Identification of Tandem Genes Involved in Lipooligosaccharide Expression by *Haemophilus ducreyi*

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A transposon insertion mutant of *Haemophilus ducreyi* 35000 possessing a truncated lipooligosaccharide (LOS) failed to bind the LOS-specific monoclonal antibody 3E6 (M. K. Stevens, L. D. Cope, J. D. Radolf, and E. J. Hansen, Infect. Immun. 63:2976–2982, 1995). This transposon was found to have inserted into the first of two tandem genes and also caused a deletion of chromosomal DNA upstream of this gene. These two genes, designated *lbgA* and *lbgB*, encoded predicted proteins with molecular masses of 25,788 and 40,236 Da which showed homology with proteins which function in lipopolysaccharide biosynthesis in other gram-negative bacteria. The tandem arrangement of the *lbgA* and *lbgB* genes was found to be conserved among *H. ducreyi* strains. Isogenic LOS mutants, constructed by the insertion of a *cat* cartridge into either the *lbgA* or the *lbgB* gene, expressed an LOS phenotype indistinguishable from that of the original transposon-derived LOS mutant. The wild-type LOS phenotype could be restored by complementation with the appropriate wild-type allele. These two LOS mutants proved to be as virulent as the wild-type parent strain in an animal model. A double mutant with a deletion of the *lbgA* and *lbgB* genes yielded equivocal results when its virulence was tested in an animal model.

Haemophilus ducreyi is the etiologic agent of chancroid, a sexually transmitted genital ulcer disease. Chancroid is endemic at relatively high levels in certain areas of Africa, Asia, and Latin America, whereas the incidence of this disease in the United States has been relatively low (1, 69). However, the number of cases of chancroid in this country has increased in the past decade (55, 59). The prevalence of this disease in both developing and developed countries is a source of concern, because chancroid has been shown to be a significant risk factor for heterosexual transmission of human immunodeficiency virus infection (8, 21, 28). This finding emphasizes the necessity for a better understanding of the pathogenic mechanisms of this bacterium.

Chancroid is a histologically well-characterized disease (18, 19, 63). The molecular basis for ulcer formation caused by *H. ducreyi* remains to be determined, although several bacterial products that could be involved in this process or in the ability of *H. ducreyi* to grow in vivo have been identified, including a hemoglobin-binding outer membrane protein (15, 67), a soluble cytotoxin (32, 39, 51), a hemolysin that acts as a contact-dependent cytotoxin (2, 43–45, 68), pili (6, 64), and lipooligosaccharide (LOS) (10, 40–42).

Analyses of *H. ducreyi* LOS indicate that it is very similar to the LOS of other gram-negative mucosal pathogens, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* (20, 34–37, 61, 62). Structural studies of *H. ducreyi* LOS have identified lactosamine and sialyllactosamine as the terminal saccharides of the LOS chain (36, 37, 61, 62). Interestingly, these structures resemble the epitopes formed by human paraglobosides, which

has led to speculation that this oligosaccharide region of LOS may function in molecular mimicry (34, 35).

While *H. ducreyi* mutants which contain altered LOS molecules (9, 66) have been constructed, the specific genes involved in LOS biosynthesis remain to be identified. Previously, we reported the isolation of an *H. ducreyi* LOS mutant which was derived from the use of a transposon-based mutagenesis system (66). We now report the identification and characterization of two genes which are essential for the expression of the wild-type oligosaccharide portion of *H. ducreyi* LOS.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. All *H. ducreyi* strains were routinely cultivated on chocolate agar (CA) plates containing 1% (vol/vol) IsoVital X (BBL Microbiological Systems, Cockeysville, Md.) as described previously (50). All *H. ducreyi* strains were grown in a humidified atmosphere of 95% air–5% CO₂ at 33°C. Antimicrobials used with *H. ducreyi* included chloramphenicol and kanamycin at final concentrations of 2 and 30 µg/ml, respectively. *Escherichia coli* XL-1 Blue, XL0LR, HB101, and DH5α were grown in Luria-Bertani (LB) medium (53). For recombinant *E. coli* strains, ampicillin, chloramphenicol, and kanamycin were used at final concentrations of 100, 30, and 50 µg/ml, respectively.

Recombinant DNA techniques. Standard recombinant DNA techniques, such as restriction enzyme digests, ligation reactions, and plasmid purifications, were performed as previously described (5, 53). Southern blot analysis of chromosomal DNA preparations from *H. ducreyi* strains was performed as described previously (67). All DNA probes were radiolabeled with [³²P]dCTP with a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). PCR was performed with a GeneAmp kit (Perkin-Elmer, Branchburg, N.J.); all reactions were carried out according to the manufacturer's instructions. To amplify products from total genomic DNA, 1 µg of *H. ducreyi* chromosomal DNA and 100 ng of each primer were used in each 100-µl reaction mixture. The final concentration of each deoxynucleoside triphosphate was 0.18 mM, and the final concentration of MgCl₂ was 2.5 mM.

