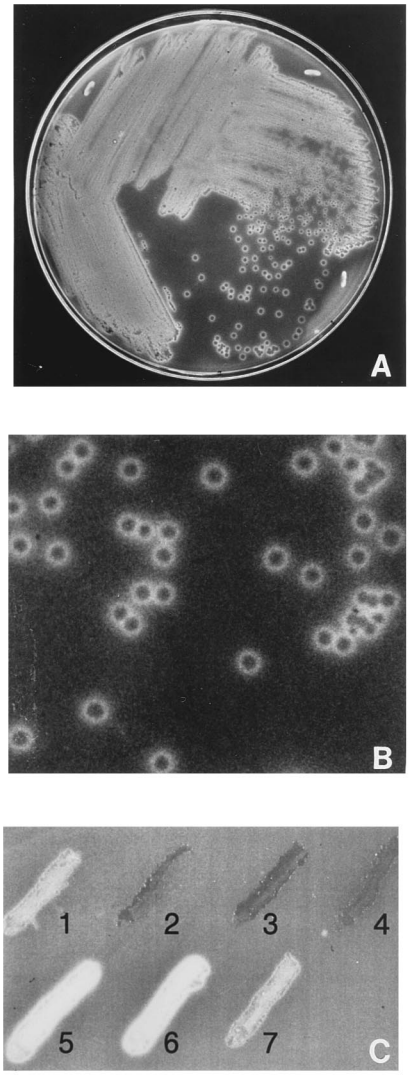

FIG. 1. Hemolytic phenotypes of *H. ducreyi* 35000 and transposon mutants on HBAPs. (A and B) Hemolysis of strain 35000 on HBAPs after 3 days of incubation. An entire HBAP showing clear zones of hemolysis surrounding growth of 35000 (A) and an enlargement of individual colonies clearly surrounded by zones of hemolysis (B) are shown. (C) Hemolytic phenotypes of 35000 and selected transposon mutants after incubation at 35°C on HBAPs for 2 days. Results are shown for bacterial strains as follows: 1, 35000; 2, 35000-TcA; 3, 35000-KmA; 4, 35000-KmB; 5, 35000-SHC; 6, 35000-SHD; and 7, 35000-KmR, a random transposon mutant. Strains 35000-TcA and 35000-KmA are nonhemolytic, and strain 35000-KmB showed a slight hemolysis that is not clearly evident in this picture but which was more visible after 3 days of incubation. Strains 35000-SHC and 35000-SCD are more hemolytic than strains 35000 and 35000-KmR, which served as controls inoculated on the same plates.

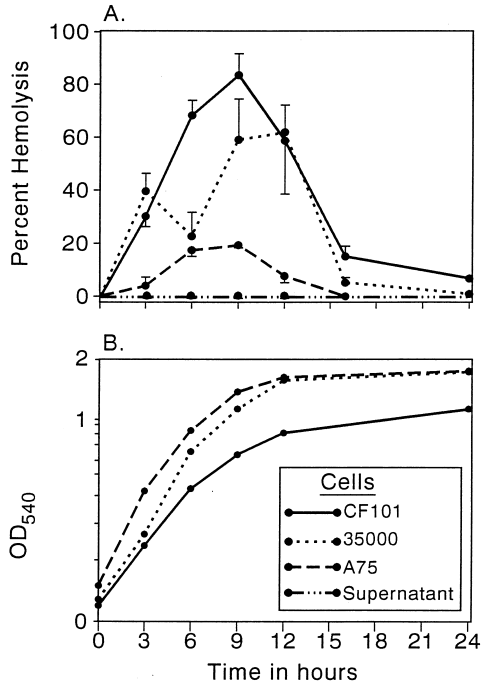

FIG. 2. Hemolytic activity produced by *H. ducreyi* 35000, CF101, and CIP A75 is growth phase dependent. (A) Hemolysis; (B) growth. All results are for 1:4 dilutions of cells. There was no activity in the undiluted supernatants. After separation from the supernatant, the cell pellet was suspended in the original volume of fresh Hd broth before analysis by liquid hemolysis assay. Values shown are averages of three determinations ± standard deviations.

