The LspB Protein Is Involved in the Secretion of the LspA1 and LspA2 Proteins by *Haemophilus ducreyi*

Christine K. Ward,† Jason R. Mock, and Eric J. Hansen*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9048

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The LspA1 and LspA2 proteins of *Haemophilus ducreyi* 35000 are two very large macromolecules that can be detected in concentrated culture supernatant fluid. Both of these proteins exhibit homology with the N-terminal region of the *Bordetella pertussis* filamentous hemagglutinin (FHA), which is involved in secretion of the latter macromolecule. The *lspA2* open reading frame is flanked upstream by a gene, *lspB*, that encodes a predicted protein with homology to the *B. pertussis* FhaC outer membrane protein that is involved in secretion of FHA across the outer membrane. The *H. ducreyi* *lspB* gene encodes a protein with a predicted molecular mass of 66,573 Da. Reverse transcription-PCR analysis suggested that the *lspB* gene was transcribed together with the *lspA2* gene on a single mRNA transcript. Polyclonal *H. ducreyi* LspB antiserum reacted with a 64-kDa antigen present in the Sarkosyl-insoluble cell envelope fraction of *H. ducreyi* 35000, which indicated that the LspB protein is likely an outer membrane protein. Concentrated culture supernatant fluids from *H. ducreyi* *lspB* and *lspA1* *lspB* mutants did not contain detectable LspA1 and detectable LspA2, respectively. However, complementation of the *lspB* mutant with the wild-type *lspB* gene on a plasmid restored LspB protein expression and resulted in release of detectable amounts of the LspA1 protein into culture supernatant fluid. When evaluated in the temperature-dependent rabbit model of infection, the *lspB* mutant was attenuated in the ability to cause lesions and was never recovered in a viable form from lesions. These results indicated that the *H. ducreyi* LspB protein is involved in secretion of the LspA1 and LspA2 proteins across the outer membrane.

*Haemophilus ducreyi* is an unencapsulated, gram-negative bacillus and is the etiologic agent of the sexually transmitted genital ulcer disease known as chancroid (2, 62). Chancroid, although rarely seen in the United States, is common in some developing countries (9). Relatively little is known about the bacterial gene products essential for virulence expression by *H. ducreyi* (52). However, several possible virulence factors of *H. ducreyi* have been identified to date. These include a number of protein genes localized to the outer membrane, including both pathogenicity island (PAI) genes and other gene products localized to the outer membrane, including both pathogenicity island (PAI) genes and other gene products localized to the outer membrane. Moreover, an *H. ducreyi* gene on a single mRNA transcript encodes a protein with a predicted molecular mass of 66,573 Da. Reverse transcription-PCR analysis suggested that the *lspB* gene was transcribed together with the *lspA2* gene on a single mRNA transcript. Polyclonal *H. ducreyi* LspB antiserum reacted with a 64-kDa antigen present in the Sarkosyl-insoluble cell envelope fraction of *H. ducreyi* 35000, which indicated that the LspB protein is likely an outer membrane protein. Concentrated culture supernatant fluids from *H. ducreyi* *lspB* and *lspA1* *lspB* mutants did not contain detectable LspA1 and detectable LspA2, respectively. However, complementation of the *lspB* mutant with the wild-type *lspB* gene on a plasmid restored LspB protein expression and resulted in release of detectable amounts of the LspA1 protein into culture supernatant fluid. When evaluated in the temperature-dependent rabbit model of infection, the *lspB* mutant was attenuated in the ability to cause lesions and was never recovered in a viable form from lesions. These results indicated that the *H. ducreyi* LspB protein is involved in secretion of the LspA1 and LspA2 proteins across the outer membrane.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The *H. ducreyi* strains and plasmids used in this study are listed in Table 1. Wild-type *H. ducreyi* strains were routinely cultivated on chocolate agar (CA) plates at 35°C in a humidified atmosphere containing 95% air and 5% CO₂ as described previously (47). Mutant or plasmid-containing *H. ducreyi* strains were grown either on CA plates containing chlor-

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* Corresponding author. Mailing address: Department of Microbiology, Hamon Biomedical Research Building, Room NA6.200, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9048. Phone: (214) 648-5974. Fax: (214) 648-5985. E-mail: eric.hansen@utsouthwestern.edu.
† Present address: Johnson & Johnson Pharmaceutical Research & Development, LLC, San Diego, CA 92121.
amphenicol (0.5 μg/ml) or on GC-heme agar plates (55) containing both chloramphenicol (0.5 μg/ml) and kanamycin (30 μg/ml) as necessary. For some experiments, *H. ducreyi* strains were grown at 33 to 34°C in a gyratory water bath at 90 rpm in a modified version of a Columbia broth-based medium (sCB) previously described for growing *Haemophilus somnus* (28, 67). sCB consisted of 35 g of Columbia broth (Difco Laboratories, Detroit, Mich.) per liter, 0.1% (wt/vol) Trizma base (Sigma Chemical Co., St. Louis, Mo.), equine hemin (25 mg of the puriﬁed chloramphenicol resistance cartridge ligated into the HpaI site; Chl' Amp'). plasmid pCW158 was grown in 2YT medium (49). plasmid pCW159 was grown in 2YT medium (49). plasmid pCW158 and the 0.7-kb Smal fragment of pCWnpCAT1 (containing a nonpolymerase chain reaction (PCR)-based ampliﬁcation of the 226-bp region spanning the *lspB-lspA2* intergenic region. A previously described primer pair that yielded a 354-bp palt-specific product (67) was included as a positive ampliﬁcation control.

