

## Expression of *Haemophilus ducreyi* Collagen Binding Outer Membrane Protein NcaA Is Required for Virulence in Swine and Human Challenge Models of Chancroid

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*Haemophilus ducreyi*, the etiologic agent of the sexually transmitted genital ulcer disease chancroid, has been shown to associate with dermal collagen fibers within infected skin lesions. Here we describe NcaA, a previously uncharacterized outer membrane protein that is important for *H. ducreyi* collagen binding and host colonization. An *H. ducreyi* strain lacking the *ncaA* gene was impaired in adherence to type I collagen but not fibronectin (plasma or cellular form) or heparin. The mutation had no effect on serum resistance or binding to HaCaT keratinocytes or human foreskin fibroblasts in vitro. *Escherichia coli* expressing *H. ducreyi* NcaA bound to type I collagen, demonstrating that NcaA is sufficient to confer collagen attachment. The importance of NcaA in *H. ducreyi* pathogenesis was assessed using both swine and human experimental models of chancroid. In the swine model, 20% of lesions from sites inoculated with the *ncaA* mutant were culture positive for *H. ducreyi* 7 days after inoculation, compared to 73% of wild-type-inoculated sites. The average number of CFU recovered from mutant-inoculated lesions was also significantly reduced compared to that recovered from wild-type-inoculated sites at both 2 and 7 days after inoculation. In the human challenge model, 8 of 30 sites inoculated with wild-type *H. ducreyi* progressed to the pustular stage, compared to 0 of 30 sites inoculated with the *ncaA* mutant. Together these results demonstrate that the collagen binding protein NcaA is required for *H. ducreyi* infection.

*Haemophilus ducreyi* is the etiologic agent of the sexually transmitted genital ulcer disease (GUD) chancroid. Chancroid is rare in the United States and Western Europe. It is prevalent in tropical resource-poor regions and is endemic in certain areas of Africa and Asia (11, 38, 39, 47), where there is also a significant confluence of chancroid infection and human immunodeficiency virus (HIV) type 1 seropositivity (17, 30, 40). In these regions, case-control studies have shown that the relative risk of acquiring HIV infection for GUD patients ranged from an odds ratio of 3 to 18.2 (20, 49, 60). Per individual sexual act, GUD produces a 4- to 23-fold enhancement of HIV acquisition (22, 33), and GUD in an HIV-positive person doubles the relative risk per coital act of viral transmission to an HIV-negative partner (22, 61). Furthermore, HIV-positive chancroid patients often present ulcers that are more severe, longer lasting, and more difficult to cure (31, 45). These observations outline the strong synergistic relationship between GUD and HIV (23, 24). Aggressive control of chancroid, a prevalent and highly transmissible GUD, may have the potential to curtail the global spread of HIV (34, 37, 42).

Chancroid presents as a painful genital ulcer with a ragged

erythematous border. This lesion emits a purulent exudate replete with macrophages, neutrophils, T and B lymphocytes, and bacteria (both *H. ducreyi* and other opportunistic species) (32). *H. ducreyi* does not cause disseminated infection in healthy or immunocompromised individuals and appears to be well adapted to survive in the skin of its obligate human host (59). However, the mechanism by which *H. ducreyi* colonizes skin remains poorly understood. *H. ducreyi* attaches to the HaCaT keratinocyte cell line in vitro (62), and this association is dependent upon expression of the outer membrane protein DsrA (15). DsrA also confers resistance to complement-mediated killing by normal human serum (19). *H. ducreyi* lacking DsrA is attenuated in both human and swine experimental chancroid models (12), but it is not clear how the keratinocyte attachment or serum resistance properties of DsrA specifically contribute to this phenotype. *H. ducreyi* also adheres to human foreskin dermal fibroblasts and forms microcolonies in vitro; this aggregative-adhesive phenotype depends on expression of the fimbria-like protein encoded by the *flp* gene cluster (41). Human volunteers inoculated with an *flp* mutant display lesions that fail to progress from the papular to the pustular stage, and live *H. ducreyi* was recovered from only 1 of 31 of these sites (53), suggesting that the above-described phenotype contributes to *H. ducreyi* survival in skin.

Despite the observation that *H. ducreyi* adheres to skin epithelial cells and fibroblasts in vitro, direct association between *H. ducreyi* and these types of cells within the chancroid lesion has not been proven. *H. ducreyi* was, however, shown to bind the extracellular matrix (ECM) proteins fibronectin, laminin,

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and collagen types I and III in vitro (9). In early-stage lesions derived from the human challenge model, *H. ducreyi* colocalized with macrophages and neutrophils within the pustule and also associated with fibrin and collagen in the dermis directly beneath the pustule (8). These findings suggest that adherence to ECM proteins, which comprise a major structural component of skin, may be an important contributing factor in *H. ducreyi* skin colonization.

We recently found that protection against *H. ducreyi* infection in the swine model of chancroid coincides with the emergence of *H. ducreyi*-specific bactericidal serum antibodies and that transfer of this immune serum protects naïve pigs against *H. ducreyi* challenge (16). Antibodies present in this immune serum recognized a distinct subset of *H. ducreyi* outer membrane proteins. Here we describe one of these proteins, termed NcaA, and its role in mediating binding of *H. ducreyi* to collagen binding and in *H. ducreyi* pathogenesis.

