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Characterization of *lbpA*, the Structural Gene for a Lactoferrin Receptor in *Neisseria gonorrhoeae*

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***Neisseria gonorrhoeae* acquires iron (Fe) efficiently from lactoferrin (LF). A 103-kDa gonococcal outer membrane LF-binding protein (Lbp) was identified previously. We isolated the structural gene *lbpA* for Lbp1 by screening a gonococcal library for a clone that could repair an LF$^-$ receptor mutant. An mTnCm3 transposon insertion mutant of *lbpA* was unable to use LF-bound Fe for growth, unable to bind LF to whole cells, and unable to express Lbp1. The DNA sequence of *lbpA* predicted a protein that shared 94% identity with the meningococcal LF receptor protein, Lbp, and was closely related to Tbp1, one of the transferrin receptor proteins. Clinical isolates of gonococci are frequently unable to acquire Fe from LF, and LF$^-$ isolates do not have a functional LF receptor. The wild-type *lbpA* gene transformed most tested LF$^-$ clinical isolates to LF$^+$, indicating that *lbpA* is defective in many clinical isolates.**

Lactoferrin (LF) is a high-affinity iron (Fe)-binding glycoprotein present abundantly in the specific granules of polymorphonuclear leukocytes and in mucosal secretions (37, 38). It is related in structure and function to transferrin (TF), an abundant serum protein (42). An important biological function of LF is probably the sequestration of free Fe from the environment of microorganisms, thus inhibiting their growth (54). LF also serves to reduce levels of free iron and thus to reduce Haber-Weiss reactions that generate tissue-damaging oxidative radicals (8, 23, 27).

There is evidence that iron acquisition plays an important role in bacterial virulence (14, 26, 45, 69). Many pathogens acquire iron by synthesis and secretion of siderophores, compounds that bind Fe with high affinity (7, 43). *Neisseria gonorrhoeae* and the closely related pathogen *Neisseria meningitidis* do not appear to synthesize soluble siderophores (41, 44, 60, 70). All tested meningococcal strains utilize LF (40, 48) as well as TF (4, 41) as an Fe source. Although all gonococcal strains can utilize TF as an Fe source (41), many cannot use LF-bound Fe (40, 72). Surveys of clinical isolates showed that 47% of 59 (40) and 70% of 44 (72) clinical isolates were unable to obtain Fe from LF. The existence of clinical isolates unable to bind LF has been questioned by Schryvers and Lee (56). Gonococci can utilize heme, hemin, hemoglobin, and certain siderophores produced by other bacteria as Fe sources, however (41, 71). The majority of nonpathogenic *Neisseria* spp. cannot use either TF or LF as an Fe source (40, 41). Thus, TF may be an essential source of Fe in vivo, not only in serum but also on mucosal surfaces, where TF is exuded during local inflammation (24). The role of LF-bound Fe utilization in survival on mucosal surfaces or in other stages of infection is unclear.

The uptake of Fe from TF, in gonococci, involves two receptor proteins, designated Tbp1 and Tbp2 (100 and 80 to 88 kDa, respectively) (2, 3, 17–19, 34, 56). The genes *tbpA* and *tbpB* encoding these proteins and their roles in TF binding and Fe acquisition have been described (2, 17–19). The integral outer membrane protein Tbp1 is required for Fe transport across the outer membrane and appears to be essential for Fe acquisition from TF. Tbp2, a surface-exposed lipoprotein, is not strictly necessary for growth on TF but makes Fe utilization more efficient (2, 18). A similar TF receptor system consisting of TBP1 (95 kDa) and TBP2 (69 to 85 kDa) has also been described in meningococci (36).

