INFECTION AND IMMUNITY, Dec. 2003, p. 7178–7182 Vol. 71, No. 12
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.12.7178–7182.2003

Haemophilus ducreyi Requires an Intact *flp* Gene Cluster for Virulence in Humans

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Received 11 June 2003/Returned for modification 11 August 2003/Accepted 25 August 2003

An intact *Haemophilus ducreyi flp* operon is essential for microcolony formation in vitro. *tadA* is the 9th of 15 genes in the operon and has homology to NTPases of type IV secretion systems. Fifteen human volunteers were experimentally infected with both *H. ducreyi* 35000HP and the *tadA* mutant, 35000HP.400. Papules developed at similar rates at sites inoculated with the mutant and parent, while pustules formed at 36.4% of parent sites and at 0% of mutant sites ($P = 0.001$). Compared to 35000HP, 35000HP.400 had only a modest but significant reduction in lesion scores in the temperature-dependent rabbit model of chancroid. These data suggest that proteins secreted by the *flp* locus are required for full expression of virulence by *H. ducreyi* in humans but have less of a role in virulence in an animal model of infection.

Haemophilus ducreyi causes chancroid, a genital ulcer disease that facilitates both acquisition and transmission of human immunodeficiency virus type 1 (7, 16). The 12.8-kb *flp* (fimbria-like protein) operon of *H. ducreyi* contains 15 genes and is essential for microcolony formation when this organism is cocultured with human foreskin fibroblasts (HFF) (23). *Actinobacillus actinomycetemcomitans* contains a very similar set of genes that encode proteins involved in the formation of surface appendages (fimbriae) (20). A comparison of the operons was reported previously (23). The major subunits of the *A. actinomycetemcomitans* fimbriae are encoded by *flp-1* and *flp-2*, the first two open reading frames (ORFs) of the operon (20). Although *H. ducreyi flp-1* and *flp-2* express proteins that are homologs of those expressed by *A. actinomycetemcomitans* (23), efforts to determine whether these proteins are assembled into surface appendages on *H. ducreyi* have been unsuccessful to date (S. Kachlany, J. Nika, and E. J. Hansen, unpublished observations). Inactivation of genes in the *flp* operon in *A. actinomycetemcomitans* results in an inability to form pili and to bind tightly to inert surfaces and in loss of virulence in an animal model of periodontal disease (20, 21, 28).

The ability of a large number of isolates of *H. ducreyi* to form microcolonies when cocultured with HFF is associated with virulence in the temperature-dependent rabbit model for experimental chancroid (1). Formation of microcolonies appears to be involved with colonization by other pathogens, including *Vibrio cholerae* and *Haemophilus influenzae* (19, 22), and formation of relatively large microcolonies in coculture with human skin fibroblasts is a feature of virulent strains of *Mycobacterium tuberculosis* (12). Inactivation of either the ninth gene (*tadA*) or the first two genes (*flp1* and *flp2*) of the *H. ducreyi flp* cluster results in mutants that cannot form microcolonies (23). However, of these mutants, only 35000.400, which contains a mutation in *tadA*, is modestly attenuated in the rabbit model, while the *flp1 flp2* double mutant is still virulent (23). These data suggest that microcolony formation by itself is not likely to be a virulence factor for *H. ducreyi* in this animal model (23).

The mutation in 35000.400 is polar and markedly reduces transcription of genes lying downstream of *tadA* in the *flp* operon as measured by real-time PCR (23). The function of the *H. ducreyi* TadA protein has not been defined on a biochemical basis. However, the TadA protein of *A. actinomycetemcomitans* is an ATPase and a member of the subfamily of the putative NTPases of type IV secretion systems (6, 25), and its activity may be necessary to energize the secretion of the Flp pili. Type IV secretion systems play an important role in the expression of certain bacterial virulence factors (11, 13). Since the mutations in the *flp1* and *flp2* genes have no apparent effect on virulence expression by *H. ducreyi* in the rabbit model, it is possible that the *tadA* gene product or genes downstream of *tadA* are involved in some other type IV secretion process that affects virulence.

