

## The *Haemophilus ducreyi* Serum Resistance Antigen DsrA Confers Attachment to Human Keratinocytes

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*Haemophilus ducreyi* is the etiologic agent of the sexually transmitted genital ulcer disease chancroid. *H. ducreyi* serum resistance protein A (DsrA) is a member of a family of multifunctional outer membrane proteins that are involved in resistance to killing by human serum complement. The members of this family include YadA of *Yersinia* species, the UspA proteins of *Moraxella catarrhalis*, and the Eib proteins of *Escherichia coli*. The role of YadA, UspA1, and UspA2H as eukaryotic cell adhesins and the function of UspA2 as a vitronectin binder led to our investigation of the cell adhesion and vitronectin binding properties of DsrA. We found that DsrA was a keratinocyte-specific adhesin as it was necessary and sufficient for attachment to HaCaT cells, a keratinocyte cell line, but was not required for attachment to HS27 cells, a fibroblast cell line. We also found that DsrA was specifically responsible for the ability of *H. ducreyi* to bind vitronectin. We then theorized that DsrA might use vitronectin as a bridge to bind to human cells, but this hypothesis proved to be untrue as eliminating HaCaT cell binding of vitronectin with a monoclonal antibody specific to integrin  $\alpha_5\beta_5$  did not affect the attachment of *H. ducreyi* to HaCaT cells. Finally, we wanted to examine the importance of keratinocyte adhesion in chancroid pathogenesis so we tested the wild-type and *dsrA* mutant strains of *H. ducreyi* in our swine models of chancroid pathogenesis. The *dsrA* mutant was less virulent than the wild type in both the normal and immune cell-depleted swine models of chancroid infection.

Chancroid is a sexually transmitted genital ulcer disease caused by the gram-negative bacterium *Haemophilus ducreyi*. Two to seven days after contact, a small tender papule surrounded by erythema develops at the site of inoculation. This papule progresses to a pustule and then to a painful soft necrotic ulcer with ragged edges. The floor of the ulcer is composed of necrotic tissue and a mixture of inflammatory cells (28). If left untreated, the ulcer can persist for weeks or even months, but it may eventually heal. *H. ducreyi* infection has never been shown to become systemic (22).

Chancroid is particularly common in Africa, Asia, and Latin America and is considered uncommon in the United States (41). There were 4,986 reported cases of chancroid in the United States in 1987; by 1999, however, the number had fallen to 143 (42). While the official number of United States chancroid cases has declined, it is suspected that chancroid is more prevalent than reported as chancroid-positive patients are often misdiagnosed (29). One reason for this is that chancroid is difficult to diagnose without specific laboratory tests that are unavailable to most health care providers (6).

The ability of certain strains of *H. ducreyi* to cause chancroid infection appears to be correlated with their ability to attach to human cells. When eight *H. ducreyi* strains were tested in an adherence assay, the six strains that adhered to over 90% of the human foreskin epithelial cells were virulent in the temperature-dependent rabbit model of *H. ducreyi* infection, while the two nonadherent strains were not virulent (40). The level

of attachment of virulent strain 35000 to human foreskin fibroblasts is significantly greater than the level of attachment of avirulent strain A77 at 25 and 35°C (2). While previous work has described various eukaryotic cells to which *H. ducreyi* binds (2, 9, 16, 24, 25, 33, 40, 44), little is known about the bacterial proteins mediating the adhesive phenotype of *H. ducreyi*.

Recently, we described the *H. ducreyi* serum resistance protein A (DsrA). This *H. ducreyi* outer membrane protein is essential for resistance to killing by normal human serum (13). DsrA is a member of a family of surface-exposed outer membrane proteins that impart both resistance to killing by complement and the tendency to form stable multimers (21, 35) (Pfam database accession number PF03895). Other members of this family include YadA of the pathogenic *Yersinia* species, the UspA proteins of *Moraxella catarrhalis*, and the Eib proteins of *Escherichia coli*. Interestingly, YadA and the UspA proteins also function as eukaryotic cell adhesins. YadA mediates the adherence of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* to intestinal tissue, to brush border membranes and polystyrene surfaces (32), to epithelial cells (19), and to fibronectin-coated coverslips (39). Expression of UspA1 and UspA2H is responsible for adherence of *M. catarrhalis* to human epithelial cell lines (1, 23, 27). The homology between DsrA and these proteins suggested that DsrA might function as a possible *H. ducreyi* adhesin.

Herein, we describe the specific role of DsrA in the attachment of *H. ducreyi* to keratinocytes by demonstrating elimination of attachment in a mutant and restoration of this defect via complementation and by conferring attachment ability to *Haemophilus influenzae* through recombinant (rDsrA) expression. We also demonstrated that DsrA is specifically responsible for binding of vitronectin and that the attachment of *H.*

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
<b>Strains</b>		
<i>H. ducreyi</i> 35000	Wild type, parent	Stanley Spinola, Indiana University
FX517	35000 <i>dsrA</i> mutant, Cm <sup>r</sup>	13
<i>H. influenzae</i> Rd	Nontypeable	American Type Culture Collection
<b>Plasmids</b>		
pLSKS	Shuttle vector without insert of <i>dsrA</i> , Cm <sup>r</sup> Kan <sup>r</sup> Str <sup>r</sup> Sul <sup>r</sup>	43
pUNCH1260	<i>dsrA</i> expression vector, Cm <sup>r</sup> Str <sup>r</sup>	13

*ducreyi* to keratinocytes occurs independent of vitronectin association.