#### MATERIALS AND METHODS

**Bacterial growth conditions.** *H. ducreyi* strains were grown on chocolate agar containing 1.5% Bacto agar, 2.5% brain heart infusion (BHI), 1% bovine hemoglobin, 1% IsoVitalX (Becton Dickinson, Cockeysville, MD), 5% newborn calf serum, and 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD) at 35°C in a humidified incubator with 5% CO<sub>2</sub>. *Escherichia coli* strains were propagated in LB broth or agar at 37°C. Ampicillin, kanamycin, vancomycin, or chloramphenicol was added to media when indicated to a final concentration of 100, 40, 3, or 1 µg/ml, respectively.

**Radioimmunoprecipitation of *H. ducreyi* outer membrane proteins.** *H. ducreyi* 35000HP was grown overnight on chocolate agar as a confluent lawn, harvested, and resuspended in phosphate-buffered saline (PBS). This suspension was added to a precoated IODO-GEN tube (Pierce Biotechnology, Rockford, IL) with 1.0 mCi of <sup>125</sup>I. Labeling was quenched after 3 min with an excess of GC medium base broth. Surface-iodinated bacteria were washed and mixed with preimmune or *H. ducreyi*-immune pig serum, incubated for 15 min at room temperature with occasional mixing, again washed with PBS, and pelleted. The resulting cell pellet was then resuspended in radioimmunoprecipitation buffer (2% Zwittergent 3-14, 50 mM Tris [pH 8.0], 5 mM EDTA, 0.15 M NaCl) and incubated with rocking for 30 min at 37°C. Insoluble matter was removed by centrifugation (5 min at 14,000 × g), and the supernatant containing solubilized antibody-labeled outer membranes was mixed with protein G-agarose (Sigma Chemical, St. Louis, MO). After overnight rocking at 4°C, the slurry was washed three times with radioimmunoprecipitation buffer and once with distilled H<sub>2</sub>O. The resulting pellet was resuspended in Laemmli sample buffer containing β-mercaptoethanol, boiled, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved bands of iodinated (outer membrane) proteins bound by preimmune or *H. ducreyi*-immune pig serum were visualized by autoradiography.

**Identification, cloning, and mutagenesis of *ncaA*.** *H. ducreyi* outer membrane proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Protein bands were excised and subjected to Edman degradation at the UNC Protein Sequencing & Peptide Synthesis Facility (University of North Carolina, Chapel Hill). Amino acid sequences were compared using EMBOSS pairwise alignment algorithms hosted by the European Bioinformatics Institute. (<http://www.ebi.ac.uk/emboss/align/index.html>). The *ncaA* gene was amplified by PCR from *H. ducreyi* 35000HP genomic DNA by using forward primer 5'CTAGGCTAATGAGAGGATATATCG3' and reverse primer 5'TTGTACGCATCGCTTGTCC3'. The resulting product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and was verified by automated DNA sequencing (UNC Genome Analysis Facility, Chapel Hill, NC). To produce an inactivated allele of *ncaA*, a 134-bp segment of *ncaA* between two NdeI sites was excised and replaced with a chloramphenicol acetyltransferase gene (*cat*) cassette derived from pUNCH40 (57). The *cat*-interrupted *ncaA* gene (*ncaA::cat*) was subcloned into plasmid pRSM2072 (a gift of Robert S. Munson, Jr.), and the resulting construct was used as a suicide vector to perform allelic exchange in *H. ducreyi* as described by Bozue et al. (14). *H. ducreyi* 35000HP was transformed with pRSM2072-*ncaA::cat* and plated on chloramphenicol to select cointegrates. Cointegrates were then plated on medium containing chloramphenicol and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 µg/ml) to counterselect for excision of the pRSM2072 vector backbone and the



FIG. 1. The *H. ducreyi* 35000HP *ncaA* locus and neighboring genes. RNA transcripts of prolyl-tRNA ligase (*proS*, HD1919), ATP-dependent DNA helicase (*recG*, HD1921), and RNA polymerase  $\omega$  subunit (*rpoZ*, HD1923) were detected by reverse transcription-PCR and found to be produced in both 35000HP and 35000HP*ncaA* (in which the *ncaA* gene is insertionally inactivated by *cat* as shown).

wild-type *ncaA* allele. Southern blot and PCR analyses of the mutant confirmed that a single copy of the *cat* cassette had been inserted into *ncaA* (data not shown). Transcriptions of genes adjacent to the *ncaA* locus (Fig. 1) in 35000HP and 35000HP*ncaA* were found equivalent by reverse transcriptase PCR, showing that *ncaA* inactivation did not exert a negative polar effect on local transcription. To examine whether 35000HP*ncaA* had phenotypes other than that caused by the mutation, 35000HP*ncaA* was compared to the master 35000HP stock used to infect human volunteers. The phenotypes included comparisons of growth rates in broth and outer membrane protein (OMP) and lipooligosaccharide (LOS) profiles, as previously described (21). 35000HP and 35000HP*ncaA* had similar generation times in broth (data not shown). OMPs and LOSs from 35000HP and 35000HP*ncaA* were analyzed by SDS-PAGE, followed by Coomassie blue and silver staining. The OMP and LOS profiles demonstrated no difference between the parent and the mutant (data not shown). Because NcaA is a low-abundance protein as determined by Coomassie blue staining, ablation of NcaA protein expression in the mutant strain was confirmed by Western blot analysis with *H. ducreyi*-immune pig serum. Whole-cell lysates of 35000HP and 35000HP*ncaA* were heated in sample buffer containing SDS and β-mercaptoethanol and then resolved by SDS-PAGE and blotted to nitrocellulose. The blot was probed with *H. ducreyi*-immune pig serum (1:1,000), followed by peroxidase-conjugated rabbit anti-pig immunoglobulin (1:32,000; Sigma Chemical, St. Louis, MO) and chemiluminescence detection (SuperSignal Pico; Pierce Biotechnology, Rockford, IL).

