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A Fibrinogen-binding Lipoprotein Contributes to Virulence of *Haemophilus ducreyi* in Humans

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Abstract

A gene expression study of *Haemophilus ducreyi* identified the hypothetical lipoprotein *HD0192*, renamed here fibrinogen binder A (*fgbA*), as preferentially expressed in vivo. To test the role of *fgbA* in virulence, an isogenic *fgbA* mutant (35000HP*fgbA*) was constructed in *H. ducreyi* 35000HP, and six volunteers were experimentally infected with 35000HP and 35000HP*fgbA*. The overall pustule formation rate was 61.1% at parent sites and 22.2% at mutant sites ($P = 0.019$). Papules were significantly smaller at mutant sites than at parent sites (13.3 versus 37.9 mm^2, $P = 0.002$) 24 h after inoculation. Thus, *fgbA* contributed significantly to virulence of *H. ducreyi* in humans. In vitro studies demonstrated that *fgbA* encodes a fibrinogen binding protein; no other fibrinogen binding proteins were identified in 35000HP. *fgbA* was conserved among clinical isolates of both class I and class II *H. ducreyi* strains, supporting the finding that *fgbA* is important for *H. ducreyi* infection.

Keywords

Haemophilus ducreyi; chancroid; human trials; fibrinogen; adhesins; virulence factors

INTRODUCTION

Haemophilus ducreyi is the causative agent of chancroid, a genital ulcer disease that contributes to the spread of human immunodeficiency virus (HIV) type 1 in endemic areas of the developing world [1]. *H. ducreyi* enters the body through microabrasions that occur during intercourse and primarily remains confined to the skin, where the organism resides extracellularly in a milieu of professional phagocytes [2,3]. *H. ducreyi* colocalizes with fibrin, collagen, neutrophils, and macrophages at the papular, pustular, and ulcerative stages of disease in both a human infection model and naturally occurring chancroid [4,5].

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A limited number of *H. ducreyi* virulence factors have been identified. Consistent with the organism's extracellular lifestyle and colocalization with collagen, full virulence of *H. ducreyi* in humans requires expression of the collagen-specific adhesin NcaA, the antiphagocytic proteins LspA1 or LspA2, and two outer membrane proteins, DsrA and DltA, which confer protection from serum-mediated killing [6–9]. Other virulence factors of *H. ducreyi* required for human infection include the hemoglobin receptor HgbA, the peptidoglycan-associated lipoprotein PAL, which is involved in outer membrane stability, an intact Flp locus, which encodes fimbria-like proteins and type IV-like secretory proteins, and a locus encoding enterobacterial common antigen-like biosynthetic genes [10–13].

With the goal of discovering new *H. ducreyi* virulence factors, we identified a panel of *H. ducreyi* genes whose corresponding mRNAs are preferentially expressed in experimental pustules [14]. The in vivo expressed genes included 133 ORFs encoding proteins of unknown function [14]. We performed in silico analyses of these in vivo-expressed hypothetical proteins, including subcellular localization algorithms and searches for conserved motifs, domains, and structural features [15–20]. From these analyses, we identified seven hypothetical outer membrane proteins (M. E. Bauer, unpublished data). In this study, we used the human model of *H. ducreyi* infection to examine the role of one such protein, the hypothetical lipoprotein HD0192, in virulence of *H. ducreyi*. We also explored a putative role for HD0192 in binding to fibrinogen; based on these studies, we named the protein FgbA (fibrinogen binder A).

METHODS

Bacterial strains and growth conditions

H. ducreyi strains (Table 1) were cultured at 33°C using media described previously [27]. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, Calif.) was used for cloning and B21 DE3/pLysS (Invitrogen) for protein expression. A recombinant clone expressing the 36-kDa Fg-binding domain of *Staphylococcus aureus* ClfA in *E. coli* was kindly provided by M. Höök (Texas A&M Health Science Center) [28]. *E. coli* strains were cultured on Luria-Bertani agar or broth supplemented with kanamycin (50 μg/ml) or ampicillin (50 μg/ml).

Construction and complementation of an *fgbA* mutant

A map of the *fgbA*-containing locus in 35000HP is shown in Fig. 1A. The upstream and downstream ORFs, *HD0193* and *HD0191*, were predicted to be transcribed from the opposite strand as *fgbA*, suggesting that *fgbA* was monocistronic (Fig. 1A). A 4.1 kb fragment containing *HD0192* (*fgbA)* was PCR amplified from 35000HP genomic DNA (see Table 2 for primers). The amplicons were digested with BamHI and XbaI and cloned into pBluescriptIISK+ (Stratagene, La Jolla, Calif.). A kanamycin resistance (KanR) cassette from pUC18K [29] was inserted into the cloned *fgbA* ORF; the mutated *fgbA* was subcloned into pRSM2072, which expresses *lacZ* and acts as a suicide vector in *H. ducreyi* [30], and introduced into *H. ducreyi* 35000HP by electroporation. KanR transformants were propagated in medium containing 5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal), as described [30], to identify colonies that had undergone allele exchange. One such colony was designated 35000HP*fgbA*. The *fgbA* mutation in 35000HP*fgbA* was confirmed by sequencing, southern blotting, and PCR analyses (data not shown). 35000HP and 35000HP*fgbA* had similar outer membrane protein (OMP) and lipooligosaccharide profiles, assessed as described [11], and demonstrated similar growth rates in broth (data not shown).