ATCC 33391, *H. aphrophilus* ATCC 33389, and *H. parainfluenzae* ATCC 33392 developed larger colonies but did not appear hemolytic on HBAPs.

Characterization of the hemolytic activity of strain 35000 in the liquid hemolysis assay. We used clear broth medium (Hd broth) (48) to monitor the hemolytic activity of *H. ducreyi* strains 35000, CF101, and CIP A75 and found that the hemolytic activity of all three strains was dependent upon the growth phase of the organism (Fig. 2). Activity increased in logarithmic phase relative to the number of organisms present, peaking late in the logarithmic phase. There was no hemolytic activity detected in any of the three strains in stationary phase. The hemolytic activity of all three strains was associated with the bacterial cells and was not detectable in the supernatant. The three strains of *H. ducreyi* analyzed by the liquid hemolysis assay differed in their peak hemolytic activities (Fig. 2); strains 35000 and CF101 were the most hemolytic and CIP A75 was less hemolytic, comparable to their relative hemolysis detected on HBAPs.

We also altered the liquid hemolysis assay to characterize the hemolytic activity of strain 35000. We found that the hemolysis produced by strain 35000 was greatest with horse RBCs, intermediate with human and rabbit RBCs, and low with sheep RBCs (Fig. 3A). The relative hemolytic activity of another *H. ducreyi* strain, CF101, on RBCs from different animals was comparable to that of strain 35000 (i.e., horse > rabbit > human > sheep).

The assay buffer and conditions used for the liquid hemolysis assay were altered to determine optimal conditions for hemolysis of horse RBCs by strain 35000 (Fig. 3B through D). We found that the addition of different divalent cations had an effect on the hemolytic activity (Fig. 3B): calcium chloride but not magnesium chloride slightly enhanced the hemolytic activity of strain 35000; sodium phosphate, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and zinc chloride had inhibitory effects; and stronchium chloride had a minimal effect on hemolysis. Hemolytic activity increased with increasing incubation temperature from 4 to 42°C (Fig. 3C) and varied over a pH range of 5.5 to 8.0, with optimal activity detected at pH 7.0 (Fig. 3D).

Detection and characterization of nonhemolytic transposon mutants. Tn*916* insertions were obtained by transformation with pAM120 and selection for tetracycline resistance. Of 1,016 tetracycline-resistant transformants screened, one non-