#### MATERIALS AND METHODS

**Cell culture.** The HaCaT keratinocyte cell line (8) was a gift from Bernard Weissman (Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill). HaCaT cells were cultured in T-75 flasks (Nuclon Δ Surface Nunc) by using RPMI 1640 (Gibco BRL catalog no. 11875-093) supplemented with 10 μM HEPES (pH 7.5) and 10% fetal bovine serum (FBS) (Gibco BRL catalog no. 26140-079) without antibiotics. The human foreskin fibroblast cell line utilized was HS27 (ATCC 1634-CRL; American Type Culture Collection, Manassas, Va.). HS27 cells were cultured in T-75 flasks by using Dulbecco's modified Eagle medium (Gibco BRL catalog no. 11995-065) supplemented with 10 μM HEPES (pH 7.5) and 10% FBS (Gibco BRL catalog no. 26140-079) without antibiotics. All cells were maintained in a 35°C humidified atmosphere with 5% CO<sub>2</sub>. Four-well dishes (Nuclon Δ Surface Nunc) were seeded with 10<sup>5</sup> cells. Attachment assays were performed as soon as the cells appeared to be confluent. Cells were grown on plastic and therefore did not differentiate.

**Bacterial culture and inoculum preparation.** For the attachment assays *H. ducreyi* and *H. influenzae* strains were cultured on chocolate agar plates. The chocolate agar plates consisted of 2.5% brain heart infusion, 1.5% agar, 1% hemoglobin, and 1% IsoVitalX (Becton Dickinson Microbiology Systems catalog no. 211876). The chocolate agar plates used for *H. ducreyi* also contained 10% FBS (Gibco BRL catalog no. 26140-079), but the plates used for *H. influenzae* did not. Plasmid-containing strains were cultured on selective media that included antibiotics (0.1 mg of chloramphenicol per 100 ml). The liquid medium used contained 3.79% brain heart infusion, 1% IsoVitalX (Becton Dickinson Microbiology Systems catalog no. 211876), and 50 μg of hemin per ml.

For the attachment assay, bacteria were recovered from freezer stocks on chocolate agar plates and incubated at 35°C in the presence of 5% CO<sub>2</sub>. After 16 to 18 h of growth, bacteria were swabbed into 2 ml of liquid medium. The suspension was vortexed for 5 s and allowed to settle for 5 min. After 5 min, the top 1 ml was removed, added to a flask containing 9 ml of liquid medium, and incubated for approximately 4 h at 35°C at 100 rpm. Turbidity was measured with a Klett meter, and the suspension was adjusted with liquid medium to obtain a final concentration of approximately 1 × 10<sup>8</sup> CFU/ml. Bacteria were pelleted and then resuspended in tissue culture medium at a concentration of approximately 1 × 10<sup>8</sup> CFU/ml and diluted into a tissue culture medium solution so that the final concentration was approximately 10<sup>6</sup> CFU/ml. Tissue culture bacterial solutions were serially diluted and plated to determine the precise number of input CFU.

For the swine experiments, *H. ducreyi* 35000 and the isogenic *dsrA* mutant strain FX517 (Table 1) were prepared as previously described (20, 36, 37). Briefly, bacteria were grown from frozen stocks on chocolate agar plates containing 10% FBS and were passaged once. Bacteria were harvested from the plates with swabs and resuspended in phosphate-buffered saline (PBS). The

bacteria were passed through a 30-gauge needle in order to reduce clumping. Serial dilutions were cultured in duplicate to determine bacterial cell concentrations.

**Attachment assays.** We added 500 μl of a 10<sup>6</sup>-CFU/ml suspension of bacteria in tissue culture medium to confluent monolayers of 10<sup>5</sup> cells in four-well tissue culture dishes (multiplicity of infection, 5 to 10) in medium supplemented with 10% FBS unless otherwise indicated. After 2 h of cocubation at 35°C in the presence of 5% CO<sub>2</sub>, the wells were randomly designated either adherent or total-count wells. Total bacterial counts were determined by removing bacteria and eukaryotic cells from the plastic with a sterile wooden stick. Suspensions were vortexed, serially 10-fold diluted, and plated in duplicate. Adherent bacteria were defined as viable bacteria that remained adherent to cells after five washes with 500 μl of PBS. After washing, cells were scraped from the plastic with a sterile wooden stick. The suspensions were then vortexed. Adherent cells counts were determined by serial dilution and plating in duplicate. The percentage of adherence was determined as follows: (number of adherent CFU after 2 h of incubation/total number of CFU) × 100.

To ensure that plasmids were maintained throughout the assay, bacterial strains were plated onto both antibiotic-containing and non-antibiotic-containing chocolate agar plates. There were no differences between the numbers of CFU.

***H. influenzae* expressing DsrA.** We introduced plasmid pUNCH1260 into *H. influenzae* strain Rd by electroporation. pUNCH1260 contains the complete *H. ducreyi dsrA* gene in broad-host-range plasmid pLSKS.

**rDsrA antiserum production.** We amplified the *dsrA* gene using 5' primer *dsrA19* (ATT AAT GCA GCA GCC GCC AAA GTT TGC TGG) and 3' primer *dsrA20* (GCG GCC GCG AAT TCA TAC CCA ACA GAA CCA CC). The 5' and 3' primers contained engineered *AseI* and *NotI* sites, respectively. PCR was performed by using Ready To Go tubes (Pharmacia), and strain 35000 chromosomal DNA was used as the template. The conditions for PCR were as follows: one initial hot start cycle of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, annealing for 1 min at 65°C, and extension for 1 min at 72°C. A single PCR product was obtained, ligated into the TA cloning vector, and transformed into DH5α. White colonies were screened for size (4.6 kb), and four clones were designated pUNCH1250A to pUNCH1250D. These clones were digested with *AseI* and *NotI*, and the insert isolated was ligated into *NdeI/NotI*-digested pET-30. Ligation mixtures were transformed into BL2(DE3)/pLysS. Transformants were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and screened for expression of rDsrA by using anti-native DsrA (13). The fusion protein did not have a signal sequence but did have a C-terminal hexahistidine tag and was directed to inclusion bodies. rDsrA was purified as previously described (14).