To study *H. ducreyi* pathogenesis in humans, we developed an experimental infection model in which strain 35000HP (HP, human passaged) and its derivatives are delivered to the epidermis and dermis of the upper arms of volunteers by puncture wounds made by the tines of an allergy testing device (7, 29). Papules form within 24 h of inoculation and evolve into pustules in 2 to 5 days or resolve spontaneously. There are significant effects of dose and gender on pustule formation (4, 9). Men are twice as likely to form pustules as women, consistent with the high male-to-female ratio in natural chancroid (9). In a subject, a pustule may develop at one site while another site resolves (5, 31). Subjects in whom all sites resolve or all sites form pustules exceed the number of subjects expected with different outcomes (30). In reinfection experiments, some volunteers are repeatedly prone to pustule formation while others are prone to resolution (30). Taken together, these data also

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suggest that there is a host effect on susceptibility to disease progression in the model (30).

To test the role of putative virulence determinants in humans, mutant-parent comparison trials have been performed using the model. In these trials, subjects are inoculated with multiple doses of the parent on one arm and an isogenic mutant on their other arm and serve as their own controls for the gender and subject effects. Of 12 mutants tested (29), mutants that lack expression of the hemoglobin receptor (HgbA), peptidoglycan-associated lipoprotein (PAL), or an outer membrane protein (OMP) that is the major known determinant of serum resistance (DsrA) form papules at rates similar to that of the parent but are attenuated in pustule formation (3, 10, 17).

Since expression of *tadA* or of genes downstream of *tadA* is required for full expression of virulence in rabbits, we speculated that *tadA* could be required for virulence in humans. Here we constructed a new *tadA* mutant (35000HP.400) in the 35000HP background. We tested the hypothesis that the *H. ducreyi tadA* mutant is attenuated in the human model and also compared the mutant and the parent in the temperature-dependent rabbit model.

Construction and characterization of 35000HP.400. Construction of *H. ducreyi* 35000HP.400 was identical to that of 35000.400, as described previously (23). Briefly, 35000HP was transformed by electroporation with linearized pJL002, and the transformants were plated on chocolate agar containing chloramphenicol (1 μg/ml). Southern blot analysis confirmed that the 1.4-kb fragment containing a chloramphenicol acetyltransferase (*cat*) cassette had inserted into *tadA* and at no other locus on the chromosome (data not shown). OMPs and lipooligosaccharides prepared from the *tadA* mutant, 35000HP.400, and the parent, 35000HP, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (17). There were no changes in OMP or lipooligosaccharide profiles when 35000HP.400 was compared to 35000HP (data not shown). The growth rates of 35000HP and 35000HP.400 in broth were identical (data not shown).

The abilities of 35000HP and 35000HP.400 to form microcolonies were assessed exactly as described previously (23), using the HFF cell line CRL 7014 (American Type Culture Collection, Manassas, Va.). A77, which does not form microcolonies in this assay, was included as a negative control (1). Both A77 and 35000HP.400 were severely impaired in their ability to form microcolonies when cocultured with HFF cells (data not shown). In contrast, 35000HP readily formed microcolonies under these same conditions in vitro (data not shown).

Human inoculation experiments. Healthy adult male and female volunteers over 18 years old were recruited for the study. Informed consent was obtained from the subjects for participation and for human immunodeficiency virus 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 Indianapolis. Enrollment procedures, exclusion criteria, preparation of the bacteria, confirmation of the identity of the inocula, method of inoculation, determination of estimated delivered dose (EDD), clinical observations, definitions of clinical endpoints, performance of surface and biopsy cultures, and antibiotic treatment were done as described in detail elsewhere (3, 5, 31, 32).

Fifteen healthy adults (11 female, 4 male; 13 white, 2 black; age range, 21 to 48 years; mean age ± standard deviation, 29 ± 8.9 years) volunteered for the study. An escalating dose response study was used to compare the virulence of 35000HP and 35000HP.400 exactly as described previously (3). The rationale for the design is described in detail elsewhere (3, 24, 29). In the first iteration, we inoculated three subjects at six sites on both arms. *H. ducreyi* clumps in vitro, and it is difficult to obtain an exact amount of CFU in an inoculum. Our goal was to inoculate one arm at two sites with an EDD (90 CFU) of the parent that causes a pustule formation rate of 70% and at a third site with the highest dose of the mutant that was heat killed. The other arm was to be inoculated at three sites with EDDs (45, 90, and 180 CFU) of the mutant that surrounded the parent dose. Unfortunately, the EDD in the first iteration was 30 CFU for 35000HP and 8, 15, and 30 CFU for 35000HP.400. Papules developed at five of six sites inoculated with the parent and at nine of nine sites inoculated with the mutant. All papules at parent and mutant sites resolved (Table 1).