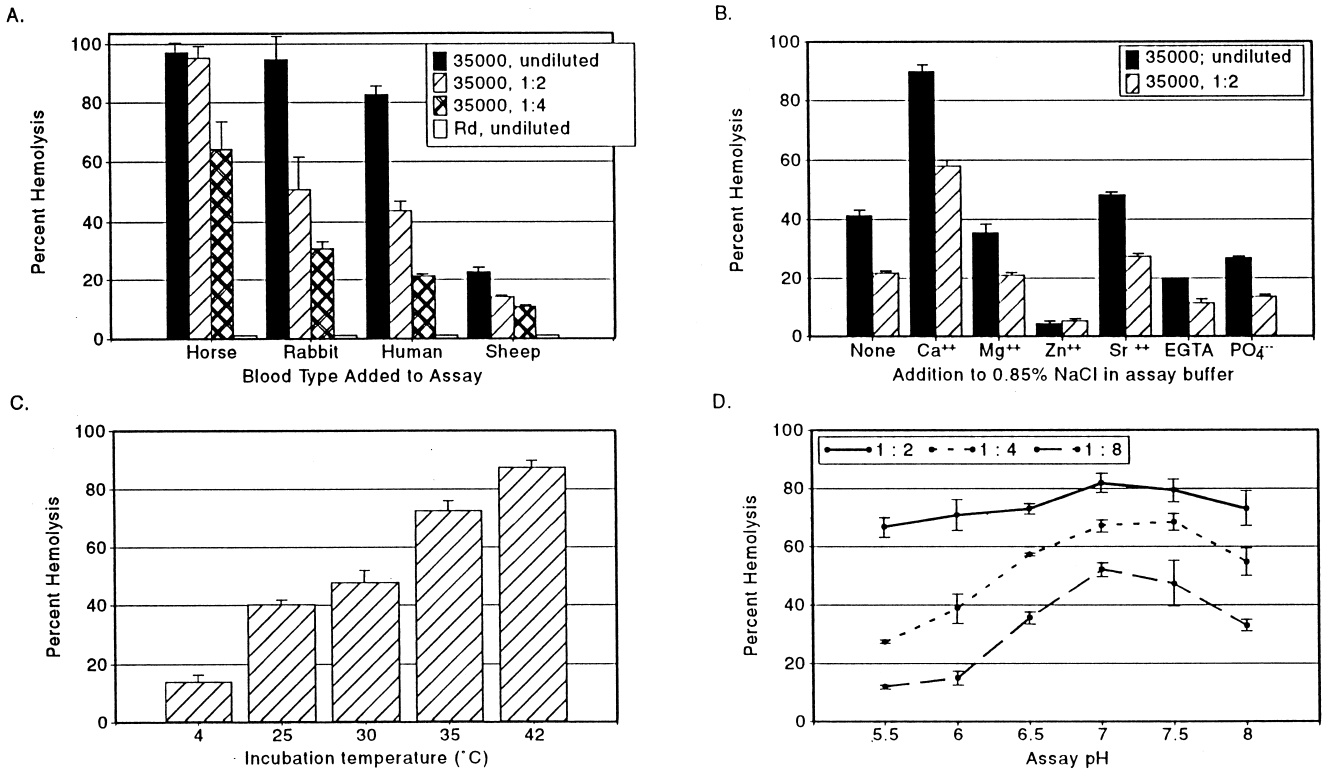

FIG. 3. Phenotypic characterization of the hemolytic activity of *H. ducreyi* 35000. Logarithmic-phase cells were harvested, centrifuged, and then suspended and diluted in the buffer to be tested. Assays were performed with the indicated dilutions of cells. (A) Effects of different sources of blood on hemolytic activity. The final concentrations of blood in these assays, indicated by the OD_{540} after complete lysis in distilled water, were as follows: horse RBCs, 1.4; rabbit RBCs, 1.1; sheep RBCs, 1.1; and human RBCs, 1.0. There was no detectable activity with *H. influenzae* Rd after overnight growth. (B) Effects of divalent cations, phosphate, EGTA, and zinc on hemolytic activity. (C) Effect of incubation temperature on hemolysis produced by strain 35000. (D) Effect of pH of assay buffer on the hemolytic activity of strain 35000. Values shown are averages of three determinations ± standard deviations. The assays in panels B through D were performed with horse blood.

hemolytic transformant, 35000-TcA, was detected and selected for further study.

Transposon Tn*1545-Δ3* insertions were obtained after transformation with pMGC20.1 and selection for kanamycin resistance. Of 6,300 kanamycin-resistant transformants screened for hemolytic activity, we detected one, designated 35000-KmA, which was nonhemolytic on HBAPs and one, designated 35000-KmB, which showed reduced but detectable hemolysis on these plates. Approximately 1% of kanamycin-resistant colonies screened on HBAPs with kanamycin displayed an enhanced hemolytic phenotype, and two, designated 35000-SHC and 35000-SHD, were characterized further. The hemolytic phenotypes of 35000 and these hemolytic mutants on HBAPs can be seen in Fig. 1C.

The hemolytic activity produced by strain 35000 and its transposon mutants was also quantitated by using the liquid hemolysis assay (Fig. 4A). In this assay, the hemolytic activity of strain 35000 was 83% and strains 35000-TcA and 35000-KmA were nonhemolytic (<5% hemolysis). Strain 35000-KmB had reduced but detectable hemolytic activity (36% hemolysis). Thus, the amount of hemolysis produced by the transposon mutants 35000-TcA, 35000-KmA, and 35000-KmB correlated with their appearance on HBAPs.