Rabbits were immunized four times with 200 μg of rDsrA per dose. The first immunization mixture contained Freund's complete adjuvant, and the remaining immunization mixtures contained incomplete Freund's adjuvant. Preimmune sera and sera present after four immunizations were obtained and screened by Western blotting.

**Vitronectin binding assay.** We used a previously described assay for detection of serum components bound by *H. ducreyi*, modified as follows. *H. ducreyi* (approximately 2 × 10<sup>8</sup> CFU) was suspended in 1 ml of gonococcal medium base broth (GCB) with 5% heat-inactivated normal human serum (NHS). After binding for 30 min at 35°C, the bacteria were extensively washed (four times) with GCB and then transferred to a new tube and washed once with PBS. Bacterial pellets with bound components of NHS (approximately 2 × 10<sup>7</sup> CFU) were subjected to Western blotting by using a sheep anti-human vitronectin (Affinity Biologicals) at a 1:5,000 dilution. The secondary antibody used was anti-sheep alkaline phosphatase (Sigma) at a 1:5,000 dilution. 5-Bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium were used as the substrates.

**Biotinylation of vitronectin.** We used the previously described method (11) for biotinylation. Briefly, 100 μg of vitronectin (kindly supplied by Jos van Putten and Tom Duensing) in PBS (pH 8.0) was mixed with 100 μg of NHS-biotin (Pierce) in a 500-μl (total volume) mixture for 2 h at room temperature. Excess aldehyde groups were blocked by addition of 20 μl of 1 M Tris (pH 8.0), followed by incubation at room temperature for an additional 30 min. Proteins were dialyzed overnight at 4°C to remove unreacted biotin.

**Whole-cell radioimmunoprecipitation with biotinylated ligands.** The assay used for whole-cell radioimmunoprecipitation with biotinylated ligands was a modification of a previously described assay used for identifying antibodies reacting with surface-exposed epitopes of gonococci (12, 38). *H. ducreyi* was surface iodinated, washed, and suspended in 0.5 ml of GCB (approximately 1 × 10<sup>8</sup> CFU per tube; 1 × 10<sup>6</sup> cpm per tube). Biotinylated vitronectin (5 μg) was added and allowed to bind at 37°C for 30 min. Control tubes lacking vitronectin were used to determine nonspecific binding. The bacteria were washed twice in PBS, and cell pellets were lysed with 1 ml of 2% Zwittergent in TEN (Tris-buffered saline with 5 mM EDTA; pH 8.0) at 37°C with mixing for 1 h. Insoluble

debris was removed by centrifugation ( $15,000 \times g$ , 10 min), and soluble material was mixed with 40  $\mu$ l of a 50% slurry of Neutravidin-agarose (Pierce). After the preparation was rocked at 4°C for 2 h, Neutravidin-agarose with bound proteins was washed five times with 2% Zwittergent in TEN (changing tubes once) and finally with TEN without detergent. Laemmli sample buffer was added, and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography performed with an intensifying screen at  $-70^\circ\text{C}$ .

**Inhibition of vitronectin binding.** Monoclonal antibody inhibition of HaCaT cell binding of vitronectin has been described previously (34). Briefly, the vitronectin binding assay was performed by allowing 100  $\mu$ l of a 10- $\mu$ g/ml solution of vitronectin (Sigma catalog no. V9881) in 50 mM sodium carbonate buffer (pH 9.6) to coat wells of a 96-well plate (Nunc-Immuno MaxiSorp plate) overnight at 4°C. Equal numbers of HaCaT cells were placed on the plates and allowed to attach to the vitronectin-coated wells in the presence of various concentrations of the anti- $\alpha_v\beta_5$  integrin antibody (mouse anti-human integrin  $\alpha_v\beta_5$  preservative-free monoclonal antibody; CHEMICON International, Inc. catalog no. MAB1961Z), the mouse immunoglobulin G1 isotype control antibody (Pharmingen catalog no. 03171D), or no antibody for 1 h at 35°C. After incubation, the wells were washed five times with PBS. The cells were then fixed with a 2.5% glutaraldehyde solution. The fixed cells were washed twice with borate buffer. After the preparations were stained with methylene blue, cell numbers were determined by reading the absorbance at 595 nm. The percentage of attachment was calculated by dividing the absorbance of a well that received antibodies by the average absorbance of the wells that were coated with vitronectin but did not receive any antibody. Wells that received HaCaT cells but were not coated with vitronectin served as the background wells.

**Animal experiments.** Crossbred (Yorkshire, Landrace, and Hampshire cross) 6- to 12-week-old female pigs were used to examine the virulence of a *dsrA* mutant of *H. ducreyi*. The methods used, including the immune cell depletion treatment, have been described previously (20, 36, 37). Briefly, after juvenile swine ears were cleaned with a mixture of water and 95% ethanol, they were inoculated dorsally with 10- $\mu$ l portions of  $10^6$ - to  $10^7$ -CFU/ml suspensions of strains 35000 and FX517 by using Multi-test multiple-skin-test applicators (Lincoln Diagnostics catalog no. MH-6).

The biopsy, recovery, and histology methods used have been described previously (20, 36, 37). Briefly, biopsies were taken 2 and 7 days after inoculation with a 6-mm punch (Acu-Punch USA; Acuderm Inc. catalog no. CE0413) and were bisected with a scalpel (Acu-Scalpel; Acuderm Inc. catalog no. S100.) One half of each biopsy was minced and plated on appropriate chocolate agar plates for recovery, and the other half was fixed for histological analysis. The sample halves used for histological observation were fixed in 4% paraformaldehyde in PBS at 4°C, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Histopathology Reference Laboratory, Richmond, Calif.). Stained biopsies from normal pigs were blindly scored for histology by using our previously developed scoring system (37). We checked that proper antibiotic resistance and susceptibility had been maintained when viable colonies were recovered by patching both 35000 and FX517 onto both plain chocolate agar plates and plates containing the correct antibiotics.