MAB and colony blot radioimmunoassay. Monoclonal antibody (MAB) 3E6 is specific for a cell surface-exposed *H. ducreyi* LOS epitope that is conserved among strains of this pathogen (26). The colony blot radioimmunoassay was performed as previously described (22) with MAB 3E6 culture supernatant as the source of the antibody.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference(s) or source
<i>E. coli</i> strains		
DH5 α	Host strain used for cloning	53
HB101	Host strain used for cloning	53
XL-1 Blue	Host strain used to screen the ZAP Express bacteriophage library	Stratagene
XLOR	Host strain used for excising the recombinant pBK-CMV plasmids from the ZAP Express bacteriophage	Stratagene
<i>H. ducreyi</i> strains		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada; binds MAb 3E6	23
35000.54	35000 with a random Tn1545- Δ 3 transposon insertion; binds MAb 3E6	This study
35000.6	LOS mutant derived from strain 35000 by mutagenesis with Tn1545- Δ 3; does not bind MAb 3E6	66
35000.4	LOS mutant derived from strain 35000 by insertion of a <i>cat</i> cartridge into the <i>AvrII</i> site of the <i>lbgA</i> gene; does not bind MAb 3E6	This study
35000.4(pLBG-A)	Strain derived from electroporation of pLBG-A into strain 35000.4 to complement the inactivated <i>lbgA</i> gene; binds MAb 3E6	This study
35000.7	LOS mutant derived from strain 35000 by insertion of a <i>cat</i> gene into the <i>HpaI</i> site of the <i>lbgB</i> gene; does not bind MAb 3E6	This study
35000.7(pLBG-B)	Strain derived from electroporation of pLBG-B into strain 35000.7 to complement the inactivated <i>lbgB</i> gene; binds MAb 3E6	This study
35000.3	LOS mutant derived from strain 35000 by deletion of an <i>AvrII-HpaI</i> fragment spanning the <i>lbgA</i> and <i>lbgB</i> genes and insertion of a <i>cat</i> cartridge into the deletion; does not bind MAb 3E6	This study
35000.3(pLBG-AB)	Strain derived from the electroporation of pLBG-AB into strain 35000.3 to complement the inactivated <i>lbgA</i> and <i>lbgB</i> genes; binds MAb 3E6	This study
1151	Wild-type strain isolated in Gambia	Allan Ronald
E1673	Wild-type strain isolated in Sweden	Allan Ronald
041	Wild-type strain isolated in Sweden	Allan Ronald
1145	Wild-type strain isolated in Amsterdam, The Netherlands	Allan Ronald
Hd13	Wild-type strain isolated in Singapore	52
Hd9	Wild-type strain isolated in Kenya	Allan Ronald
STD101	Wild-type strain isolated in Dallas, Tex.	67
Cha-1	Wild-type strain isolated in Dallas, Tex.	25
1352	Wild-type strain isolated in Kenya	Allan Ronald
512	Wild-type strain isolated in Thailand	Allan Ronald
Hd12	Wild-type strain isolated in Korea	38
Plasmids		
pMS1	Plasmid delivery system for mutagenesis of <i>H. ducreyi</i> with Tn1545- Δ 3; Cm ^r Kan ^r	66
pMKS6	Plasmid pACYC184 containing a 10-kb <i>NcoI</i> chromosomal DNA fragment from <i>H. ducreyi</i> 35000.6; Tet ^r Kan ^r	66
pBK-CMV	Plasmid excised from the Zap Express cloning vector; Kan ^r	Stratagene
pBluescriptII SK+	Cloning vector; Amp ^r	Stratagene
pSL4035	pBK-CMV with a 4.9-kb insert of <i>H. ducreyi</i> chromosomal DNA; this insert contains the <i>lbgA</i> and <i>lbgB</i> genes	This study
pSL4114	pBluescript with a 3.2-kb <i>XbaI-NcoI</i> fragment from plasmid pSL4035; this insert contains the <i>lbgA</i> and <i>lbgB</i> genes	This study
pSL4114.1A	pSL4114 with a <i>cat</i> cartridge inserted into the <i>AvrII</i> site within <i>lbgA</i>	This study
pSL4114.2H	pSL4114 with a <i>cat</i> cartridge inserted into the <i>HpaI</i> site within <i>lbgB</i>	This study
pSL4114.12AH	pSL4114 from which a 1.35-kb <i>AvrII-HpaI</i> fragment was deleted and replaced with a <i>cat</i> cartridge	This study
pLS88	Shuttle vector capable of replication in <i>H. ducreyi</i> and <i>E. coli</i> ; Sm ^r Sul ^r Kan ^r	14 and 74
pCAT88-BC	pLS88 in which a <i>cat</i> cartridge was inserted into the <i>SacI</i> site and a <i>BglII</i> site was constructed within the <i>EcoRI</i> site; Kan ^r Cm ^r	This study
pLBG-A	pCAT88-BC with a 0.8-kb <i>AvaI-SnaBI</i> fragment containing the <i>lbgA</i> gene	This study
pLBG-B	pCAT88-BC with a 2.1-kb <i>HhaI-BglI</i> fragment containing the <i>lbgB</i> gene	This study
pLBG-AB	pCAT88-BC with a 2.6-kb <i>AflIII-BglI</i> fragment containing both the <i>lbgA</i> and <i>lbgB</i> genes	This study

Construction of an *H. ducreyi* genomic library. A genomic library from *H. ducreyi* 35000 was constructed in the ZAP Express vector (Stratagene Cloning Systems, La Jolla, Calif.) as previously described (67).

Nucleotide sequence analysis. Both strands of the 3.2-kb *NcoI-XbaI* insert in pSL4114 were sequenced in their entirety. DNA sequence information was analyzed through the National Center for Biotechnology Information (NCBI) with the BLAST network service to search GenBank (4) and with programs from the University of Wisconsin Genetics Computer Group (13).

Identification of the Tn1545- Δ 3 insertion site in the chromosome of strain 35000.6. The insertion site of transposon Tn1545- Δ 3 in the 10-kb *H. ducreyi* DNA insert in pMKS6 was determined by nucleotide sequence analysis. The initial oligonucleotide primers used for sequencing were derived from the published

nucleotide sequences of the extremities of Tn1545- Δ 3 (48). The oligonucleotide primer for the integration-excision gene was 5'-GACGCTGAACCTATTACGC AC-3', and that for the *aphA-3* gene was 5'-CGTCGTATCAAGCTCATTC-3'.

Construction of *H. ducreyi* isogenic mutants. Plasmid pSL4114 was either (i) linearized by digestion with the restriction enzyme *AvrII* or *HpaI*, which cut exclusively within *lbgA* or *lbgB*, respectively, or (ii) digested with both *AvrII* and *HpaI* to excise a 1.35-kb DNA fragment containing portions of both the *lbgA* and *lbgB* genes. The digested plasmids were then subjected to agarose gel electrophoresis followed by purification with a GeneClean II kit (Bio 101, La Jolla, Calif.) and treatment with Klenow fragment and calf alkaline phosphatase. The *cat* cartridge was excised from plasmid pUC Δ ECAT with *EcoRI* (67), treated with Klenow fragment, and blunt-end ligated with the three digested forms of

pSL4114 described above with T4 DNA ligase. The ligation mixtures were used to transform *E. coli* HB101, and the desired recombinants containing the *cat* gene were selected by growth on LB agar containing chloramphenicol. Plasmids containing a *cat* cartridge inserted into the *lbgA* gene (pSL4114.1A), the *lbgB* gene (pSL4114.2H), or the deletion site (pSL4114.12AH) were identified by restriction enzyme analysis.

Plasmids pSL4114.1A, pSL4114.2H, and pSL4114.12AH were purified by cesium chloride density gradient centrifugation, linearized by digestion with *Pst*I, and used to electroporate wild-type *H. ducreyi* 35000 as previously described (24). Transformants were selected on CA plates containing chloramphenicol.