Hybridization analysis of strain 35000 and its transposon derivatives. Strain 35000 and its transposon derivatives were screened for homology to genes from known hemolysin operons, including those of the AaLt probe (containing *A. actinomycetemcomitans* leukotoxin genes) and the Hpm probe (containing the *P. mirabilis* hemolysin genes) (Fig. 5 and data not shown). To determine if homologs of either of these two hemolysin genes were disrupted in the transposon mutants, whole-cell DNAs from 35000, 35000-TcA, 35000-KmA, 35000-KmB, 35000-SHC, and 35000-SHD were digested with *Cla*I, transferred to Nytran filters, and then probed with the radiolabelled AaLt and Hpm probes. In these experiments, the AaLt probe hybridized to the same-size fragments in strain 35000 and its transposon derivatives (data not shown), indicating that in none of these strains had the transposons inserted into a fragment that was homologous to the AaLt probe.

A different pattern of hybridization was seen with similar blots reacted with the Hpm probe. In these experiments, 35000, 35000-KmB, 35000-SHC, and 35000-SHD showed two fragments (approximately 9.7 and 8.5 kb) that hybridized with the Hpm probe (Fig. 5A, lanes 1, 4, 5, and 6). However, 35000-TcA and 35000-KmA had homologous 8.5-kb fragments, no 9.7-kb fragment, and a new fragment of >10 kb (Fig. 5A, lanes 2 and 3). These experiments imply that 35000-TcA and 35000-KmA, but not the other transposon mutants, have a transposon insertion in the fragment that is homologous to pWPM177. Because neither Tn*916* nor Tn*1545-Δ3* contains *Cla*I sites (7, 20), the *Cla*I restriction fragment containing the transposon should be increased in size. The fragment containing the transposon in 35000-TcA is larger than that in 35000-KmA, consistent with the relative sizes of Tn*916* and Tn*1545-Δ3*.

The locations of the transposon insertions in the mutants were determined by hybridization of Southern blots with radiolabelled Tn*1545-Δ3*, which also detects the homologous

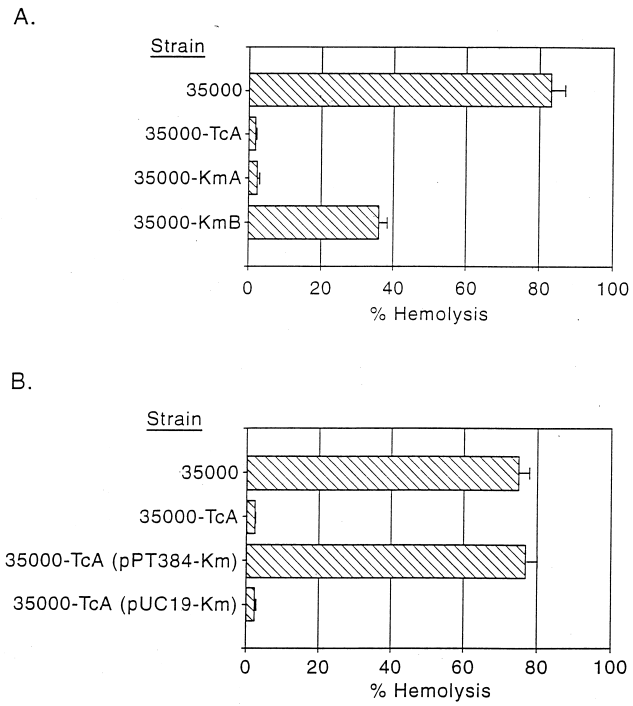

FIG. 4. Hemolytic activities of selected *H. ducreyi* strains as determined by liquid hemolysis assay. (A) Strain 35000 and its transposon derivatives. (B) Complementation of the hemolytic defect in strain 35000-TcA by pPT384-Km. All liquid hemolysis assays were performed with undiluted samples after 6 h of incubation of a 1:10 dilution of bacteria from an overnight culture. The differences in hemolytic phenotype were not due to differences in the OD_{540}s of the different strains used in the assay, which were as follows: 35000, 0.7; 35000-TcA, 0.7; 35000-KmA, 0.6; and 35000-KmB, 0.7 (A) and 35000, 0.9; 35000-TcA, 0.7; 35000-TcA(pPT384-Km), 8.7; and 35000-TcA(pUC19-Km), 0.7 (B). The mean of three determinations ± the standard deviation for each test is shown.