**Statistical analysis.** Statistical analysis was performed by using Sigma Stat, version 2.0 (Jandel Scientific, San Rafael, Calif.). The specific methods utilized are indicated below.

## RESULTS

**DsrA expression is necessary for efficient attachment of *H. ducreyi* to HaCaT cells but not to HS27 cells.** We compared the attachment of the wild-type parent strain (35000) and the attachment of the isogenic *dsrA* mutant strain (FX517) to HaCaT cells in parallel. HaCaT cells are a spontaneously transformed human keratinocyte epithelial cell line that maintains its full epidermal differentiation capacity (8). Approximately 10-fold less FX517 than 35000 adhered to HaCaT cells (Fig. 1) ( $P \leq 0.001$ , as determined by the Mann-Whitney rank sum test). A non-*dsrA* cryptic mutation was not responsible for FX517's attachment defect as FX517 complemented with pUNCH1260, a plasmid containing *dsrA* (13), attached to HaCaT cells at wild-type levels (Fig 1). DsrA expression was responsible for the restoration of attachment, as the pLSKS vector did not enhance attachment (Fig. 1).

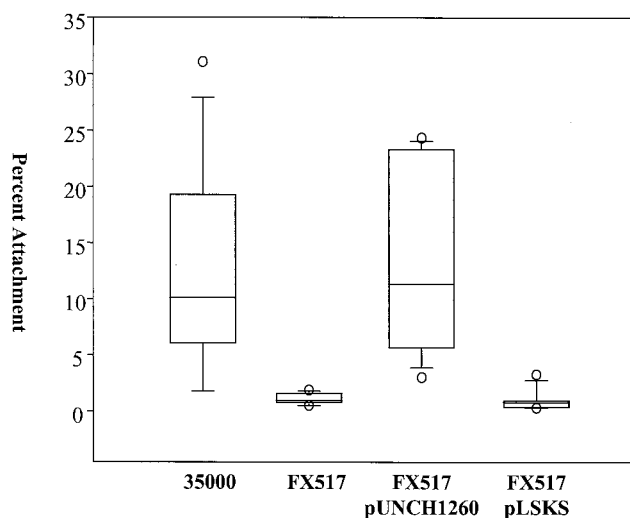


FIG. 1. Effect of DsrA expression on *H. ducreyi* attachment to HaCaT cells. In each assay, approximately  $10^6$  CFU of each strain was incubated with  $10^5$  HaCaT cells for 2 h. The percentage adherence was determined as follows: (number of adherent CFU after 2 h of incubation/total number of CFU after 2 h of incubation)  $\times$  100. The data are presented in a box plot due to unequal variance. Each box indicates the median, 25th, and 75th percentiles, and the error bars indicate the 10th and 90th percentiles.

To investigate the role of DsrA in the attachment of *H. ducreyi* to fibroblasts, we repeated the attachment assays using HS27, a human foreskin fibroblast cell line. FX517 and 35000 adhered equally well to this fibroblast cell line (Fig. 2). This result indicated that DsrA was not required for attachment of *H. ducreyi* to fibroblasts.

**DsrA is sufficient for attachment to HaCaT cells.** The related bacterial strain *H. influenzae* Rd does not express DsrA and does not readily attach to HaCaT cells (40). We transformed *H. influenzae* with either pUNCH1260 or pLSKS to examine the effect of DsrA expression on adherence. *H. influenzae* transformed with pUNCH1260 expressed a protein whose size was consistent with the size of DsrA, and the iden-

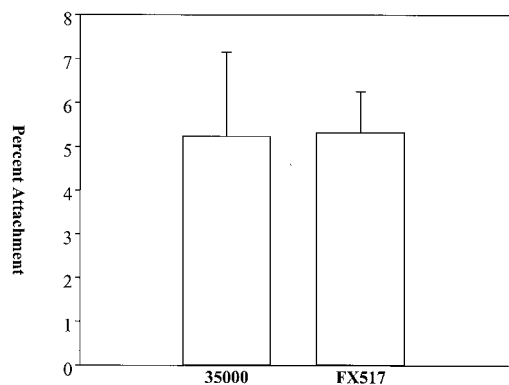


FIG. 2. Effect of DsrA expression on *H. ducreyi* attachment to HS27 cells. In each assay, approximately  $10^6$  CFU of each strain was incubated with  $10^5$  HS27 cells for 2 h. The percentage of adherence was determined as follows: (number of adherent CFU after 2 h of incubation/total number of CFU after 2 h of incubation)  $\times$  100. The data are means and standard deviations.

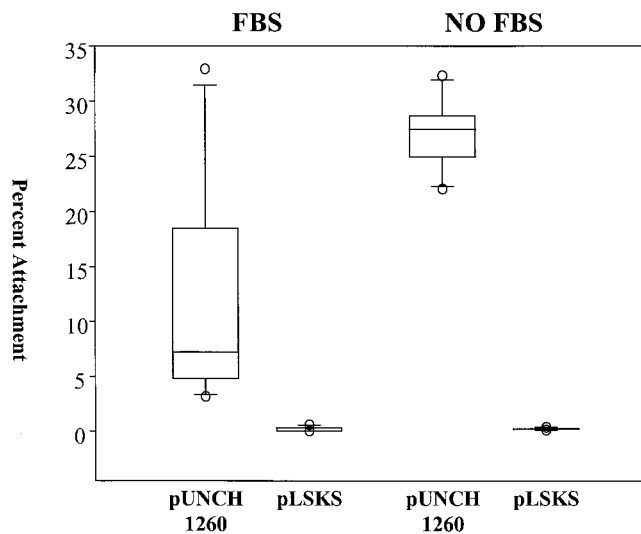


FIG. 3. rDsrA enhanced the attachment of *H. influenzae* to HaCaT cells. rDsrA confers an attachment phenotype to *H. influenzae* in the presence and in the absence of FBS. The attachment data are presented in a box plot due to unequal variance. Each box indicates the median, 25th, and 75th percentiles, and the error bars indicate the 10th and 90th percentiles.