**Construction of *H. ducreyi* mutants.** (i) Construction of an *lspB* mutant. A 2.88-kb portion of the *H. ducreyi* *lspB* gene (nucleotides 797 to 3679 in the sequence deposited in the GenBank database under accession number AF289079) was ampliﬁed by PCR using primers 5'-TTGGATACCGTCGTTAAGGGTTTTG G-3' and 5'-TCAAGCATGAGTTAATAGTCTGCT-3' yielding a 431-bp product that spanned the *lspB-lspA2* intergenic region, and from the *H. ducreyi* pal gene (53). The two primers described above for ampliﬁcation of the 226-bp region from *lspB* encoding amino acids 27 to 100 of the *LspB* protein yielded a 226-bp palt-speciﬁc product. Primers 5'-TGGAATACCGTTAGAAGGTATTG G-3' and 5'-TCAAGCATGAGTTAATAGTCTGCT-3' yielded a 431-bp product that spanned the *lspB-lspA2* intergenic region. A previously described primer pair that yielded a 354-bp palt-specific product (67) was included as a positive ampliﬁcation control.

(ii) Construction of an *lspB* mutant. A 2.88-kb portion of the *H. ducreyi* *lspB* gene (nucleotides 797 to 3679 in the sequence deposited in the GenBank database under accession number AF289079) was ampliﬁed by PCR using primers 5'-TTGGATACCGTCGTTAAGGGTTTTG G-3' and 5'-TCAAGCATGAGTTAATAGTCTGCT-3' yielding a 431-bp product that spanned the *lspB-lspA2* intergenic region. A previously described primer pair that yielded a 354-bp palt-specific product (67) was included as a positive ampliﬁcation control.
A. Construction of *H. ducreyi* *lspB* mutants:

![Diagram of construction of mutants and recombinant plasmid pCW225 used in this study.](image)

- **pCW158** (8.3 kb)
- **pCW159** (9.0 kb)

Digest with *HpaI* and ligate to 0.7 kb *SmaI* fragment from pCWnpCAT1 (*cat* cartridge)

**H. ducreyi 35000.88** (*lspB* mutant; chlor^R^)

**H. ducreyi 35000.188** (*lspA1 lspB* mutant; kan^R^ chlor^R^)

B. Construction of the *lspB* complementation plasmid pCW225:

![Diagram of construction of the *lspB* complementation plasmid pCW225](image)

**IspB PCR Product** (2.9 kb)

- Digest with *SalI* and *BamHI* and ligate to pACYC184 digested with *SalI* and *BamHI*

**pCW173** (6.6 kb)

- Digest with *PvuII* and *NruI* and ligate 4.3 kb *PvuII-NruI* fragment to 1.2 kb *MamI-EcoRV* fragment from pLS88 (*kan* gene)

**pCW225** (5.5 kb)

![Diagram of construction of the *lspB* complementation plasmid pCW225](image)

**FIG. 1.** Construction of the mutants and recombinant plasmid pCW225 used in this study. (A) Construction of the *H. ducreyi* *lspB* and *lspA1 lspB* mutants; (B) construction of recombinant plasmid pCW225. Restriction sites in parentheses are not present in pCW225 and reflect cloning junctions.
pACYC184 (14) with forward primer 5′-TTTCCCGGGTGTCACTAGAGG AAGCTAATGGAGAAGAAAAATCTTG-3′ and reverse primer 5′-TTTCCG GGGTTCCTAATCCGAGAATGACCCCG CCCTGCC-3′ (Small sites underlined) and ligation the product into pBluecript KS+ (+). These primers were essentially the same as those used by Lukomski and colleagues to generate the nonpolar promoterless cat cassette contained in pSFL1 (38). Plasmid pCW159 was linearized by digestion with PstI and used to electropropagate H. ducreyi 35000 as previously described (27). H. ducreyi transformants were selected on CA containing chloramphenicol. One mutant, 35000.88, was randomly selected and used for further analysis.

(b) Construction of an lspa1 lspb mutant. Plasmid pCW159 (Fig. 1A) was linearized by digestion with PstI and was used to electropropagate the H. ducreyi lspa1 mutant 35000.1 (66). H. ducreyi transformants were selected on CA containing chloramphenicol. One mutant, 35000.188, was randomly selected and used for further analysis.