Relatively less is known about Fe uptake from LF than from TF in either gonococci or meningococci. The requirement for direct contact of cells with LF-bound Fe suggested that a cell surface receptor for LF was present (39, 57). An LF-specific gonococcal receptor was identified by Lee and Schryvers by use of a solid-phase binding assay (35). Affinity purification enabled Schryvers and Lee (56), Lee and Bryan (34), and Cornelissen et al. (18) to isolate a protein of 101 or 105 kDa as the putative LF receptor in the gonococcus. Recently, a meningococcal gene designated *iroA* encoding a 98-kDa Fe-regulated outer membrane protein was cloned and sequenced (49). The *iroA* gene product was identified subsequently as an LF receptor, and the gene *iroA* was renamed *lbpA* (48).

In this study, we sought molecular evidence for the identity of the gonococcal LF receptor and for the defect in isolates unable to use LF as a sole Fe source. Results showed that gonococcal *lbpA* was very similar to the meningococcal homolog and that *lbpA* mutations were common in clinical isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *N. gonorrhoeae* strains used were wild-type FA19 (39) and MS11 (M. So, Oregon Health Sciences University, Portland), LF$^-$ clinical isolates F62 (*lrf-2*), FA1012 (*lrf-3*), FA1090 (*lrf-8*), FA1092 (*lrf-5*), FA1095 (*lrf-6*), FA3000 (*lrf-4*), and FA3002 (*lrf-7*), an LF$^-$ ethyl methanesulfonate-induced mutant FA6353 (*lrf-1*), and a TF$^-$ mutant FA6838 (13, 16, 32). In addition, 34 strains of *N. gonorrhoeae* isolated from female prostitutes in Nairobi, Africa, were obtained through the courtesy of Ian Maclean, University of Manitoba, Manitoba, Winnipeg, Canada. Gonococci were maintained routinely on GCB agar (Difco Laboratories, Detroit, Mich.) containing Kellogg supplements I and II (32). Cultures were incubated at 37°C with a 5% CO_2 atmosphere. Chemically defined medium (CDM) has been described previously (13). Free Fe was removed from the medium by adsorption through Chelex-100 (Bio-Rad, Richmond, Calif.). Determination of the ability of a mutant to grow on LF was accomplished by streaking colonies from GCB agar plates onto CDM agar plates containing 2.5 μM either LF or TF, each saturated to about 20% with Fe, and then incubating the plates for about 40 h. Chelated CDM broth supplemented with 5 μM partially Fe-saturated LF also was used to test the ability to use LF as an Fe source. Growth in CDM broth was monitored

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by optical density with a Klett-Summerson colorimeter with a green filter. LF and TF were purchased from U.S. Biochemical Corporation, Cleveland, Ohio, and from Sigma Chemical Co., St. Louis, Mo., respectively. *Escherichia coli* cells were grown in LB medium supplemented with appropriate antibiotics at 37°C.

Membrane preparation. Membranes were prepared in accordance with an established procedure (18, 70).

SDS-PAGE and Western blot (immunoblot) analysis. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% gels (33). Protein bands were visualized by staining with either silver (73) or Coomassie brilliant blue. For Western blot analysis, proteins from either total or outer membranes were separated by SDS-PAGE and then transferred electrophoretically to nitrocellulose (67). Immunoblotting was performed as described previously (18) with a 1:2,000 dilution of the monoclonal antibody (MAb) A4FA7, which is specific for meningococcal Lbp (49) and which was kindly donated by Jan T. Poolman, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Whole-cell LF binding assay. Overnight cultures of gonococci in CDM broth were diluted in fresh CDM medium to an adjusted Klett reading of approximately 30, added to duplicate cultures containing either no added Fe or 100 μM ferric citrate, and incubated in a rotary shaker with 5% CO_2 for about 5 h until they reached stationary phase. About 10^8 CFU were applied to a nitrocellulose filter by use of a dot blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.). For dot blot assays of LF receptor function, filters were probed with 10 μg of partially Fe-saturated human LF per ml, washed three times for 5 min each time in high-salt buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl), and then detected with horseradish peroxidase (HRP)-conjugated human LF antibody (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Dot blot assays for TF receptor activity utilized HRP-conjugated TF and have been described previously (13).