tity of the protein was confirmed by Western blotting (data not shown). rDsrA clearly fractionated into the Sarkosyl-insoluble outer membrane preparations, suggesting that it was correctly localized to the outer membrane of *H. influenzae* (data not shown). Transformation with pUNCH1260 significantly enhanced the ability of *H. influenzae* to adhere to HaCaT cells compared to the results obtained after transformation with the vector, pLSKS, alone ( $P \leq 0.001$ , as determined by the Mann-Whitney rank sum test) (Fig 3). In fact, rDsrA-expressing *H. influenzae* attached to HaCaT cells at levels similar to the levels of attachment of *H. ducreyi* 35000.

**DsrA directly binds vitronectin.** Since DsrA exhibits homology to UspA2 and UspA2 mediates *M. catarrhalis* binding of vitronectin (27), we investigated the possibility that DsrA binds vitronectin. We attempted to pull vitronectin out of NHS with various *H. ducreyi* strains. Only 35000, a strain that expressed DsrA, bound vitronectin (Fig 4A). As wild-type *H. ducreyi* could bind vitronectin from NHS, we used vitronectin to affinity purify its receptor from *H. ducreyi*. Biotinylated human vitronectin, recombinant human vitronectin, and bovine vitronectin purified only one radiolabeled outer membrane protein from 35000 and no radiolabeled outer membrane protein from FX517 (Fig. 4B). The protein from 35000 was the approximately the same size as DsrA. Since it was possible that a nonradiolabeled protein was the vitronectin receptor and DsrA was just in a complex with this protein, we expressed rDsrA in *H. influenzae* Rd, a strain that normally does not bind vitronectin. Cloned *dsrA*, but not the plasmid vector alone, conferred upon *H. influenzae* the ability to bind vitronectin in solution (Fig. 4A). As only bacteria expressing DsrA bound vitronectin and vitronectin affinity purified an outer membrane protein receptor only from DsrA-expressing *H. ducreyi*, we concluded that DsrA was solely responsible for the binding of vitronectin by *H. ducreyi*.

**DsrA-mediated attachment to HaCaT cells does not require vitronectin.** DsrA is required for both *H. ducreyi* adherence to keratinocytes and vitronectin binding. Accordingly, we considered the possibility that *H. ducreyi* might use the vitronectin present in FBS (18) as a bridge to attach to HaCaT cells. Since *H. ducreyi* died in tissue culture media lacking FBS (data not shown), we performed side-by-side *H. influenzae* attachment experiments with tissue culture media that contained and lacked FBS. A lack of FBS had no impact on the ability of *H. influenzae* Rd transformed with pUNCH1260 to attach to HaCaT cells (Fig. 3). Similarly, a lack of FBS did not enhance the ability of *H. influenzae* Rd transformed with pLSKS to adhere. When the attachment percentages obtained in the non-FBS-

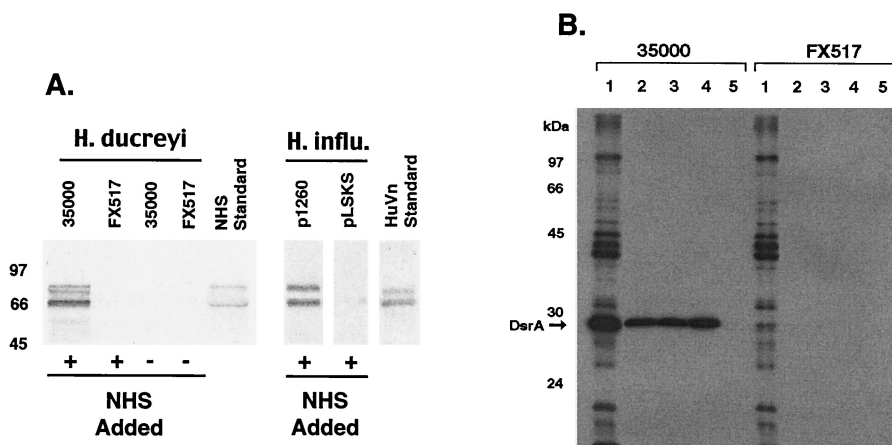


FIG. 4. DsrA is responsible for the binding of vitronectin by *H. ducreyi*. (A) Western blot of DsrA-expressing and non-DsrA-expressing strains that were exposed to NHS, solubilized, transferred, and probed with anti-human vitronectin antibody. (B) Autoradiograph of affinity-purified vitronectin binding proteins from iodinated *H. ducreyi* strains 35000 and FX517. Lanes 1, iodinated *H. ducreyi* whole cells; lanes 2 to 5, affinity purification using human vitronectin, recombinant human vitronectin, bovine vitronectin, and no vitronectin, respectively. The position of DsrA is indicated by the arrow. HuVn, human vitronectin.

