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Trimeric Autotransporter DsrA Is a Major Mediator of Fibrinogen Binding in *Haemophilus ducreyi*

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Haemophilus ducreyi **is the etiologic agent of the sexually transmitted genital ulcer disease chancroid. In both natural and experimental chancroid, *H. ducreyi* colocalizes with fibrin at the base of the ulcer. Fibrin is obtained by cleavage of the serum glycoprotein fibrinogen (Fg) by thrombin to initiate formation of the blood clot. Fg binding proteins are critical virulence factors in medically important Gram-positive bacteria. *H. ducreyi* has previously been shown to bind Fg in an agglutination assay, and the *H. ducreyi* Fg binding protein FgbA was identified in ligand blotting with denatured proteins. To better characterize the interaction of *H. ducreyi* with Fg, we examined Fg binding to intact, viable *H. ducreyi* bacteria and identified a novel Fg binding protein. *H. ducreyi* bound unlabeled Fg in a dose-dependent manner, as measured by two different methods. In ligand blotting with total denatured cellular proteins, digoxigenin (DIG)-Fg bound only two *H. ducreyi* proteins, the trimeric autotransporter DsrA and the lectin DltA; however, only the isogenic *dsrA* mutant had significantly less cell-associated Fg than parental strains in Fg binding assays with intact bacteria. Furthermore, expression of DsrA, but not DltA or an empty vector, rendered the non-Fg-binding *H. influenzae* strain Rd capable of binding Fg. A 13-amino-acid sequence in the C-terminal section of the passenger domain of DsrA appears to be involved in Fg binding by *H. ducreyi*. Taken together, these data suggest that the trimeric autotransporter DsrA is a major determinant of Fg binding at the surface of *H. ducreyi*.**

The Gram-negative bacterium *Haemophilus ducreyi* is the causative agent of the sexually transmitted genital ulcer disease chancroid (reviewed in references 1 to 3). Chancroidal lesions are soft, purulent, and painful ulcers that bleed easily upon contact (3, 4). In both natural and experimental chancroid, *H. ducreyi* is found mostly in the dermis, colocalizing with neutrophils and fibrin, but it can also be found in the extracellular space of the epidermis (5, 6). Survival of *H. ducreyi* in this hostile environment is thought to be dependent on many factors, including resistance to phagocytosis and to the bactericidal activity of normal human serum (NHS). One outer membrane protein (OMP) of *H. ducreyi*, the ducreyi serum resistance A (DsrA) protein, has been shown to be involved in serum resistance by preventing binding of naturally occurring IgM at its surface (7, 8). DsrA is required for full virulence in human experimental *H. ducreyi* infection (9).

DsrA belongs to the family of trimeric autotransporter adhesin (TAA), multifunctional outer membrane proteins involved in adhesion and serum resistance (10). Structurally, TAA proteins contain three domains: an N-terminal signal peptide, a passenger domain, and a C-terminal translocator region (11). The highly conserved translocator domain of TAA, which serves as the criterion for inclusion of proteins in this family, anchors TAA in the outer membrane. The passenger domain of TAA is involved in its different functions. As the name implies, TAA proteins are expressed as trimers at the surface of bacterial cells, and trimerization of the passenger domain is required for structural stability, adhesive activity, and virulence (12, 13). The prototypical protein for this group of proteins, YadA from *Yersinia enterocolitica*, is lollipop shaped, with the head of YadA forming the lollipop portion. In YadA, both fibronectin (Fn) and collagen binding domains are found in the head section (14). DsrA does not bind collagen but interacts with Fn and vitronectin (Vn) (15, 16). Fn and Vn binding are mediated by amino acids in the C-terminal region of the DsrA passenger domain (17).

There are two clonal populations of *H. ducreyi* strains, termed class I and class II (18–21), based on variant outer membrane determinants, including DsrA (18–21). Each class of *H. ducreyi* strains expresses a different DsrA protein, termed $DsrA_I$ and $DsrA_{II}$ for class I and class II strains, respectively (18). Despite low homology in their passenger domains, the two classes of DsrA proteins possess the same functions: both $DsrA_I$ and $DsrA_{II}$ confer serum resistance and Fn and Vn binding (16).

Fibrinogen (Fg) is a large serum glycoprotein involved in coagulation, wound healing, neoplasia, angiogenesis, and the inflammatory response (22, 23). Fg interacts with the extracellular matrix (ECM) and cells. The 340-kDa Fg molecule is composed of three nonidentical peptide chains, termed α, β, and γ, pairs of which are joined together by disulfide bonds (23). Fg binding proteins of Gram-positive bacteria have been well characterized and have been shown to be involved in pathogenesis (24, 25); however, there is less evidence about the presence of Fg binding proteins in Gram-negative bacteria and their role in disease. *H. ducreyi* was first shown to interact with Fg in a latex bead agglutination assay, where all 21 isolates tested bound Fg (26). In 2009, a lipoprotein termed FgbA (HD0192) was shown to bind digoxigenin (DIG)-Fg in ligand blotting of denatured proteins from prototypical class I *H. ducreyi* strain 35000HP. FgbA was shown to be involved in the pathogenesis of experimental chancroid (27). Because *H. ducreyi* colocalizes with fibrin in both natural and experimental chancroid, we sought to identify the cell surface receptor of *H. ducreyi* that interacts with the precursor of fibrin, Fg. In two

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype[a]	$DsrA_I$ protein expressed	Reference(s) or source
Strains			
H. ducreyi			
35000HP	Wild-type, human-passaged variant of strain 35000	$DsrA_I$	37, 38
35000HP Δ*dsrA* (FX517)	35000HP *dsrA*::CAT	No $DsrA_I$	7
35000HP Δ*ncaA*	35000HP *ncaA*::CAT	$DsrA_I$	30
35000HP Δ*dltA*	35000HP *dltA*::*kan*	$DsrA_I$	29
35000HP Δ*momp*	35000HP *momp*::ΩKm2; 35000HP-SMS2	$DsrA_I$	39
35000HPΔ*ompA2*	35000HP *ompA2*::ΩKan2; 35000HP-SMS3	$DsrA_I$	16
35000HP Δ*ftpA*	35000HP *ftpA*::mTn*3*(Cm); 35000HP-SMS1	$DsrA_I$	39
35000HP Δ*fgbA*	35000HP *fgbA*::*kan*	$DsrA_I$	27
HMC21 (425)	Wild type	$DsrA_I$	8
HMC21Δ*dsrA* (FX529)	HMC21*dsrA*::CAT	No $DsrA_I$	8
HMC50 (010-2)	Wild type	$DsrA_I$	8
HMC50Δ*dsrA* (FX530)	HMC50*dsrA*::CAT	No $DsrA_I$	8
HMC54	Wild type	$DsrA_I$	8
HMC54Δ*dsrA*	HMC54*dsrA*::CAT	No $DsrA_I$	8
H. influenzae			
Rd KW20	Wild type	No $DsrA_I$	ATCC
Plasmids			
pLSSK	*H. ducreyi* shuttle vector; Sm^r	No $DsrA_I$	40
pUNCH1260	Complete *dsrA* ORF in pLSSK (774 bp; 257 amino acids)	$DsrA_I$	7
pUNCH1424	bp 73 to 510 deleted in *dsrA*	$DsrA_I$Δ25–170	17
pUNCH1425	bp 73 to 474 deleted in *dsrA*	$DsrA_I$Δ25–158	17
pUNCH1426	bp 73 to 438 deleted in *dsrA*	$DsrA_I$Δ25–146	17
pUNCH1427	bp 73 to 399 deleted in *dsrA*	$DsrA_I$Δ25–133	17
pUNCH1428	bp 73 to 363 deleted in *dsrA*	$DsrA_I$Δ25–121	17
pUNCH1429	bp 73 to 330 deleted in *dsrA*	$DsrA_I$Δ25–110	17
pUNCH1430	bp 73 to 291 deleted in *dsrA*	$DsrA_I$Δ25–97	17
pUNCH1431	bp 73 to 195 deleted in *dsrA*	$DsrA_I$Δ25–65	17
pUNCH1706	Deletion of MEQNTHNINKLS from $DsrA_I$	$DsrA_I$ΔMEQNTHNINKLS	This study
pUNCH1707	Deletion of NTHNINKLSKEL from $DsrA_I$	$DsrA_I$ΔNTHNINKLSKEL	This study
pUNCH1764	Deletion of LKVLDARISKNKQ from $DsrA_I$	$DsrA_I$ΔLKVLDARISKNKQ	This study
pUNCH2105	bp 73 to 156 deleted in *dsrA*	$DsrA_I$Δ25–51	This study
pUNCH2106	bp 73 to 123 deleted in *dsrA*	$DsrA_I$Δ25–42	This study

