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Transcription of Candidate Virulence Genes of *Haemophilus ducreyi* during Infection of Human Volunteers

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Haemophilus ducreyi **expresses several putative virulence factors in vitro. Isogenic mutant-to-parent comparisons have been performed in a human model of experimental infection to examine whether specific gene products are involved in pathogenesis. Several mutants (*momp, ftpA, losB, lst, cdtC*, and *hhdB*) were as virulent as the parent in the human model, suggesting that their gene products did not play a major role in pustule formation. However, we could not exclude the possibility that the gene of interest was not expressed during the initial stages of infection. Biopsies of pustules obtained from volunteers infected with *H. ducreyi* were subjected to reverse transcription-PCR. Transcripts corresponding to *momp, ftpA, losB, lst, cdtB*, and *hhdA* were expressed in vivo. In addition, transcripts for other putative virulence determinants such as *ompA2, tdhA, lspA1*, and *lspA2* were detected in the biopsies. These results indicate that although several candidate virulence determinants are expressed during experimental infection, they do not have a major role in the initial stages of pathogenesis.**

Haemophilus ducreyi is a gram-negative bacterium that is the etiologic agent of chancroid, a genital ulcer disease that facilitates acquisition of human immunodeficiency virus type 1 (17, 35). During sexual intercourse, breaks in the epithelium may provide a portal of entry for *H. ducreyi*. After an incubation period of 1 to 7 days, a small erythematous papule develops. Pustules form 2 to 3 days later and eventually progress into a soft painful ulcer (25).

A human model of *H. ducreyi* infection was developed by our laboratory to study *H. ducreyi* pathogenesis (4, 5, 27, 32). Volunteers are inoculated with bacteria at multiple sites on the skin of the upper arm via puncture wounds made with an allergy testing device. In the human model, 1 to 100 CFU are sufficient to initiate infection. Papules develop in 24 h, and pustules usually form 2 to 5 days later, consistent with the natural course of disease. The histopathology of pustules resembles that of naturally occurring ulcers. For subject safety, infection is limited to the pustular stage of disease, and subjects are typically infected for 7 to 14 days.

Several putative virulence factors of *H. ducreyi* have been identified including pili, outer membrane proteins, toxins, and lipooligosaccharide (LOS) (8–10, 14, 15, 20, 22, 23, 26, 29, 33, 36). To study their role in pathogenesis, isogenic mutants were constructed and compared to the parent in the human model. To date, we have performed 10 mutant-to-parent comparison trials. Isogenic mutants of *pal, hgbA*, and *dsrA* were attenuated in their ability to form pustules compared to the parent (3, 7, 18). Seven mutants, including those with disruptions in *hhdB, cdtC, hhdB* and *cdtC, momp, ftpA, lst*, and *losB*, caused pustule formation rates that were similar to those caused by the parent (2, 28, 34, 38, 39). These results were surprising in that some of these candidate virulence factors (*hhdB, cdtC, momp, losB*) have roles in adherence, cell death, or serum resistance in vitro (10, 11, 19–21, 37). Although the results of human challenge trials suggested that these gene products were not required for pustule formation, we could not exclude the possibility that the gene of interest was simply not transcribed in the initial stages of infection. If a gene was transcribed and its isogenic mutant was still virulent, then it would be very unlikely that the gene product played a major role in virulence.

Previous studies indicated that sera from patients with chancroid contained elevated levels of antibodies to HgbA, TdhA, D15, Hlp, hemolysin, cytolethal distending toxin (CDT), and LOS of *H. ducreyi* when compared to control sera, suggesting that these factors were expressed during natural infection (1, 12, 16, 22, 24). Confocal microscopy performed on lesions from experimentally infected subjects indicated that *ftpA, pal, hlp, momp*, and/or *ompA2* was expressed in vivo (6). However, detection of gene expression by serology and confocal microscopy is limited by the availability of appropriate antigens or antibodies.