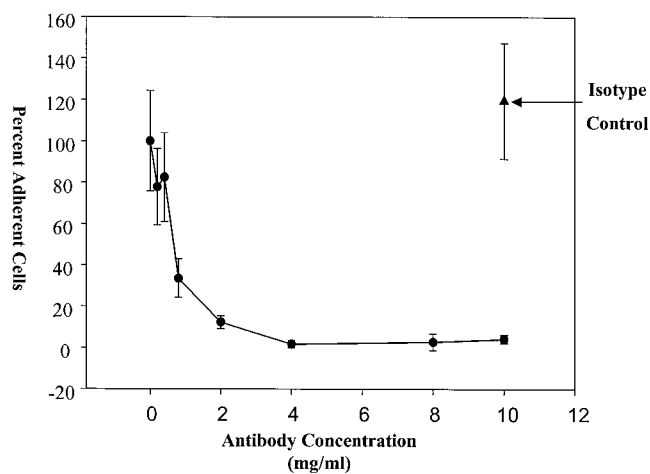


FIG. 5. Effect of a blocking  $\alpha_v\beta_5$  monoclonal antibody on HaCaT cells binding to vitronectin. Symbols: ●, percentage of HaCaT cell attachment in the presence of various concentrations of the  $\alpha_v\beta_5$  antibody; ▲, percentage of HaCaT cell attachment in the presence of the isotype control. The data are normal and are means and standard deviations.

containing *H. influenzae* experiments were compared, there was once again a statistically significant difference between the attachment of the DsrA-expressing bacteria and the attachment of the non-DsrA-expressing bacteria ( $P \leq 0.001$ , as determined by the Mann-Whitney rank sum test). This result suggested that vitronectin was not required for attachment mediated by DsrA.

Additional evidence that vitronectin does not play a role in the attachment of *H. ducreyi* to HaCaT cells was obtained from experiments performed with a function-blocking monoclonal antibody to integrin  $\alpha_v\beta_5$ . Integrin  $\alpha_v\beta_5$  is the only vitronectin receptor in HaCaT cells (34); accordingly, a monoclonal antibody to integrin  $\alpha_v\beta_5$  completely blocked HaCaT cell binding to immobilized vitronectin in a concentration-dependent fashion (Fig. 5). *H. ducreyi* effectively adhered to HaCaT cells in 5- and 1- $\mu$ g/ml solutions of this monoclonal antibody (Fig. 6). These antibody concentrations inhibited HaCaT cell binding to vitronectin by over 65 and 99%, respectively (percentages based on two-parameter exponential decay regression analysis of the data). Since the monoclonal antibody to  $\alpha_v\beta_5$  blocked the binding of HaCaT cells to vitronectin but did not affect the attachment of *H. ducreyi* to HaCaT cells, we concluded that *H. ducreyi* does not use vitronectin as a bridge to attach to HaCaT cells and in fact binds to HaCaT cells independent of vitronectin.

**Expression of DsrA is essential for complete virulence in the swine model of chancroid infection in both normal and immune cell-depleted animals.** We multiply inoculated the ears of three normal and three cyclophosphamide (CPA)-treated immune cell-depleted animals with strains 35000 and FX517. Lesion biopsies were collected 2 and 7 days after inoculation. Lesion biopsies, not animals, were considered independent units. After bisection, one half of each biopsy was analyzed for organism recovery, and the other half was fixed, sectioned, and stained with hematoxylin and eosin. We scored the stained samples by using a previously described histology scoring sys-

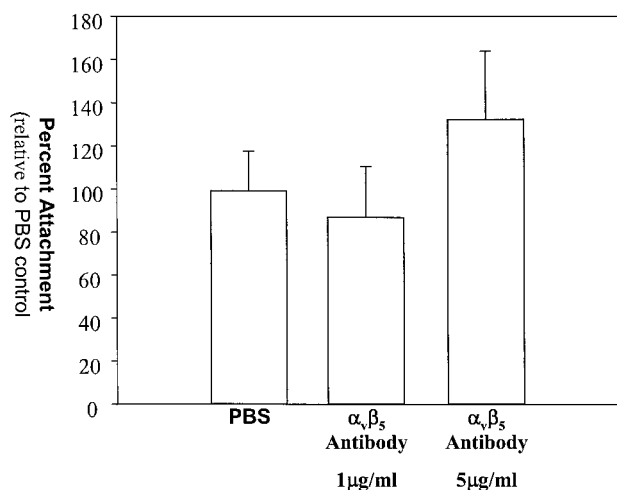


FIG. 6. Effect of a blocking  $\alpha_v\beta_5$  monoclonal antibody on *H. ducreyi* attachment to HaCaT cells. The concentrations of the  $\alpha_v\beta_5$  antibody are indicated. The data are normal and are means and standard deviations.

tem (37). Briefly, a score of 1 indicates normal skin, a score of 2 indicates the presence of dermal perivascular and interstitial mononuclear cell infiltrate, a score of 3 indicates the presence of intraepidermal pustules, a score of 4 indicates the presence of epidermal pustules plus keratinocyte cytopathology and acanthosis, and a score of 5 indicates ulceration or epidermal necrosis with dermal erosion and a confluence of immune cells. The histology slides were coded and scored blindly to prevent introduction of bias. Only lesions from normal animals were scored with our five-point system as it is not possible to score histology with our scale in immune cell-depleted animals since it is the immune cells themselves which contribute almost entirely to lesion severity (36).

**Normal pigs.** FX517 was impaired in its ability to infect normal pigs, as measured by its decreased recovery and histology scores compared to those of 35000. The average number of CFU recovered from FX517-infected lesion biopsies was 10-fold lower than the average number of CFU recovered from 35000-infected lesion biopsies on day 2, and there were no viable bacteria in any of the FX517-inoculated lesions on day 7 (Fig. 7). The differences in recovery between 35000- and FX517-inoculated lesions were statistically significant on day 2 ( $P = 0.014$ , as determined by the Mann-Whitney rank sum test) and day 7 ( $P = 0.014$ , as determined by the Mann-Whitney rank sum test). These results suggested that the *dsrcA* mutant was cleared faster from the normal pigs as there were fewer FX517 culture-positive biopsies and the few positive biopsies contained far fewer live organisms. The histology scores for the FX517-inoculated lesions were significantly lower on day 7 than the histology scores for the 35000-infected lesions ( $P \leq 0.001$ , as determined by a *t* test) (Fig. 8). The mean lesion histology score for 35000 on day 7 was 3.96, while the mean histology score for FX517 was 2.28 (Fig. 8). The decreased recovery on day 2, the lack of recovery of viable organisms on day 7, and the significantly reduced histology scores for strain FX517 relative to the scores for 35000 on day 7 suggested that by day 7 the FX517 infection had experienced