[a] CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol; Km2, Kan2 or *kan*, kanamycin resistance cassette; ORF, open reading frame.

different binding assays using intact bacterial cells, only *H. ducreyi* lacking expression of *dsrA* had significantly reduced amounts of cell-associated Fg compared to the wild-type parental strain. Furthermore, complementation of an isogenic *dsrA* mutant restored Fg binding and conferred binding to a *Haemophilus influenzae* strain incapable of binding Fg. A 13-amino-acid sequence in the translocator domain of DsrA was shown to be involved in the interaction of Fg at the surface of *H. ducreyi* cells. Our findings indicate that Fg interacts specifically and in a dose-dependent manner with DsrA at the surface of viable *H. ducreyi* cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. ducreyi* strains (Table 1) were routinely cultured and passaged on chocolate agar plates (CAP) containing GC medium base (Difco, Detroit, MI) and 1% bovine hemoglobin (Becton, Dickinson, Sparks, MD), supplemented with 5% FetalPlex (Gemini Bio-Products, West Sacramento, CA) and 1× GGC (0.1% glucose, 0.001% glutamine, 0.026% cysteine) (28) at 34.5°C in 5% CO_2. *H. ducreyi* strains were also cultured on heme plates containing GC medium base and 50 μg/ml heme (H5533; Sigma-Aldrich, St. Louis, MO) and supplemented with 1× GGC. *H. influenzae* strains expressing different plasmids (Table 1) were cultured on CAP supplemented with 1% IsoVitalex (BD, Franklin Lakes, NJ). Streptomycin (100 μg/ml) was added to the growth medium when appropriate.

Construction of in-frame *dsrA*$_I$ deletion mutants. To complement a panel of truncated DsrA proteins already constructed (17), two more in-frame *dsrA*$_I$ deletion mutants were created in the N-terminal region of the passenger domain of $DsrA_I$. Two forward primers, DsrAxH and DsrAxI (Table 2), were each used separately in combination with primer DsrAxE (17) and pUNCH1260 as the template to generate two separate PCR products using the following conditions: 95°C for 5 min for 1 cycle, followed by 30 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final polishing step at 72°C for 5 min. The products from these PCRs (DsrAxH-E and DsrAxI-E) were digested overnight with XmaI, cleaned, and ligated for 30 min at room temperature with XmaI-treated pUNCH1424, as previously described (17), to form pUNCH2105 and pUNCH2106, respectively.

Smaller deletions were also made in the passenger (pUNCH1764) and translocator (pUNCH1706 and pUNCH1707) domains of DsrA. For each of these three constructs, overlap extension PCR with *Pfx* polymerase (Grand Island, NY) was used to generate three separate PCR products. Two separate PCRs were performed to generate the 5′ and 3′ portions of the constructs, using primers 1 and 2 and primers 3 and 4 (Table 2), respectively, with the appropriate primers 2 and 3 used for each construct. pUNCH1260 was used as the DNA template. The conditions for the first

TABLE 2 List of primers

Name	Sequence (5′ to 3′)	Construct(s)
DsrAxH	TCCCCCGGGTTCGATATTAAAGTGCCAGGG	pUNCH2105
DsrAxI	TCCCCCGGGAAGGGTAAGTGGACTTGGTCT	pUNCH2106
DsrA#1	GCGGGATCCCCCGACAGCATTCAGTG	pUNCH1706, pUNCH1707, pUNCH1764
#2ND3	AGTTTGCAATTCTTTCATACGATAAGAATCATCTAAATA	pUNCH1706
#3ND3	GATTCTTATCGTATGAAAGAATTGCAAACTGGTTTAGCC	pUNCH1706
DsrA#4	GGCAAGCTTAATTAATAAAAGTGGC	pUNCH1706, pUNCH1707, pUNCH1764
#2ND4	GGCTAAACCAGTTTGTTGTTCCATCATACGATAAGAATC	pUNCH1707
#3ND4	CGTATGATGGAACAACAAACTGGTTTAGCCAACCAATCA	pUNCH1707
SrDsrARev#2	TACATTCAAAATCTCATTAAGCCTATTCTTCGCTTGTAC	pUNCH1764
SrDsrA2For#3	AAGAATAGGCTTAATGAGATTTTGAATGTAGGTAATCAT	pUNCH1764

two PCRs were as follows: for the 5′ region, 1 cycle at 95°C for 2 min, followed by 30 cycles of denaturation (95°C, 15 s), annealing (43°C for pUNCH1706, 43°C for pUNCH1707, and 45°C for pUNCH1764) for 30 s, and extension (68°C, 1 min), with a final 1-cycle polishing step at 68°C for 5 min; for the 3′ region, the conditions for PCRs were identical to those for the 5′ region, except that the annealing step was carried out at 41°C for pUNCH1707. The third PCR was use to make the final construct containing the 5′ and 3′ regions next to the deletion. For this reaction, primers 1 and 4 were used with PCR products obtained with the first and second PCRs as templates. The conditions for this last PCR are identical to those described above, except that the annealing step for all of the constructs was performed at 45°C, and the extension step lasted 90 s instead of 60 s.

All PCR products were subsequently ligated and electroporated into the single isogenic *dsrA* mutant FX517 (7). Clones expressing recombinant proteins of the expected size were selected based on protein expression, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The DNA sequence of the chosen clones was confirmed by sequencing at the University of North Carolina (UNC) sequencing facility. Expression of truncated DsrA proteins at the surface of *H. ducreyi* cells was verified with a rabbit polyclonal antibody (Ab) developed to the passenger domain of $DsrA_I$ (rNT-$DsrA_I$) in a whole-cell binding assay (17).