For complementation, the *fgbA* ORF and 197 bp of upstream flanking sequence was PCR-amplified (Table 2) and cloned into shuttle vector pLSKS [31]. The resulting construct was introduced into 35000HP*fgbA* by electroporation and selected on plates containing kanamycin and streptomycin.

Human Inoculation Protocol

Six healthy volunteers (5 men, 1 woman; 5 whites, 1 black; age range: 21–54 years, mean ± standard deviation, 32 ± 14) 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. All aspects of the mutant-parent trial and determining colony phenotypes were performed exactly as described previously [7,10, 13]. Statistical analysis compared papule and pustule formation rates using a logistical regression model with generalized estimating equations to adjust for within subject correlation, as described [12]. Papule size was compared between strains using a mixed effects model with a random subject effect.

Recombinant fusion protein construction and expression

Genes encoding HD0192, HD0581, and HD1218 were PCR-amplified (Table 2), starting immediately downstream of their lipidation sites, cloned into pCR-XL-TOPO (Invitrogen), and subcloned into pRSET B (Invitrogen) to express the ORFs fused in frame with an N-terminal 6xHis tag. The constructs were expressed in B21 DE3/pLysS, following the manufacturer's directions. Recombinant proteins were purified with the QIAexpressionist System, following the manufacturer's instructions (Qiagen Inc, Valencia, Calif).

Fibrinogen binding ligand blot assay

Purified, human Fg (Enzyme Research Laboratories, Inc., South Bend, Ind.) was labeled with digoxigenin (dig) using the DIG protein labeling kit from Roche Applied Sciences (Indianapolis, Ind.), following the manufacturer's instructions. Bacterial proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 2% nonfat milk in phosphate-buffered saline pH 7.4 (PBS), and probed with a 1:1000 dilution of dig-Fg (10 μg/ml) in 2% milk-PBS overnight. Dig-Fg was detected using digoxigenin detection reagents (Roche), following the manufacturer's protocols.

Bacterial protein preparations

Whole cell lysates and OMPs of 35000HP were prepared as described previously [27]. Triton X-114 phase partitioning was performed on mid-logarithmic cultures of 35000HP as described [32]. Following separation, both phases were precipitated with acetone to collect proteins. Aqueous and detergent phase protein samples were suspended in identical volumes of Laemmli sample buffer for comparison by standard SDS-PAGE or in rehydration buffer containing 9 M urea, 4% Igepal CA-630, 1% dithiothreitol, and 2% carrier ampholytes (pH 4–7) [33] for two-dimensional gel electrophoresis (2DE).

2DE and tandem mass spectrometry

2DE was performed by the Indiana Center for Applied Protein Sciences as described previously [33,34]. Briefly, detergent phase proteins from 35000HP were separated by isoelectric focusing with a pH 4–7 linear gradient, followed by molecular size through 4–20% gradient gels (Bio-Rad). Samples were analyzed in parallel on two gels; one gel was stained with Coomassie [34], and the other was transferred to PVDF and probed with dig-Fg by ligand blot. Coomassie-stained spots corresponding to positive signals in the ligand blot were excised and subjected to tandem mass spectrometry (MS-MS) for peptide sequence analysis. The results indicated multiple protein IDs in each spot; proteins with at least two distinct matching peptides were considered for further analysis (Table 3).

Conservation of *fgbA* sequence in *H. ducreyi* strains

H. ducreyi strains and class designations are listed in Table 1. For strains whose phenotypic class had not been previously reported, we PCR-amplified the *dsrA* locus from genomic DNA using class-specific primer pairs described by White et al. [Table 2 and ref. 24]. All strains tested contained a *dsrA* locus that amplified with class I-specific but not class II-specific primers (data not shown) and were therefore considered class I strains (Table 1).

fgbA-containing sequences, including 145 bp 5' and 130 bp 3' to the *fgbA* ORF, were PCR-amplified from genomic DNA of all listed *H. ducreyi* strains (Table 2). The amplicons were sequenced, and the resulting sequences aligned by the ClustalW algorithm in the Lasergene software package (DNASTAR, Madison, WI).