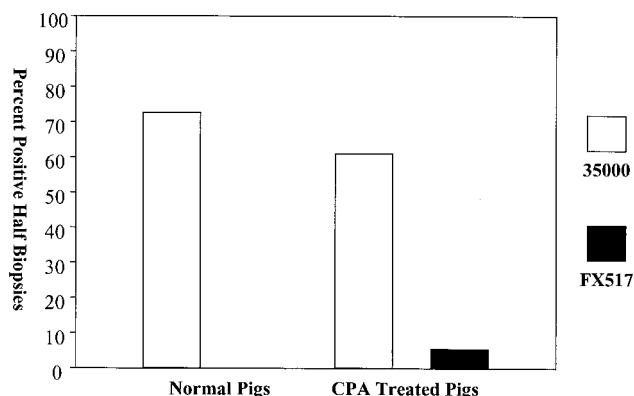


FIG. 7. Percentages of day 7 lesion biopsies from normal and CPA-treated, immune cell-depleted pigs that were culture positive. The percentages were determined as follows: (number of culture-positive lesion biopsies/total number of lesion biopsies) × 100.

much greater clearance than the 35000 infection. These results are in agreement with the findings obtained with the human challenge model (7), as Bong et al. concluded that the *dsrA* mutant was attenuated in virulence in the human challenge model.

**CPA-treated pigs.** The results obtained with the CPA-treated, immune cell-depleted animals were similar to the results obtained with the normal pigs. The percentage of FX517 culture-positive biopsies was greatly reduced compared to the percentage of 35000 culture-positive biopsies on both days 2 and 7 (Fig. 7). While there was not a statistically significant difference in the recovery of viable organisms between the 35000- and FX517-infected lesions on day 2, there was a statistically significant difference on day 7 ( $P = 0.012$ , as determined by a *t* test). Furthermore, in the CPA-treated, immune cell-depleted animals only one of the FX517 lesions contained viable *H. ducreyi* on day 7, while more than one-half of the 35000-inoculated lesions contained viable bacteria. This is a significant finding as FX517 died even when immune cell-mediated clearance was greatly impaired, as the numbers of

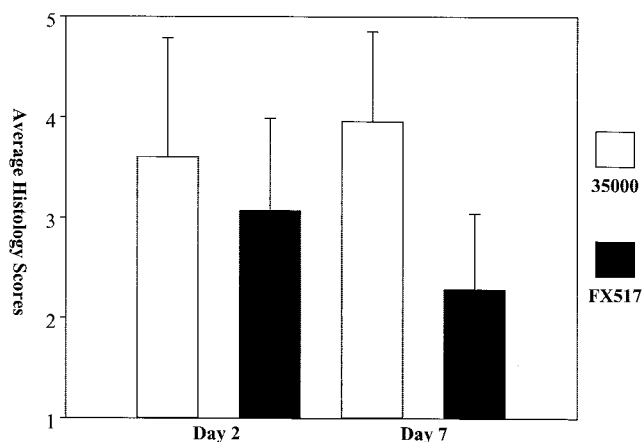


FIG. 8. Average histology scores from day 2 and 7 lesion biopsies from normal pigs. The data are normal and are means and standard deviations.

TABLE 2. Effect of CPA treatment on pigs

Day	% Reduction in cell no. after CPA treatment <sup>a</sup>		
	Neutrophils	Monocytes	Lymphocytes
0	97.707 ± 4.157	97.955 ± 3.542	83.469 ± 11.921
2	99.710 ± 0.502	97.754 ± 3.889	87.220 ± 5.555
7	93.253 ± 3.789	83.535 ± 7.447	84.660 ± 2.328

<sup>a</sup> The values are the mean percent reductions in cell number ± standard deviations. The percentages were based on data obtained from blood drawn prior to the start of treatment. Treatment was begun 4 days prior to inoculation.

neutrophils, monocytes, and lymphocytes were severely reduced after CPA treatment (Table 2).

DISCUSSION

Without adhesion to host cells, initiation of natural infection is highly unlikely (5, 31), yet little is known about how *H. ducreyi* adheres to human cells. Similarity to other bacterial adhesins inspired us to investigate DsrA as a possible *H. ducreyi* adhesin. We measured the adherence of wild-type *H. ducreyi* strain 35000 and the isogenic *dsrA* mutant FX517 to HaCaT cells and HS27 cells. While there was no difference in the percentages of attachment of FX517 and 35000 to HS27 cells, there was consistently a 10-fold difference in the percentages of attachment to HaCaT cells. Before we concluded that DsrA was both necessary and sufficient for attachment of *H. ducreyi* to HaCaT cells, we had to rule out two competing possibilities. The first possibility was that there was an undetected mutation elsewhere in the genome and that this mutation was responsible for the reduced attachment of FX517. The second possibility was that the loss of DsrA, a major outer membrane protein, induced the component actually responsible for mediating adherence to be aberrantly localized or expressed. To test the first hypothesis, we measured the attachment ability of FX517 transformed with a plasmid containing the *dsrA* gene. To test the second hypothesis, we measured the attachment phenotype conferred by rDsrA expression in the closely related strain *H. influenzae* Rd.

DsrA expression successfully restored attachment of the mutant to wild-type levels. From these experiments, we concluded that DsrA was necessary for attachment and that an undetected mutation was not responsible for the attachment defect. Expression of rDsrA in *H. influenzae* Rd dramatically increased attachment to HaCaT cells, while transformation with the control plasmid had no discernible effect. The ability to transfer the attachment phenotype along with protein expression demonstrated that DsrA was sufficient for attachment to HaCaT cells.