Complementation of the *lbgA* and *lbgB* mutations. Plasmid pSL4114 was digested with the following enzyme combinations: *Ava*I and *Sna*BI, which excised the intact *lbgA* gene as a 0.8-kb fragment; *Hha*I and *Bgl*II, which excised the intact *lbgB* gene as a 2.1-kb fragment; and *Afl*III and *Bgl*II, which excised the *lbgA* and *lbgB* gene pair as a 2.6-kb fragment. After the addition of *Bgl*II oligonucleotide (dephosphorylated) linkers (New England Biolabs, Beverly, Mass.), these fragments were ligated into the *Bgl*II site in plasmid pCAT88-BC. The 6.1-kb plasmid pCAT88-BC was derived from pLS88 (14, 74) by (i) the addition of a *Bgl*II site into the *Eco*RI site and (ii) the insertion of a *cat* cartridge into the *Sac*I site. These ligation mixtures were used to transform either *E. coli* DH5 α or HB101, and the desired recombinants were selected by growth on LB agar containing kanamycin. The recombinant plasmids pLBG-A, pLBG-B, and pLBG-AB, containing the *lbgA*, *lbgB*, and *lbgAB* genes, respectively, were purified with the Wizard DNA purification system (Promega Corp., Madison, Wis.) and used to electroporate selected *H. ducreyi* strains. Plasmid-containing transformants were selected by growth on CA containing kanamycin.

Analysis of *H. ducreyi* LOS. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGE) of *H. ducreyi* LOS from proteinase K-treated whole-cell lysates was performed as previously described (30, 46). SDS-PAGE-resolved LOS bands were stained with silver (70). Western blot analysis with Mab 3E6 was performed as described previously (66).

Virulence testing. Determination of the ability of wild-type and mutant *H. ducreyi* strains to produce dermal lesions in a temperature-dependent rabbit model was accomplished as described previously (3, 50, 67). Briefly, eight New Zealand White male rabbits were used in each experiment. Serial dilutions of two or three *H. ducreyi* strains were injected intradermally into each animal. Lesion scores were recorded on days 2, 4, and 7 postinfection. Recovery of viable *H. ducreyi* from lesion material and statistical analyses were performed as described previously (67). A total of 48 animals were used in six independent virulence tests. To confirm the identities of *H. ducreyi* strains recovered from lesions, organisms recovered from CA plates were tested for their abilities to grow on CA containing either chloramphenicol (for isogenic mutants) or kanamycin (for strains carrying recombinant plasmids derived from pCAT88-BC). In addition, the LOS phenotype of the recovered strains was confirmed by the use of Mab 3E6 in the colony blot assay and, when appropriate, plasmid DNAs were isolated from these strains.

Nucleotide sequence accession number. The nucleotide sequences of the *lbgA* and *lbgB* genes were deposited at GenBank and assigned the accession number U58147.

RESULTS

Characterization of the LOS mutant strain 35000.6 in model systems. While developing a mutagenesis system for *H. ducreyi*, we constructed an *H. ducreyi* LOS mutant via transposon-mediated mutagenesis (66). Mutant strain 35000.6 lacks reactivity with the LOS-specific Mab 3E6 and contains a truncated LOS molecule relative to the LOS of the wild-type strain 35000 (66). A preliminary experiment in a temperature-dependent rabbit model with mutant strain 35000.6 indicated that this LOS mutant had a reduced ability to produce dermal lesions (Table 2, experiment A). As a control, a random Tn1545- Δ 3 transposon insertion mutant was also included in this experiment. The lesion scores of this control strain (35000.54) were equivalent to those of the wild-type strain (Table 2, experiment A), suggesting that the presence of the Tn1545- Δ 3 transposon alone was not responsible for the reduction in the ability of the LOS mutant strain 35000.6 to produce lesions. Additionally, assessment in vitro of the ability of this LOS mutant to adhere to or invade HEP-2 cells revealed no differences relative to that of the wild-type parent strain (data not shown).

Cloning of the mutated LOS locus. To identify the genetic locus affected by the transposon, a bacteriophage-based genomic DNA library constructed from *H. ducreyi* 35000 was probed with a 2.35-kb *Hind*III-*Sna*BI fragment from pMKS6.

The 10-kb *Nco*I chromosomal DNA fragment in pMKS6 was originally cloned from the LOS mutant strain 35000.6 and contains the transposon Tn1545- Δ 3 (66). The *Hind*III-*Sna*BI fragment encompasses part of this transposon and approximately 1 kb of *H. ducreyi* chromosomal DNA flanking the transposon insertion site. A pBK-CMV-based plasmid designated pSL4035, excised from a recombinant clone that hybridized with this probe, was found to contain a 4.9-kb *H. ducreyi* DNA insert (Fig. 1). A 3.2-kb *Xba*I-*Nco*I fragment from this insert was subcloned into the *Bam*HI site in pBluescriptII to generate plasmid pSL4114 (Fig. 1).

Nucleotide sequence analysis. The 3.2-kb DNA insert of pSL4114 was found to contain four predicted open reading frames (ORFs) (Fig. 1). Preliminary restriction mapping of pMKS6 and pSL4114 indicated that the insertion site for Tn1545- Δ 3 was likely located in either the second or third ORF, which were arranged in tandem. The first ORF in this pair was nearly 700 nucleotides in length (nucleotides 511 to 1182 in Fig. 2). Immediately following the termination codon of this ORF was a second, slightly larger ORF (nucleotides 1186 to 2214 in Fig. 2). Presumptive association of these ORFs with LOS expression led to their tentative designations as LOS biosynthesis genes A and B (*lbgA* and *lbgB*) (Fig. 1).

The putative protein products encoded by *lbgA* and *lbgB* have predicted molecular masses of 25,788 and 40,236 Da and isoelectric points of 9.23 and 9.46, respectively. A BLAST search of nonredundant DNA and protein databases identified proteins involved in LOS or lipopolysaccharide (LPS) biosynthesis with homology to the protein products of *lbgA* and *lbgB*. The deduced amino acid sequence of LbgA exhibited 23 to 25% identity and 48 to 52% similarity to that of a predicted protein from *H. influenzae* KW20 (PIR, locus F64091) (17) and to the product of the *H. influenzae* type b *lex-1/lic2A* gene, which is involved in LOS biosynthesis (12, 27). Additionally, LbgA was 52% similar to the LpsA protein of *Pasteurella haemolytica* (47). The deduced amino acid sequence of LbgB was 24% identical and 52% similar to that of the RfaK protein (1,2-*N*-acetylglucosamine transferase) of *E. coli* (31).