Complementation of the H. ducreyi lspa1 lspb mutant. A 2.88-kb DNA fragment containing the H. ducreyi lspa1 gene was amplified by PCR with Pfu DNA polymerase (Stratagene) and primers 5′-TTTCTGCGATTTAAGACTGCACCCC C-3′ (PstI site underlined) and 5′-TTGATCCGTAATTTTTGTGTTAAAAACG GATTG-3′ (BamHI site underlined). After digestion with SalI (the SalI site is present in the amplified fragment) and BamHI, the resultant 2.65-kb DNA fragment was ligated into pCVC184, and the ligation reaction mixture was used to electropropagate H. ducreyi strain A777 to obtain pCW173 (Fig. 1B). Plasmid pCW173 was digested with PvuII and NruI to excise the chloramphenicol resistance gene, and the 4.3-kb fragment from the digest was ligated to the 1.2-kb Maml-EcoRV fragment (containing the kanamycin resistance gene) of pLS88 to produce plasmid pCW225. Plasmid pCW225 was transformed into the lspa1 lspb mutant 35000.88, and the resulting transformants were selected on GC-heme agar containing kanamycin and chloramphenicol.

Isolation and fractionation of cell envelopes. The Sarkosyl-insoluble cell envelope fraction was isolated as previously described (36) from H. ducreyi strains grown overnight in sCB.

SDS-PAGE and Western blot analysis. To detect LspA1 and LspA2, samples containing H. ducreyi CCS were heated at 100°C for 5 min in sample buffer (45), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 7.5% (wt/vol) polyacrylamide separating gel, and transferred to nitrocellulose as described previously (67). The membranes were blocked with phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 and 3% (wt/vol) skim milk and incubated with monoclonal antibodies (MAbs) in the form of hybridoma supernatants. The MAbs used in this study have been described previously (67). MAb 40A4 is LspA1 specific, MAb 1BH9 is LspA2 specific, and MAb 11B7 recognizes both LspA1 and LspA2. MAbs bound to proteins on nitrocellulose membranes were detected by using 125I-labeled goat anti-mouse immunoglobulin G (G2) followed by autoradiography.

To detect LspB, Sarkosyl-insoluble H. ducreyi cell envelope fractions (20 μg of protein/lane) or H. ducreyi whole-cell lysates (37) were heated in sample buffer (45) containing 5% (vol/vol) 2-mercaptoethanol, resolved by SDS-PAGE with a 10% (wt/vol) polyacrylamide separating gel, and transferred to nitrocellulose. For Western blot analysis, the membranes were blocked and processed as described above except that a 1:2,000 dilution of the polyclonal mouse LspB antiserum was used as the source of primary antibody.

Serum bactericidal assay. The serum bactericidal assay was performed as previously described (65), except that normal human serum was used as the source of complement.

Virulence testing. The temperature-dependent rabbit model for experimental chancroid (47) was used to evaluate the virulence of the H. ducreyi strains described in this study. Lesions were scored on days 2, 4, and 7 postinfection by using the following scoring system: 0, no change; 1, erythema; 2, induration; 3, nodule; 4, pustule or necrosis. A statistical analysis of lesion scores was performed as described previously (5, 56). On day 7 postinfection, the animals were euthanized, and the lesions which had been initially inoculated with 106 CFU were excised from each rabbit, bisected with a sterile scalpel blade, and rinsed with PBS to recover putstular material. PBS washes were spread onto CO to recover viable H. ducreyi.

Nucleotide sequence accession number. The nucleotide sequence of the H. ducreyi 35000 lspb locus has been deposited in the GenBank database under accession number AF289079.

RESULTS

Determination of the nucleotide sequence of the H. ducreyi lspb gene. It was previously determined that the lspa2 gene of H. ducreyi 35000 was flanked upstream by an ORF (designated lspb) that encoded a protein with homology to the protein encoded by the B. pertussis flaC gene (67, 68). Because only the 3′ portion of the H. ducreyi lspb gene had been sequenced previously (67), we determined the complete nucleotide sequence of the H. ducreyi 35000 lspb gene, as well as that of the upstream flanking DNA. Approximately 2 kb of DNA upstream of the available sequence of the partial H. ducreyi lspb gene was amplified by PCR by using a previously described method (40) and was sequenced. The resultant nucleotide sequence was confirmed by subsequent sequence analysis of a PCR product produced independently by amplifying this region from the H. ducreyi 35000 chromosome with different oligonucleotide primers and the high-fidelity thermostable DNA polymerase Pfu (data not shown).

Nucleotide sequence analysis revealed that the complete lspb ORF contained 1,770 bases (nucleotides 1900 to 3669 in Fig. 2) and encoded a protein with a predicted molecular mass of 66,573 Da. Immediately upstream of the lspb ORF were three small putative ORFs which encoded predicted polypeptides with homology to different regions of the glycerophosphodiester phosphodiesterase GlpQ (2). The predicted LspB protein contained a putative 26-amino-acid signal peptide sequence and had a carboxy-terminal phenylalanine residue; the latter fact was consistent with the possibility that this protein might be associated with the outer membrane of H. ducreyi (58). PHI-BLAST analysis revealed significant similarity of the predicted H. ducreyi LspB protein to the H. somnus Lpb protein (GenBank accession no. BAC76848; Expect = e-148), as well as to the FhaC proteins of Bordetella bronchiseptica (GenBank accession no. AAF21946; Expect = e-64) and B. pertussis (GenBank accession no. NP_880575; Expect = e-62). An alignment of the predicted H. ducreyi LspB protein with the Lpb protein of H. somnus and the FhaC protein of B. pertussis is shown in Fig. 3.