SDS-PAGE and Western and ligand blotting. Total cellular proteins prepared from strains of *H. ducreyi* were subjected to SDS-PAGE under reducing conditions (except for the experiment shown in Fig. 2) at 150 V using a mini-Protean II system (Bio-Rad, Hercules, CA). Gels were transferred onto nitrocellulose membranes for 2 h at 200 mA and blocked overnight in 0.5% Tween 20 in phosphate-buffered saline (PBS) (blocking solution). Membranes were treated in either of two ways: ligand blotting with DIG-labeled Fg (for the experiment shown in Fig. 2 only) or Western blotting. For ligand blotting, 100 μg of Fg (depleted of plasminogen, von Willebrand factor, and Fn) (Enzyme Research Laboratories, South Bend, IN) was labeled with DIG (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The membranes were thereafter incubated with DIG-Fg (1:10,000) in blocking solution for 1 h at room temperature. After three washes with 0.05% Tween 20 in PBS (wash buffer), the membranes were incubated with an anti-DIG secondary Ab (Roche Diagnostics, Mannheim, Germany) for 1 h at room temperature with mixing. After three more washes, the membranes were developed by chemiluminescence using the substrate LumiPhos^{WB} (Thermo Fisher Scientific, Pittsburgh, PA). For Western blotting, membranes were incubated with either anti-Fg (1:10,000; Sigma-Aldrich, St. Louis, MO) in the Fg binding assay (described below), recombinant full-length $DsrA_I$ (rFL-$DsrA_I$) (18), or a polyclonal rabbit Ab to recombinant DltA (29) at a dilution of 1:25,000 for 30 min at room temperature. After three washes, the membranes were incubated with an anti-goat or anti-rabbit alkaline phosphatase-conjugated secondary Ab (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature and developed using chemiluminescence.

Fg binding assay. The Fg binding assay was carried out exactly as described for the Fn binding assay (16), using suspensions of intact, viable *H. ducreyi* bacteria and purified Fg; however, in this instance, cell-associated Fg was measured both by Western blotting and flow cytometry. Briefly, suspensions (1 ml at an optical density at 600 nm [OD_{600}] of 0.5; approximately 2.5×10^8 CFU/ml) of bacteria grown on CAP or heme plates (15 to 16 h) were prepared in GC broth (GCB) and incubated with amounts of Fg indicated in Fig. 1 or the legends of the other figures for 30 min at 34.5°C (5% CO_2 and humidified atmosphere), with mixing every 10 min. Bacterial cells were thereafter washed three times for 30 min with GCB. The resulting pellet was transferred to a new microcentrifuge tube and washed a fourth time. After the whole-cell binding assay, we assessed Fg bound to the surface of *H. ducreyi* using two methods. Bacteria pellets were suspended in Laemmli sample buffer with β-mercaptoethanol, and a 3-μl aliquot was subjected to SDS-PAGE (12%) and Western blotting with anti-Fg as described above. We also used flow cytometry to assess *H. ducreyi*-associated Fg. Bacterial suspensions were incubated with fluorescein isothiocyanate (FITC)-Fg (labeled according to the manufacturer's instructions) (FluoReporter FITC Protein Labeling Kit; Life Technologies, Grand Island, NY) as described above and analyzed using an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). A total of 100,000 events were recorded, and a threshold of 10,000 was used. FCS Flow Cytometry data analysis (De Novo Software, Los Angeles, CA) was used to create the histograms from data obtained with the C6 cytometer.

For competition assays, Fg was first labeled with DIG (Roche Diagnostics GmbH, Mannheim, Germany). Unlabeled Fg was then incubated with suspensions of intact, viable *H. ducreyi* cell suspensions for 30 min prior to addition of DIG-Fg. Cell-associated Fg was measured as described above. Previous data from the unlabeled Fn binding assay as well as other unpublished data from our laboratory indicate that the viability of *H. ducreyi* cells is conserved after four washes of 30 min and that *H. ducreyi* can survive at least 6 h at room temperature (data not shown). Therefore, bacterial cells analyzed by flow cytometry were viable at the time of analysis.

Serum susceptibility. *H. ducreyi* resistance to 50% NHS was determined as previously described (18). Results are expressed as percent survival of *H. ducreyi* bacteria in fresh NHS compared to survival in heated NHS (ΔNHS) [(number of CFU in fresh NHS/number of CFU in ΔNHS) × 100].

Densitometry and statistical analyses. Three bands on Western blots were used for density measurements: the thick band above the 66.2-kDa marker, which represents the α chain (63.5 kDa), and the doublet below the 66.2-kDa molecular mass standard, which encompasses the β (56 kDa) and the γ (47 kDa) chains of Fg. Density was determined using either ImageJ (version 1.62 [http://rsb.info.nih.gov/nih-image/]) or AlphaVIEW SA (version 3.2.4, build 1021 [ProteinSimple, Santa Clara, CA]). Data obtained using densitometry were analyzed using a single sample *t* test using the VassarStats website (http://vassarstats.net/). Binding was expressed as the percentage of full-length wild-type (WT) DsrA or absence of competing unlabeled protein, defined as 100% binding. A P value of <0.05 was considered statistically significant.