Recent findings obtained with the human challenge model call into question the significance of keratinocyte binding in chancroid infection as *H. ducreyi* is not often found in association with keratinocytes (3, 4). However, attachment to keratinocytes may be an important part of chancroid pathogenesis as both the human challenge model and the swine model use Multi-test multiple-skin-test applicators. These devices deliver the majority of organisms through the epidermis to the dermis (unpublished results) (3). Even in human challenge samples examined immediately after inoculation, the majority of the

bacteria are located in the dermis (3). As this inoculation method appears to bypass the epithelial layer, it is possible that this procedure does not accurately reflect the natural mode of transmission or inoculation. Therefore, the lack of association between *H. ducreyi* and keratinocytes in the human challenge model does not eliminate the possibility that bacterial interactions with keratinocytes are a significant part of *H. ducreyi* pathogenesis.

After we determined that DsrA was required for *H. ducreyi* to attach to HaCaT cells, our next goal was to determine the mechanism of attachment. As DsrA binds vitronectin, we thought that DsrA might use vitronectin as a bridge to connect to the HaCaT cell surface. Support for this idea came from the fact that other bacteria, such as *Neisseria gonorrhoeae* (10, 11, 17) and *Pneumocystis carinii* (26), effectively use vitronectin as a bridging molecule to attach to and invade human cells. Vitronectin is a major cell attachment-promoting protein present in FBS (18), so to test the role of vitronectin in the attachment of *H. ducreyi*, we first attempted to perform the attachment assays in the absence of FBS. Unfortunately, these attachment assays were not successful as *H. ducreyi* did not survive in tissue culture media lacking FBS (data not shown). Fortunately, *H. influenzae* persisted without a problem in tissue culture media lacking FBS. Therefore, we decided that parallel to performing the *H. influenzae* Rd experiments in the presence of FBS, we would also perform experiments in the absence of FBS. Expression of DsrA, regardless of the presence of FBS in the tissue culture media, significantly enhanced the attachment of *H. influenzae* to HaCaT cells.

These data did not support our vitronectin bridging model, but we did not discard our model yet. We concluded that we needed to block vitronectin association with HaCaT cells and then observe its effect on the adherence of *H. ducreyi* to HaCaT cells. As integrin  $\alpha_v\beta_5$  is solely responsible for the binding of vitronectin by HaCaT cells (34), we used an anti-human integrin  $\alpha_v\beta_5$  monoclonal antibody to inhibit HaCaT cell binding to vitronectin in a concentration-dependent fashion. This antibody had no discernible effect on the adherence of *H. ducreyi* to HaCaT cells. As the  $\alpha_v\beta_5$  monoclonal antibody blocked the binding of HaCaT cells to vitronectin but did not affect the attachment of *H. ducreyi* to HaCaT cells, we concluded that *H. ducreyi* binds HaCaT cells independent of an association with vitronectin.

The *dsrA* mutant is avirulent in the human challenge model of chancroid infection (7). Nevertheless, we decided to test the importance of DsrA expression in *H. ducreyi* pathogenesis with the swine model of chancroid infection. These experiments were done both as a validation of the swine model and because of the unique opportunities for experimentation that exist with the swine model. With pigs, we can observe the progression of pustules to ulcers and perform experiments on immunosuppressed animals. These latter experiments allow examination of the bacterial contribution to infection independent of the host response (37).

FX517 was impaired in virulence in both the normal and immunosuppressed swine models of chancroid infection. Decreased virulence was demonstrated in the CPA-treated pigs by the decreased recovery of FX517 relative to the recovery of 35000 and by the lower number of lesions that contained any viable FX517 cells on day 7. The loss of DsrA left *H. ducreyi*

sufficiently attenuated in virulence that even in the absence of immune cell-mediated clearance, FX517 was not as virulent as 35000.

In normal pigs, the average recovery of FX517 from lesion biopsies and the percentage of FX517 culture-positive biopsies were significantly reduced compared to the data obtained with 35000 on both day 2 and day 7. In fact, no viable FX517 cells were found in any lesions biopsied 7 days after inoculation. These data and the reduced average histology scores from the day 7 biopsies suggested that normal animals cleared the *dsrA* mutant faster than they cleared the wild type. As DsrA is also required for serum resistance in *H. ducreyi* (13), the decreased survival of the bacteria in the animals could be due to either the decreased attachment ability or the increased serum susceptibility or both. Determination of the specific effects of the two phenotypes awaits mapping of the individual functions to distinct areas of the protein and generation of specific mutants in which the two functions attributed to DsrA are separated.

While we have identified a means of attachment, we do not suggest that this is the only means of host association. It is likely that *H. ducreyi* employs multiple means of attachment. In fact, there are at least two other bacterial factors that have demonstrated roles in *H. ducreyi* attachment. One is the *H. ducreyi* homolog of GroEL, a 58.5-kDa heat shock protein (Hsp), which partially influences the adherence of *H. ducreyi* to HEp-2 cells (15). The other ligand involves the *flp* operon. Products of the *flp* operon mediate the attachment of *H. ducreyi* to certain nonbiological surfaces and human foreskin fibroblasts (30). Interestingly, while products of the *flp* operon mediate attachment to human foreskin fibroblasts, they are not involved in the attachment of *H. ducreyi* to HaCaT cells (30). DsrA exhibited opposite specificities as it was necessary and sufficient for attachment to HaCaT cells but was not required for the attachment of *H. ducreyi* to HS27 fibroblasts. The fact that these two adhesins have complementary specificities suggests that they might function in different stages of infection. Bauer et al. have shown in the human challenge model that at the pustular stage of the disease the majority of the bacteria are found in the dermis (3, 4). Perhaps DsrA is required during an early stage of infection, while the products of the *flp* operon are essential later during the pustular and ulcerative stages. In conclusion, we think that DsrA is not only an important adhesin for *H. ducreyi* but also an important virulence factor and possible vaccine candidate.

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