In this study, we examined whether transcripts of candidate virulence genes of *H. ducreyi* were present in lesions of experimentally infected human subjects. We also estimated the number of CFU in an entire lesion and the number of CFU that allowed detection of bacterial transcripts in human tissue.

MATERIALS AND METHODS

Bacteria and culture conditions. *H. ducreyi* 35000HP is a human-passaged variant of 35000 described previously (5, 31). *H. ducreyi* 35000HP was grown on chocolate agar plates supplemented with 1% IsoVitaleX and incubated at 33°C with 5% CO_2 or in brain heart infusion broth containing 50 μg of hemin per ml, 1% IsoVitaleX, and 5% heat-inactivated fetal calf serum and incubated with aeration at 33°C.

Human subjects and biopsies. Tissue was collected from eight adults (two female and six male; mean age ± standard deviation [SD], 33.6 ± 10.4 years) who participated in several mutant-to-parent comparison trials (7, 18, 39) or who were specifically infected for this 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 at Indianapolis. Enrollment procedures and exclu-

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TABLE 1. Primers

Primer	Gene	Sequence (5′ to 3′)	Amplicon size (bp)	Limit of detection (CFU)[a]
18set5′	*pal*	TTGAGAATTCAGTAGTTCATCAGGTAAAACAGATG	400	10^1
18set3′		TAAAGAATTCAAATTAGTACTCTAATACTGCACGG		
28set5′	*hlp*	TTGAGAATTCGATAAGCCTGCAAACAACCCTGTAG	578	10^3
28set3′		CCAAGAATTCGGTTTATTTGTTTTCGTCAGCTTC		
Pilin1	*ftpA*	GACCACACAGTGCCAGGT	220	10^1–10^2
PilinR2		GCGTTGCCATACGTTCAGC		
Momp-for-2	*momp*	CTGCACCAGATGCCAATACC	700	10^3
Momp-rev-2		TGATAACCCTACAGAAACAGAATG		
OmpA2-for-5	*ompA2*	TGGATTAGCGCGTAACGATTATAGTG	315	10^1–10^2
OmpA2-rev-5		GCTCGTCCATCATCATTTGCG		
TdhA-for-2	*tdhA*	TGATAAAGGCAACCACTACCCAC	550	10^2
TdhA-rev-2		CTGCGCGACAGTATTAATCCAAGG		
HgbA-for-2	*hgbA*	GCTCAGGTTATGCTATTCGTGGTGT	549	10^1–10^2
HgbA-rev-2		CATAGCCTAGGGTAAAGCGGTTGGT		
Lst-for-1	*lst*	GGTTACGTGGTTTAGATGTTTATGGTGC	423	10^1–10^2
Lst-rev-1		CGATATATATCAATCTTTTCTGCTTCTGTC		
LosB-for-1	*losB*	CGTAATAGGGATTTATTGCTTTTGAGAG	513	10^2–10^3
LosB-rev-1		CAGCAATATGCACAATAGAAGTATCAGGTG		
Cdt3	*cdtB*	AGGCGGTTCTGATGCGGTAAG	359	10^2
Cdt4		AGCGATCACGAACAAAACTAACAG		
HhdA-for-1	*hhdA*	GGCGGTCAAAATATAATGGAAAGAG	565	10^2–10^3
HhdA-rev-1		TCGTGCCCGGCTTGGATACTAC		
P10	*lspA1*	TTGAATTCAAGTTTCAGCAGGGACAGCAAATATC	264	10^1–10^2
P11		TTTCTCGAGTCTAATTTTTCGGCGACAAGG		
P12	*lspA2*	AAGTTTCAGCAAGAGCGGC	320	10^2–10^3
P13		TATTGGCTGCAAGCTCTG		
β-actin 5′	β-*actin*	ATCTGGCACCACACCTTCTACAATGAGCTCCG	837	ND
β-actin 3′		CGTCATACTCCTGCTTGCTGATCCACATCTGC		

[a] Value represents the range of limits of detection for three experiments. ND, not determined.

sion criteria are described elsewhere in detail (5, 32). Six volunteers were inoculated on the upper arm with estimated delivered doses (EDD) of 60 to 100 CFU of *H. ducreyi* 35000, 35000HP, or 35000.304, an isogenic *cdtC* and *hhdB* double mutant. Subjects were observed until they achieved clinical endpoint, defined as resolution of disease at all sites, 14 days of infection, or the development of a painful pustule. At clinical endpoint, a total of eight pustules were biopsied with a punch forceps.