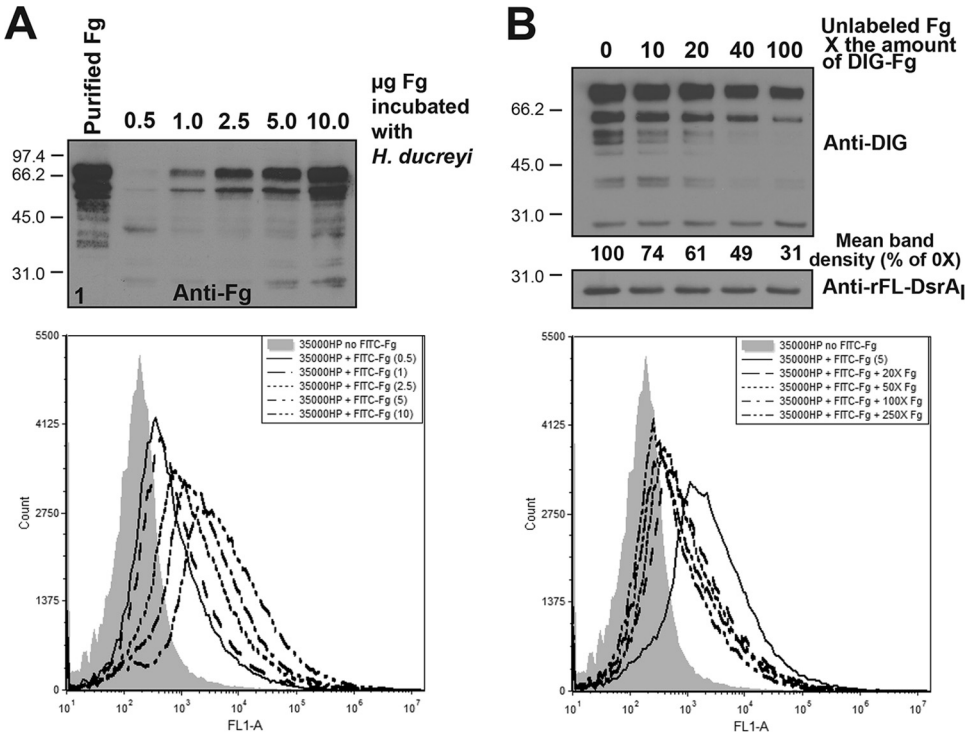

FIG 1 Fg interacts with *H. ducreyi* at its cell surface. Fg binding by intact, viable cells of *H. ducreyi* strain 35000HP was assessed using two different methods, Western blotting with anti-Fg (top) and flow cytometry (bottom). (A) Standard curve with different amounts of Fg (top) or FITC-Fg (bottom) incubated with intact *H. ducreyi* bacteria. (B) Competition assay with unlabeled and DIG- or FITC-labeled Fg. Viable bacteria of *H. ducreyi* strain 35000HP were incubated with unlabeled Fg prior to incubation with DIG-labeled (Western blot, top) or FITC-labeled (flow cytometry, bottom) Fg. The numbers at the bottom of the blot refer to densitometry of the Fg bands and indicate percent binding compared to results with no addition of unlabeled protein (0×), defined as 100% binding. For all Western blotting and flow cytometry experiments, representative blots and histograms from at least two independent experiments are shown. For Western blotting, Ponceau S staining of the nitrocellulose membranes was performed to ensure equal loading of total proteins in all lanes (not shown).

RESULTS

Fg binds the surface of viable *H. ducreyi* bacteria in a dose-dependent manner. We used an Fg binding assay using suspensions of intact, viable bacteria and purified soluble plasma Fg, similar to the one previously described for Fn binding (16), to determine if purified Fg interacts with the surface of *H. ducreyi* cells. We detected cell surface-bound Fg by Western blotting and flow cytometry following Fg incubation with viable bacteria. In Western blotting, proteins solubilized from viable bacteria incubated with Fg were separated by SDS-PAGE and transferred onto nitrocellulose, and *H. ducreyi*-bound Fg was identified with an anti-Fg Ab. For flow cytometry, suspensions of *H. ducreyi* incubated with FITC-Fg were assayed directly to detect FITC bound to the bacterial surface. Because *H. ducreyi* binds Fn and because Fn interacts with Fg (23), this study was performed using Fg depleted of plasminogen, von Willebrand factor, and Fn to ensure that the interaction between *H. ducreyi* and Fg was not mediated via Fn or other factors that bind Fg. Fg bound the surface of *H. ducreyi* in a dose-dependent manner (Fig. 1A), as measured using Western blotting (top) and flow cytometry (bottom).

We previously showed that the type of medium used for growth affects Fn binding by *H. ducreyi* (16). Fn binding occurs only on heme plates as hemoglobin blocks the interaction of Fn at the surface of *H. ducreyi*. This was not the case for Fg binding as results similar to those shown in Fig. 1A were obtained with bacteria grown on heme plates (data not shown). Thus, hemoglobin does not block the interaction of Fg at the surface of *H. ducreyi* cells.

In order to show specificity of Fg binding at the surface of *H. ducreyi* bacteria, we used a competition assay with unlabeled and DIG- or FITC-labeled Fg. Addition of increasing amounts of unlabeled Fg prior to incubation of *H. ducreyi* with DIG- or FITC-Fg reduced ligand binding to the bacterial cell surface (Fig. 1B). This was consistent regardless of the method used to detect the interaction between Fg and *H. ducreyi*. Addition of excess irrelevant ligand did not affect binding of DIG- or FITC-Fg to *H. ducreyi* (data not shown).

DIG-Fg binds the *H. ducreyi* DsrA in ligand blotting. To identify the bacterial proteins involved in Fg binding by *H. ducreyi*, total cellular proteins from a panel of *H. ducreyi* strains, including prototypical isolate 35000HP and isogenic mutants in several OMPs (Table 1), were subjected to ligand blotting probed with DIG-Fg. These mutants were chosen because their targeted mutant proteins are either surface exposed and/or involved in binding proteins of the ECM. DsrA and NcaA are two TAA proteins expressed by *H. ducreyi* shown to interact with Fn/Vn and collagen, respectively (16, 30). DltA is a lectin that binds Fn only in ligand blotting but not in whole-cell binding assays (29). Also included were mutants of the two major OMPs of *H. ducreyi*, *momp* and *ompA*, since the former was shown to be involved in Fn binding by *H. ducreyi* (16). Finally, an isogenic mutant lacking the Fg binding protein FgbA (27) was also tested in the Fg binding

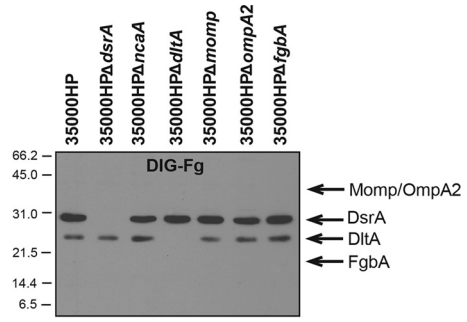

FIG 2 DIG-Fg binds to denatured DsrA. Total cellular proteins prepared from a panel of *H. ducreyi* strains grown on CAP, including 35000HP and isogenic mutant strains in this background, were subjected to ligand blotting with DIG-Fg under nonreducing conditions. Shown is a representative blot from at least two independent experiments. The location (molecular mass) of the different proteins on the blot is indicated using arrows (18, 27). The presence of the indicated proteins on the blot was confirmed by Ponceau S staining prior to incubation of the membrane with DIG-Fg.

assay to determine if *fgbA* binds Fg at the bacterial surface. These mutants were previously characterized for the absence of expression of the targeted protein (Table 1). The ligand blotting method used in this single experiment is different from the Western blot technique described above and used throughout these studies because interactions between DIG-Fg and *H. ducreyi* proteins occurs in a denatured environment apart from the protein's location in *H. ducreyi*. Under nonreducing conditions, DIG-Fg bound the TAA DsrA and the *H. ducreyi* lectin DltA (Fig. 2). DIG-Fg binding to DltA was lost upon reduction with β-mercaptoethanol (data not shown). DIG-Fg did not bind FgbA, as previously reported (27). These results suggest that both *H. ducreyi* DltA and DsrA may bind Fg at the bacterial surface.

