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A DltA Mutant of *Haemophilus ducreyi* Is Partially Attenuated in Its Ability To Cause Pustules in Human Volunteers

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Haemophilus ducreyi **produces two outer membrane proteins, called DltA (*H. ducreyi* lectin A) and DsrA (*H. ducreyi* serum resistance A), that contribute to the ability of the organism to evade complement-mediated serum killing. In contrast to their isogenic parent strain, 35000HP, the DsrA mutant FX517 exhibits 0% survival in 50% normal human serum and the DltA mutant FX533 exhibits 23% survival. Compared to 35000HP, FX517 does not cause pustule formation in human volunteers. To test whether DltA was required for virulence in humans, seven volunteers were experimentally infected with 35000HP and FX533. Four subjects were inoculated with fixed doses of 35000HP (101 CFU or 130 CFU) at three sites on one arm and escalating doses of FX533 (range, 46 CFU to 915 CFU) at three sites on the other arm. Pustules only developed at mutant-injected sites at doses nearly twofold higher than that of the parent, suggesting that FX533 was partially attenuated. Three subjects were inoculated with similar doses of the parent (67 CFU) and mutant (104 CFU) at three sites. Pustules formed at five of nine parent sites and one of nine mutant sites. Overall, the papule and pustule formation rates for 35000HP and FX533 were similar for the trial. However, for the five subjects who received similar doses of the parent and mutant, pustules developed at 7 of 15 sites (46.7%; 95% confidence interval [CI], 16.9% to 76.5%) inoculated with the parent and at 1 of 15 (6.7%; 95% CI, 0.1% to 18.4%) sites inoculated with the mutant ($P = 0.043$). We concluded that the DltA mutant was attenuated in its ability to cause disease at doses similar to that of the parent.**

Haemophilus ducreyi is a gram-negative, unencapsulated bacterium that causes the genital ulcer disease chancroid, which is characterized by painful genital ulcers and inguinal lymphadenitis. Chancroid is rare in the United States (9) but remains prevalent in developing countries (32). Because chancroid facilitates the acquisition and transmission of human immunodeficiency virus type 1 (13, 19, 24, 32), *H. ducreyi* remains an important pathogen.

To study *H. ducreyi* pathogenesis in humans, we developed an experimental infection model in which strain 35000HP and its derivatives are inoculated into the skin of the upper arms of healthy volunteers (27, 31). The bacteria are delivered by puncture wounds made by the tines of an allergy testing device, simulating abrasions that occur during intercourse, the presumed natural route of infection. In the model, papules form within 24 h of inoculation and either evolve into pustules in 2 to 5 days or resolve spontaneously. Men and women form papules at similar rates, but the odds ratio of men developing pustules is 2.8-fold (95% confidence interval [CI], 1.6- to 5.0-fold) higher than that of women (7; unpublished observations). For an individual inoculated at multiple sites, a pustule may develop at one site while another site resolves (4, 30). Outcomes (pustule formation versus resolution) at multiple sites within a subject tend to be similar, suggesting that there is a host effect on susceptibility to disease progression (28). When subjects are challenged twice, they tend to have the same outcome as their first infection, confirming the existence of a host effect (3, 28).

To test the role of putative virulence determinants in humans, isogenic mutant-parent comparison trials have been performed with the strain 35000HP (HP, human-passaged) background (27). Volunteers are inoculated with multiple doses of 35000HP on one arm and mutant derivatives of 35000HP on the other arm and serve as their own controls for gender and host effects. To date, all 16 mutants tested in the human model are able to initiate papule formation. Mutants have fallen into the following two categories: those that cause pustules at doses similar to that of the parent and those unable to form pustules at doses up to 10-fold higher than that of the parent. Mutants of the *flp* operon, the hemoglobin receptor gene (*hgbA*), the peptidoglycan-associated lipoprotein gene (*pal*), the *H. ducreyi* serum resistance protein gene (*dsrA*), large supernatant protein genes (*lspA1* and *lspA2*), and a collagen binding protein gene (*ncaA*) are each attenuated for pustule formation (2, 8, 12, 17, 29; R. A. Fulcher, L. E. Cole, D. M. Janowicz, K. L. Toffer, K. R. Fortney, B. P. Katz, P. E. Orndorff, S. M. Spinola, and T. H. Kawula, unpublished data).