Detection of lspb sequences in H. ducreyi strains. To determine the prevalence of the lspb gene in H. ducreyi strains, we performed a Southern blot analysis with genomic DNA from 16 H. ducreyi strains (67) isolated in diverse geographic regions (Fig. 4A). These 16 strains included 4 strains (A77, 6V, E1673, and 78226) (Fig. 4A, lanes 13 to 16) whose CCS were previously shown to not contain detectable LspA1 and LspA2 (67) and which were found to be essentially avirulent in an animal model of experimental chancroid (5). An lspb-specific probe (see Fig. 2 for location) hybridized to a single band in all strains examined, indicating that the lspb gene was conserved among these strains and was present as a single copy. The lspb probe was observed to hybridize to restriction fragments that were approximately five different sizes in the H. ducreyi strains examined: 4.8 kb (Fig. 4A, lanes 1 and 11), 4.6 kb (Fig. 4A, lane 16), 4.0 kb (Fig. 4A, lane 12), 3.9 kb (Fig. 4A, lanes 3, 5 to 7, 9, 13, and 15), and 3.6 kb (Fig. 4A, lanes 2, 4, 8, 10, and 14). This indicated that there was some genetic diversity among these strains in this region of the chromosome.

The H. ducreyi 35000 lspb and lspa2 genes are present on a single transcript. Because the translation initiation codon of the lspa2 ORF was separated from the translational stop codon of the lspb ORF by only 46 nucleotides, we hypothesized that the lspb and lspa2 ORFs could be cotranscribed, yielding a single mRNA. Attempts to determine the size of the
transcript(s) containing the lspB and lspA2 sequences by Northern blot analysis were not successful (data not shown), probably because of the extremely large size (>17,000 nucleotides) of the predicted transcript. Therefore, we sought to localize the lspB and lspA2 sequences to the same RNA transcript by RT-PCR analysis of RNA isolated from broth-grown H. ducreyi. Using primers that spanned the lspB-lspA2 junction, we amplified a 431-bp product from both strain 35000 (Fig. 5, lane 8) and the lspB mutant (Fig. 5, lane 7) that is known to express the LspA2 protein at readily detectable levels (66). A 226-bp lspB-specific product was also obtained from H. ducreyi 35000 (Fig. 5, lane 8) and the lspA1 mutant (Fig. 5, lane 9). No PCR products were obtained from RNA samples not subjected to RT prior to PCR amplification (Fig. 5, lanes 10 to 13), indicating that the PCR products shown in lanes 6 to 9 of Fig. 5 were not a result of DNA contamination of the RNA samples. Primers to reverse transcribe and amplify a 354-bp region of the H. ducreyi pal gene transcript (53) were included to eliminate potential polar effects on expression of the downstream lspB transcript and the 5' region of the lspA2 transcript were present on the same mRNA molecule.

Detection of LspB protein expression by H. ducreyi strains. To detect and localize the expression of the LspB protein by various H. ducreyi strains, we produced a polyclonal LspB antiserum by immunizing mice with a six-His–LspB fusion protein (described in Materials and Methods). This antiserum was used in a Western blot analysis to probe whole-cell lysates prepared from the same strains that were analyzed by Southern blotting as described above. This polyclonal LspB antiserum reacted with an antigen having an apparent molecular weight of approximately 64,000 from 11 H. ducreyi strains (Fig. 4B, lanes 1 to 11), including strain 35000. The immunoreactive antigen from strain CIP 542 (Fig. 4B, lane 12) was slightly smaller and had an apparent molecular weight of approximately 58,000. The functional significance of the size difference, if any, is not apparent, and CIP 542 has previously been shown to release LspA1, but not LspA2, into CCS (67). This polyclonal LspB antiserum also reacted with an antigen of the same apparent size that was present in the Sarkosyl-insoluble cell envelope fraction of H. ducreyi 35000 (Fig. 6, lane 1), a result which indicated that the LspB protein is likely present in the outer membrane of H. ducreyi.

The same antiserum did not react with the four avirulent strains (A77, 6V, E1673, and 78226) (Fig. 4B, lanes 13 to 16) whose CCS lacked detectable LspA1 or LspA2 (5, 59). Interestingly, when we PCR amplified and sequenced the lspB gene from strain A77, we found that the lspB ORF in this strain contained a 7-nucleotide (5’-CTTATAT-3’) insertion 894 nucleotides downstream from the ATG start codon. This insertion altered the reading frame, resulting in a premature translational stop codon in this gene (data not shown). This mutation is likely responsible for the lack of LspB protein expression in strain A77 (Fig. 4B, lane 13).