The amino acid sequence of the predicted protein encoded by the ORF upstream of *lbgA* (ORF C in Fig. 1) had significant homology (>80%) with an *H. influenzae* ribosomal protein (7, 16, 17). The ORF located immediately downstream from *lbgB* (ORF D in Fig. 1) encoded a predicted protein with greater than 70% homology to exonuclease III of *H. influenzae* (17, 54). Neither of these two genes was analyzed further.

Conservation of *lbgA* and *lbgB* among *H. ducreyi* strains. PCR analysis was used to investigate whether the tandem arrangement of *lbgA* and *lbgB* was conserved among other *H. ducreyi* strains. When oligonucleotide primers derived from the 5' end of the *lbgA* ORF and the 3' end of the *lbgB* ORF were used with chromosomal DNAs from 12 *H. ducreyi* strains from diverse geographic regions, a 1.4-kb PCR product was obtained from all 12 strains, including 35000 (data not shown).

Analysis of the site of insertion of Tn1545- Δ 3 within the chromosome of *H. ducreyi* 35000.6. Preliminary mapping of the DNA insert in pMKS6 had placed Tn1545- Δ 3 within either *lbgA* or *lbgB*. To determine which gene contained the transposon, oligonucleotide primers were designed for sequencing out from Tn1545- Δ 3 into the adjacent *H. ducreyi* chromosomal DNA in pMKS6. Approximately 500 nucleotides were sequenced from the right extremity of Tn1545- Δ 3 in pMKS6, and a nucleotide sequence contained in *lbgA* (beginning at nucleotide 670 in Fig. 2) was identified approximately 100 nucleotides from the start of the primer sequence (data not shown).

Approximately 1.4 kb of nucleotide sequence was obtained from the *H. ducreyi* DNA flanking the left extremity of

TABLE 2. Lesion formation by the wild-type, LOS mutant, and complemented LOS mutant strains in a temperature-dependent rabbit model^a

Expt	Bacterial strain	Inoculum size (CFU)	Mean lesion score (SD) on day:			P value ^b
			2	4	7	
A	35000 (wild type)	10 ⁵	3.63 (0.52)	3.75 (0.71)	3.75 (0.71)	0.0109 0.1705
	35000.6 (transposon mutant)	10 ⁵	2.75 (0.71)	2.75 (0.89)	3.25 (1.04)	
	35000.54 (random transposon)	10 ⁵	3.88 (0.35)	3.88 (0.35)	3.88 (0.35)	
B	35000 (wild type)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	0.1540
	35000.4 (<i>lbgA</i> mutant)	10 ⁵	3.88 (0.35)	4.00 (0.00)	4.00 (0.00)	
	35000	10 ⁴	3.50 (0.54)	3.88 (0.35)	3.75 (0.46)	
	35000.4	10 ⁴	3.25 (0.46)	3.00 (0.93)	2.75 (0.89)	
C	35000 (wild type)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	0.5858
	35000.7 (<i>lbgB</i> mutant)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	
	35000	10 ⁴	3.50 (0.54)	3.63 (0.74)	3.50 (1.07)	
	35000.7	10 ⁴	3.25 (1.03)	3.38 (0.92)	3.25 (1.17)	
D	35000 (wild type)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	0.0108
	35000.3 (<i>lbgAB</i> mutant)	10 ⁵	3.63 (0.74)	3.75 (0.46)	3.63 (1.06)	
	35000	10 ⁴	3.50 (0.54)	3.63 (0.74)	3.50 (1.07)	
	35000.3	10 ⁴	2.75 (0.71)	2.13 (1.25)	2.00 (1.07)	
E	35000 (wild type)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	0.0467 0.0971
	35000.3 (<i>lbgAB</i> mutant)	10 ⁵	3.75 (0.46)	3.75 (0.71)	3.75 (0.71)	
	35000.3(pLBG-AB)	10 ⁵	4.00 (0.00)	4.00 (0.00)	4.00 (0.00)	
	35000	10 ⁴	3.25 (0.46)	3.38 (0.52)	3.75 (0.46)	
	35000.3	10 ⁴	2.75 (0.46)	2.38 (1.30)	2.50 (1.31)	
	35000.3(pLBG-AB)	10 ⁴	3.13 (0.64)	3.13 (0.84)	3.13 (0.84)	
F	35000 (wild type)	10 ⁵	3.88 (0.35)	3.63 (0.74)	4.00 (0.00)	0.1899 0.0027
	35000.3 (<i>lbgAB</i> mutant)	10 ⁵	3.50 (0.54)	3.50 (0.76)	3.50 (0.76)	
	35000.3(pLBG-AB)	10 ⁵	3.25 (0.46)	3.00 (0.76)	2.88 (0.99)	
	35000	10 ⁴	2.38 (0.52)	1.75 (1.39)	1.88 (1.25)	
	35000.3	10 ⁴	2.50 (0.54)	1.38 (1.30)	1.38 (0.74)	
	35000.3(pLBG-AB)	10 ⁴	2.25 (0.46)	1.50 (1.41)	1.25 (1.28)	

^a Eight rabbits were used in each experiment.^b P values were calculated for the differences between wild-type and test strain lesion scores. With the exception of experiment A, P values were calculated with the lesion scores from both inoculum sizes and from all three days.

Tn1545-Δ3 in pMKS6. However, no identity was found between this sequence and that of the 3.2-kb DNA insert in pSL4114. Therefore, it appeared that the transposon insertion event in the LOS mutant strain 35000.6 deleted not only the 5' end of *lbgA* but also the upstream ORF (i.e., ORF C in Fig. 1) and additional DNA.

Construction of isogenic *H. ducreyi* mutants deficient in expression of LOS. The discovery of the deletion involving the 5' end of *lbgA* and the immediately adjacent (upstream) chromosomal DNA in strain 35000.6 prompted us to question whether the LOS phenotype of this mutant resulted from the inactivation of the *lbgA* gene or from the deletion of upstream DNA. Therefore, specific mutations were created in the *lbg* genes to determine which gene(s) was necessary for expression of wild-type LOS. The *lbgA* and *lbgB* genes were both independently and simultaneously inactivated by insertion of a *cat* cartridge (see Materials and Methods and Fig. 1) into the

appropriate restriction site. Plasmids pSL4114.1A (with a mutated *lbgA* gene), pSL4114.2H (with a mutated *lbgB* gene), and pSL4114.12AH (with both *lbgA* and *lbgB* deleted) were linearized and used to electroporate wild-type *H. ducreyi* 35000. All chloramphenicol-resistant transformants from each electroporation lacked reactivity with the LOS-specific MAb 3E6 (data not shown). A single, representative strain from each electroporation experiment was chosen at random for further study.