RESULTS

fgbA contributed to virulence in the human model of infection

fgbA transcripts are expressed during human infection [14]. To determine whether FgbA played a role in disease, *fgbA* was inactivated in *H. ducreyi* 35000HP by insertion of a kanamycin resistance cassette (Fig. 1A). The resulting mutant, 35000HP*fgbA*, was directly compared with 35000HP for virulence in the human model of *H. ducreyi* infection. Six healthy adults volunteered for the study (Table 4). In the first iteration, each of two subjects was infected with a fixed estimated delivered dose (EDD) (88 CFU) of 35000HP at three sites on one arm and varying EDDs (59, 118, and 256 CFU) of 35000HP*fgbA* at three sites on the other arm. Pustules formed at 3 of 6 parent sites and 1 of 6 mutant sites (Table 4). In the second iteration, 3 volunteers were inoculated with 98 CFU of 35000HP on one arm and 43, 85, and 169 CFU of 35000HP*fgbA* on the other arm. Pustules formed at 5 of 9 parent sites and 3 of 9 mutant sites (Table 4). In the third iteration, the volunteer was inoculated with 50 CFU of 35000HP at 3 sites and 78 CFU of 35000HP*fgbA* at 3 sites; pustules formed at all 3 parent sites and at 0 of 3 mutant sites.

Overall, the pustule formation rate was 61.1% (95% CI, 32.7%–89.6%) at 18 parent sites and 22.2% (95% CI, 0.1%–51.7%) at 18 mutant sites ($P = 0.019$). Papules were significantly smaller at mutant sites (mean, 13.3 mm^2) than at parent sites (mean, 37.9 mm^2) 24 h after inoculation ($P = 0.002$). These results suggested that expression of FgbA facilitated the ability of *H. ducreyi* to initiate disease and progress to pustule formation in humans.

For the parent and mutant broth cultures used to prepare the inocula, all 107 parent and 107 mutant colonies tested were phenotypically correct for kanamycin resistance. Ten of 15 sites (67.7%) inoculated with the parent yielded positive surface cultures, while 2 of 15 mutant sites (13%) yielded a positive surface culture. All colonies obtained from surface cultures (n = 406 and n = 43) and biopsy specimens (n = 72 and n = 36) from parent sites and mutant sites, respectively, had the expected antibiotic susceptibility.

Recombinant FgbA bound to human fibrinogen

To discern possible functions for FgbA, we performed in silico analysis of the deduced protein sequence (Fig. 1B). The N-terminus contained a signal II peptide and lipidation site consistent with a lipoprotein (Fig. 1B).The C-terminus contained 3.5 direct, tandem repeats of a 29-amino acid motif with a consensus sequence of N-EMKDAAKAKLEDMKESAAEAKESLAEKAN-C. The repetitive region was predicted to form an alpha-helical coiled-coil structure (Fig. 1B). Coiled coil motifs are found in a wide variety of proteins with structural or regulatory functions and frequently involve homomeric or heteromeric protein-protein interactions [18]. Several bacterial proteins with coiled coils interact with host proteins [35–37]. The only known host protein that *H. ducreyi* colocalizes with, and for which no adhesin has yet been identified, is

fibrin. Many bacteria interact with fibrin via Fg-binding proteins with coiled coil motifs. Thus, we hypothesized that FgbA may bind Fg.

To determine whether FgbA could bind Fg, the *fgbA* ORF was expressed, without its signal peptide, as a recombinant N-terminal 6xHis tagged fusion protein (designated r-FgbA) and probed with dig-Fg in a ligand blot assay. The Fg-binding domain of ClfA from *Staphylococcus aureus* served as a positive control [28]. As shown in Fig. 2A, r-FgbA demonstrated Fg binding activity, both within an *E. coli* whole cell lysate (Fig. 2A lane 2) and after purification over a nickel column (Fig. 2A lane 3). These data suggested that FgbA was able to bind human Fg.

Identification of Fg-binding protein(s) in *H. ducreyi*

Because many pathogens express redundant adhesins for important ligands, we examined 35000HP for additional Fg-binding proteins using the ligand blot assay. We first probed whole cell lysates and OMP preparations of *H. ducreyi* 35000HP with dig-Fg. As shown in Fig. 2B, dig-Fg bound to a single, approximately18 kDa band present in whole cell lysates and in OMPs.

To partially purify the Fg-binding protein, *H. ducreyi* 35000HP cells were subjected to Triton X-114 phase partitioning (Fig. 2C). Fg binding activity partitioned to the detergent phase; no activity was observed in the aqueous phase (Fig. 2C). Thus, the Fg binding activity was likely due to a protein with hydrophobic domains or components.

To identify the Fg-binding band, detergent phase proteins were separated by 2DE and subjected to ligand blot analysis. Two Fg binding spots were detected by ligand blot; each migrated to the same molecular size and slightly different pIs, in the range of pI 4–5.5 (data not shown), suggesting one protein with heterogeneous post-translational modification. Coomassie-stained spots corresponding to the Fg binding spots were excised and subjected to MS-MS analysis. Peptides were identified that corresponded to seven proteins, each with a predicted molecular weight between 10 and 20 kDa and acidic pI (Table 3). One of the proteins identified was FgbA (Table 3). Two additional identified proteins, lipoprotein HlpB and hypothetical protein HD1218, had no known function; the remaining four proteins were orthologous to bacterial proteins with assigned functions (Table 3). Three of the proteins, including FgbA, lipoprotein HlpB, and hypothetical protein HD1218, contained predicted signal peptides indicating an extracytoplasmic location [15,16]; the remaining four proteins were predicted to be cytoplasmic (Table 3).