Construction of isogenic H. ducreyi lspB and lspA1 lspB mutants. To investigate the role of the H. ducreyi LspB protein in the secretion of the LspA1 and LspA2 proteins, we constructed two isogenic mutants as described in Materials and Methods (Fig. 1A). An lspB mutant (35000.88) was constructed to investigate the role of the LspB protein in the secretion of the LspA1 protein. Similarly, an lspA1 lspB mutant (35000.188) was constructed to investigate the role of the LspB protein in the secretion of the LspA2 protein (because an lspA1 mutant expresses readily detectable levels of LspA2 in CCS [66]). A nonpolar promoterless chloramphenicol resistance cassette (38) was used to construct the lspB mutations in order to eliminate potential polar effects on expression of the downstream lspA2 gene. Mutants were initially identified from pools of antibiotic-resistant transformants by PCR analysis and were subsequently confirmed by Southern blot analysis to contain the desired lspB mutations (data not shown).
Characterization of membrane proteins and CCS from wild-type and mutant *H. ducreyi* strains. Western blot analysis of whole-cell lysates (data not shown) and Sarkosyl-insoluble cell envelope fractions of the 35000.88 (lspB) and 35000.188 (lspA1-lspB) mutants with the polyclonal LspB antiserum confirmed that these two mutants did not produce the LspB protein (Fig. 3, lanes 2 and 3, respectively). Western blot analysis was also performed with CCS prepared from wild-type and *lspB* mutant strains; these CCS were probed with LspA1- and LspA2-specific MAbs to evaluate the effect of this mutation on the secretion of the LspA1 and LspA2 proteins by *H. ducreyi* (Fig. 7).

**FIG. 3.** Alignment of the amino acid sequences of *H. ducreyi* LspB (Hd LspB), *H. somnus* IbpB (Hs IbpB), and *B. pertussis* FhaC (Bp FhaC). Identical amino acids are indicated by asterisks, and conserved amino acids are indicated by periods.

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**FIG. 4.** A summary of the membrane proteins and CCS from wild-type and mutant *H. ducreyi* strains. Western blot analysis of whole-cell lysates (data not shown) and Sarkosyl-insoluble cell envelope fractions of the 35000.88 (lspB) and 35000.188 (lspA1-lspB) mutants with the polyclonal LspB antiserum confirmed that these two mutants did not produce the LspB protein (Fig. 3, lanes 2 and 3, respectively). Western blot analysis was also performed with CCS prepared from wild-type and *lspB* mutant strains; these CCS were probed with LspA1- and LspA2-specific MAbs to evaluate the effect of this mutation on the secretion of the LspA1 and LspA2 proteins by *H. ducreyi* (Fig. 7). CCS from the *lspB* mutant 35000.88 (Fig. 7A, lane 2) did not present any bands for LspA1 or LspA2. The wild-type strain and the *lspB* mutant 35000.188 (Fig. 7A, lane 3) did present bands for LspA1 and LspA2.
ferred to nitrocellulose, and probed with an EcoRV, electrophoresed through a 0.8% (wt/vol) agarose gel, trans-

analysis, chromosomal DNA from each strain was digested with

mixtures: lane 1, no template (negative control); lanes 2 and 4, 100 ng 

transcripts. The following templates were included in the reaction 

contain detectable LspA1. Similarly, CCS from the lspA1 lspB mutant 35000.188 (Fig. 7B, lane 3) did not contain detectable 

LspA2 protein, whereas the CCS from the 

lspA1 mutant 35000.1 contained readily detectable levels of LspA2 (Fig. 7B, lane 6). RT-PCR analysis of total RNA from the 

lspA1 lspB mutant revealed the presence of a transcript derived from 

lspA2 gene did not eliminate transcription of the downstream lspA2 gene. In ad-

dition, Western blot analysis with MAb 11B7 showed that whole-cell lysates of both the lspB and lspA1 lspB mutants contained immunoreactive LspA proteins (data not shown), a result which suggested that the LspA protein(s) accumulated inside these mutants. Taken together, these results indicated that the H. ducreyi LspB protein is probably involved in the 

secretion of both the LspA1 and LspA2 proteins.

The growth of the lspB mutant 35000.88 and the lspA1 lspB mutant 35000.188 in sCB was no different from the growth of 

the wild-type strain 35000 (data not shown), indicating that the 

lspB mutation did not affect the ability of these strains to grow 

in vitro. We also evaluated the bactericidal activity of normal 

human serum against the lspB mutant and the lspA1 lspB mu-

tant and found that these two mutants were as serum resistant 

as wild-type strain 35000 (data not shown). Western blot anal-

ysis confirmed that both of these mutants expressed the DsrA 

protein (data not shown) that is responsible for the expression 

of serum resistance by H. ducreyi (20).

Complementation of the H. ducreyi lspB mutant. Comple-

mentation of the lspB mutation in strain 35000.88 with the H.