DNA isolation, labeling, colony hybridization, and Southern blotting. Chromosomal DNA was isolated and purified by CsCl gradient ultracentrifugation as described previously (18). Plasmid DNA was isolated by use of Qiagen (Studio City, Calif.) columns. DNA was digested with enzymes in accordance with the manufacturer's instructions. Plasmid probes were labeled with [α-^{32}P]dCTP (ICN Radiochemicals) by use of the random prime DNA labeling kit from Boehringer Mannheim Biochemicals. Oligonucleotides were labeled with [γ-^{32}P]ATP (ICN) by use of polynucleotide kinase by the method of Sambrook et al. (53). Colony hybridization was carried out as described previously with ^{32}P-labeled DNA (53). For Southern hybridization, DNA was cut with restriction enzymes, and fragments were separated by electrophoresis on a 0.8% agarose gel and then transferred to Zetabind nylon membranes (Cuno, Inc., Meriden, Conn.) as described previously (53). The Southern blot was hybridized and washed under high-stringency conditions (11).

^{55}Fe uptake. The Fe uptake protocol was described previously (13, 39).

Transformation. *E. coli* was transformed by the calcium chloride shock procedure (28). Gonococci were transformed as described previously (10, 13, 61). LF^+ or TF^+ transformants were selected on CDM supplemented with either 2.5 μM LF or 2.5 μM TF, each about 20% saturated with Fe.

FA19 chromosomal library construction and screening for clones. A complete *Mlu*I digest of FA19 (LF^+) chromosome was fractionated into 2- to 4-, 4- to 6-, or 6- to 10-kb fragments on an agarose gel. Subsequently, purified DNA from each size class was tested for transformation of LF^- strain FA6353 to the LF^+ phenotype. The results showed that the 4- to 6-kb fragments produced the most LF^+ transformants (data not shown). *Mlu*I-digested FA19 DNA fragments of 4 to 6 kb were excised and purified with the Geneclean II kit (Bio 101, Inc., La Jolla, Calif.) and then ligated to pBluescript derivative pUNCH615 (66) digested previously with *Mlu*I and dephosphorylated with shrimp alkaline phosphatase as described in the manufacturer's instructions (U.S. Biochemical). Other libraries were constructed in pBR322 (Bethesda Research Laboratories [BRL]) with 1.8- to 2.5-kb and 2.5- to 3.2-kb complete *Ava*I digests and 2- to 3-kb complete *Ssp*I digests ligated separately into the corresponding sites of linearized pBR322 (BRL) dephosphorylated with shrimp alkaline phosphatase.

The ligation mixtures were used to transform *E. coli* DH5α MCR (BRL). Ampicillin-resistant (Ap^r) transformants were screened for clones containing the LF receptor gene by a method adapted from Spratt (62). Agar plates containing about 70 colonies, about half of which were recombinant plasmids, were replicated onto a similar medium and then incubated at 37°C for about 12 h. Pools of colonies were then scraped from the entire plate, and from each pool, plasmid DNA was isolated by the Qiascreen protocol (Qiagen). The original plates were stored. DNA from each pool at a concentration of 10 μg/ml was used to transform piliated cells of a gonococcal LF^- strain, FA6353, selecting for growth on CDM-LF agar. Pools of DNA that produced LF^+ transformants were identified, and progressively smaller pools consisting of fewer colonies were screened in a similar manner until a single recombinant LF^+ clone was identified.

Transposon mutagenesis. The shuttle mutagenesis method developed by Seifert et al. (58) was used to insert mTnCm3 into pUNCH123.