Since the doses in the first iteration were lower than what we desired, we enrolled three more subjects and attempted to increase the doses. Three volunteers were inoculated with an EDD of 45 CFU of 35000HP and 22, 44, and 87 CFU of 35000HP.400 in the second iteration. Six of six parent sites and four of nine mutant sites developed papules (Table 1). At clinical endpoint, one parent site and four mutant sites resolved. Pustules developed at five of six parent sites and at none of the mutant sites.

Since the mutant seemed impaired in its ability to cause pustules, we attempted to confirm the observation with another group of subjects using increased doses of the mutant. We challenged three additional volunteers in the third iteration. Each subject was inoculated with an EDD of 97 CFU of 35000HP and 41, 82, and 163 CFU of 35000HP.400. Papules developed at three of six parent sites and three of nine mutant sites. At endpoint, all parent and mutant sites resolved (Table 1). We challenged three more volunteers in a fourth iteration. Each subject was inoculated with an EDD of 38 CFU of 35000HP and 216, 432, and 864 CFU of 35000HP.400. Papules developed at three of six parent sites and three of nine mutant sites. At endpoint all parent and mutant sites resolved. Since all six of these subjects were not susceptible to pustule formation with the parent, we could not confirm that there was a difference in virulence between the mutant and the parent.

In the fifth iteration, we amended our infection protocol and inoculated the subjects with three doses of the live parent and three doses of the live mutant and omitted the heat-killed control. The EDDs were 135 CFU of 35000HP and 291, 581, and 1163 CFU of 35000HP.400. Papules developed at all nine parent sites and nine mutant sites. At endpoint, two parent sites and all mutant sites resolved. Pustules developed at seven of nine parent sites and at zero of nine mutant sites (Table 1). Thus, the mutant did not form pustules, even at doses that were 10-fold greater than that of the parent.

Comparisons of papule and pustule formation rates between the two strains were performed using a logistic regression model with generalized estimating equations (GEE) (38) to

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

Subject	Gender	No. of days of observation	Isolate	No. with initial papule	Final outcome of initial papule	
					No. with pustule	No. resolved
206	F	8	35000HP	2		2
			35000HP.400	3		3
207	F	5	35000HP	1		1
			35000HP.400	3		3
208	M	6	35000HP	2		2
			35000HP.400	3		3
209	F	8	35000HP	2	1	1
			35000HP.400	1		1
210	F	6	35000HP	2	2	
			35000HP.400	0		
211	F	6	35000HP	2	2	
			35000HP.400	3		3
212	F	7	35000HP	2		2
			35000HP.400	1		1
213	F	6	35000HP	1		1
			35000HP.400	0		
216	F	5	35000HP	0		
			35000HP.400	2		2
217	F	5	35000HP	0		
			35000HP.400	1		1
218	F	6	35000HP	1		1
			35000HP.400	2		2
219	F	14	35000HP	2		2
			35000HP.400	3		3
224	M	7	35000HP	3	2	1
			35000HP.400	3		3
225	M	7	35000HP	3	2	1
			35000HP.400	3		3
226	M	7	35000HP	3	3	
			35000HP.400	3		3

[a] Volunteers 206, 207, and 208 were inoculated in the first iteration, 209, 210, and 211 were inoculated in the second iteration, 212, 213, and 216 were inoculated in the third iteration, 217, 218, and 219 were inoculated in the fourth iteration, and 224, 225, and 226 were inoculated in the fifth iteration. Each volunteer was inoculated at two sites with 35000HP and at three sites with 35000HP.400 except in the fifth iteration, where three sites were inoculated with 35000HP. F, female; M, male.

account for the correlation among sites within the same subject. The GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals for these rates except when the rate was zero and the GEE estimate does not exist. For those cases (pustule formation rates for the 35000HP.400 strain) we used a conservative approach and calculated the exact binomial confidence intervals based on the number of subjects rather than the number of sites.

The cumulative results for the five iterations showed that papules developed at 26 (78.8%; 95% confidence interval [CI]; 61.5 to 96.0%) of 33 sites inoculated with live 35000HP (dose range, 30 to 135 CFU), and at 31 (68.9%, 95% CI; 49.1 to 88.7%) of 45 sites inoculated with live 35000HP.400 (dose range, 8 to 1,163 CFU) ($P = 0.38$). Pustules formed at 12 (36.4%, 95% CI; 14.7 to 58.1%) sites inoculated with the parent compared to 0% (95% CI; 0 to 18.1%) of sites inoculated with the mutant ($P = 0.001$). For the six subjects who were susceptible to pustule formation with the parent (iterations two and five), pustules formed at 80% (95% CI; 64.8 to 95.2%) of the 15 parent sites compared to 0% (95% CI; 0 to 39.4%) of the 18 mutant sites ($P < 0.0001$). These data suggest that the mutant was avirulent relative to the parent in its ability to progress to pustule formation.