FIG. 4. Southern and Western blot analyses of H. ducreyi strains to 
detect lspB genes and LspB protein expression. (A) For Southern blot 
analysis, chromosomal DNA from each strain was digested with EcoRV, electrophoresed through a 0.8% (wt/vol) agarose gel, trans-

FIG. 5. Multiplex RT-PCR analysis of H. ducreyi lspB-containing 
transcripts. The following templates were included in the reaction 
mixtures: lane 1, no template (negative control); lanes 2 and 4, 100 ng of H. ducreyi wild-type strain 35000 genomic DNA (positive control); lanes 3 and 5, 100 ng of H. ducreyi lspA1 mutant genomic DNA (positive control); lanes 6, 8, 10, and 12, 1 µg of H. ducreyi wild-type strain 35000 total RNA; lanes 7, 9, 11, and 13, 1 µg of H. ducreyi lspA1 mutant total RNA. The primer sets included in the reaction mixtures were as follows: lanes 1 to 3, 6, 7, 10, and 11 contained both pal-specific (354-bp product) and lspB-lspA2 (431-bp product) primers; lanes 4, 5, 8, 9, 12, and 13 contained both pal-specific (354-bp product) and lspB-specific (226-bp product) primers. The reaction mixtures loaded in lanes 10 to 13 were not subjected to the RT step of the RT-PCR procedure and served as controls to detect DNA contamination of RNA preparations. Lane M contained DNA size markers.

FIG. 6. Western blot analysis of the Sarkosyl-insoluble cell enve-

lope fraction from wild-type, mutant, and complemented H. ducreyi 

strains with a polyclonal LspB antiserum as described in Materials and Meth-

ods. The positions of molecular size markers are indicated on the 

left.

FIG. 7. Western blot analysis of CCS from wild-type, mutant, and 
complemented H. ducreyi strains with LspA1-specific MAb 40A4 (A) and LspA2-specific MAb 1H9 (B). Lane 1, wild-type strain 35000; lane 2, lspB mutant 35000.88; lane 3, lspA1 lspB mutant 35000.188; lane 4, 35000.88(pCW177) (vector-only control); lane 5, 35000.88(pCW225). The positions of molecular mass markers are indicated on the left.
**TABLE 2. Lesion formation by wild-type and mutant *H. ducreyi* strains in the temperature-dependent rabbit model**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>Inoculum size (CFU)</th>
<th>Lesion score (mean ± SD) on day:</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>35000</td>
<td>10&lt;sup&gt;⁵&lt;/sup&gt;</td>
<td>4.00 ± 0.00</td>
<td>4.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>35000.88 (<em>lspB</em> mutant)</td>
<td>10&lt;sup&gt;⁵&lt;/sup&gt;</td>
<td>3.00 ± 0.00</td>
<td>2.75 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>35000</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.63 ± 0.53</td>
<td>3.63 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>35000.88 (<em>lspB</em> mutant)</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.00 ± 0.00</td>
<td>2.25 ± 0.49</td>
</tr>
<tr>
<td>2</td>
<td>35000</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.00 ± 0.00</td>
<td>3.88 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>35000.88(pCW177)</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.00 ± 0.00</td>
<td>2.88 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>35000.88(pCW225)</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.13 ± 0.35</td>
<td>3.13 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>35000</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.13 ± 0.35</td>
<td>3.13 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>35000.88(pCW177)</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>2.88 ± 0.35</td>
<td>2.00 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>35000.88(pCW225)</td>
<td>10&lt;sup&gt;⁴&lt;/sup&gt;</td>
<td>3.00 ± 0.00</td>
<td>2.25 ± 0.46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Seven rabbits were used in experiment 1, and eight rabbits were used in experiment 2.

<sup>b</sup> *P* value calculated for the difference between wild-type and test strain lesion scores for the three scoring days by using both inoculum-sizes.

<sup>c</sup> Significant difference.

<sup>d</sup> The *P* value of 0.0088 refers to the difference between the 35000.88(pCW177) and 35000.88(pCW225) strains.

ducreyi *lspB* gene provided in trans on plasmid pCW225 (Fig. 1B) was performed to confirm that the *lspB* gene was responsible for the phenotypic effects described above. Strain 35000.88(pCW225) (Fig. 6, lane 5), but not the vector-only control 35000.88(pCW177) (Fig. 6, lane 4), expressed LspB protein that was detectable in the Sarkosyl-insoluble cell envelope fraction, and it also was able to secrete the LspA1 protein (Fig. 7A, lanes 5 and 4, respectively). These results confirmed that an undetected secondary mutation was not responsible for the lack of detectable LspA1 in CCS from the *lspB* mutant strain 35000.88. It should also be noted that the presence of large number of repeated proteins members in the secretion domain, there is relatively limited overall identity at the primary amino acid sequence-structure. The genes for the secretion system is characterized by a channel-forming β-barrel outer membrane protein (TpsB) and a cognate large exoprotein (TpsA) synthesized as a preprotein that undergoes extensive proteolytic processing during secretion. The large exoprotein is probably transported across the cytoplasmic membrane in a Sec-dependent manner and then guided by the conserved N-proximal secretion domain comprised of approximately 110 amino acids across the periplasm in an extended conformation (with the C-terminal region of the TpsA preprotein acting as an intramolecular chaperone) directly to the transporter such that translocation across both membranes is coupled (33).