Recently, Leduc et al. described a 22-kDa outer membrane protein (OMP), DltA (*H. ducreyi* lectin A), that binds to fibronectin and was present in seven different *H. ducreyi* strains tested (20). DltA shares conserved domains with β-ricin chains and an R-type lectin and binds to lactose, GalNAc, and several N-glycosylated glycoproteins (20). DltA also contributes to the ability of the organism to evade complement-mediated serum

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killing. 35000HP is resistant to normal human serum (NHS) at concentrations up to 50% (11, 14). In contrast to 35000HP, the isogenic *dsrA* mutant FX517 exhibits 0% survival in 50% normal human serum, while the isogenic *dltA* mutant FX533 exhibits 23% survival (20). A double *dsrA dltA* mutant, FX534, is more susceptible to 10% NHS than FX517, confirming that both proteins contribute to serum resistance (20). DsrA mediates resistance to serum by abrogating binding of immunoglobulin M (IgM) to the bacteria and preventing the initiation of the classical complement cascade (1). How DltA contributes to serum resistance is under investigation.

In experimental human infection, *H. ducreyi* colocalizes with polymorphonuclear leukocytes, macrophages, collagen, and fibrin but does not colocalize with laminin or fibronectin (5). The bactericidal activity of human serum likely plays an important role in *H. ducreyi* pathogenesis, in that blood and plasma, which contain fibrin, transude into the wounds. The isogenic *dsrA* mutant FX517 does not cause pustules in human volunteers, presumably because it is killed by serum (8). The role of DltA in the pathogenesis of chancroid is currently unknown. Here we tested the ability of FX533 to cause disease in humans.

Comparison of 35000HP and FX533. 35000HP is a human-passaged variant of strain 35000 and was reported previously (4). FX533, which contains a kanamycin resistance cassette inserted into the *dltA* locus, was constructed in the 35000HP background as described previously (20). Both strains were grown on chocolate agar plates supplemented with 1% IsoVitaleX and incubated at 35°C with 5% CO_2 or in broth consisting of proteose peptone, 50 μg of hemin per ml, 1% IsoVitaleX, and 5% heat-inactivated fetal calf serum. Where appropriate, the media were supplemented with kanamycin (30 μg/ml).

To examine whether FX533 had phenotypes other than that caused by the *dltA* mutation, FX533 was compared to the dedicated 35000HP stock used to infect volunteers. The comparison included growth rates in broth and OMP and lipooligosaccharide (LOS) profiles, as described previously (12). 35000HP and FX533 had similar generation times in broth (data not shown). OMP and LOS from 35000HP and FX533 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The OMP and LOS profiles demonstrated no differences between the parent and the mutant (data not shown). By Coomassie blue staining, DltA is a low-abundance lipoprotein, and these results are similar to those reported previously (20).

Human inoculation experiments. Healthy adult male and female volunteers over 18 years of age were recruited for the study. Subjects gave informed consent for participation and for human immunodeficiency virus (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), and clinical observations were done exactly as described previously (17, 29). When a papule was present, the area of erythema was calculated by measuring the greatest dimensions vertically and horizontally in millimeters and then multiplying the two measurements. The areas were measured and recorded by a physician who was blinded to the identity of the inoculum used at each site. 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 >6 mm in diameter, or resolution of infection at all sites. Once a clinical end point was achieved, the code was broken. Up to four sites with active disease (inoculated with the parent or the mutant), if present, were biopsied with punch forceps. The subjects were then treated with two doses of oral ciprofloxacin as described previously (4, 31).

TABLE 1. Response of patients to inoculation with live *H. ducreyi* strains[a]

Volunteer no.	Gender[b]	Days of observation	Isolate	No. of initial papules	Final outcome of sites[c]	
					No. of pustules	No. of resolved sites
273	F	8	35000HP	3	1	2
			FX533	3	0	3
274	M	6	35000HP	3	1	2
			FX533	2	0	2
276	M	6	35000HP	3	2	1
			FX533	3	3	0
277	F	8	35000HP	3	0	3
			FX533	3	1	2
278	F	8	35000HP	3	0	3
			FX533	2	1	1
280	F	7	35000HP	3	2	1
			FX533	3	0	3
281	F	6	35000HP	3	3	0
			FX533	3	0	3

[a] Volunteers 273 and 274 were inoculated in the first iteration; volunteers 276 and 277 were inoculated in the second iteration; and volunteers 278, 280, and 281 were inoculated in the third iteration.
[b] M, male; F, female.
[c] There were no papules as a final outcome for any of the patients.