To determine whether HlpB or HD1218 was able to bind Fg, the genes corresponding to both proteins were recombinantly expressed as N-terminal 6xHis tagged fusion proteins and subjected to ligand blot analysis. Neither recombinant HlpB nor recombinant HD1218 bound to dig-Fg in the ligand blot assay (data not shown). Thus, FgbA was the only *H. ducreyi* OMP identified by ligand blot to bind human Fg. The predicted lipidation of FgbA was consistent with the hydrophobic nature and heterogeneous pI observed for the Fg-binding activity in *H. ducreyi*.

A *fgbA* mutant did not bind Fg

To confirm that *fgbA* conferred *H. ducreyi* binding to Fg by ligand blot, we compared 35000HP and 3500HP*fgbA* for Fg binding. 35000HP*fgbA* did not express the 18 kDa Fg binding protein; however, *trans*-complementation with the *fgbA* ORF restored Fg binding activity to the mutant (Fig. 2D). The complemented mutant showed a much stronger Fg-binding band than did the parent strain. Although FgbA levels in 35000HP and the complemented mutant have not been quantitatively compared, the increased activity is likely from overexpression of *fgbA* on the

multi-copy shuttle vector. The mutagenesis and complementation data demonstrate that FgbA encodes the *H. ducreyi* Fg binding protein detected by ligand blot.

fgbA was conserved among *H. ducreyi* strains

Two phenotypic classes of *H. ducreyi* strains have been identified, based on differences in OMPs and LOS migration patterns [24]. To examine conservation of *fgbA* among strains of both classes, we PCR-amplified the *fgbA* locus from 10 clinical *H. ducreyi* isolates, including six class I and four class II strains (Table 1). *fgbA*-containing amplicons were obtained from all strains; however, amplicons from six strains migrated faster than that of 35000HP, indicating size variations in the *fgbA* loci (Fig. 3A).Sequencing showed that the faster migrating amplicons contained deletions within the repetitive motif of *fgbA*; however, no strains harbored frameshift mutations or premature stop codons (Fig. 3B).

Class-specific changes were noted among the sequenced *fgbA* loci. Compared with *fgbA* of 35000HP, three class I strains had identical *fgbA* ORFs, and two strains contained a 58-amino acid deletion but were otherwise identical (Fig. 3B). This deletion removed the first two copies of the 29-amino acid repeat in 35000HP FgbA (Fig. 1B). No class I loci had changes in the sequenced regions outside the ORF (data not shown).

The four class II isolates tested were identical to each other and all contained the same 58-amino acid deletion observed in two of the class I strains (Fig. 3B). However, compared with the class I *fgbA* ORFs, the class II ORFs harbored two conservative amino acid substitutions and a 7-amino acid insertion (Fig. 3B). Upstream of the ORF, the class II strains harbored a 1-bp deletion (data not shown). Although this deletion did not affect the predicted promoter elements of *fgbA*, it did produce a frameshift mutation in the 5' gene, *HD0193*. The class II strains also harbored a T–C base change 98 bp downstream of the 3' end of the *fgbA* ORF that was outside the predicted Rho-independent terminator and *HD0191* (Fig. 1A). Overall, the *fgbA* ORF was conserved among class I and class II strains of *H. ducreyi* but with differences in the length of the repetitive motif.

DISCUSSION

In this study, we identified a novel Fg-binding protein, FgbA, that contributes significantly to the disease process of *H. ducreyi* in human volunteers. We originally identified *fgbA* among *H. ducreyi* transcripts expressed during human infection [14] and, because of its predicted outer membrane location, we hypothesized that FgbA is involved in virulence. Using the human model of *H. ducreyi* infection, we demonstrated that an isogenic *fgbA* mutant, although able to cause pustules, did so at a significantly lower rate than that of 35000HP; thus, 35000HP*fgbA* was partially attenuated [9,13]. Biosafety considerations preclude testing a *trans*-complemented mutant in human subjects because of potential horizontal transfer of resistance plasmids to skin flora. With this caveat, the human challenge experiments demonstrate that FgbA is important for the organism's virulence.