transposon, Tn*916*. As can be seen in Fig. 5C, the restriction fragments that hybridized to Tn*1545*-Δ*3* were different sizes in the different transposon mutants, except for 35000-SHC and 35000-SCD, which have similar-size homologous fragments. No fragments hybridized in strain 35000, which had no transposon insertions (Fig. 5C, lane 1). Strain 35000-TcA had two fragments that hybridized, indicating that this strain had two transposon insertions (Fig. 5C, lane 2).

We compared the sizes of the fragments that hybridized with the Hpm probe with those that hybridized to Tn*1545*-Δ*3* in each mutant. These fragments were comparable sizes in strain 35000-TcA (compare lanes 2 in Fig. 5A and C) and strain 35000-KmA (compare lanes 3 in Fig. 5A and C), indicating that the transposon had inserted into the fragment that was homologous to pWPM177 in these mutants. The smaller fragment that hybridized did not change in size in these mutants, indicating that *H. ducreyi* contains other DNA sequences that are homologous to the Hpm probe. There was no hybridization with vector alone (pBS+; data not shown).

Cloning and characterization of the *H. ducreyi* fragment homologous to the Hpm genes. Southern blots of strain 35000 digested with *Bgl*II indicated that a fragment of approximately 6.0 kb hybridized to the Hpm probe at low stringency. Thus, *Bgl*II fragments from strain 35000 of approximately 6 kb were purified and cloned into pUC19 which had been treated with *Bam*HI and calf intestinal phosphatase. Plasmids from the resulting clones were digested with *Sst*I and *Hin*dIII, and Southern blots of these digests were tested for homology to the Hpm probe. Of five homologous clones detected by this method, one, pPT376 (Fig. 6), was selected for further study.

To test whether we had cloned the appropriate DNA into pPT376, the insert DNA from this clone was excised by digestion with *Sst*I and *Sal*I, purified, radiolabelled, and hybridized with Southern blots of whole-cell DNAs from strain 35000 and its transposon derivatives (Fig. 5B). In these experiments, the probe DNA from pPT376 hybridized to fragments which were similar in size to those that hybridized to the Hpm probe (compare Fig. 5A and B). Most importantly, the fragments that hybridized to the pPT376 probe in the nonhemolytic mutants, 35000-TcA and 35000-KmA, were larger than those from strain 35000. The above experiments indicated that the insert in pPT376 was homologous to the *P. mirabilis* hemolysin genes and that DNA from this fragment contains the transposon insertion in the nonhemolytic mutants.

DH5α(pPT376) was nonhemolytic on HBAPs, suggesting that this clone did not contain the intact hemolysin gene or that it was not properly expressed in *E. coli*. Therefore, *Sau*3A permutations of the insert in pPT376 were selected from a pUC19 gene bank of 35000 in DH5α by using colony hybridization and radiolabelled insert DNA from pPT376 as described in Materials and Methods. Seven clones, containing different *Sau*3A permutations of the DNA homologous to pPT376, were detected. These seven clones extended both 5′