**Serum susceptibility assay.** Serum susceptibility assays were performed as previously described (19). Briefly, bacteria grown on solid medium for 16 to 18 h were collected and suspended in 2 ml of BHI, vortexed for 5 seconds, and allowed to settle for 5 min. After settling, the upper 1 ml was removed and the cell density was adjusted such that the final concentration of bacteria was approximately  $1 \times 10^3$  to  $5 \times 10^3$  CFU/ml. Bacteria were mixed with fresh or heat-inactivated normal swine serum to a final concentration of 25% or 50%. Following incubation for 45 min (35°C, 5% CO<sub>2</sub>), the bacterial suspensions were plated to count viable CFU. Percent survival was calculated by dividing the number of CFU from fresh serum by the number from heat-inactivated serum and multiplying by 100. Assays were performed in triplicate and on three separate days.

**Fluorescein labeling of bacterial strains.** Overnight cultures of *H. ducreyi* grown on chocolate agar or of *E. coli* grown in LB broth were collected and washed three times in PBS, followed by passage through a 30-gauge needle to disperse large aggregates. Carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) in dimethyl sulfoxide was added to a final concentration of 12 µM, and the suspension was incubated with gentle mixing at 35°C for 25 min. *H. ducreyi* labeling reactions were quenched with an equal volume of BHI medium supplemented with 1% IsoVitalX (Becton Dickinson, Cockeysville, MD), 1% hemoglobin (Becton Dickinson, Cockeysville, MD), and 10% fetal bovine serum (Life Technologies, Rockville, MD). *E. coli* labeling reactions were quenched by addition of bovine serum albumin to a final concentration of 1%. The resulting fluorescein-labeled bacteria were collected by centrifugation at 1,040 × g for 10 min and resuspended in LB (*E. coli*) or in BHI supplemented with 1% IsoVitalX and 1% hemoglobin (*H. ducreyi*). Bacteria were then incubated for 40 to 60 min at 35°C with shaking. The doubling times of fluorescein-labeled and unlabeled *H. ducreyi* were similar (data not shown).

**Extracellular matrix protein attachment assay.** White 96-well plates (Fluoronunc; Nalge Nunc, Rochester, NY) were coated with bovine or human type I collagen (Chondrex, Redmond, WA), human cellular fibronectin, or human plasma fibronectin (Sigma-Aldrich, St. Louis, MO) diluted in collagen dilution buffer (Chondrex, Redmond, WA) or 50 mM sodium carbonate, pH 9.6 (fibronectins). Similar results were obtained in binding assays using bovine- and human-derived type I collagens. Plates were incubated overnight at 35°C

(fibronectin) or 4°C (collagen), washed three times with PBS containing 0.05% Tween 20, and blocked with 300  $\mu$ l of 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS for 2 hours at 35°C. Wells were washed three times in PBS. Fluorescein-labeled or nonlabeled bacteria in BHI (200  $\mu$ l) were added to appropriate wells. Separate aliquots of the bacterial suspensions were serially diluted and plated to determine CFU input. After 4 h at 35°C, the wells were washed three times with PBS containing 0.05% Tween 20, and 200  $\mu$ l of PBS was added to all wells prior to reading fluorescence. Fluorescence emission at 520 nm was measured following excitation at 490 nm, using a microplate spectrometer (model LS50B; Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England). Standard curves were established for each strain in each assay to allow calculation of attached CFU from fluorescence intensity.

**Animal care.** Pigs were housed in an American Association for Accreditation of Laboratory Animal Care-accredited P2 containment facility at North Carolina State University College of Veterinary Medicine. Animals were sedated for all procedures with 0.3 ml per 22.7 kg of body weight of an anesthetic formulation consisting of: Telazol (tiletamine HCl [50 mg/ml] and zolazepam HCl [50 mg/ml]; Fort Dodge Laboratories, Fort Dodge, IA), 50 mg/ml ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA), and 50 mg/ml xylazine (Miles Laboratories, Shawnee Mission, KS). Atropine sulfate (Phoenix Scientific Inc., Joseph, MO) was given at 0.5 to 1 ml to slow salivary and bronchial secretion. Pigs were maintained in the same room but in separate enclosures during the course of the study.

**Virulence testing in pigs.** Inoculum preparation, inoculation, and biopsy procedures were previously described (27, 52). Briefly, *H. ducreyi* grown on chocolate agar was suspended in PBS and passed through a 30-gauge needle to disperse aggregates. Multi-Test skin test applicators (Lincoln Diagnostics, Decatur, Ill.) were loaded with 10  $\mu$ l of inoculum and pressed into the dorsal side of the ear. Each round of inoculations entailed four to six application sites per ear per strain tested. Biopsies of experimental lesions collected 2 and 7 days after inoculation using 6-mm skin biopsy punches (Acuderm, Ft. Lauderdale, FL) were minced and plated on chocolate agar containing vancomycin to detect viable *H. ducreyi* within lesions.