Southern blot analysis was used to confirm that proper allelic exchange had occurred in each of these three chloramphenicol-resistant strains, designated 35000.4 (*lbgA* mutant), 35000.7 (*lbgB* mutant), and 35000.3 (*lbgA* and *lbgB* double mutant). An *lbgA*-containing 0.87-kb *Afl*III-*Ban*II DNA fragment from pSL4114 (probe 1 in Fig. 1) was used to probe an *Afl*III-*Ban*II digest of chromosomal DNA from both strain 35000 and strain 35000.4. This probe hybridized with a 0.8- to 0.9-kb fragment from the wild-type chromosomal DNA and a

FIG. 1. Partial restriction maps of the recombinant plasmid pSL4035 and its derivatives. A 3.2-kb *XbaI*-*NcoI* DNA fragment from the 4.9-kb insert in pSL4035 was subcloned into pBluescript II to construct pSL4114; this plasmid contains the complete *lbgA* and *lbgB* genes. Restriction sites in parentheses indicate vector DNA. C and D indicate irrelevant ORFs upstream and downstream of *lbgA* and *lbgB*, respectively; the arrows indicate the predicted direction of transcription. Plasmid pSL4114.1A is plasmid pSL4114 with a 1.4-kb *cat* cartridge inserted into the *AvrII* site in *lbgA*. Plasmid pSL4114.2H is pSL4114 with the *cat* cartridge inserted into the *HpaI* site in *lbgB*. Plasmid pSL4114.12AH is pSL4114 from which a 1.35-kb *AvrII*-*HpaI* fragment (containing all of *lbgA* and most of *lbgB*) was deleted and replaced with the *cat* cartridge. Probe 1 indicates the 0.87-kb *AflIII*-*BlnI* restriction fragment used to identify *lbgA* in Southern blot analysis. Probe 2 indicates the 1.36-kb *HpaI*-*NcoI* restriction fragment generated to identify *lbgB*. Plasmid pLBG-A is pCAT88-BC containing the intact *lbgA* gene. Plasmid pLBG-B is pCAT88-BC containing the intact *lbgB* gene, whereas pLBG-AB is pCAT88-BC containing the *lbgAB* gene pair.

2.3-kb DNA fragment from the mutant strain 35000.4 (Fig. 3, upper panel, lanes A and B, respectively). Similarly, when chromosomal DNA preparations from strains 35000 and 35000.7 were digested with *AflIII*-*BlnI* and probed with an *lbgB*-containing 1.35-kb *HpaI*-*NcoI* DNA fragment from pSL4114 (probe 2 in Fig. 1), a 2.3-kb DNA fragment from strain 35000 and a 3.6-kb DNA fragment from mutant strain 35000.7 (Fig. 3, upper panel, lanes C and D, respectively) bound this probe. Therefore, either an *AflIII* or a *BlnI* site must be present in the wild-type chromosome 2.3 kb downstream from the *BlnI* site in *lbgA* (Fig. 1). The presence of the larger DNA fragments in the mutants is consistent with the presence of the 1.4-kb *cat* cartridge inserted into the respective target genes.

The presence of the *cat* cartridge in the *lbgA* and *lbgB* genes of these two mutants was also confirmed by Southern hybridization. A *cat* gene probe hybridized only with a 2.2-kb *AflIII*-*BlnI* fragment from the *lbgA* mutant strain 35000.4 and a 3.6-kb *AflIII*-*BlnI* fragment from the *lbgB* mutant strain 35000.7 (Fig. 3, lower panel, lanes B and D, respectively). Wild-type chromosomal DNA did not hybridize with the *cat* gene probe (Fig. 3, lower panel, lanes A and C).

Allelic exchange in the *lbgAB* double-mutant strain 35000.3 was more difficult to detect, because the size of the deleted *AvrII*-*HpaI* fragment (1.35 kb) was approximately the same as that of the inserted *cat* cartridge (1.4 kb). Therefore, chromosomal DNA from the wild-type strain 35000 and *lbgAB* mutant strain 35000.3 was digested with *NcoI*, which cuts once within

the *cat* cartridge. A 1.35-kb *HpaI*-*NcoI* DNA fragment from pSL4114 that encompassed the 3' half of the *lbgB* gene (probe 2 in Fig. 1) hybridized with a 7-kb *NcoI* fragment from wild-type chromosomal DNA (Fig. 3, upper panel, lane E). Therefore, an *NcoI* site must be located 7 kb 5' from the *NcoI* site depicted in Fig. 1. This same probe hybridized with a 1.8-kb *NcoI* fragment from the double-mutant strain 35000.3 (Fig. 3, upper panel, lane F). This smaller fragment from the double mutant is consistent with the presence of the *cat* cartridge and its *NcoI* site as depicted in Fig. 1 (see pSL4114.12AH). This was confirmed by the hybridization of the *cat* gene probe with only the 1.8- and 5.1-kb *NcoI* fragments from the double-mutant strain 35000.3 (Fig. 3, lower panel, lane F).

Analysis of LOS expression by the isogenic mutants. SDS-PAGE and Western blotting were used to analyze LOS expression by the wild-type strain 35000 and the isogenic LOS mutant strains 35000.4, 35000.7, and 35000.3. LOS-specific silver staining revealed a difference in the rates of migration of the LOSs of the three mutant strains compared to that of the wild-type LOS. The LOS of the mutant strains 35000.4, 35000.7, and 35000.3 (Fig. 4, upper panel, lanes C, E, and G, respectively) migrated at similar rates but faster than the LOS of the wild-type strain 35000 (Fig. 4, upper panel, lane A), suggesting an alteration or deletion in the LOSs of the mutant strains. Western blot analysis showed that the LOS of the wild-type strain readily bound MAb 3E6 (Fig. 4, lower panel, lane A) whereas the LOSs of the mutant strains 35000.4, 35000.7, and 35000.3 (Fig. 4, lower panel, lanes C, E, and G,