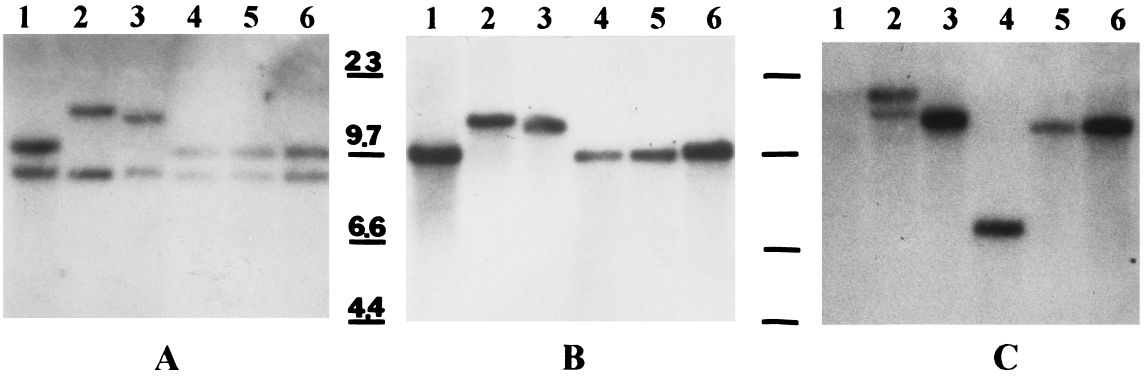

FIG. 5. Hybridization of whole-cell DNAs from strain 35000 and selected transposon mutants with the following radiolabelled probes: Hpm (A), pPT376 (B), and Tn*1545*-Δ*3* (C). DNAs were derived from 35000 (lanes 1), 35000-TcA (lanes 2), 35000-KmA (lanes 3), 35000-KmB (lanes 4), 35000-SHC (lanes 5), and 35000-SHD (lanes 6). Hybridization with Hpm was performed at low stringency (35% formamide); hybridizations with pPT376 and *1545*Δ*3* were performed at high stringency (50% formamide). The Hpm and Tn*1545*-Δ*3* probes are described in Materials and Methods; the pPT376 probe, derived from insert DNA from this clone, is described in Results. Positions of molecular size markers of lambda DNA digested with *Hin*dIII (in kilodaltons) are indicated between the panels.

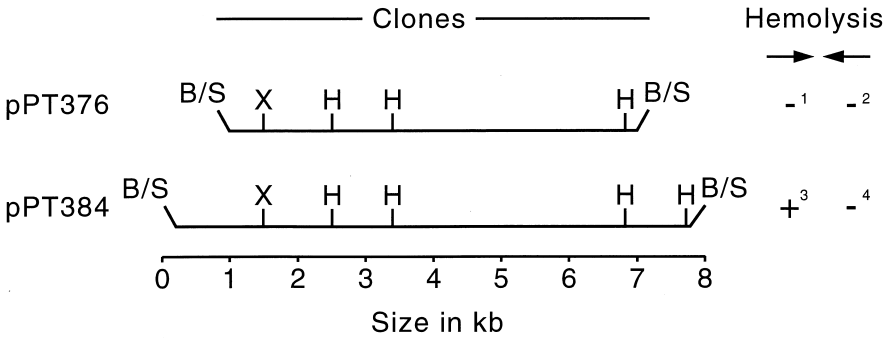

FIG. 6. Restriction maps and hemolytic phenotypes of pPT376 and pPT384. The inserts in these two clones are both contained in pUC19, and their positions relative to each other are shown. Hemolysis was tested on HBAPs after 2 days of growth. The plasmids generated from ligation into pTZ18R and pTZ19R are indicated as follows: 1, pPT376-TZ18; 2, pPT376-TZ19; 3, pPT384-TZ18; and 4, pPT384-TZ19. The orientations of the pPT19R constructs are the same as those of the original clones in pUC19. The direction of the *lac* promoter in these clones (arrows) is indicated. Restriction enzyme sites are as follows: B/S, *Bam*HI-*Sau*3A hybrid site; X, *Xho*I; and H, *Hin*dIII.

and 3′ of the insert in pPT376, but all were inserted in the same orientation as pPT376 and were either weakly hemolytic or nonhemolytic on HBAPs.

Expression of the *H. ducreyi* hemolysin gene in *E. coli*. To locate the position and orientation of the hemolysin gene in pPT376, the ends of this clone were sequenced and the predicted amino acid sequence was compared with that of the Hpm genes of *P. mirabilis*. We found that the predicted amino acid sequence of the first 120 nucleotides of pPT376 (as it is drawn in Fig. 6) was 33% identical to the amino acid sequence 544 nucleotides downstream from the translational start of the first gene (*hpmB*) in the Hpm gene cluster. These experiments indicated that the *H. ducreyi* hemolysin gene extends 5′ of pPT376 as it is shown in Fig. 6.