Our in vitro assays sought to identify a function for this novel virulence factor. The predicted coiled coil structure suggested that FgbA may interact with host proteins. *H. ducreyi* colocalizes with collagen and fibrin in vivo, and a collagen-specific adhesin has already been identified in *H. ducreyi* [4–6]. We therefore tested the ability of FgbA to bind Fg, the soluble precursor of fibrin. r-FgbA bound to Fg, and FgbA was subsequently identified as the major *H. ducreyi* protein binding Fg in the ligand blot assay. Although FgbA bears no sequence homology with other known Fg binding proteins, several other extracellular pathogens, including *Staphylococcal* and *Streptococcal* species, express Fg binding proteins with coiled coils and repetitive motifs [38–40]. Another notable feature of FgbA is the number of charged residues, which represent 45% of the mature peptide and confer a net charge of -7. Charged residues

frequently indicate sites of protein-protein interaction; however, the large number of charges in FgbA is unusual. The role of these charged residues in interactions with Fg is being investigated.

The predicted outer membrane location of FgbA was based on sorting rules for *E. coli* lipoproteins, since lipoprotein sorting has not been well studied in *H. ducreyi* [17,19,20]. Consistent with an outer membrane location, Fg-binding activity was observed in sarkosyl-insoluble protein preparations, which are enriched for OMPs (Fig. 2B); however, this is not proof of outer membrane location. Further, even if anchored to the outer membrane by its lipid portion, the protein portion of FgbA could face either the periplasm or the external milieu. Thus, additional work is needed to define the location and possible surface exposure of FgbA.

The *fgbA*-containing locus is conserved among class I and class II strains of *H. ducreyi*. Although the strains differed in the lengths of their ORFs, all strains maintained an intact *fgbA* ORF. This conservation among clinical isolates supports the conclusion that *fgbA* is important for *H. ducreyi*. The major difference in the deduced FgbA sequences was the length of the repetitive coiled coil region. Despite the smaller size, the strains with deletions relative to 35000HP were still predicted to form coiled coils. Whether these deletions, or the class II-specific amino acid changes, affect the capacity to bind Fg is under investigation.

A BLASTP search with FgbA or its 29-amino acid repetitive motif showed homology with hypothetical proteins in several *Pasteurellaceae*, including *Actinobacillus pleuropneumoniae* Aple 020001366, *H. somnus* HS1338, *Pasteurella multocida* PM0442, and NTHI1667 from nontypeable *H. influenzae* 86-028NP. All four homologs are predicted lipoproteins of 15–22 kDa with pI of 4.6–4.9 containing a disproportionate number of charged amino acids and a net negative charge. The C-terminal portions of these homologs encoded 3–5 direct, tandem repeats of 22–29 amino acids Thus, FgbA may represent a family of lipidated virulence factors in several pathogenic genera of the *Pasteurellaceae*.

What role might FgbA play for *H. ducreyi*? Although not a component of normal skin, Fg transudates from serum into *H. ducreyi*-infected lesions and is cleaved to form a matrix of fibrin strands. Fg and fibrin are plentiful in *H. ducreyi*-infected lesions throughout the disease process [4,5]. Thus, FgbA may act as an adhesin to anchor the bacterium to the fibrin matrix. Although the ligand blot assay used in this study does not define FgbA as an adhesin, the assay demonstrated that FgbA can bind Fg.

An alternative role of Fg binding proteins in pathogenesis is to protect extracellular bacteria from phagocytosis, as exemplified by *S. pyogenes* M protein [39,41]. Another possible role of Fg binding proteins is to occlude opsonization with antibodies or complement, although this function is controversial [42–45]. Because *H. ducreyi* associates with Fg in vivo and resists phagocytosis, we hypothesize that FgbA may be involved in adherence or antiphagocytic activity for *H. ducreyi*. Studies to detail the mechanism(s) by which FgbA contributes to virulence in vivo are underway.

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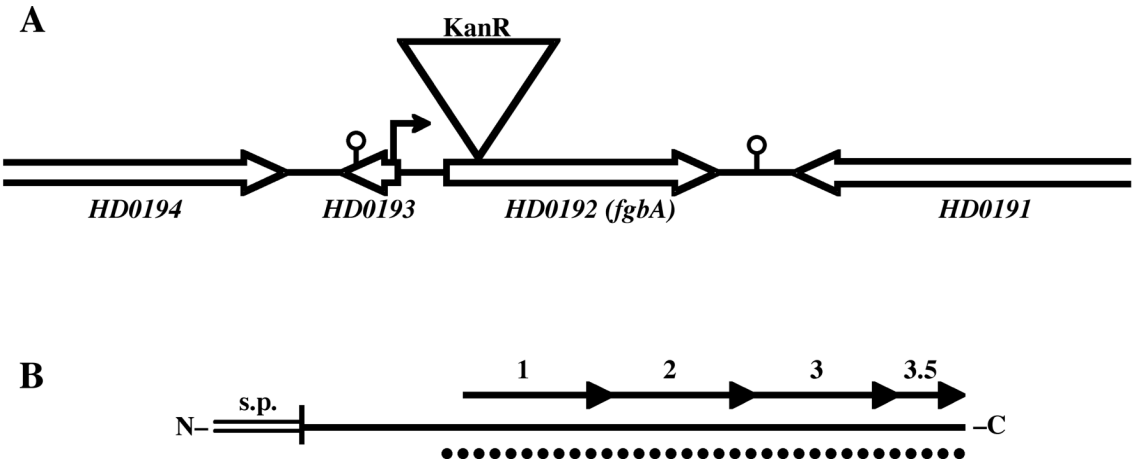