**DISCUSSION**

The predicted protein product of the *H. ducreyi* *lspB* gene exhibits homology (42% similarity) to *B. pertussis* FhaC, which is the sole outer membrane accessory protein involved in the secretion of FHA (encoded by *fhaB*) (29, 31, 68). *B. pertussis* FhaB and FhaC are the prototypic components of the recently described two-partner secretion system involved in the export of large (100- to 500-kDa) virulence factors (33). This specialized secretion system is characterized by a channel-forming β-barrel outer membrane protein (TpsB) and a cognate large exoprotein (TpsA) synthesized as a preprotein that undergoes extensive proteolytic processing during secretion. The large exoprotein is probably transported across the cytoplasmic membrane in a Sec-dependent manner and then guided by the conserved N-proximal secretion domain comprised of approximately 110 amino acids across the periplasm in an extended conformation (with the C-terminal region of the TpsA preprotein acting as an intramolecular chaperone) directly to the transporter such that translocation across both membranes is coupled (33).

Despite the high levels of homology among the TpsA exoprotein members in the secretion domain, there is relatively limited overall identity at the primary amino acid sequence level. The presence of a large number of repeated β-strands that fold into amphipathic β-helices is common among proposed TpsA members, including *H. ducreyi* LspA1 and LspA2 (34), suggesting that a specialized secretion system is required for large proteins rich in β-structure. The genes for the secreted TpsA exoprotein and its cognate TpsB outer membrane transporter are typically present in the same operon (33, 34). The TpsB exporter proteins are typically approximately 60-kDa proteins, contain several transmembrane β-strands, including an amphipathic C-terminal 10-amino-acid region, and are predicted to form an integral transmembrane β-barrel...
channel in the outer membrane through which they translocate the cognate exoprotein (31). Each TpsB protein appears to be specific for secreting only its cognate TpsA exoprotein (30), although the FhaC exporter proteins of B. pertussis and B. bronchiseptica appear to be functionally interchangeable for the secretion of B. pertussis FHA (32), probably because of the high level of primary amino acid sequence identity between these two proteins.

PHI-BLAST analysis revealed that orthologs of the H. ducreyi LspB protein are encoded in a large number of bacterial genomes, including many genomes that have been recently sequenced but have yet to have their TpsAB systems functionally characterized (data not shown). Members of the TpsAB family that have been characterized previously include systems that produce and export the Ca\(^{2+}\)-independent cytolysins of Serratia marcescens, Proteus mirabilis, and H. ducreyi, the HsxA heme-hemopexin-binding protein of Haemophilus influenzae, and several adhesions, including the HMW1 and HMW2 proteins of H. influenzae and the FHA proteins of B. pertussis and B. bronchiseptica (reviewed in references 33 and 34). Genes that encode members of the TpsAB family have also been identified in the genomes of H. somnus (GenBank accession no. BAC78648), Pasteurella multocida (39), E. coli (46), Pseudomonas aeruginosa (57), Neisseria meningitidis (60), Fusobacterium nucleatum (35), Xanthomonas campestris (17), Yersinia pestis (44), Photobacterium luminescens (18), and Ralstonia solanacearum (48), but they have yet to be functionally characterized. In addition, several of these genomes, including those of H. ducreyi, B. pertussis, P. multocida, P. luminescens, and R. solanacearum, encode two or more TpsA/B systems.

The H. ducreyi lspB gene is located directly upstream of the lspA2 ORF and encodes a protein which, consistent with the characteristics of TpsB transporters (33, 34), is present in the Sarkosyl-insoluble cell envelope fraction of H. ducreyi and has a predicted molecular mass of 66,573, an extensive \(\beta\)-sheet conformation, and a carboxy-terminal phenylalanine residue typical of outer membrane proteins. The majority of the identity between the H. ducreyi LspB protein and the well-studied B. pertussis FhaC transporter was found in the \(\beta\)-strand regions and in loops 1 and 8 (L1 and L8) (24). Southern blot analysis with an lspB-specific DNA probe resulted in identification of a single hybridizing band in the 16 H. ducreyi strains included in this study, indicating that a single lspB gene was conserved among strains of this pathogen. Among the bacterial proteins with homology to LspB, the IbpB outer membrane transporter protein of H. somnus (GenBank accession no. BAC78648) exhibited the highest degree of similarity (61%). Interestingly, the H. ducreyi hhaB gene product, a putative TpsB exporter involved in the secretion of the HhA hemolysin (cytolysin) (43), exhibited only 30% similarity to the LspB protein.