On the basis of the sequencing data, clone pPT384 (Fig. 6), which extends approximately 1 kb both 5′ and 3′ of the insert in pPT376, was selected for further study. The inserts from pPT384 and pPT376 were inserted in both orientations relative to the *lac* promoter in pTZ18R and pTZ19R, transformed into DH5α, and tested for their hemolytic phenotypes on HBAPs. In these experiments, the clones containing the insert from pPT384 in pTZ18R were hemolytic but those with the insert in pTZ19R were not (Fig. 6). The plasmids resulting from these experiments were designated pPT384-TZ18 and pPT384-TZ19, respectively. Clones of pPT376 inserted in both orientations in pTZ18R and pTZ19R were nonhemolytic. These experiments implied that pPT384 contains the intact *H. ducreyi* hemolysin gene(s) and that expression of this gene(s) in *E. coli* requires an exogenous promoter. Thus, the *H. ducreyi* promoter may be missing in the clones or the existing *H. ducreyi* promoter may be present but under repression or lacking a required positive effector in *E. coli.*

Cloning of hemolysin constructs into pLS88 and complementation of the hemolysin defect in 35000-TcA. Recombinants of pPT384 and pUC19 cloned into pLS88 were obtained as described in Materials and Methods, designated pPT384-Km and pUC19-Km, respectively, and tested for their ability to complement the hemolysin defect in 35000-TcA (Fig. 4B). In these experiments, pPT384-Km fully restored the hemolytic phenotype of 35000-TcA, while 35000-TcA and 35000-TcA-(pUC19-Km) showed <5% hemolysis in this assay.

DISCUSSION

We have characterized the hemolytic activity of *H. ducreyi* 35000 and found that the *H. ducreyi* hemolysin gene is a homolog of the calcium-independent hemolysin of *P. mirabilis*. The *H. ducreyi* hemolysin gene is expressed in *E. coli* and complements a nonhemolytic transposon mutant of *H. ducreyi*. The homology of the *H. ducreyi* hemolysin gene with the *hpmA* and *hpmB* genes of *P. mirabilis* implies that it may have a similar function and structure.

The *P. mirabilis* hemolysin, similar to its homolog in *Serratia marsescens*, is encoded by two genes: *hpmA*, which is the structural gene for the hemolysin, and *hpmB*, which encodes a protein that allows the secretion of the structural gene from the bacterial cell (24, 35, 49). The *hpmB* gene product is also thought to modify the *hpmA* gene product, although the nature of this modification is unknown. Discovery of a homolog of the calcium-independent hemolysin of *P. mirabilis* in *H. ducreyi* suggests that this toxin has spread to species other than members of the family *Enterobacteriaceae*, possibly by horizontal gene transfer. Further characterization of the *H. ducreyi* hemolysin will reveal the degree of similarity to the *P. mirabilis* and *S. marcescens* hemolysins at both the genetic and the functional levels. Hybridization probes derived from the *H. ducreyi* hemolysin gene could be used to assess its dissemination to other members of the *Pasteurellaceae* family.

The histological characteristics of chancroidal ulcers, including necrotic tissue, an influx of inflammatory cells, and persistence of viable *H. ducreyi* organisms, suggest the production of a cytolysin by this organism. Hemolysins produced by other organisms, identified by their ability to lyse RBCs, are able in many cases to lyse or impair the cellular functions of other cell types, including inflammatory and epithelial cells (8, 11, 51). Studies performed with the *Proteus* hemolysin indicate that it is toxic to many different cell types (43). The *Serratia* hemolysin alters the release of inflammatory mediators from mast cells and neutrophils, suggesting that it may increase vascular permeability, edema formation, and granulocyte accumulation in vivo (14). Thus, an *H. ducreyi* cytolysin could be important in the destruction of epithelial cells seen in chancroidal lesions, eliciting the inflammatory response, and in enhancing survival of the organism in the presence of inflammatory cells. Additionally, lysis of host cells may be an important mechanism by which *H. ducreyi* acquires heme, which is required for the growth of this organism.