Fig. 1. Maps of the *HD0192 (fgbA)*-containing genetic locus (A) and the Fg-binding protein FgbA (B)

(A) Scale map of the *fgbA* locus in 35000HP. Open arrows indicate direction of transcription of ORFs; single lines indicate intergenic sequences. Triangle denotes site of insertional mutagenesis with KanR cassette in 35000HP*fgbA*. Small arrow indicates putative promoter upstream of the *fgbA* ORF; stalked circles indicate predicted Rho-independent transcriptional terminators. (B) Scale map of the protein product of *fgbA*. The horizontal solid line represents the 1° structure of the mature protein, the double line indicates the signal 2 peptide, and the vertical line between them denotes the site of signal peptide cleavage and lipidation. Arrows represent the 29-amino acid direct repeats. Dotted line indicates the predicted alpha-helical coiled-coil structure.

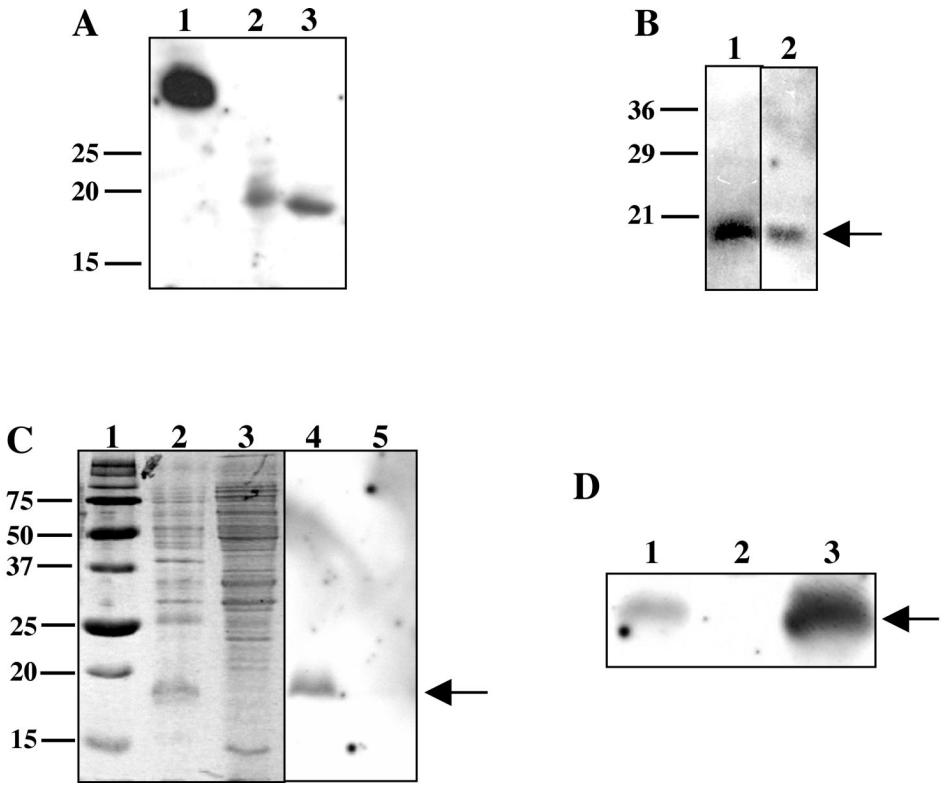

Fig. 2. FgbA conferred fibrinogen binding in a ligand blot assay

(A) Lane 1, lysate of *E. coli* strain expressing recombinant Fg-binding portion of *S. aureus* ClfA; lane 2, lysate of *E. coli* strain expressing recombinant HD0192; lane 3, recombinant HD0192 after purification over a Ni column. (B) Whole cell lysate (lane 1) and OMP preparation (lane 2) of 35000HP probed with dig-Fg and developed to detect dig. Note the Fg-binding bands at 18 kDa (arrow). (C) Fg-binding activity was enriched in a hydrophobic protein preparation. Triton X-114 detergent (lanes 2, 4) or aqueous (lanes 3, 5) phase proteins from whole cell lysates of 35000HP were stained with Coomassie (lanes 2, 3) or probed with dig-Fg in the ligand blot (lanes 4, 5). Lane 1, molecular size markers. Note the 18 kDa bands (arrow) present in detergent phase samples. (D) FgbA conferred Fg-binding activity in *H. ducreyi*. Whole cell lysates of parent strain 35000HP (lane 1), mutant 350000HP*fgbA* (lane 2), and complemented mutant (lane 3) were probed with dig-Fg in the ligand blot assay. Note the FgbA band present in the parent, absent in the *fgbA* mutant, and restored in the complemented mutant.