**Virulence testing in human volunteers.** Healthy adult male and female volunteers over 18 years of age were recruited for the study. Subjects gave informed consent for participation and for HIV serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University-Purdue University of Indianapolis. The experimental challenge protocol, preparation and inoculation of the bacteria, calculation of the estimated delivered dose (EDD), surface cultures, and clinical observations were done exactly as described previously (4, 5, 54, 55, 58). Each subject was inoculated on one arm with three identical doses of the parent strain and on the other arm with three doses of twofold serial dilutions of the mutant. Subjects were observed until they reached a clinical end point, defined as either 14 days after inoculation, development of a pustule that was either painful or greater than 4 mm in diameter, or resolution of infection at all sites. Once a clinical end point was achieved, the code was broken and sites with clinical disease, if present, were biopsied. The subjects were then treated with two doses of oral ciprofloxacin (5, 55).

To confirm that the inocula were correct and that no phenotypic changes occurred during infection, individual colonies from the inocula, surface cultures, and biopsy specimens were picked, suspended in freezing medium, and frozen in 96-well plates. The colonies were scored for susceptibility to chloramphenicol. If available, sufficient colonies ( $n \geq 30$ ) from an individual specimen were scored so that there was a 95% probability that  $\leq 11\%$  of the colonies would have the incorrect phenotype (1).

**Statistical analysis.** Data from the pig model were analyzed using SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA) and SAS version 9 (SAS Institute Inc., Cary, NC). Numbers of viable wild-type and mutant *H. ducreyi* CFU recovered from lesion biopsies were analyzed using Poisson regression. Percentages of culture-positive biopsies were analyzed via logistic regression modeling of probabilities of recovery. Both regression models were adjusted for variability among pigs by using generalized estimating equations (GEE). Comparisons of papule and pustule formation rates between the two strains in the human challenge model were performed using a logistic regression model with GEE to account for the correlation among sites within the same subject, as described previously (53). The GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals for these rates. When a rate was zero, the exact binomial confidence intervals were calculated based on the number of subjects rather than the number of sites. The  $z$ -significance test of two means was used to compare the adherence of different strains of bacteria to type I collagen, and the  $t$  test was used to determine the significance of NcaA-expressing *E. coli* bound to collagen.

## RESULTS

**Identification of a putative oligomeric coiled adhesin in *H. ducreyi*.** We recently demonstrated that pigs repeatedly inoculated with *H. ducreyi* develop a modest antibody-mediated protective response against reinfection (16). Targets of this antibody response were identified by immunoprecipitation of  $^{125}$ I-labeled *H. ducreyi* OMPs with immune sera from multiply inoculated pigs. Seven distinct *H. ducreyi* outer membrane proteins were precipitated exclusively by immune sera (data not shown). One of these proteins, with an apparent molecular mass of  $>100$  kDa, was isolated and identified via N-terminal amino acid sequencing and comparison to the published *H. ducreyi* genome sequence. The N-terminal sequence, ITTES IPT, matched predicted amino acids 21 to 28 of a previously uncharacterized *H. ducreyi* OMP (hypothetical protein HD1920; BLAST accession no. NP\_874254) with a predicted molecular mass of  $\sim 33$  kDa. We named this protein NcaA.

The NcaA amino acid sequence is similar to those of the *H. ducreyi* OMP DsrA (25.1% identity and 42.6% similarity), *Yersinia enterocolitica* YadA (23.0% identity and 39.9% similarity), *Moraxella catarrhalis* UspA proteins (21.0 to 22.0% identity and 35.6 to 39.3% similarity), and *Escherichia coli* Eib (20.7 to 27.2% identity and 38.0 to 43.2% similarity) proteins. These proteins have been described as oligomeric coiled adhesins (Oca) (48), a subfamily of the type Vc surface-attached oligomeric autotransporter family (25). They are typified by a signature C-terminal 9-amino-acid stretch of alternating hydrophobic amino acids terminating with phenylalanine or tryptophan which comprises an outer membrane localization motif. The C-terminal region of these proteins also appears to mediate the formation of multimers that can remain stable even when heated with detergent and reducing agents (48). The disparity between the hypothetical molecular weight of monomeric NcaA and its dramatically slower migration in SDS-PAGE (Fig. 2) is consistent with similar proteins of this nature. The sequence of the 79 C-terminal amino acids of NcaA is 98% similar to the consensus Oca sequence and also possesses the 9-amino-acid terminus motif. Oca proteins share other properties in addition to the C-terminal signature sequence, including involvement in adherence, binding of ECM proteins, and resistance to complement-mediated killing. These common functions led us to investigate whether NcaA mediates adherence to eukaryotic cells and ECM proteins.

**Extracellular matrix protein binding properties of NcaA.** To determine whether NcaA binds ECM proteins, we compared the ECM binding properties of wild-type *H. ducreyi* 35000HP with those of a mutant unable to express NcaA due to insertion-deletion inactivation of the *ncaA* gene. Fluorescein-labeled 35000HP and the *ncaA* mutant (35000HP*ncaA*) were incubated in ECM-coated microplate wells. Assays were performed by incubating a bacterial suspension of constant density in wells coated with a range of ECM concentrations from 0 to 80  $\mu$ g/ml. A minimum *H. ducreyi* density of  $10^7$  CFU/ml was sufficient to ensure that binding saturation was achieved at the highest ECM concentrations. Bacterial binding was determined by measuring the fluorescence intensity of each well following incubation and washing and then relating that intensity to an assay-specific standard curve correlating fluorescence to CFU.