TABLE 2. Lesion formation by *H. ducreyi* strains in the temperature-dependent rabbit model[a]

Strain	Inoculum size (CFU)	Mean lesion score ± SD on day:		
		2	4	7
35000HP	10^5	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
35000HP.400	10^5	3.9 ± 0.4	4.0 ± 0.0	4.0 ± 0.0
35000HP	10^4	3.1 ± 0.4	3.1 ± 0.6	3.0 ± 0.8
35000HP.400	10^4	3.0 ± 0.0	2.3 ± 0.7	2.1 ± 0.4

[a] Eight rabbits were used in this experiment. The *P* value was 0.0041 for the difference between parent and mutant strain lesion scores over the three scoring days using both inoculum sizes; P values of ≤0.0167 are significant.

Recovery and confirmation of bacteria from human lesions. No bacteria were recovered from sites inoculated with the heat-killed bacteria. *H. ducreyi* was recovered intermittently from surface cultures of sites inoculated with live bacteria. Of the 26 sites with active disease that were inoculated with the parent, 11 (42.3%; 95% CI; 19.9 to 58.3%) yielded at least one positive surface culture, while only 1 of 31 (3.2%, 95% CI; 0.8 to 9.3%) mutant sites with disease yielded a positive culture ($P < 0.0001$). The proportion of positive surface cultures obtained from sites when papules or pustules were present was 20 of 106 (18.9%) for the 26 parent sites and was 1 of 74 (1.4%) for the 31 mutant sites. Biopsies were done on all 12 parent pustules, and *H. ducreyi* was recovered from all of the parent biopsies cultured. Our protocol does not permit biopsy of sites where disease has resolved, and no mutant sites were biopsied.

All colonies obtained from surface cultures ($n = 411$) and biopsies ($n = 232$) of parent sites were phenotypically correct (Cm^s). Three colonies obtained from a surface culture of a mutant site were phenotypically correct (Cm^r). Thus, all colonies tested from surface cultures and biopsies had the expected phenotype.

Evaluation of 35000HP.400 and 35000HP in the temperature-dependent rabbit model. The results of the human challenge experiments were somewhat surprising in that the previous *tadA* mutant, 35000.400, is only modestly attenuated in the temperature-dependent rabbit model (23). The cultures used to infect the subjects in the second iteration of the human challenge experiments were used for virulence testing in the rabbit model, exactly as described previously (26). Inoculum size was determined by colony count and adjusted to the nearest order of magnitude. Skin lesions were scored 2, 4, and 7 days postinfection according to the following scoring system: 0, no change; 1, erythema; 2, induration; 3, nodule; 4, pustule or necrosis. Lesion scores were subjected to statistical analysis exactly as described previously (1, 35). 35000HP.400 was less virulent than 35000HP in this rabbit model (Table 2; $P < 0.0041$), although the difference in lesion scores was small, similar to what was reported previously for 35000.400 (23).

Evaluation of 35000HP.400 for secondary mutations in other known virulence determinants of *H. ducreyi*. In the human challenge model, mutants that lack expression of HgbA, PAL, or DsrA are attenuated in pustule formation (3, 10, 17). We therefore evaluated 35000HP.400 for the presence of secondary mutations in the genes encoding these proteins. In

Western blot analysis, whole-cell lysates of 35000HP and 35000HP.400 contained proteins of the expected size that bound PAL-, DsrA-, and HgbA-specific antibodies (14, 17, 35) (data not shown).

To confirm that the genes encoding these proteins did not contain mutations that could have affected the function of these macromolecules, the ORF of each gene from both 35000HP and 35000HP.400 was sequenced, using the cultures used to inoculate the subjects in the second iteration. The following primers were used in PCR together with chromosomal DNA from 35000HP and 35000HP.400 to amplify three genes for nucleotide sequence analysis: for *dsrA*, 5′-CGTCAT TGACATTTTTTTAATG-3′ and 5′-ATAACAGCAAAAGC TATAACAAAG-3′; for *pal*, 5′-TAACTTGCCAGGAGCAG AG-3′ and 5′-GGCTTATCTAACCGCTTA-3′; for *hgbA*, 5′-TTTTTTATCTTTTGGGGC-3′ and 5′-AAATATCGGGGA TGTTGC-3′. The nucleotide sequences of both strands of the entire ORFs of the *pal*, *dsrA*, and *hgbA* genes were identical between these two strains (data not shown).