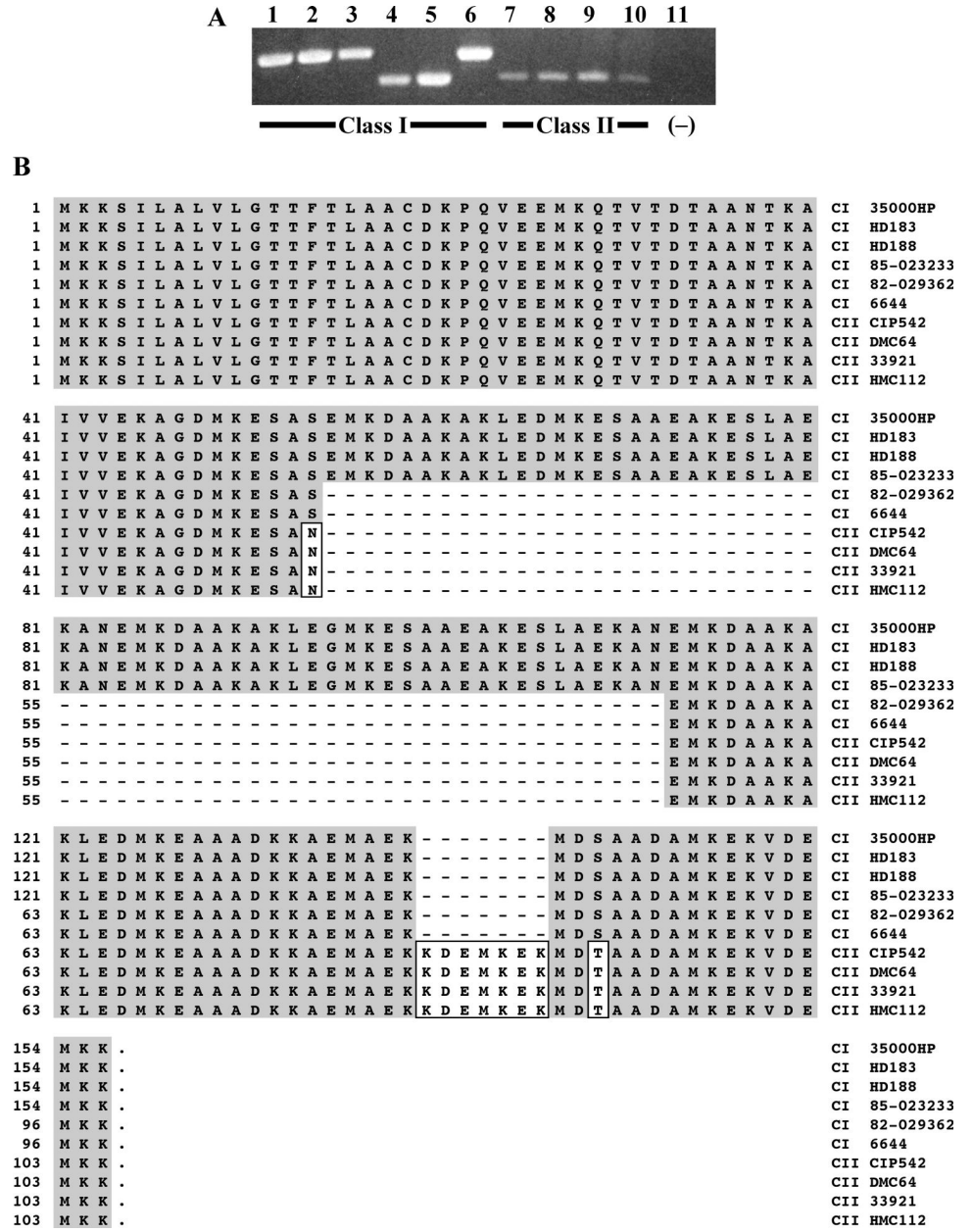

Fig. 3. *fgbA* varied in size but was conserved among class I and class II clinical isolates of *H. ducreyi*

(A) PCR amplicons of the *fgbA* locus from genomic DNA of clinical isolates listed in Table 1. Lanes 1–6, class I strains 35000HP, HD183, HD188, 82-029362, 6644, and 85-023233; lanes 7–10, class II strains CIP542 ATCC, DMC64, 33921, and HMC112. Lane 11, negative control (no template added). (B) Alignment of deduced FgbA sequences among *H. ducreyi* clinical isolates. Strain names are listed on the right-hand side. C I, class I strain; C II, class II strain. Shaded residues match those of 35000HP, boxed residues differ from those of 35000HP, and dashes indicate missing residues.