Biopsies of uninfected skin were also obtained from two volunteers. Each biopsy of uninfected skin was divided longitudinally. Either 10^6 or 10^7 CFU of 35000HP grown to mid-logarithmic phase was added to one portion of each uninfected biopsy to serve as a positive control, while the remainder of the specimen served as a negative control.

After biopsy, all specimens were immediately placed in either 1 ml of freezing media (3% [wt/vol] tryptic soy broth–10% glycerol–10% heat-inactivated fetal calf serum) or proteose peptone saline (77 mM NaCl–1% [wt/vol] proteose peptone) and weighed. Tissue was homogenized for 2 min on ice and a portion (220 μl) of the homogenate was quantitatively cultured on chocolate agar with and without vancomycin (3 μg/ml) as described previously (31, 32).

RNA isolation and reverse transcription (RT). The remaining homogenate was recovered and usually 600 to 700 μl was centrifuged at 16,000 × *g* for 5 min at 4°C. Pellets were suspended in 1 ml of Ultraspec RNA (Biotecx Laboratories, Inc., Houston, Tex.), and RNA was isolated according to the manufacturer's instructions. RNA was suspended in diethyl pyrocarbonate-treated H_2O and incubated with 15 U of DNase I (GenHunter Corp, Nashville, Tenn.) at 37°C for 45 min. DNase I was heat inactivated by incubating the reaction mixture at 75°C for 6 min. For in vitro experiments, RNA was isolated as described above from approximately 10^8 CFU of 35000HP grown to mid-logarithmic phase.

The Advantage RT-for-PCR kit (Clontech Laboratories, Palo Alto, Calif.) was used to make cDNA according to the manufacturer's instructions from either approximately 600 ng of RNA from biopsies or RNA that represented approximately 10^7 CFU of 35000HP. All cDNA reaction mixtures were paired with an equal amount of RNA that was not subjected to reverse transcriptase to control for DNA contamination.

PCR. Amplification of most target cDNAs was performed with $MgCl_2$ (5 mM) and the appropriate primers (30 pM) in 100-μl reaction mixtures using the PCR Core Kit Plus (Roche Molecular Biochemicals, Indianapolis, Ind.) as per the manufacturer's instructions. *ompA2* cDNA was amplified in the presence of $MgCl_2$ (2.5 mM). The synthetic primers used to amplify target cDNA were based on published sequences (9, 22, 27, 30, 36) (Table 1). Robert Munson, Jr. (Ohio State University, Columbus), kindly provided the primers Cdt3 and Cdt4 (Table 1). Reaction mixtures were denatured at 95°C for 5 min, then cycled 45 times through 95°C for 1 min, 60°C for 25 s, and 72°C for 2 min, with a final extension at 72°C for 7 min and then held at 4°C using a thermal cycler (Perkin-Elmer PCR System 2400). The annealing temperature for most primers was 60°C except for *ompA2* and *lspA1* primers, which annealed at 63°C, and *ftpA* and β-actin primers, which annealed at 50 and 55°C, respectively. Genomic DNA from 35000HP and H_2O were used as templates for positive and negative controls, respectively. Ten microliters of cDNA or RNA was used as template for PCRs. Amplicons were analyzed by electrophoresis of 10 μl of each PCR on 1.2% agarose gels stained with ethidium bromide.

Nucleotide sequence analysis. Amplicons from PCRs using 35000HP as template and each primer set were sequenced on an ABI Prism 310 (Applied Biosystems) automated sequencing system employing dye terminators. Sequencing data were compared to published sequences for each gene of interest.