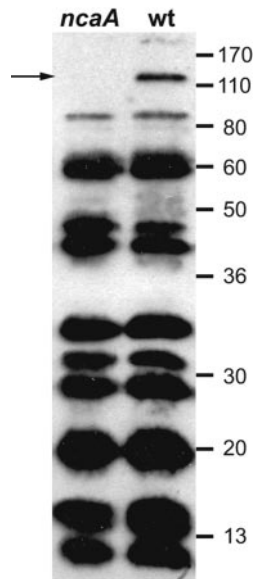


FIG. 2. Immunoblot of *H. ducreyi* 35000HP (wild type [wt]) and 35000HP*ncaA* (*ncaA*) whole-cell lysates with immune pig serum. The arrow indicates the position of an apparently multimeric form of NcaA, which migrated as a >100-kDa protein despite heating in the presence of SDS and  $\beta$ -mercaptoethanol. Other bands represent the constellation of *H. ducreyi* proteins recognized by polyclonal immune pig serum. Numbers indicate molecular mass standards in kilodaltons. The immunoblot film was scanned with an HP ScanJet 3970. The contrast of the scanned image was adjusted using the auto contrast command within Adobe Photoshop CS, and annotations were added with Adobe Illustrator CS.

Binding of the *ncaA* mutant strain to type I human collagen was significantly less than that of its isogenic parent strain *H. ducreyi* 35000HP (Fig. 3), with statistically significant differences in attachment at all ECM concentrations tested except 0.002  $\mu$ g/ml. In contrast, there was no difference in the level of binding of 35000HP or 35000HP*ncaA* to heparin (an analog of the cell surface receptor heparan sulfate proteoglycan), plasma fibronectin, or cellular fibronectin (data not shown). These data indicate that NcaA promotes specific adherence to type I collagen and not to related ECM proteins or cell surface adhesion molecules.

To determine if NcaA was sufficient to confer adherence to type I collagen, we tested the binding phenotype of *E. coli* expressing *H. ducreyi* NcaA. *E. coli* TOP10 containing the *ncaA* gene in pCR2.1 (TOP10 *pncaA*) or with pCR2.1 vector alone (TOP10 pCR2.1) was fluorescently labeled and incubated in microplate wells coated with type I collagen. Approximately  $10^6$  TOP10 *pncaA* CFU bound to collagen following incubation with  $8 \times 10^6$  CFU, whereas fewer than 100 TOP10 pCR2.1 CFU remained after incubation with  $3.2 \times 10^7$  CFU (Fig. 4). TOP10 *pncaA* did not bind wells coated with plasma fibronectin (data not shown). These data indicate that NcaA is both necessary and sufficient to elicit specific association with type I collagen.

**Effect of NcaA expression on *H. ducreyi* serum resistance and adherence to keratinocytes and foreskin fibroblasts.** The Oca proteins UspA2, (2) and YadaA (7, 35) and the Eib proteins A, C, D, E, and F (50, 51) impart resistance in their

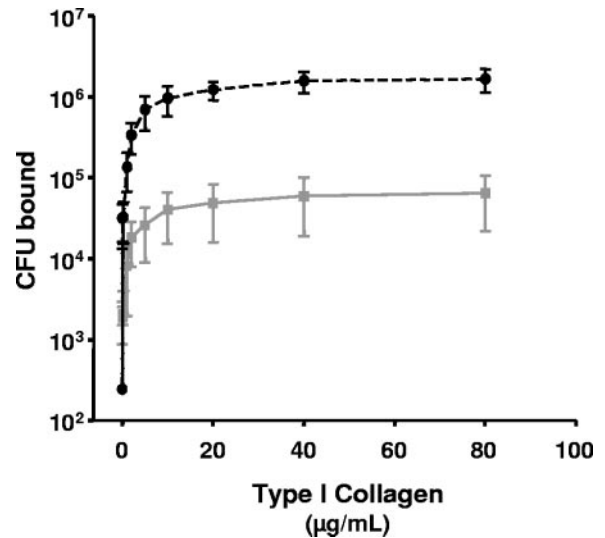


FIG. 3. Adherence of fluorescently labeled *ncaA* mutant (gray plot) or wild-type (black dashed plot) *H. ducreyi* to immobilized type I collagen. Adherence at each concentration of collagen was tested eight times in each assay, and adherence assays were performed three times for each strain. The data presented were averaged from the three assays, and error bars depict standard errors of the means. All values except the lowest (0.002  $\mu$ g/ml collagen) were significantly different (all  $P < 0.05$ ).

respective organisms to complement-mediated killing in the absence of opsonizing antibody and have also been shown to mediate binding to host receptors. NcaA has significant amino acid sequence similarity to DsrA, an *H. ducreyi* outer mem-