Expression of a cytolysin may enable *H. ducreyi* to enter and survive in host cells. Many cytolysins, such as those produced by *Shigella* and *Listeria* spp., are required for escape of the organism from the phagocytic vacuole during cellular invasion of epithelial cells. These cytolysins are required for lysis of the

host vacuole after entry into epithelial cells and possibly for release from the host cell (27, 34). We have demonstrated previously that *H. ducreyi* is also able to invade cultured epithelial cells (46), but the role of the hemolysin in this process is not known. Cytolysins from other organisms have been associated with survival in other cell types. For example, a cytolysin from *Salmonella* spp. has also been associated with survival of these organisms in macrophages and is associated with virulence in a mouse animal model (4).

The phenotypic characterization of the hemolytic activity of *H. ducreyi* allowed us to achieve enhanced expression and detection of the activity by using the liquid hemolysis assay. Hemolytic activity was greatest in late logarithmic phase and nondetectable in stationary phase, a pattern of expression similar to those of other hemolysins (31). The greatest hemolytic activity was achieved when the assay was performed at a neutral pH and was slightly enhanced by the addition of calcium. Because the tests were done with whole cells, these results may reflect differences in release, expression, or degradation of the hemolysin rather than the activity of the hemolysin itself. Further characterization of purified hemolysin will clarify its phenotypic characteristics, including the effect of pH, heat lability, and the effect of divalent cations.

Our observations regarding the specificity of the *H. ducreyi* hemolysin for RBCs of different species and its association with the cell are similar to the findings of Palmer et al. (25). The specificity of hemolysis for different animal RBCs may reflect differences in expression of hemolysin receptors on the surface of these cells. The association of the *H. ducreyi* hemolysin with cells is surprising, because colonies of this organism on plate media were surrounded by a zone of hemolysis, implying that the hemolysin is released into the media. These results may indicate that the *H. ducreyi* hemolysin is proteolytically degraded in the supernatants in liquid assays, as is the *Serratia* hemolysin (35). Alternatively, lysis of bacterial cells in the plate medium could explain the release of the hemolysin into the agar medium surrounding bacteria colonies. Further studies will clarify the cellular location and host cell specificity of the hemolysin in *H. ducreyi.*

Hybridization experiments with *H. ducreyi* DNA revealed other interesting homologies. The fragments hybridizing with the RTX hemolysin operon of *A. actinomycetemcomitans* were unchanged in size in the nonhemolytic transposon mutants, implying that this locus is not responsible for the hemolytic phenotype in strain 35000. Similar homologs are present in *A. actinomycetemcomitans*, which contains an RTX toxin with major activity on leukocytes (16), and in *Neisseria meningitidis*, which has an RTX-like toxin with unknown target activity (45). In addition to the RTX homology, two restriction fragments from *H. ducreyi* DNA hybridized to the *P. mirabilis* hemolysin gene, but only one of these fragments was altered in size in the nonhemolytic transposon mutants. The other fragment may contain another (nonhemolytic) cytolysin gene or a gene encoding a protein with a different function, such as the high-molecular-weight adhesion proteins from *H. influenzae* which share limited homology with both the *Serratia* hemolysin ShlA and ShlB genes (4).

The nature of the *H. ducreyi* hemolysin, its ability to lyse different human cell types relevant to disease, and its role in the pathogenesis of chancroid are all important directions for future research. The isolation of nonhemolytic transposon mutants will allow studies of the effect of the *H. ducreyi* hemolysin on those cell types encountered in a chancroidal ulcer and will clarify its possible role in disease. Evaluation of the relative abilities of the wild type and mutants to invade epithelial cells in our tissue culture invasion model (46) will determine the role of the hemolysin in cellular invasion. Finally, the role of the *H. ducreyi* hemolysin in the pathogenesis of chancroidal disease should be studied by using isogenic mutant strains in primate (47) and rabbit (29) animal models, the human model for experimental chancroid (40), and tissue culture invasion models.

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