An *H. ducreyi* isogenic *dsrA* mutant binds significantly less Fg at the cell surface than parental *H. ducreyi* 35000HP. To determine whether Fg directly binds DsrA at the surface of *H. ducreyi* cells, we tested the same panel of individual OMP mutants described above using the Fg binding assay with intact, viable *H. ducreyi* bacteria, assessing surface-associated Fg by Western blotting and flow cytometry. Among this panel of *H. ducreyi* mutants lacking demonstrated virulence factors, only the *dsrA* mutant bound significantly less Fg than the parent strain 35000HP (67 to 95% reduction in Western blotting; $P < 0.05$), whether the strains were grown with or without hemoglobin (Fig. 3A and data not shown). These results were confirmed by flow cytometry as FITC-Fg binding to the surface of *H. ducreyi* cells was altered only in the *dsrA* mutant (Fig. 3B). Binding by the 35000HP Δ*fgbA* isogenic mutant strain in the previously identified Fg binding protein FgbA was not significantly reduced compared to the level of

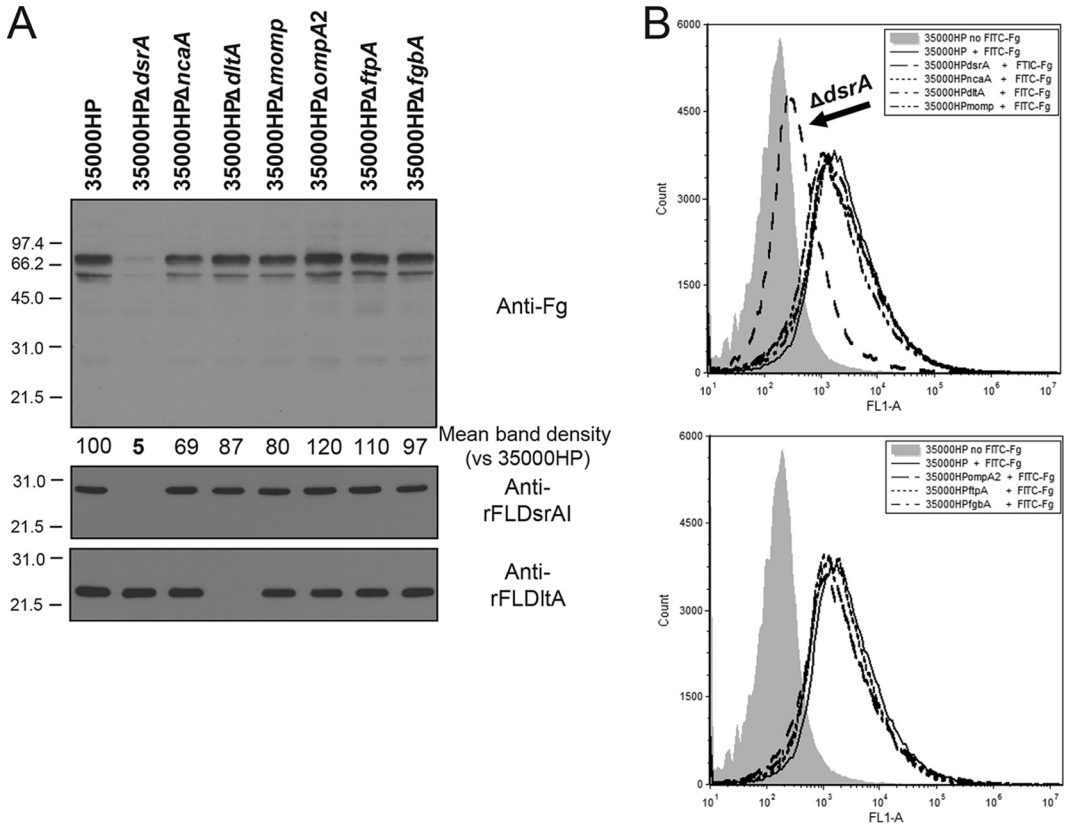

FIG 3 DsrA is a major mediator of Fg binding by *H. ducreyi*. A panel of *H. ducreyi* strains, including prototypical class I isolate 35000HP and several isogenic mutants, was subjected to the Fg binding assay. (A) Cell-associated Fg was measured by Western blotting. The mean band density, calculated from the three Fg bands on Western blots obtained in three independent experiments, is indicated underneath the blots as a percentage of Fg binding by 35000HP, defined as 100%. Bolded mean band density indicates significantly reduced Fg binding compared to strain 35000HP ($P < 0.05$). Ponceau S staining of the nitrocellulose membranes and Western blotting using antisera to full-length $DsrA_I$ and full-length DltA were used to confirm equal loading of lanes. (B) Cell-associated FITC-Fg (5 μg) was measured using flow cytometry. Representative histograms from at least two independent experiments are shown.

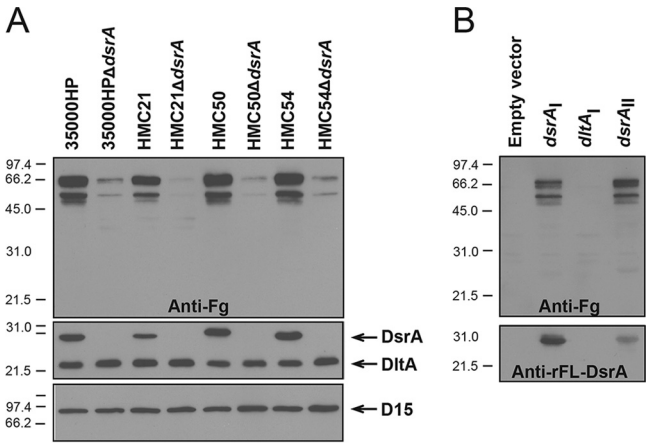

FIG 4 Both classes of DsrA proteins confer Fg binding. (A) Four different class I *H. ducreyi* strains, along with their respective *dsrA* mutants, were assayed for Fg binding using Western blotting with 2.5 μg of unlabeled Fg. (B) Plasmids encoding full-length $dsrA_I$, $dltA_I$, $dsrA_{II}$, or an empty plasmid (pLSSK) were expressed in the non-Fg-binding strain *H. influenzae* Rd and tested for binding to Fg by Western blotting. Ponceau S staining of the nitrocellulose membranes (data not shown) and Western blotting with anti-recombinant D15 (rD15) protein were used to ensure equal loading in lanes (36). Shown are representative blots from at least two independent experiments. Anti-rFL-$DsrA_I$, rabbit polyclonal antibody elicited to full-length recombinant $DsrA_I$ (18).

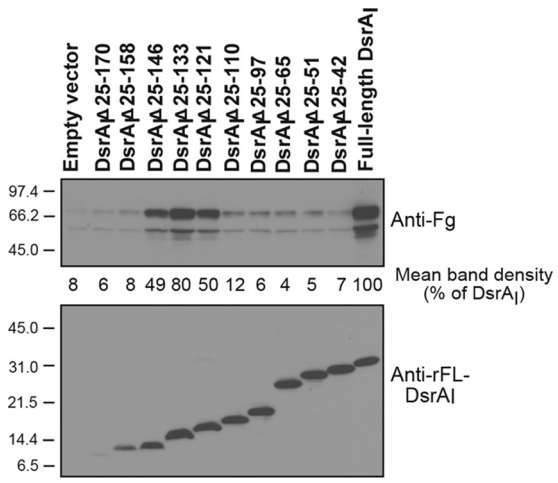

FIG 5 A sequence in the passenger domain of DsrA may be involved in Fg binding by *H. ducreyi*. A panel of truncated DsrA proteins, expressed in an *H. ducreyi dsrA* mutant, was tested for Fg binding by Western blotting with 2.5 μg of unlabeled Fg. (Top) Representative Western blot from three independent Fg binding assays with identical results. The band density from all three assays is expressed as an average and shown as a percentage of binding by full-length $DsrA_I$, assigned as 100% binding. (Bottom) Expression of truncated $DsrA_I$ proteins was assessed using a polyclonal Ab developed to full-length $DsrA_I$ (anti-rFL-$DsrA_I$). Ponceau S staining of the nitrocellulose membranes was used to ensure equal loading of total proteins in all lanes (data not shown).