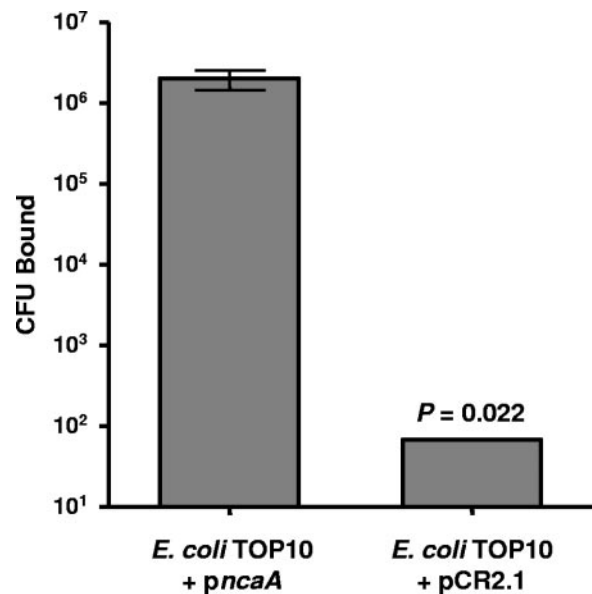


FIG. 4. Impact of NcaA expression on *E. coli* adherence to type I collagen. Fluorescently labeled *E. coli* TOP10 containing empty vector or *ncaA* plasmids was incubated in microplate wells coated with 40  $\mu$ g/ml bovine type I collagen. Data presented are mean *E. coli* CFU bound (error bars show standard errors of the means) following incubation with  $1.6 \times 10^7$  and  $8.0 \times 10^6$  vector- or *ncaA*-containing CFU, respectively.

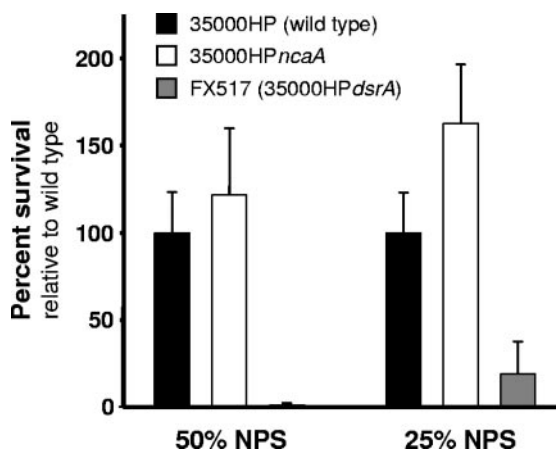


FIG. 5. Serum sensitivity of *H. ducreyi* mutants. Survival of wild-type (black bars), *ncaA* mutant (white bars), and *dsrA* mutant (gray bars) *H. ducreyi* after incubation in 25% or 50% normal pig serum (NPS) is shown. Serum survival was assayed in triplicate in three independent experiments (error bars show standard errors of the means).

brane protein and Oca protein that confers resistance to killing by normal human serum (19) as well as attachment to keratinocytes (15). We therefore tested the sensitivity of 35000HPncaA to normal human serum. The serum-sensitive strain *H. ducreyi* FX517 (35000HPdsrA) was effectively killed by 25% and 50% naïve pig serum with complement, whereas 35000HP and 35000HPncaA were both resistant to complement-mediated killing (Fig. 5). We also determined if NcaA mediates adhesion to keratinocytes and fibroblasts, which are the predominant cell types in skin. Both 35000HP and 35000HPncaA adhered to HaCaT cells, a human keratinocyte cell line (13), and HS27 cells, a human foreskin fibroblast cell line (1634-C; American Type Culture Collection, Manassas, VA) (data not shown). From these findings we concluded that NcaA neither contributes to serum resistance nor promotes adhesion to cultured skin fibroblasts or keratinocytes.

**NcaA expression is necessary for virulence in the swine model of chancroid infection.** To determine if NcaA is important in skin lesion formation in vivo, we inoculated pigs with 35000HP or 35000HPncaA and assessed bacterial recovery from biopsies taken 2 or 7 days postinoculation (p.i.). These data were generated from 14 animals across six experimental iterations. The percentages of biopsies containing viable *H. ducreyi* at day 2 p.i. were not significantly different for sites

inoculated with 35000HP or 35000HPncaA (60.3% versus 56.4% culture positive, respectively) (Table 1). However, there was a statistically significant difference ( $P < 0.0001$ ) between the proportions of culture-positive biopsies taken at day 7 p.i. from sites inoculated with 35000HP (72.9%) or 35000HPncaA (20.0%). Despite similar numbers of culture-positive biopsies at day 2 p.i., the average number of CFU recovered from 35000HP-inoculated sites was over fivefold higher than the number recovered from mutant-inoculated sites (161.0 versus 30.4 CFU per biopsy). This span increased to over 24-fold at day 7 p.i. (146.8 versus 6.1). Differences in the number of CFU recovered per biopsy were statistically significant ( $P < 0.0001$ ) at both days of biopsy. We also inoculated pigs with the cointegrate merodiploid strain generated during construction of 35000HPncaA in order to determine if the phenotype of 35000HPncaA was due to a spurious attenuating mutation rather than the targeted inactivation of the *ncaA* locus. Unlike 35000HPncaA, the cointegrate was recovered from pig ear lesions with similar frequency and in similar amounts as wild-type 35000HP at days 2 and 7 p.i. (Table 1), demonstrating that this strain is not attenuated.