Limit of detection. To determine the limit of detection for each *H. ducreyi* primer set, three samples of RNA prepared from 35000HP grown to mid-logarithmic phase were used to make cDNA that was subsequently serially diluted and used as template in PCR. Amplicons were analyzed as described above. The number of CFU that gave rise to each RT-PCR sample loaded on the gel was calculated. The limit of detection was defined as the lowest number of CFU whose cDNA yielded a visible RT-PCR product on an ethidium bromide-stained gel.

Analysis of amplicons prepared from biopsies. All RT-PCR products obtained from biopsies were subjected to agarose gel electrophoresis and Southern blot analysis. DNA was transferred to a nylon membrane by a standard capillary method. Probes were prepared from amplicons that were obtained using 35000HP genomic DNA as template and the appropriate primer set. Probe labeling, hybridization, and detection were performed as described previously (34). If a band of the expected size was detected by agarose gel and Southern blotting or Southern blotting alone, the gene of interest was considered to have been transcribed in vivo. For β-*actin*, RT-PCR products were only analyzed by agarose gel electrophoresis and stained with ethidium bromide. The number of CFU corresponding to the RT-PCR sample loaded on the gel was calculated

TABLE 2. Bacterial recovery

Sample[a]	EDD (CFU)	Duration of infection (days)	Total CFU present in biopsy	Estimated minimal doubling time (h)
142a	60	7	1.9×10^5	15
142b	60	7	1.7×10^4	21
165	93	6	6.0×10^5	12
164	93	7	7.0×10^4	18

[a] Volunteer number. Samples a and b were collected from different sites on the same volunteer.

based on the quantitative culture for each biopsy and the portion of each biopsy that was converted to cDNA.

RESULTS AND DISCUSSION

Specimens. Eight pustules were biopsied from six volunteers who were infected for 7.0 ± 1.1 days (mean ± SD). Two additional biopsies were obtained from two uninfected volunteers. Biopsies were homogenized, and a portion was cultured on selective and nonselective media. While *H. ducreyi* was the only bacterium recovered from pustules, no bacteria were recovered from uninfected skin.

Of the eight biopsies, four were obtained in a way that allowed us to estimate the number of CFU in an entire lesion. In other words, the biopsy encompassed the entire pustule, and the entire specimen was homogenized and quantitatively cultured. We recovered on average 2.3×10^5 CFU per biopsy (range, 1.7×10^4 to 6.0×10^5 CFU). Due to the fact that *H. ducreyi* aggregates, the CFU are minimal estimates of the number of bacteria present in a sample. Since the EDD and duration of infection were known, the doubling time for *H. ducreyi* in vivo was calculated to be 16.5 ± 3.8 h (Table 2). Since the rate of bacterial cell death in vivo is unknown, the doubling time is a minimal estimate.

RT-PCR of *H. ducreyi* mRNA in vitro. To verify that the target sequences were amplified, PCR products obtained from 35000HP genomic DNA and each of the *H. ducreyi* primer sets were sequenced. The sequence of each amplicon matched that of the expected product (data not shown).

To determine the sensitivity of each *H. ducreyi* primer pair, we amplified 10-fold serial dilutions of cDNA prepared from broth-grown 35000HP. Experiments were repeated with three preparations of RNA. Under the conditions studied, the limits of detection for different primer pairs varied from 10^1 to 10^3 CFU (Table 1). The results indicated that our methods had sufficient sensitivity to amplify *H. ducreyi* cDNA from patient samples.

Detection of *H. ducreyi* mRNA in control samples. To detect potential inhibitors or false-positive signals, we performed RT-PCR on homogenates prepared from two biopsies of uninfected skin with and without *H. ducreyi*. No products were detected in samples from uninfected skin, but the expected PCR products were amplified from uninfected skin homogenized with *H. ducreyi*. To verify that cDNA was present in the PCR, β-*actin* was amplified from uninfected skin with and without *H. ducreyi* (data not shown). Although uninfected skin did not contain the same cells or genes expressed as inflamed tissue, the data suggested that the RT-PCR products were *H. ducreyi* specific and not eukaryotic in origin.