35000HP (Fig. 3A and B). The levels of expression of DsrA and DltA were similar in all strains and under all culture conditions tested (Fig. 3A and data not shown).

The data presented in Fig. 3 show that DsrA appears to be a major determinant of Fg binding at the surface of viable bacteria of *H. ducreyi* class I strain 35000HP. To determine if Fg binding by DsrA is a general phenomenon among *H. ducreyi* strains, we examined class I parent and *dsrA* mutant strain pairs (8) using the unlabeled Fg binding assay, measuring cell-associated Fg by Western blotting. In each case, mutational inactivation of *dsrA* reduced binding of Fg at the surface of *H. ducreyi* cells (Fig. 4A). Moreover, strain HMC21, whose expression of DsrA was lower than that of the other isolates, bound less Fg than the other tested strains. The results of the unlabeled Fg binding assay with multiple *dsrA* mutants strongly suggest that DsrA is the primary mediator of Fg binding by class I *H. ducreyi* strains.

Expression of full-length $DsrA_I$ or $DsrA_{II}$ renders *H. influenzae* strain Rd capable of binding Fg. The two classes of *H. ducreyi* strains express DsrA proteins (encoded by $dsrA_I$ or $dsrA_{II}$) that are highly similar in their C-terminal translocator domains (86% identity) but different in their passenger domains (18). We previously showed that both classes of DsrA proteins bind Fn and Vn in the C-terminal regions of their passenger domains (17). To determine whether class II DsrA proteins are also involved in binding Fg at the surface of viable bacteria, full-length $dsrA_I$ or $dsrA_{II}$ was expressed in *trans* from plasmid pUNCH1260 or pUNCH1296, respectively, in *H. influenzae* strain Rd, which does not bind Fn, Vn, or Fg (16). Expression of both $DsrA_I$ and $DsrA_{II}$ conferred Fg binding to *H. influenzae* Rd, whether it was grown on CAP or heme plates (Fig. 4B and data not shown). In contrast, expression of $dltA_I$ or an empty vector did not affect Fg binding to *H. influenzae* Rd. These findings indicated that despite dissimilar primary amino acid sequences in their passenger domains, both $DsrA_I$ and $DsrA_{II}$ mediate Fg binding.

A region in the C terminus of the passenger domain of DsrA interacts with Fg. A panel of $DsrA_I$ proteins truncated in their passenger domains was previously used to determine amino acid sequences of $DsrA_I$ involved in serum resistance as well as Fn and Vn binding by *H. ducreyi* (17). These same truncated $DsrA_I$ proteins expressed in the *H. ducreyi* isogenic *dsrA* mutant were therefore used to identify the domain of DsrA bound by Fg using the Fg binding assay. The two smallest truncated proteins, $DsrA_I$ with a deletion of residues 25 to 170 ($DsrA_I$Δ25–170) and $DsrA_I$Δ25–158, bound Fg to the same level as a *dsrA* mutant expressing an empty vector (Fig. 5). The next longest construct, $DsrA_I$ Δ25–146, bound Fg to 49% of the level of a strain expressing full-length $DsrA_I$, and $DsrA_I$ Δ25–133 bound 80% of that level (Fig. 5). Surprisingly, Fg binding by the next-longest truncated $DsrA_I$ protein, $DsrA_I$Δ25–121, was reduced to 50% of the binding of the parent strain (Fig. 5). Furthermore, larger truncated $DsrA_I$ proteins bound Fg at levels similar to the level of the *dsrA* mutant expressing an empty vector (Fig. 5). These results were not related to the expression levels of the truncated $DsrA_I$ proteins since all but the smallest constructs, $DsrA_I$Δ25–170 and $DsrA_I$Δ25–158, appeared to be expressed at levels similar to the level of the full-length $DsrA_I$ (Fig. 5). Reduced surface exposure of truncated $DsrA_I$ proteins also did not correlate with the data obtained in the Fg binding assay as all truncated $DsrA_I$ proteins, except the two smallest ones, were expressed at levels similar to the level of full-length $DsrA_I$ at the bacterial surface (17; also data not shown).

The above-described results could be explained by improper folding of the larger truncated DsrA proteins at the surface of *H. ducreyi* cells. We postulated that improperly folded DsrA proteins might block the Fg binding site present closer to the bacterial membrane. To test this hypothesis, we removed a 13-amino-acid sequence (LKVLDARISKNKQ) in the C-terminal region of the passenger domain of DsrA (Fig. 6A). The truncated protein