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100
GGATCTTCCCTAGAACTACTCTCGAGAAGCTTTTGAATTCCTTGGATCTTTATCTAAAAATAAAATAGAATTGCTTTCTTTTGGCGTTGAGTAATTTA
200
CTCAACATATTTTGTAGTTACATATGGTACTTATCAGCGGATGTCAATAAATATATAATCATTTAACTGCAACAAGATAAGTGGCGATATGGCTTCATC
300
AGAGGTTCTCCAATGAACAAGGTATTACCCAGAAATATAAGAAATACGGCAACATGTTCTTGGCGTAATGTAATTAACACCGCTCAACAGTAGAGA
400
AAAATTTAATTTAGAGCTATGTGGTAATGGCAACCATTCATACAGGCAACAGCGTGTAGTTGACACCGCGGTCGTGTTGAACGTTTCAATAAACG
500
TTTAAATATCCCGAGCACTAAATAATTAGTGTGTTGTCAATAAAACCTGCAATAGCAGGGTTTATTGTAATAAGTAAAAATTTTCTTATTAATAAAA
600
GTGAAATAATAGGATGAATTCCTCCCTATTTTATTATTAGTTTAAAAGATTACCTAGGAAAAATATTATGCAACGTTTAAATGCATTAGGATTA
700
M D E F P P I F I I S L K D S P R K N I I A E R L N A L G L>
800
TCTTTTAATTTTGTGATGCGATTATGGCAAGAAGTTCTTAAAGAAATGTGCGAAATAAATATGATTTTATATACAAAAATTTAATGCAAGAA
S F N F F D A I Y G K E L S K K Q L S E I N Y D F Y I Q K F N A R>
900
AGCGCTTACTTTAGGAGAAATGGTGTGCAATTAGTCATATAAATGTATGAATATATTGTAATAAATAATTAACGAGGCAATTATTTTAGAGGA
K R L T L G E I G C A I S H I K L Y E Y I V K N N I N E A I I L E D>
1000
TGATGCTATAGTATCTATTTTAAAGATATTATATGTGATGCATTAAAAAGATTCTTGTAAATAGAGATATTATTTTAGATCATGGTAAGGCT
D A I V S I Y F K D I I C D A L K K I S C K Y E I L F L D H G K A>
1100
AAAATATTCCTCTTTTACTAGGAGTTTGTGAAAGATATAGATTAGCTAAATATATTCTCCATCGAAAAATCTAAAAGAATTATTTTCGTACTACAG
K I F P F T R S L V E R Y R L A K Y I S P S K K S K R I I I R T T>
1200
CATATATTATACGTTAAATGGTGTCTGTAAGCTACTAAATTTAGCGTATCTTATCGTATGCCATCAGATTATTTAACTGGGACACTTCAATTAACGGG
A Y I I T L N G A R K L L N Y A Y P I R M P S D Y L T G T L Q L T G>
1300
AATAAATGCTTATGGTATTGAGCCCTTGCCTTTTGGTGGGCCAATTTCTGAAATGATCAATAGAGATAGATATTGTTAAATGAATCTTAAAGAA
I N A Y G I E P P C V F G G P I S E I D Q I E D R Y C * M N L K E>
1400
AAATTAAGAAATATCGTATAGCATTGGTAAATGGTTTAGATAGAAAAATATCATTTACTAAATTAAGGTTGCTCTCTAAAAAATCTTTTCTTCTTA
K L R K L R I A F G K L V L D R K I S F T K L R L P P K K I L F L>
1500
GGCAAGATGGAAGATAGGTGATTATATTGTAGTCTTTTGGTGGTAAATAAGCAATTAACCTAATATTGAGATAGGCGTTATTGTTCTCGGA
R Q D G K I G D Y I V S S F V F R E I K K Q L P N I E I G V I C S D>
1600
TAAATATGCTTATTTTAAATAAAATGCTTACATAGACCATTTGTTATATTGTTGCAACAAGGAATATATTAGACTATTTGAAATGCTGATGAAGTA
K N A Y L F N K N A Y I D H C Y I V R T R N I L D Y L K C G M K L>
1700
GCCAATCAGAAATATGATTAGTTATTTGATCTACTATAGCATTACGTAATAGGGATTATGCTTTTGGAGATTATTAATGCTAAACATTATATAGGTT
A N Q K Y D L V I D F T I A L R N R D L L L L R V I N A K H Y I G>
1800
ATGAGAAAATTAATTAATCAATTTGATTTTAAATTAAGAAATGAGAAAAAGCATATTTCAACAGTCTATAAAGAGCCCTTAGAATTAATAGGACTAAA
Y E K T N Y Q L F D F N I K N E K K H I S T V Y K R A L E L I G L N>
1900
CCATATAGATATAAGCTATGATATTCATTAACAAAGACAGTCATGAAAGAAATAAAATATATCTATCTGAGTCTTGTTTAGATAGCTTTATCGCACTT
H I D I S Y D I P L N K D S H E R I K I Y L S E S C L D S F I A L>
2000
AATTTATTTGGAGCTAGTTCTACTAGGTCTTCAATGATAAAGATATATGAATGCTTGAATATTGGAAGAAAAATACAAATAAACCAATTTTATGTT
N L F G A S S T R S F N D N K I Y E L L E Y L K K N T N K P I L L>
2100
TAACATTCCCTGCAATAGCTAGAAAAATAAAAAAATGCTTGTCTATTTTCGGAAGTATTATCTATGAACATACGAAACAGTGTGATGACTATAGA
L T F P A I A R K L K K I A C H F S E V F I Y E H T K T V F D T I E>
2200
ATTAATACGTTTTCGTGATTGGTATTTCACCTGATCTCTATTGTGCATATTGCTGTTGGACTTAATAAAAAATTAATTGCATTTTATAGTCTTGAT
L I R F A D L V I S P D T S I V N I A V G L N K K L I A F Y S L D>
2300
GAACAAATTTTCATTCACCTGCAACCAATAATTTAGTTGAAACACATATTTGCGTTTGAACATAACATAAATGAAATAAATATTTTCAGAAATTCAG
E Q N F I H W Q P N N L V E T H I L R F E H N I N E I N I S E I Q>
2400
CAAAATGTTAAATTAATAATACGTTTGAATTTTAAATACTGCCCAATAGGGGCTGATCAGAGGGTTTTCATAGCTCTGATTTCTAAATCAAT
A K W L N * *
2500
TCCTGTTTCTACACAATGGTTGTAGTGTGTTGAAGCTAAAAATTAAGTCAATTGTAACCCACGGTTGTCATTAAACCTTTAGAGCGATAATCAAAC
2600
CATGAGAAATGATCAGTTGTAGTTGGATGCAATGCACGGAAGTATCTACTAAACCATAGTGATATAAACGGTCTAACCATTCGCGTCTCTCTGCTAAGA
2700
AAGAATATTTGCGCTCTCTTAGCCAACGTTTACGATTGCTTACCAATCCCAATATCTAAATCCGTAGAGCAGATATTATATACCCATAATAATAAT
2800
AGGGTTGTATGCTGTAGGGCGGTTCTTAAATAATGTTAGGTACGATAGAAATTTGCTTTAGCAGGAAATTTTGTTCATGCTGCGGATTTTCACTT
2900
TGTGGGAAGTAGCCATTTAGTACGGTTAGGATACCAAAATCAGTGTTAATCTCTGCCATAATCATTCGCTTTTGGGCATCAGCTGTATCATTTGGAAAGC
3000
CTTTACGAATGGTAAGCGGTGTTTCTTAAATAGTACGCCATAATGGCCTTTTGGCCATGATAAAAAACGTGATAGCCAGATGTTCTACAAG
3100
TTGATGTGGGAAATCTTCATCTGCAACTTTGATTTCTGTAAGCCGATCACATCAGGCTGATGTTTATGATGATGCTTCTAATTGATGTGGCTAGCA
3200
CGTAAGCCATTGATATTAAGAGATAAATTCATAAAACCTTAATAGGGAAAAATGTTATAACGTCATCGCAGCAATCCAACCAAGATTAATAATGGA
ATGCTAAAAATGTAAGAAAGTAGGACGACAGAATCCCAATGTGATCATGTTTGGCATCAGCATTTAAGCCTGATGTAGGGCTTAATGTTGAGTCAGAAG
CTGGCGAGCTGCACTGCGCTAAGCGCGCGCAACGCCACAATCGCCATGGGAAGATCC