**Human inoculation experiments.** The human challenge model allowed us to assess the importance of NcaA in establishing infection in the natural host of *H. ducreyi*. An escalating dose-response study was used to compare the virulences of 35000HP and 35000HPncaA. Eleven healthy adults (seven females and four males; age range, 20 to 53 years; mean age  $\pm$  standard deviation, 38.6  $\pm$  11.3 years) volunteered for the study. One subject (no. 245) withdrew prior to inoculation. In the first iteration, one subject was inoculated at three sites with an EDD of 34 CFU of the parent strain and at three sites with EDDs of 23, 46, and 92 CFU of the mutant. Papules developed at two of three sites inoculated with the parent and at two of three sites inoculated with the mutant, but all the papules resolved (Table 2). In the second iteration, three subjects were inoculated at three sites with an EDD of 85 CFU of the parent and at three sites with EDDs of 31, 62, and 124 CFU of the mutant. Papules developed at four of nine sites inoculated with the parent strain and at five of nine sites inoculated with the mutant (Table 2). A pustule developed at one parent site, while all mutant sites resolved (Table 2). In the third iteration, the subjects were inoculated with an EDD of 92 CFU of the parent and with EDDs of 74, 147, and 294 CFU of the mutant. Papules developed at all nine parent sites and at seven of nine mutant sites (Table 2). Pustules developed at six of nine parent sites, while all mutant papules resolved. In the fourth iteration,

TABLE 1. Response to experimental inoculation of *H. ducreyi* 35000HP, 35000HPncaA, and 35000HPncaA merodiploid in pigs

| Strain                  | <i>H. ducreyi</i> CFU per lesion biopsy <sup>a</sup> at day p.i.: |                             | % <i>H. ducreyi</i> -positive biopsies <sup>b</sup> at day p.i.: |                               |
|-------------------------|---|-----------------------------|--|-------------------------------|
|                         | 2   | 7                           | 2  | 7                             |
| 35000HP                 | 161.0 (90.5–497.4)  | 146.8 (111.2–320.8)         | 60.3 (30.9–75.7)   | 72.9 (57.5–96.8)              |
| 35000HPncaA             | 30.4 (19.5–82.3) <sup>c</sup>                                     | 6.1 (2.2–28.6) <sup>c</sup> | 56.4 (31.6–68.7)   | 20.0 (17.1–61.7) <sup>c</sup> |
| 35000HPncaA merodiploid | 280.9 (181.0–1390.2)  | 320.3 (148.3–1080.7)        | 70.0 (24.9–85.4)   | 75.0 (64.3–95.7)              |

<sup>a</sup> Values indicate the observed mean CFU per biopsy, with 95% confidence intervals in parentheses, as modeled by poisson regression and GEE adjustment to account for variability between pigs.

<sup>b</sup> Values indicate the observed mean proportion of biopsies harboring viable *H. ducreyi*, with 95% confidence intervals in parentheses, as modeled by logistic regression and GEE adjustment.

<sup>c</sup> Significantly different ( $P < 0.0001$ ) from values for wild-type or merodiploid lesions on same day postinoculation.

TABLE 2. Human volunteer response to inoculation of live *H. ducreyi* strains<sup>a</sup>

| Volunteer | Gender <sup>b</sup> | Days of observation | Strain      | No. of initial papules | Final outcome of initial papule |              |
|-----------|---------------------|---------------------|-------------|------------------------|---------------------------------|--------------|
|           |                     |                     |             |                        | No. of pustules                 | No. resolved |
| 244       | F                   | 5                   | 35000HP     | 2                      |                                 | 2            |
|           |                     |                     | 35000HPncaA | 2                      |                                 | 2            |
| 243       | F                   | 9                   | 35000HP     | 3                      | 1                               | 2            |
|           |                     |                     | 35000HPncaA | 2                      |                                 | 2            |
| 247       | M                   | 3                   | 35000HP     | 1                      |                                 | 1            |
|           |                     |                     | 35000HPncaA | 0                      |                                 |              |
| 248       | F                   | 5                   | 35000HP     | 0                      |                                 |              |
|           |                     |                     | 35000HPncaA | 2                      |                                 | 2            |
| 249       | M                   | 8                   | 35000HP     | 3                      | 1                               | 2            |
|           |                     |                     | 35000HPncaA | 2                      |                                 | 2            |
| 250       | F                   | 7                   | 35000HP     | 3                      | 3                               |              |
|           |                     |                     | 35000HPncaA | 3                      |                                 | 3            |
| 251       | M                   | 8                   | 35000HP     | 3                      | 2                               | 1            |
|           |                     |                     | 35000HPncaA | 3                      |                                 | 3            |
| 242       | F                   | 6                   | 35000HP     | 3                      |                                 | 3            |
|           |                     |                     | 35000HPncaA | 3                      |                                 | 3            |
| 252       | F                   | 9                   | 35000HP     | 3                      | 1 <sup>c</sup>                  | 1            |
|           |                     |                     | 35000HPncaA | 3                      |                                 | 3            |
| 253       | F                   | 5                   | 35000HP     | 2                      |                                 | 2            |
|           |                     |                     | 35000HPncaA | 2                      |                                 | 2            |

<sup>a</sup> Volunteer 244 was inoculated in iteration 1. Volunteers 243, 247, and 248 were inoculated in iteration 2. Volunteers 249, 250, and 251 were inoculated in iteration 3. Volunteers 242, 252, and 253 were inoculated in iteration 4.