Conclusions. In the volunteers, 35000HP.400 formed papules at a rate similar to that of 35000HP but did not progress to the pustular stage of disease. In previous studies, isogenic *hgbA*, *pal*, and *dsrA* mutants were impaired in their ability to form pustules in humans (3, 10, 17). The *tadA* mutant contained no mutations in the genes encoding these proteins and expressed these proteins. National and local biosafety committees have precluded our testing a mutant complemented in *trans* in human subjects because they do not want human skin flora to acquire a plasmid encoding a virulence determinant. We did not test a repaired mutant because a challenge with a repaired mutant would not address the possibilities that the mutant was impaired due to polar effects of the *cat* insertion in *tadA* on the downstream genes of the *flp* operon or that another mutation occurred during the repair. With the caveat that we did not test a repaired or complemented mutant, this is the fourth demonstration that a putative virulence factor of *H. ducreyi* facilitates pustule formation in humans.

Since the mutation in *tadA* has polar effects on its downstream genes (23), we do not know which gene is responsible for decreased virulence in humans. Interestingly, mutations in the *tadD* gene of *Pasteurella multocida* and in the *tadA* gene of *A. actinomycetemcomitans* also reduced the virulence of these pathogens in their respective animal models (18, 28). Therefore, mutations in the *tad* genes affect the virulence of at least three different bacterial species, although the specific virulence mechanism(s) affected by these mutations remain to be identified in most cases.

Nine of the 15 volunteers who participated in this trial were not susceptible to pustule formation with the parent strain. Three of these subjects were inoculated with low doses of the parent strain, and six were inoculated with doses that usually lead to pustule formation (4, 9). All six of the latter were women, who are known to progress to pustule formation less often than men (9). The results of this trial underscore our recent findings that there is a host effect on susceptibility to pustule formation in the model (30). Since each subject is inoculated with the parent and the mutant, we controlled for the host effect, and our statistical analysis accounted for the correlation of outcome among multiple sites inoculated within the same subject. Whether these observations have implications for other human challenge models is unclear.

In previous studies, eight mutants tested with the human model were also evaluated with the temperature-dependent rabbit model (*ftpA*, *lbgB*/*losB*), the swine model (*cdtC hhdB* double mutant; *dsrA*, *sodC*), or both (*cdtC*, *hhdB*, *hgbA*/*hupA*) (29). The results obtained from the animal models are consistent with those in the human model (2, 3, 14, 15, 24, 29, 33–37; I. Leduc, D. W. Cameron, and S. M. Spinola, Prog. Abstr. 12th Meet.Int. Soc. Sex. Transm. Dis. Res., abstr. P386, p. 126, 1997), except that the *sodC* mutant is virulent in humans and attenuated in swine (8, 27). In the present study, the *tadA* mutant was unable to produce pustules in subjects who were susceptible to pustule formation by the parent strain. In contrast, this mutant was only slightly less virulent than its parent in the temperature-dependent rabbit model. We had hypothesized previously that *H. ducreyi* is not as efficient a pathogen for animals as for humans and that a relatively small decrease in virulence may lead to a greater change in the animal models than in the human model (29). Based on the data in the present study, it is apparent that some mutants may be more attenuated in humans than in animals.

In summary, this trial shows that the 35000HP.400 *tadA* mutant is impaired in pustule formation in humans but is only slightly attenuated in an animal model. The mechanism for reduced virulence is unclear. Future studies will center on whether the decrease in virulence of 35000HP.400 is replicated by mutants deficient in production of the Flp proteins in humans.

This work was supported by grants AI31494 and AI27863 (to S.M.S.) and AI32011 (to E.J.H.) from the National Institutes of Allergy and Infectious Diseases. The human challenge trials were also supported by grant MO1RR00750 to the GCRC at Indiana University.

We thank Christopher Elkins for providing rabbit polyclonal sera to recombinant DsrA, Martha Greenwald and Beth Zwickl for monitoring the subjects, Stacy Bennett for manuscript preparation, Margaret Bauer and Tricia Humphreys for their thoughtful criticism of the manuscript, and the volunteers who participated in the trial.

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Editor: J. T. Barbieri