In preliminary experiments utilizing three biopsies, specimens were homogenized in either proteose peptone saline or freezing medium. When biopsies were homogenized in proteose peptone saline, no signal was recovered. However, homogenizing biopsies in freezing medium allowed for the detection of *H. ducreyi* mRNA. Preliminary experiments indicated that RNA was not stable with storage at −70°C, consistent with the instability of prokaryotic mRNA. However, cDNA stored at −70°C was stable for at least 4 months. Only biopsies that were homogenized in freezing medium and whose cDNA was made on the same day as the biopsy were included in the final analysis.

Detection of transcripts of candidate virulence genes in vivo. Five biopsies obtained from sites inoculated with the parent strain were analyzed for the presence of *H. ducreyi* cDNAs. Since the products of *pal, hlp*, and *ftpA* were detected by confocal microscopy in vivo (6), these genes served as positive controls for the specimens. *pal, hlp*, and *ftpA* cDNAs were detected in all the specimens. Thus, the biopsies were also analyzed for the presence of other cDNAs.

Isogenic mutants of *ftpA, momp, hhdB, cdtC, hhdB* and *cdtC, lst*, and *losB* caused pustule formation rates that were similar to the parent in the human model of *H. ducreyi* infection (2, 34, 38, 39). The results suggested that the gene products were not required for pustule formation. Except for *ftpA*, we did not know whether these genes were transcribed in the initial stages of infection. The hemolysin and CDT are encoded by *hhdBA* and *cdtABC* gene clusters, respectively (10, 26). Thus, transcription of *hhdA* and *cdtB* were likely to represent transcription of each gene cluster. RT-PCR and Southern blotting were used to analyze five biopsies for the presence of *momp, hhdA, cdtB, lst*, and *losB* cDNAs (Fig. 1). *lst, losB*, and *cdtB* cDNAs were present in five of five biopsies, and *momp* and *hhdA* cDNAs were detected in four of five biopsies. Since the genes encoding *ftpA, momp, lst, losB, cdtB*, and *hhdA* were transcribed in vivo, these data validate the results of the human challenge trials, which indicated that these gene products made minimal contributions to virulence. The data exclude the possibility that the genes were simply not transcribed during the initial stages of infection. However, expression of these genes may be important at the ulcerative stage.

H. ducreyi expresses two outer membrane proteins designated HgbA and TdhA, which bind hemoglobin and heme, respectively (13, 33). An isogenic *hgbA* mutant was attenuated in the human model of *H. ducreyi* infection (3). This suggested that *hgbA* was expressed during infection and loss of *hgbA* impaired the ability of the organism to survive in vivo. Thus, *tdhA* may not be expressed in vivo, or *tdhA* expression may not compensate for the loss of *hgbA*. *hgbA* cDNA was found in four of five biopsies, and *tdhA* cDNA was amplified from five of five biopsies. Although the isogenic *hgbA* mutant grew in the pres-