Nine healthy adults (six females and three males; age range, 23 to 68 years; mean age ± standard deviation, 40 ± 16 years) volunteered for the study. One subject (275) failed to meet the entry criteria, and one subject (279) withdrew prior to inoculation. A total of seven subjects participated in the trial. Two subjects (273 and 274) were inoculated in the first iteration, two subjects (276 and 277) were inoculated in the second iteration, and three subjects (278, 280, and 281) were inoculated in the third iteration (Table 1).

An escalating dose-response study was initially used. In the first iteration, we inoculated two subjects on one arm at three sites with 35000HP, with an EDD of 101 CFU, and on the other arm at three sites with FX533, with EDDs of 46, 92, and 183 CFU. Papules developed at six of six sites injected with the parent strain (parent sites) and five of six sites injected with the mutant (mutant sites) (Table 1). Two parent sites developed pustules, while no mutant sites developed pustules (Table 1). Since the mutant seemed impaired in the ability to cause pustules, we infected an additional two subjects and increased the dose of the mutant. For subjects 276 and 277, the EDDs of the mutant strain were 229, 457, and 915 CFU and the EDD of the parent strain was 130 CFU. All six parent sites and all six

mutant sites developed papules (Table 1). Pustules developed at all three mutant sites in subject 276 and at the mutant site inoculated with the highest dose in subject 277 (Table 1).

The results of the first two iterations suggested that FX533 was partially attenuated in the ability to form pustules. In the first iteration, at doses comparable to that of the parent, the mutant was unable to cause pustules. In the second iteration, the mutant caused pustules at doses nearly twofold higher or more than that of the parent. To test whether FX533 was partially attenuated, we inoculated the third group of subjects (278, 280, and 281) with similar EDDs of both the parent and mutant strains. The EDD of the parent was 67 CFU, and the EDD of the mutant was 104 CFU. Papules formed at nine of nine sites inoculated with the parent and at eight of nine sites inoculated with the mutant (Table 1). Pustules formed at five of nine sites inoculated with the parent and at one of nine sites inoculated with the mutant (Table 1).

Comparisons of papule and pustule formation rates and proportions of positive surface cultures between the two strains were performed by using a logistic regression model with generalized estimating equations to account for the correlation among sites within the same subject, as described previously (29). The generalized estimating equation sandwich estimate for the standard errors was used to calculate 95% confidence intervals for these rates. The cumulative results for the three iterations showed that papules developed at 95.2% (95% CI, 79.3% to 99.9%) of sites inoculated with 35000HP and at 90.5% (95% CI, 79.3% to 99.9%) of sites inoculated with FX533 ($P = 0.55$). The surface area of papule erythema at each site 24 h after inoculation for mutant papules (mean, 17.4 mm^2) was not significantly different from that for parent papules (mean, 24.8 mm^2) ($P = 0.065$). Overall, pustules formed at 9 of 21 (42.9%; 95% CI, 17.4% to 68.3%) sites inoculated with 35000HP and at 5 of 21 (23.8%; 95% CI, 0.1% to 49.3%) sites inoculated with FX533 ($P = 0.31$).

Five subjects (273, 274, 278, 280, and 281) were inoculated with similar parent (75.0 ± 17.2 CFU [mean ± standard deviation]) and mutant (104.6 ± 37.3 CFU) EDDs. For these five subjects, papules formed at 14 of 15 sites (93.3%; 95% CI, 81.7% to 99.9%) inoculated with 35000HP and at 13 of 15 sites (86.7%; 95% CI, 81.7% to 99.9%) inoculated with FX533 ($P = 0.55$). The mean papule surface area for sites inoculated with the parent (25.1 mm^2) was similar to that for sites inoculated with the mutant (15.1 mm^2) ($P = 0.15$). Pustules formed at 7 of 15 sites inoculated with the parent (46.7%; 95% CI, 16.9% to 76.5%), in contrast to 1 of 15 sites inoculated with the mutant (6.7%; 95% CI, 0.1% to 18.4%) ($P = 0.04$). Thus, the mutant was attenuated in the ability to cause disease at doses similar to that of the parent.

Recovery and characterization of bacteria from human lesions. To confirm that the inocula were correct and that the mutant had not lost the kanamycin resistance insertion 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 kanamycin on kanamycin-containing chocolate agar plates. If available, sufficient colonies ($n \geq 30$) from an individual specimen were scored so that there was a 95% probability that ≤11% of the colonies would have the incorrect phenotype (2).