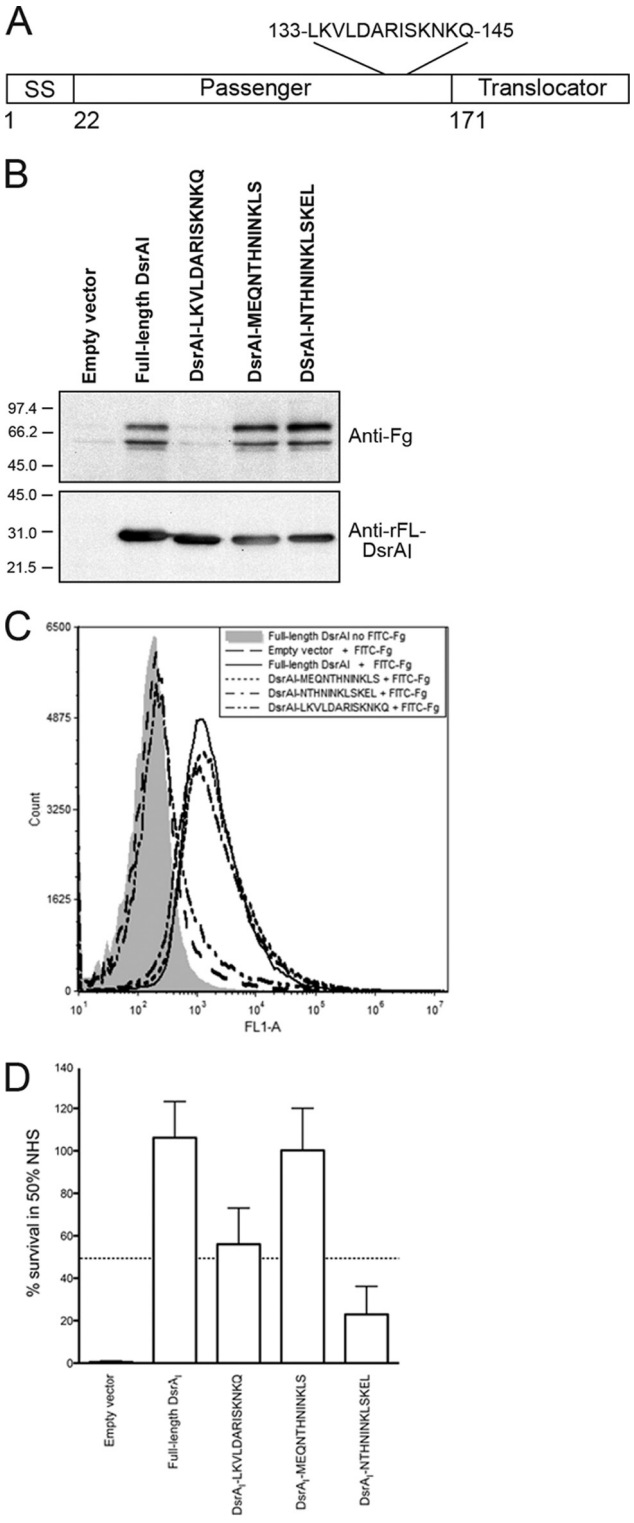

FIG 6 A 13-amino-acid sequence in the C-terminal section of the passenger domain of $DsrA_I$ is involved in Fg binding by *H ducreyi*. (A) Schematic showing the location of the 13-amino-acid mutation in the DsrA protein. Fg binding at the surface of *dsrA* mutants was investigated using both Western blotting with unlabeled Fg (5 μg) (B) and flow cytometry with FITC-Fg (5 μg) (C). Shown are representative blots and histograms from at least three independent experiments with similar results. (D) Bactericidal assay with 50% NHS. Shown are means ± standard deviations of three independent experiments. Survival below the dotted line indicates a serum-sensitive strain. SS, signal sequence.

$DsrA_I$Δ25–133, which contains this sequence, bound the most Fg of all truncated proteins, with 80% of WT levels (Fig. 5). Removal of these amino acids abrogated binding of *H. ducreyi* to Fg, as measured using Western blotting (Fig. 6B) and flow cytometry (Fig. 6C). In contrast, removal of 12-amino-acid sequences in the translocator domain (MEQNTHNINKLS and NTHNINKLSKEL) did not affect Fg binding (Fig. 6B and C).

We previously suggested that serum resistance by DsrA might be due to shielding of *H. ducreyi* surface determinants by the interaction of DsrA with large serum components (17). To determine whether Fg binding is involved in *H. ducreyi* serum resistance mediated by DsrA, we measured the survival of the mutants shown in Fig. 6B and C in a bactericidal assay with 50% NHS. Removal of the sequence LKVLD ARISKNKQ did not affect survival of *H. ducreyi* in NHS (Fig. 6D). Conversely, removal of one of the sequences in the translocator domain, NTHNINKLSKEL, rendered the *H. ducreyi* strain expressing this mutation susceptible to the bactericidal activity of NHS (Fig. 6D). Based on these data, we conclude that Fg binding by *H. ducreyi* is not involved in serum resistance.

DISCUSSION

In both natural and experimental chancroid, *H. ducreyi* colocalizes with fibrin (5, 6). Fibrin is a vital part of the coagulation process, which originates from the transformation of the serum glycoprotein Fg by the action of thrombin (22). Several strains of *H. ducreyi* had previously been shown to bind Fg in an agglutination assay (26). Furthermore, an Fg binding protein had been identified in the prototypical class I strain 35000HP in an assay with denatured *H. ducreyi* proteins (27). Based on this information, we sought to determine if viable *H. ducreyi* bacteria interacted with Fg at the cell surface by incubating soluble Fg with a suspension of *H. ducreyi* and measuring the cell-associated Fg using Western blotting and flow cytometry. We showed that Fg depleted of Fn, plasminogen, and von Willebrand factor bound the bacterial cell surface in a dose-dependent manner, regardless of the medium used to grow *H. ducreyi* (Fig. 1A). This is different from Fn binding by *H. ducreyi*, which occurs only when bacteria are grown on heme plates, due to the presence of Hb in the growth medium that partially inhibits Fn binding by *H. ducreyi* (16). Furthermore, the interaction between viable *H. ducreyi* bacteria and Fg was specific as unlabeled Fg competed for binding of FITC- and DIG-labeled Fg to the bacterial cell (Fig. 1B). These findings indicated that the serum glycoprotein Fg interacts specifically at the cell surface of *H. ducreyi*.

In search of bacterial surface proteins that interacted with Fg, we showed that DIG-Fg bound only two *H. ducreyi* proteins, DsrA and DltA (Fig. 2), in ligand blotting against denatured, total cellular proteins from a panel of *H. ducreyi* OMP mutants. However, ligand blots may not reflect what occurs at the surface of viable *H. ducreyi* bacteria. For example, DltA was shown to interact with DIG-Fn in ligand blotting, but a *dltA* mutant was not impaired in its interaction with Fn in a Fn binding assay using viable *H. ducreyi* cells (16). We found the same to be true with Fg since only the isogenic *dsrA* mutant was significantly impaired in its binding to Fg compared to the parent strain in the Fg binding assay with intact *H. ducreyi* bacteria (Fig. 3). None of the five other isogenic mutants tested had reduced binding to Fg, including the mutant that lacks the previously identified Fg binding protein FgbA. Furthermore, mutation of the *dsrA* gene in three other class I strains of *H. ducreyi* also reduced Fg binding by these strains (Fig. 4A). These results differ from those for some Gram-positive bacteria like *Staphy-*

lococcus aureus or group A *Streptococcus* (GAS) because these pathogens express several different Fg binding proteins, and removal of one Fg binding protein does not alter its surface interactions with Fg (24). Therefore, our data strongly suggest that $DsrA_I$ is a major mediator of Fg binding at the surface of class I *H. ducreyi* strains.

Our data also show that $DsrA_{II}$ can mediate Fg binding in class II *H. ducreyi* strains (Fig. 4B). Because we have been unable to make a mutation in class II *H. ducreyi* strains, we have examined $DsrA_{II}$ functions by expressing proteins in the *dsrA* class I *H. ducreyi* mutant or *H. influenzae* strain Rd. Even though $DsrA_I$ and $DsrA_{II}$ share little homology in their passenger domains, our data show that both proteins can mediate Fg binding in *H. influenzae* strain Rd (Fig. 4B). Thus, we conclude that DsrA not only interacts with Fg at the surface of *H. ducreyi* but also can confer Fg binding to a non-Fg-binding heterologous host.

We previously showed that ECM proteins Fn and Vn bind DsrA in the C-terminal portion of the passenger domain (17). A short, 53-residue section of the passenger domain of $DsrA_I$, expressed along with the translocator domain (a truncated DsrA protein with residues 25 to 121 removed, where amino acids 1 to 21 encompass the leader peptide and 22 to 170 comprise the passenger domain) (Fig. 6A), bound 56 and 100%, respectively, the levels of Vn and Fn binding by full-length $DsrA_I$ (17). Truncated $DsrA_I$ proteins with larger passenger domains bound Vn and Fn at the same levels as the full-length protein, while constructs with smaller passenger domains bound Vn and Fn at levels similar to those of a *dsrA* isogenic strain expressing an empty vector (17). Data presented here show that this does not appear to be the case for Fg binding. Curiously, only the truncated protein with residues 25 to 133 missing ($DsrA_I\Delta$25-133) bound Fg at 80% of the binding levels of full-length $DsrA_I$ (Fig. 5). Interestingly, all other truncated $DsrA_I$ proteins bearing a larger passenger domain than $DsrA_I\Delta$25-133 bound less Fg, with most constructs binding Fg at background levels. Based on these data, we speculated that the Fg binding site of DsrA is in the C-terminal portion of the passenger domain, present in proteins $DsrA_I\Delta$25-146 and $DsrA_I\Delta$25-133, encompassing residues LKVLDARISKNKQ (17). When the truncated DsrA protein is small, this portion of the protein either folds properly and/or is exposed to the surface, enabling its interaction with Fg. However, as the truncations of the passenger domains get smaller and the DsrA constructs get larger, their three-dimensional structures are not native and perhaps are subject to improper folding that hides the Fg binding sites. Only when full-length DsrA is expressed at the bacterial surface is it capable of adopting a native conformation, and the Fg binding site on DsrA is once again available for interaction with Fg.