<sup>b</sup> M, male; F, female.

<sup>c</sup> One site remained a papule at the time the subject reached the end point.

the subjects were inoculated with an EDD of 140 CFU of the parent and EDDs of 490, 960, and 1,920 CFU of the mutant. Papules developed at eight of nine sites inoculated with the parent and at eight of nine sites inoculated with the mutant (Table 2). A pustule developed at one parent site, while all mutant sites resolved (Table 2).

The cumulative results for the four iterations showed that papules developed at 76.7% (95% confidence interval [CI], 55.9% to 97.4%) of sites inoculated with 35000HP and at 74.3% (95% CI, 55.3% to 91.3%) of sites inoculated with 35000HPncaA. Overall, pustules formed at 8/30 (26.7%; 95% CI, 6.4% to 46.9%) sites inoculated with 35000HP, compared to 0 of 30 (0%; 95% CI, 0% to 25.9%) sites inoculated with 35000HPncaA ( $P = 0.005$ ). Thus, the mutant was impaired in its ability to form pustules compared to the parent.

For the four parent and four mutant broth cultures used to prepare the inocula, all 192 parent colonies and all 192 mutant colonies tested were phenotypically correct (mutant, chloramphenicol resistant [Cm<sup>r</sup>]; parent, Cm<sup>s</sup>). Of the 30 sites that were inoculated with the parent, 6 (20%) yielded at least one positive surface culture, while 0 of 18 mutant sites yielded a positive surface culture. All colonies obtained from surface cultures ( $n = 346$ ) and biopsy specimens ( $n = 9$ ) from parent sites were phenotypically correct (Cm<sup>s</sup>). Thus, all tested colonies from the inocula, surface cultures, and biopsy specimens

had the expected phenotype. We were unable to test the mero-diploid strain for complementation of the 35000HPncaA phenotype because inoculation of human volunteers with strains that harbor plasmids is not permitted.

## DISCUSSION

During the course of identifying targets of immune serum that conferred protection against *H. ducreyi* infection in the swine chancroid model, we found that at least one of the antibodies bound a previously uncharacterized outer membrane protein. We named this protein NcaA because our findings have shown it to be necessary for collagen association in *H. ducreyi*. The sequence of the C-terminal 79 amino acids of NcaA is similar to that of the oligomeric coiled adhesin (Oca) subfamily of autotransporter proteins. Oca proteins form oligomeric lollipop-shaped surface projections on the bacterial outer membrane, consisting of N-terminal head and neck domains followed by a coiled-coil stalk and a YadA-like C-terminal domain that anchors the protein in the outer membrane (28, 48). The amino acid sequence of NcaA is consistent with this proposed structure, but its three-dimensional structure has not been determined. Oca proteins also migrate as heat-stable oligomers during SDS-PAGE. This peculiarity is thought to reflect the avid oligomerization property of these proteins on the bacterial surface (3, 19, 36, 48, 51). YadA exists as a trimer both when anchored to the bacterial outer membrane and during migration through SDS-PAGE (48). Monomeric NcaA has a predicted molecular mass of 33 kDa but shows an apparent size of >100 kDa in SDS-PAGE (Fig. 2), suggesting that it also oligomerizes, though it is not known how many NcaA monomers assemble to form a functional complex.

Chancroid transmission occurs when *H. ducreyi* penetrates the keratinized skin surface layer and colonizes the dermis or epidermis of the skin. Type I collagen is the predominant structural component of skin (6), and *H. ducreyi* is found associated with type I collagen in the dermis of biopsy samples collected from human volunteers 48 h postinoculation (8). Other ECM attachment studies also demonstrated that *H. ducreyi* binds to immobilized type I collagen in vitro (1, 9). Our results indicate that specific association of *H. ducreyi* with type I collagen is mediated by NcaA. Collagen binding is a crucial factor in the pathogenesis of other organisms that inhabit skin during infection. For example, loss of YadA-mediated collagen binding is associated with loss of *Yersinia enterocolitica* virulence in a mouse model of infection (56). In addition, the *Staphylococcus aureus* collagen adhesin Cna is required for virulence in multiple animal models of infection (18, 26, 44, 46). Our finding that NcaA binds collagen is consistent with the nature of *H. ducreyi* as a dermal pathogen.

Our in vitro experiments showed that NcaA neither mediates adherence to cultured skin cells nor confers resistance to complement-mediated killing. We also demonstrated that NcaA is necessary and sufficient for bacterial association with type I collagen. Bacterial adherence to host tissues is generally considered to be an essential step in establishing infection (10, 43). Our studies here illustrate that adhesins mediating association of bacteria with structural ECM proteins in contrast to adherence to host cells can also be important virulence determinants. NcaA appears to have such a role in *H. ducreyi* patho-



genesis. In pigs, the *ncaA* mutant was recovered from significantly fewer lesions than the wild type. In human volunteers, the *ncaA* mutant was unable to form pustules, making NcaA the seventh *H. ducreyi* protein shown to be essential for virulence in the native human host (4, 12, 21, 29, 53). Whereas these findings demonstrate that NcaA is required for infection of humans and pigs, we are conducting further studies to elucidate the collagen binding property of NcaA and to determine whether it is directly responsible for the contribution of NcaA to *H. ducreyi* pathogenesis. We are also determining if an antibody response against NcaA can confer protection against *H. ducreyi* infection.

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