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FIG. 2. Nucleotide sequences and the deduced amino acid sequences of the *lbgA* and *lbgB* genes from *H. ducreyi* 35000 and their predicted protein products. Putative -35 and -10 regions are overlined. The asterisks indicate the termination codon(s) after *lbgA* and *lbgB*.

FIG. 3. Southern blot analysis of chromosomal DNA preparations from the wild-type strain 35000 and the isogenic LOS mutant strains 35000.4, 35000.7, and 35000.3. Chromosomal DNAs from these strains were digested with the appropriate restriction enzymes, resolved by agarose gel electrophoresis, and processed for Southern blot analysis. Lanes A, C, and E, wild-type strain 35000; lanes B, *lbgA* mutant strain 35000.4; lanes D, *lbgB* mutant strain 35000.7; lanes F, *lbgAB* double-mutant strain 35000.3. Lanes A to D contain double digests with *Afl*III and *Ban*II, whereas lanes E and F contain digests with *Nco*I. Lanes in the upper panel were probed with *lbgA*- or *lbgB*-containing gene fragments, whereas lanes in the lower panel were probed with the *cat* cartridge. Lanes A and B in the upper panel were probed with an *Afl*III-*Ban*II DNA fragment from pSL4114 (to detect *lbgA*). Lanes C to F were probed with an *Hpa*I-*Nco*I DNA fragment from pSL4114 (to detect *lbgB*). Size markers are on the right side of each panel.

respectively) did not react detectably with this antibody. It should be noted that this same altered LOS migration pattern and absence of reactivity with MAb 3E6 were also observed for the LOS of the original transposon-containing LOS mutant strain 35000.6 (Fig. 4, lanes B).

Complementation of the *lbgA* and *lbgB* mutations. The plasmid constructs pLBG-A, pLBG-B, and pLBG-AB (Fig. 1) were used to electroporate the mutant strains 35000.4, 35000.7, and 35000.3, respectively. Several kanamycin-resistant transformants were isolated from each electroporation and tested in a colony blot assay for reactivity with MAb 3E6. All transformants tested regained reactivity with this MAb (data not shown). Furthermore, the plasmids isolated from each of the complemented transformants still contained the cloned insert of *lbgA*, *lbgB*, or *lbgAB*. One complemented mutant strain from each electroporation was chosen at random for further characterization.

SDS-PAGE of the LOSs of the complemented mutant strains 35000.4(pLBG-A), 35000.7(pLBG-B), and 35000.3 (pLBG-AB) (Fig. 4, upper panel, lanes D, F, and H, respec-

FIG. 4. SDS-PAGE analysis of LOSs expressed by the wild-type strain, isogenic LOS mutant strains, and the complemented mutant strains. LOSs present in proteinase K-treated whole-cell lysates of these strains were resolved by SDS-PAGE and either silver stained (upper panel) or blotted to nitrocellulose and probed with the LOS-specific MAb 3E6 (lower panel). Lanes: A, wild-type strain 35000; B, Tn1545- Δ 3-containing mutant strain 35000.6; C, *lbgA* mutant strain 35000.4; D, complemented mutant strain 35000.4(pLBG-A); E, *lbgB* mutant strain 35000.7; F, complemented mutant strain 35000.7(pLBG-B); G, *lbgAB* double-mutant strain 35000.3; H, complemented-mutant strain 35000.3(pLBG-AB).

tively) revealed that the LOSs of these three strains migrated at a rate indistinguishable from that of the wild-type LOS (Fig. 4, upper panel, lane A). Similarly, in the corresponding Western blot, the LOSs of the complemented mutant strains 35000.4(pLBG-A), 35000.7(pLBG-B), and 35000.3(pLBG-AB) (Fig. 4, lower panel, lanes D, F, and H, respectively) bound the LOS-specific MAb 3E6, confirming the restoration of expression of this epitope.

Virulence expression by the isogenic mutants. The isogenic LOS mutant strains were tested for their abilities to produce lesions in a temperature-dependent rabbit model (50). Both the *lbgA* mutant 35000.4 and the *lbgB* mutant 35000.7 were as virulent as the wild-type strain 35000 (Table 2, experiments B and C). The *lbgAB* double-mutant strain 35000.3 was tested in three separate experiments (Table 2, experiments D, E, and F). Statistical analysis confirmed that in two (Table 2, experiments D and E) of these three experiments, the double-mutant 35000.3 produced lesions that were less severe than those of the wild-type strain. In the third experiment with this double mutant (Table 2, experiment F), the mutant was as virulent as the wild-type parent strain.

The complemented double-mutant 35000.3(pLBG-AB) was used in this animal model in two experiments (Table 2, experiments E and F). In the first instance, complementation of the double mutant restored virulence to wild-type levels (Table 2, experiment E). However, in the experiment where the double mutant was as virulent as the wild-type parent strain (Table 2, experiment F), the complemented double mutant proved to be less virulent than the wild-type parent strain.