We tested this hypothesis by removing the 13-amino-acid sequence LKVLDARISKNKQ, thought to represent the Fg binding domain in full-length $DsrA_I$, and keeping the rest of the protein intact (Fig. 6A). Removal of this sequence in DsrA significantly reduced binding of Fg by *H. ducreyi* (Fig. 6B and C). Fg binding was not affected when similar-sized sequences were removed in the translocator domain of DsrA (Fig. 6B and C). These data indicate that LKVLDARISKNKQ is involved in Fg binding by DsrA. We are currently investigating the roles of individual amino acids in this region of DsrA in Fg binding.

The results presented here are consistent with the Fg binding domains identified in Fg binding proteins from Gram-positive bacteria. Three staphylococcal proteins, ClfA, FnpbA, and SdrG, which are critical to pathogenesis of this bacteria, have been shown to interact with Fg through subdomains N of their A regions (31, 32). Although these subdomains share low amino acid sequence homology, their secondary structures are highly conserved (24). This suggests that the binding of Fg by ClfA, FnbpA, and SdrG involves a conformational epitope (24). This may also be the case for $DsrA_I$ and $DsrA_{II}$; despite their highly different passenger domains, both are capable of mediating Fg binding. However, it is not known at this time if both classes of DsrA proteins share similar secondary structures.

Bauer et al. reported that *H. ducreyi* expresses an Fg binding protein termed FgbA (27). The Fg binding phenotype of this lipoprotein was demonstrated using a ligand blot in which DIG-Fg bound to solubilized, denatured whole cells and OMP preparations of class I *H. ducreyi* strain 35000HP. As shown above, we were unable to reproduce these results. In our hands, DIG-Fg did not bind the 18-kDa FgbA protein in ligand blotting, and an *fgbA* mutant was not impaired in its interaction with Fg at the surface of *H. ducreyi* cells in our unlabeled Fg assay (Fig. 3). The reasons for these conflicting results are still unclear, especially since the source of Fg for all experiments in both reports was the same. Differences in DIG labeling may have denatured Fg, leading to binding of DIG-Fg to FgbA in the results presented by Bauer et al. However, a more likely explanation is that differences in *H. ducreyi* culture conditions affected protein expression in the two studies. Bauer et al. used mid-log-phase liquid cultures, whereas we used 16-h-old *H. ducreyi* bacteria from CAP. It is possible that FgbA is only expressed in certain media and/or during certain phases of the growth cycle of *H. ducreyi* that were not replicated under the conditions used in our studies.

H. ducreyi is highly resistant to the bactericidal activity of NHS (7). The presence of the $DsrA_I$ protein at the surface of the *H. ducreyi* strain prevents binding of bactericidal IgM at the bacterial cell surface (8). Because classical inhibitors of the complement system such as Vn and C4BP do not appear to be involved in this process (8) and because there is a correlation between the size of DsrA and the amount of IgM bound at the surface of *H. ducreyi* (17), we hypothesized that the mechanism of serum resistance by DsrA involves shielding of target epitopes at the bacterial cell surface. Our results with truncated $DsrA_I$ proteins indicate that serum resistance is not associated with Fn or Vn binding to $DsrA_I$ because $DsrA_I\Delta$25-133, which retained serum resistance, did not interact with Fn or Vn (17). However, $DsrA_I\Delta$25-133 is the only truncated protein to interact with Fg at levels similar to the level of full-length $DsrA_I$ (Fig. 5). It was tempting to speculate that a section of DsrA encompassing the peptide LKVLDARISKNKQ is involved in serum resistance as Fg binding and serum resistance appear to coincide in that one region of DsrA. However, our results indicate that larger truncated proteins, which are serum resistant, do not bind Fg. Furthermore, removal of this peptide resulted in loss of Fg binding but retention of serum resistance although these levels were on the edge between serum resistance and susceptibility (53% survival) (Fig. 6D). We therefore conclude that Fg binding is not involved in *H. ducreyi* serum resistance mediated by DsrA.

The 236-amino-acid DsrA protein is therefore capable of binding several serum and ECM proteins, along with binding keratinocytes. Although this multifunctionality has rarely been reported for an adhesin in a Gram-negative bacteria (33), the M protein of group A *Streptococcus* (GAS) has been ascribed multiple functions, some shared with DsrA, including interaction with many host proteins, like Fg, Fn, IgG, plasminogen, albumin, factor H,

collagen IV, and C4BP, and inhibition of phagocytosis (24). There are several similarities between the *H. ducreyi* DsrA protein and the M protein of GAS. First, both proteins are relatively small, with a highly conserved C-terminal domain and more variable N-terminal domain (34, 35). Like many TAAs, M proteins appear as hair-like fibrils at the surface of the cells although this has not been specifically shown for DsrA (34, 35). Finally, both proteins have a coiled-coil domain that appears to be involved in its multiple functions. M and DsrA proteins also differ. Not all M proteins appear to have the same functions (35), while both classes of DsrA proteins have so far been shown to mediate Fg, Fn, and Vn binding and resistance to the bactericidal activity of NHS. Furthermore, M proteins contain repeat sequences, whereas DsrA does not appear to have distinct repeated domains (although many TAAs do) (35). Finally, both proteins are anchored differently to the bacterial surface as they are expressed in bacteria with (*H. ducreyi*) and without (GAS) an outer membrane.

Using two different methods, we show that the Gram-negative pathogen *H. ducreyi* interacts with Fg at its bacterial cell surface. This interaction was specific to Fg since addition of unlabeled Fg, but not an irrelevant protein, competed for binding of DIG- and FITC-labeled Fg at the bacterial surface. This binding was mediated through the multifunctional TAA DsrA in four different class I *H. ducreyi* strains. Furthermore, both classes of DsrA proteins conferred Fg binding to a heterologous host that cannot bind Fg. A 13-amino-acid sequence from DsrA was involved in the interaction of Fg with *H. ducreyi*. Since Fg binding is critical in the pathogenesis of medically important Gram-positive bacteria, providing definitive proof that intact, viable *H. ducreyi* bacteria interact with Fg at their surface furthers our understanding of the pathogenesis of chancroid. These data suggest that *H. ducreyi* interacts with serum Fg present in chancroidal ulcers. These results may also hint that DsrA is the protein that helps *H. ducreyi* colocalize with fibrin in the base of the ulcer as fibrinogen is the precursor to fibrin. We conclude that DsrA is a multifunctional, surface-exposed TAA involved in binding to several host ligands, including Fg.

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