Table 1

H. ducreyi strains used in this study

Strain Designation	Geographic Origin	Year of Isolation	Class	References
35000HP[a]	Winnipeg, Canada	1975	I[b]	[21,22]
HD183	Singapore	1982	I[c]	[23]
HD188	Kenya	1982	I[c]	[23]
82-029362	California, USA	1982	I[c]	[23]
6644	Boston, USA	1989	I[c]	[23]
85-023233	New York City, USA	1985	I[c]	[23]
CIP542 ATCC	Hanoi, Vietnam	1954	II[b]	[22,24]
HMC112	not reported	1984	II[b]	[24]
33921	Nairobi, Kenya	not reported	II[d]	[25]
DMC64	Bangladesh	not reported	II[b]	[24]

[a] Human passaged version, isolated in 1996, of strain 35000.

[b] Phenotypic class determined by White et al.[24].

[c] Phenotypic class determined in this study (see Materials and Methods).

[d] Phenotypic class determined by Post and Gibson [26].

Table 2

Oligonucleotides Used in This Study

Uses	Forward Primer[a]	Forward Primer[a]
recombinant expression of *fgbA (HD0192)*	CCTCAAGTTGAAGAAATGAAACA AACGG	CGAATTCGTATTTGGTAATAA ATGACCGC
recombinant expression of *HD0581*	TGTAATAAACCTGATCCTGCTACA GA	AGTCATTTGAAAGTCCTATGTC AG
recombinant expression of *HD1218*	ACCATCTCTGCACACGCAACCATA	GGGACCATTCCTAATATGCAA ATCC
mutagenesis of *fgbA*	catatcggatccCCGCCAACGTTTAAGC CCATCATT	catatctctagaTCGGGCGACTTTAG CGCAATATCA
trans–complementation with *fgbA*; survey of *fgbA* loci in *H. ducreyi* strains	taagaatgcggccgcTACGCTGCGCCACA GACTACTAAA	taagaatgcggccgcTGACCGCGATA AGCGGTCTTT
class I-specific primers *dsrA* 14 and *dsrA* 24[b]	GACAGCATTCAGTGAATAATGGC	AATGAAGTCCGCACCTTTAAC GGC
class II-specific primers *dsrA* 42 and *dsrA* 43[c]	TGCCTTGCTCTTAATGACG	TAAAAGCACATAAACAAGCG

[a] Lowercase lettering indicates linkers with non-*H.ducreyi* sequences

[b] Primers were exactly the same as the class I-specific primer pair of White et al. [24].

[c] Primers were the *H. ducreyi*-specific portions, excluding linker sequences, of the class II-specific primer pair of White et al. [24].

Table 3

H. ducreyi proteins identified by MS-MS of Fg-binding spots

Protein Annotation	Accession no.	No. matching peptides	predicted MW, kDa	predicted pI	predicted signal peptide[a]
hypothetical protein HD0192 (FgbA)	AAP95185	4	16.8[b]	5.03[b]	residues 1–18
lipoprotein HlpB	AAP95513	2	17.8[c]	5.33[c]	residues 1–19
NifU-like protein	AAP95949	5	13.6	4.66	none
hypothetical protein HD1218	AAP96060	2	12.6[d]	4.72[d]	residues 1–21
universal stress protein UspA	AAP96234	2	15.7	4.5	none
10 kDa chaperonin GroES	AAP96537	3	10.3	4.88	none
50S ribosomal protein L7/L12	AAP96610	4	12.2	4.60	none

[a] Signal peptide prediction based on the SignalP algorithm [15].

[b] Predicted mature FgbA protein, excluding signal peptide, is 14.9 kDa with pI = 4.85.

[c] Predicted mature HlpB protein, excluding signal peptide, is 15.9 kDa with pI = 4.99.

[d] Predicted mature HD1218 protein, excluding signal peptide, is 10.4 kDa with pI = 4.39.

Table 4

Response to Inoculation of live *H. ducreyi* Strains

Volunteer no.[a]	Gender[b]	Days of Observation	Strain	No. of Initial Papules	No. of Pustules	Final Outcomes of Sites:		
						Papule	Pustule	Resolved
297	F	14	35000HP	3	1	0	1	2
			35000HP*fgbA*	3	1	0	1	2
300	M	8	35000HP	2	2	0	2	0
			35000HP*fgbA*	2	0	0	0	2
301	M	7	35000HP	3	2	1	2	0
			35000HP*fgbA*	2	0	0	0	2
302	M	7	35000HP	3	3	0	3	0
			35000HP*fgbA*	3	3	0	3	0
303	M	6	35000HP	3	0	0	0	3
			35000HP*fgbA*	3	0	0	0	3
304	M	6	35000HP	3	3	0	3	0
			35000HP*fgbA*	2	0	0	0	3

[a] Volunteers 297 and 300 were inoculated in iteration one. Volunteers 301, 302 and 303 were inoculated in iteration two. Volunteer 304 was inoculated in iteration three.

[b] F, female; M, male.