Viable *H. ducreyi* cells were isolated from the lesions resulting from the injection of 10^5 CFU for all of the strains tested

in this animal model. Colony blot analysis of several colonies recovered from each lesion confirmed that the mutant strains had retained the MAb 3E6-negative phenotype and that the wild-type and complemented strains had retained the MAb 3E6-positive phenotype. Testing of the sensitivities of the recovered strains to kanamycin as well as plasmid isolation confirmed that the complemented double-mutant strain 35000.3 (pLBG-AB) had retained its plasmid.

DISCUSSION

LPS plays a critical role in disease caused by gram-negative pathogens (49). Although the lipid A portions of LPS and LOS molecules have been shown to be highly toxic, the contributions of the non-lipid A regions of these amphipathic molecules to pathogenesis have only recently begun to be investigated. The oligosaccharide components of the LOS of the mucosal pathogens *N. gonorrhoeae* and *H. influenzae* likely contribute to the virulence of the former (57, 58, 60, 72) and are known to be involved in virulence expression by the latter, at least in an animal model (11, 12, 73). The involvement of LOS in virulence expression by *H. ducreyi* has not been extensively investigated to date, even though LOS was the first *H. ducreyi* antigen to be associated with a proposed virulence mechanism for this organism (i.e., serum resistance) (40–42). Later, it was established that injection of relatively large numbers of *H. ducreyi* (ca. 10^8 to 10^9 CFU) readily produced inflammation and necrosis in different animal models (10, 71) and that this effect could be attributed to the LOSs of these bacteria. An *H. ducreyi* LOS mutant selected for its resistance to pyocins contained a truncation in its oligosaccharide side chain (9); whether this pyocin-resistant LOS mutant was less virulent than its parent strain has not been reported.

The availability of the transposon-bearing LOS mutant strain 35000.6 allowed us to clone and characterize, for the first time, genes that are essential for expression of wild-type LOS by *H. ducreyi*. The *lbgA* and *lbgB* genes were shown to be arranged in tandem in the *H. ducreyi* chromosome, and inactivation of either or both genes resulted in loss of reactivity with the LOS-specific MAb 3E6. These mutations could be readily complemented with wild-type alleles supplied in *trans* (Fig. 4).

The similarity between the putative protein product of the *H. ducreyi* *lbgB* gene and the *N*-acetylglucosamine transferase (RfaK) protein of *E. coli* (31) suggested that *lbgB* may be the *H. ducreyi* homolog of the *rfaK* gene. In *E. coli*, RfaK catalyzes the addition of *N*-acetylglucosamine to an inner core heptose of the LPS (56). The *H. ducreyi* LbgB protein also exhibits strong similarity to the RfaK proteins of *Neisseria meningitidis* (29) and *Salmonella typhimurium* (33) (46.5 and 48.9%, respectively). As reported for other Rfa proteins, LbgB is highly basic and lacks any significant potential transmembrane-spanning sequences (data not shown), consistent with the proposed cytoplasmic or peripheral membrane location on the inner face of the cytoplasmic membrane for this protein (31, 56). However, in contrast to the multiple, closely linked genes involved in enteric LPS biosynthesis (i.e., the *E. coli* *rfa* genes) (31, 56), the *H. ducreyi* *lbgB* gene exists together with only a single other gene (*lbgA*), whose product is now known to be involved in LOS biosynthesis in *H. ducreyi*.

Although the *H. ducreyi* *lbgA* gene product does not possess homology with any *E. coli* or *S. typhimurium* LPS biosynthetic enzymes, this *H. ducreyi* protein is similar to proteins involved in LOS expression by *H. influenzae* (12, 27). A specific function in LOS biosynthesis has not been established for the product of the *H. influenzae* type b *lex-1/lic2A* gene; however, an *H. influ-*

enzae type b *lex-1* mutant contained an altered LOS and was less virulent than the wild-type parent strain in an infant rat model (12). It should be noted, however, that the tandem repeats of the nucleotide tetramer CAAT which are present in the 5' region of the *H. influenzae* type b *lex-1/lic2A* gene (12, 27) and which are involved in LOS phase variation in *H. influenzae* (27) are not present in *lbgA*. While the absence of these nucleotide repeats in *lbgA* does not preclude phase variation by *H. ducreyi* LOS epitopes, studies from this laboratory suggest that at least the LOS epitope defined by its reactivity with MAb 3E6 does not exhibit phase variation in *H. ducreyi* 35000 (26). Similarly, structural analyses of purified *H. ducreyi* LOS indicate that phase variation of *H. ducreyi* LOS epitopes is minimal (37, 61, 62).

The diminished virulence of the LOS (transposon) mutant strain 35000.6 (Table 2, experiment A) suggested that the altered LOS structure was directly related to the attenuated virulence phenotype. However, the discovery of a deletion of chromosomal DNA upstream of the transposon insertion site in *lbgA* prompted reevaluation of the involvement of LOS in this virulence difference. Construction of the isogenic *lbgA* (35000.4) and *lbgB* (35000.7) mutants and complementation of these mutations confirmed that both the *lbgA* and *lbgB* gene products were necessary for the expression of the LOS epitope recognized by MAb 3E6. Inactivation of each of these genes independently, although imparting structural and phenotypic alterations to the LOS, had no detectable effect on the ability of these LOS mutants to produce lesions in an animal model (Table 2, experiments B and C).

Deletion of both the *lbgA* and *lbgB* genes (strain 35000.3) likewise resulted in the loss of the MAb 3E6-reactive epitope, and the LOS of this double mutant phenotypically resembled the LOSs of the individual mutants (Fig. 4). However, equivocal results were obtained in virulence testing of this mutant. Two of three experiments indicated that the double-mutant 35000.3 had a reduced capacity to form dermal lesions (Table 2, experiments D and E), although the difference in lesions scores in one of these experiments (Table 2, experiment E) barely achieved statistical significance. In a third experiment (Table 2, experiment F), the double mutant was no less virulent than the wild type. Previous experiments with this same animal model with both wild-type (3) and mutant (67) *H. ducreyi* strains indicated that these different strains yielded reproducible results from one experiment to the next. Therefore, it may be that certain mutations affecting LOS expression, or at least those involved in this study, have a subtle effect on virulence that can be assessed accurately only with a much larger sample population. Alternatively, it is possible that a deleterious effect on virulence by certain mutations affecting LOS expression is detectable only in the human model for experimental chancroid (65).

In conclusion, the results of this study indicate that two tandem genes (*lbgA* and *lbgB*) are essential for expression of a conserved LOS surface epitope defined by its reactivity with MAb 3E6. Loss of the ability to express this epitope, at least when caused by independent mutations in the *lbgA* and *lbgB* genes, does not affect the virulence of *H. ducreyi* 35000 in an animal model.

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