Of the 21 sites inoculated with the parent, 8 (38%) yielded at least one positive surface culture, while 1 of 21 (5%) mutant sites yielded a positive culture ($P = 0.04$). Our protocol only permits biopsy of sites where disease is present at the end point. For subjects 273, 274, 280, and 281, pustules formed only at parent sites. One parent biopsy yielded 3.4×10^5 CFU; five additional biopsies were processed for other purposes and were not cultured. For subject 276, one mutant biopsy yielded 3.4×10^5 CFU and one parent biopsy yielded 1.5×10^2 CFU. For subject 277, one mutant pustule was processed for histopathology, which was identical to that reported previously for parent pustules (22, 30, 31; data not shown). For subject 278, one mutant pustule yielded 0 CFU.

For the three parent and three mutant broth cultures used to prepare the inocula, all 141 parent colonies and all 144 mutant colonies tested were phenotypically correct (the mutant was kanamycin resistant [Kan^r], and the parent was kanamycin sensitive [Kan^s]). All colonies obtained from surface cultures ($n = 245$) and biopsy specimens ($n = 49$) from parent sites were phenotypically correct, as were all colonies obtained from surface cultures ($n = 1$) and biopsy specimens ($n = 47$) from mutant sites.

Conclusions. Serum resistance is an important feature in the pathogenesis of *H. ducreyi* infection (1, 8, 11, 20). Our previous results demonstrated that the serum-susceptible *dsrA* mutant was highly attenuated in human volunteers and prompted our present experiments with the *dltA* mutant, which is also susceptible to serum. Overall, FX533 formed papules and pustules at rates similar to those for 35000HP. However, at doses comparable to that of the parent, FX533 was impaired in the ability to form pustules. Because FX533 led to pustule formation at sites inoculated with doses twofold higher or more than those of the parent but not with comparable doses, we classified FX533 as partially attenuated. This is the seventh demonstration that a putative virulence factor of *H. ducreyi* facilitates pustule formation in humans, and FX533 is the first partially attenuated mutant described for 35000HP.

National and local biosafety committees have precluded our testing of a mutant complemented in *trans* in human subjects because of the possibility of horizontal transfer of a broad-host-range plasmid encoding a virulence determinant to skin flora. When FX533 was complemented with *dltA* in *trans*, its survival in 50% serum was similar to that of 35000HP (20). Thus, the partial attenuation of FX533 observed in the human model is very likely due to the lack of expression of DltA and not to a secondary mutation.

The bactericidal activity of NHS is an important component of innate host defenses against many bacterial pathogens (16, 21, 23). The major determinant of serum resistance in *H. ducreyi* is the outer membrane protein DsrA (11). DsrA is a member of a family of outer membrane proteins that confer resistance to killing and prevent binding of IgM and complement to the bacterial surface (1, 15, 25). The *dsrA* mutant FX517 was unable to form pustules in human volunteers (8). Compared to FX517, FX533 demonstrates partial serum susceptibility (20), consistent with its partial attenuation for pustule formation in human volunteers. The serum resistance of 35000HP is also mediated in part by the major OMP (MOMP) (14), an OmpA homologue, which in other organisms also plays a role in serum resistance. A MOMP mutant was not

attenuated for pustule formation in human volunteers, perhaps because 35000HP expresses a second OmpA homologue, OmpA2 (18, 33).

DltA confers the ability of 35000HP to attach to fibronectin. DltA is affinity purified on lactose and *N*-acetylglucosamine, and N-glycosylated glycoproteins bind to DltA in ligand blots (20). DsrA mediates attachment to keratinocytes (10). However, confocal microscopy on experimental lesions failed to demonstrate 35000HP binding to fibronectin or keratinocytes (6). Throughout the course of experimental infection, 35000HP colocalizes with collagen, fibrin, macrophages, and polymorphonuclear cells in the upper dermis (5, 6, 27). We did not perform localization studies with FX533-infected pustules due to the paucity of samples. Lectins play important roles as adhesion molecules between bacteria and host cells during infection (26). DltA may recognize glycosylated receptors on host cells and play a role in the adhesion of *H. ducreyi* to host tissues.

In summary, these data show that the loss of expression of DltA results in partial attenuation of 35000HP. The mechanism by which DltA confers virulence, whether through serum resistance or attachment to lactose-related carbohydrates on host cell membranes, is unclear. Studies are ongoing to delineate how DltA affects virulence.

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