It is interesting that H. ducreyi strain A77, which has an lspB gene but which does not secrete the LspA1 or LspA2 protein (67), contains a 7-nucleotide insertion in the lspB ORF that results in a premature translational stop codon. Strain A77 has been reported previously to be serum sensitive (20, 41), to be deficient in adherence to human foreskin fibroblasts (3, 5), to be deficient in microcolony formation (5), to lack a galactose residue in the N-acetyllactosamine portion of its lipooligosaccharide (59), and to be avirulent in the temperature-dependent rabbit model (5). In light of the reports of other phenotypic changes accumulated by A77, the discovery of the lspB mutation in this strain raises the possibility that A77 has a hypermutator phenotype that has rendered this strain avirulent.

Northern blot analysis of H. ducreyi total RNA with lspB- and lspA2-specific probes was not successful in determining the size of the transcript(s) derived from these two genes (data not shown), likely because of the extremely large size predicted for this transcript (>17,000 nucleotides) if the genes were cotranscribed. The lack of a discrete hybridizing band on a Northern blot probed with lspB suggested that the lspB gene was not transcribed as part of a monocistronic operon but likely was cotranscribed with lspA2. Therefore, we confirmed by RT-PCR analysis that the H. ducreyi lspB gene was, in fact, cotranscribed with the lspA2 gene. This finding was notable considering our observation that LspA2 is very difficult (66) and sometimes impossible (67) to detect in Ccs from wild-type strain 35000, whereas LspB can be readily detected by Western blot analysis (Fig. 6). Why LspA2 is present at barely detectable levels in Ccs from wild-type strain 35000 is not apparent, but it could be due to some type of posttranscriptional regulation or posttranslational processing.

We constructed two independent mutants to investigate the role of the LspB protein in the secretion of the LspA1 and LspA2 proteins. An lspB mutant was constructed to address the role of LspB in the secretion of the LspA1 and LspA2 proteins. Similarly, an lspA1 lspB double mutant was constructed to address the role of LspB in the secretion of the LspA2 protein because LspA2 can be readily detected in Ccs from an lspA1 mutant (66). Ccs from the lspB mutant and the lspA1 lspB mutant did not contain detectable LspA1 and LspA2, respectively, indicating that the single LspB protein encoded by the H. ducreyi genome is involved in the secretion of both of these proteins across the outer membrane. Furthermore, the lspB mutant was significantly less virulent in the temperature-dependent rabbit model than the wild-type parental strain 35000, suggesting that the ability to secrete the LspA1 and LspA2 proteins was required for the full expression of virulence by H. ducreyi in this animal model. These results confirm that these two proteins are involved in virulence expression by this pathogen, which was first demonstrated by the finding that an lspA1 lspA2 double mutant of H. ducreyi is substantially attenuated in the temperature-dependent rabbit model (66).

Complementation of the lspB mutation in strain 35000.88 with the wild-type lspB gene on a plasmid restored the ability of this mutant to secrete LspA1 in vitro (Fig. 7). We were unable to perform a similar complementation analysis of lspA1 lspB mutant 35000.188 because this mutant and the plasmid containing the wild-type lspB gene both possessed a kanamycin resistance gene, a condition that precluded stable maintenance of the plasmid. Nonetheless, RT-PCR analysis confirmed that the lspA2 gene was transcribed in the lspA1 lspB mutant, indicating that the presence of the nonpolar promotorless cat cartridge in the lspB gene did not prevent transcription of the downstream lspA2 gene.

Complementation with the wild-type lspB gene in the lspB mutant 35000.88 could only partially restore the defect in virulence expression in the animal model (Table 2). However, the complemented mutant [35000.88(pCW225)] did yield higher lesion scores than the vector-only control strain 35000.88(pCW177), and the difference was significant. The
failure of complementation with the wild-type lspB gene to fully restoring virulence to this mutant may involve the relative level of expression of the LspB protein by this strain. It appeared that LspB was overexpressed by this complemented mutant (Fig. 6, lane 5), and the increased abundance of LspB may have had a detrimental effect on the structure or function of the outer membrane in this strain. Moreover, we were never able to successfully clone the full-length lspB gene on the higher-copy-number plasmid pLS88 in H. ducreyi (data not shown), a result which suggested that the LspB protein was toxic when it was overexpressed from this multicopy plasmid in H. ducreyi. Therefore, it seemed possible that the 35000.88 (pCW225) strain was under stress and would not exhibit complete restoration of virulence in vivo since it did not truly represent the wild-type state under stress and would not exhibit complete restoration of virulence in vivo since it did not truly represent the wild-type state.

Collectively, the data obtained in the present study indicate that H. ducreyi contains a single lspB gene whose protein product is involved in secretion of both the LspA1 and LspA2 proteins across the outer membrane. It will be interesting to determine the exact mechanism of secretion of H. ducreyi LspA1 and LspA2, to characterize the manner in which these large proteins interact with the LspB transporter in the outer membrane, and to compare this mechanism with that described for the secretion of FHA across the outer membrane of B. pertussis.

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