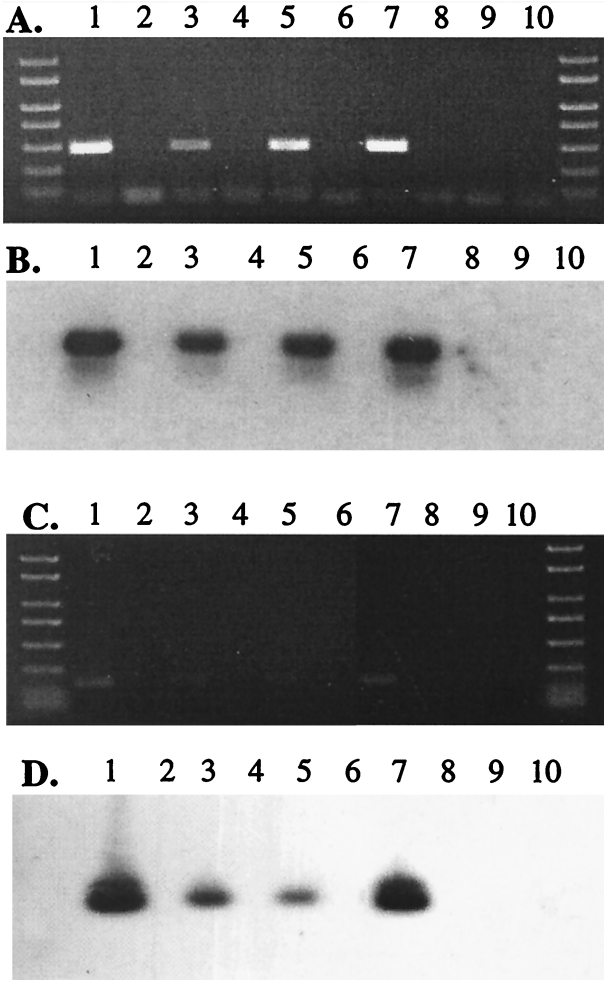

FIG. 1 Composite agarose gels stained with ethidium bromide (A and C) and Southern blot (B and D) of PCR and RT-PCR products. PCR was performed with either *losB* primers (panels A and B) or *ftpA* primers (panels C and D). Lanes 1 and 2 contain 35000HP genomic DNA and no template, respectively. cDNA (lanes 3, 5, 7, and 9) and RNA that was not reverse transcribed (lanes 4, 6, 8, and 10) were prepared from biopsy 165 (lanes 3 and 4), biopsy 164 (lanes 5 and 6), uninfected skin homogenized with *H. ducreyi* (lanes 7 and 8), and uninfected skin (lanes 9 and 10).

ence of heme in vitro (14), the data suggest that *tdhA* may not compensate for the loss of *hgbA* during infection.

ompA2, lspA1, and *lspA2* are candidate virulence genes that may play a role in adherence or serum resistance (21, 23, 36). A monoclonal antibody that specifically recognizes the gene product of *ompA2* is not available (23), and the gene products of *lspA1* and *lspA2* are secreted in vitro (36). Thus, confocal microscopy could not be used to determine if these genes were expressed in vivo. Transcripts for *ompA2, lspA1*, and *lspA2* were detected in all five biopsies examined. Similarly, Ward et al. detected *lspA1* and *lspA2* cDNAs in aspirates of lesions obtained from rabbits infected with *H. ducreyi* (36). Since *ompA2, lspA1*, and *lspA2* are transcribed during experimental infection, evaluation of their respective isogenic mutants in the human model will be relevant.

The actual samples amplified by RT-PCR and analyzed by agarose gel electrophoresis and Southern blotting were estimated to contain 122 ± 159 CFU (mean ± SD; range, 7 to 400 CFU). Despite an abundance of eukaryotic RNA, RT-PCR products were detected in samples estimated to represent as few as 7 CFU of *H. ducreyi*. Our ability to detect *H. ducreyi* transcripts expressed in vivo was variable in samples with <100 CFU but was more consistent when a sample contained >100 CFU (data not shown). The RNA prepared from in vitro-grown organisms lacked eukaryotic RNA, and the experiments done on the biopsies and in vitro grown bacteria were not performed simultaneously. Thus, we could not compare in vitro and in vivo levels of expression.

In summary, we amplified specific *H. ducreyi* transcripts in human lesions and estimated a minimal doubling time for *H. ducreyi* in vivo. Although all the candidate virulence genes examined in this study were transcribed in vivo, we cannot conclusively state that the mRNAs were translated. Nevertheless, these results strengthen the findings of several human challenge trials that suggested that several of these putative virulence determinants, while expressed in vivo, did not play a major role in pustule formation. Since we detected *H. ducreyi* transcripts from very few organisms, our methods should be applicable to other human skin infections such as those caused by *Treponema pallidum*, group A streptococci, *Staphylococcus aureus, Mycobacterium leprae*, and *Leishmania*. Future studies will be directed towards examining whether specific *H. ducreyi* genes are differentially expressed during human infection.

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