DNA sequencing and sequence analysis. DNA was sequenced directly from double-stranded plasmid DNA at the Automated DNA Sequencing Facility of the University of North Carolina—Chapel Hill with an Applied Biosystems model 373A DNA sequencer by use of the Taq DyeDeoxy Terminator Cycle Sequencing kit or by the dideoxynucleotide chain termination method (55) with Sequenase version 2.0 (U.S. Biochemical). The sequence was determined by use of a set of nested deletions generated by exonuclease III (46), allowing the use of universal sequence primers as well as designed synthetic primers to complete desired regions. The DNA sequence was determined for both strands of the *lbpA* sequence. Computer analysis of the resulting sequence information was performed with the Genetics Computer Group (University of Wisconsin, Madison) package (21).

Nucleotide sequence accession number. The nucleotide sequence of *lbpA* reported here has been deposited in GenBank under the accession number U16260.

RESULTS

Isolation of the LF receptor gene. To isolate the gene for the wild-type LF receptor, we constructed a gonococcal library enriched for LF^+ DNA fragments and screened for the ability to transform an LF receptor-deficient mutant to LF^+ (see Materials and Methods). Approximately 1,800 *E. coli* transformants obtained from the *Mlu*I FA19 library in pUNCH615 were divided into 26 pools and screened for their ability to transform FA6353 (LF^-) to LF^+. One of these 26 pools yielded a significant number of LF^+ transformants. The screening procedure was repeated with individual colonies from the positive pool, and one *E. coli* isolate whose DNA transformed FA6353 to LF^+ at a frequency of 10^{-4}/CFU was identified. Restriction analysis and hybridization studies showed that pUNCH122 contained a 2-kb gonococcal insert (data not shown). The recombinant plasmid pUNCH122 presumably contained at least a portion of the LF receptor gene. Further studies described later in this section confirmed this observation.

The entire LF receptor gene was then cloned (Fig. 1). Oligonucleotide primers close to the ends of the 2.0-kb insert in pUNCH122 were used as probes for Southern hybridization of FA19 chromosomal DNA digested with several different restriction enzymes. A 17-mer oligonucleotide primer (GBL22) homologous to the 5′ end of pUNCH122 hybridized to a 2.7-kb *Ava*I fragment and a 2.5-kb *Ssp*I fragment, while the other probe, a 17-mer oligonucleotide primer (GBL21) homologous to the 3′ end of pUNCH122, hybridized to a 2.3-kb *Ava*I fragment on Southern blots (data not shown). Three separate size-fractionated libraries containing either 2.3- or 2.7-kb *Ava*I fragments or 2.5-kb *Ssp*I fragments were constructed (see Materials and Methods). Clones from each library that hybridized with the appropriate oligonucleotide were isolated. One positive clone from each library, designated pUNCH124, pUNCH126, and pUNCH127, was selected for further study. The clones were shown subsequently by sequencing and restriction mapping to overlap partially (Fig. 1).

Transposon mutagenesis of the LF receptor gene. To determine whether the recombinant plasmid pUNCH122 contained the desired gene, we constructed an mTnCm3 insertion mutant of pUNCH122 designated pUNCH129 and used pUNCH129 to transform FA19 to Cm^r. Transformants were scored for the LF^- phenotype, and one designated FA6775 was analyzed in Southern blots for the site of the mTnCm3 insertion. DNA from FA19 and FA6775 was digested with *Ava*I and probed with the 2.7-kb *Ava*I fragment from pUNCH126. Examination revealed hybridization to a 4.3-kb *Ava*I fragment in FA6775 (Fig. 2A, lane 2) but to a 2.7-kb fragment in FA19 (Fig. 2A, lane 1). The 1.6-kb increase in the size of the *Ava*I fragment in FA6775 corresponded to the expected size of the transposon. Furthermore, hybridization with an mTnCm3-specific probe (Fig. 2B, lane 2) confirmed that the 4.3-kb *Ava*I fragment in FA6775 was due to a double crossover event between the mTnCm3 mutagenized insert in pUNCH129 and the desired chromosomal fragment.

LF utilization. Growth of FA6775 was reduced significantly in CDM supplemented with LF, although growth was normal

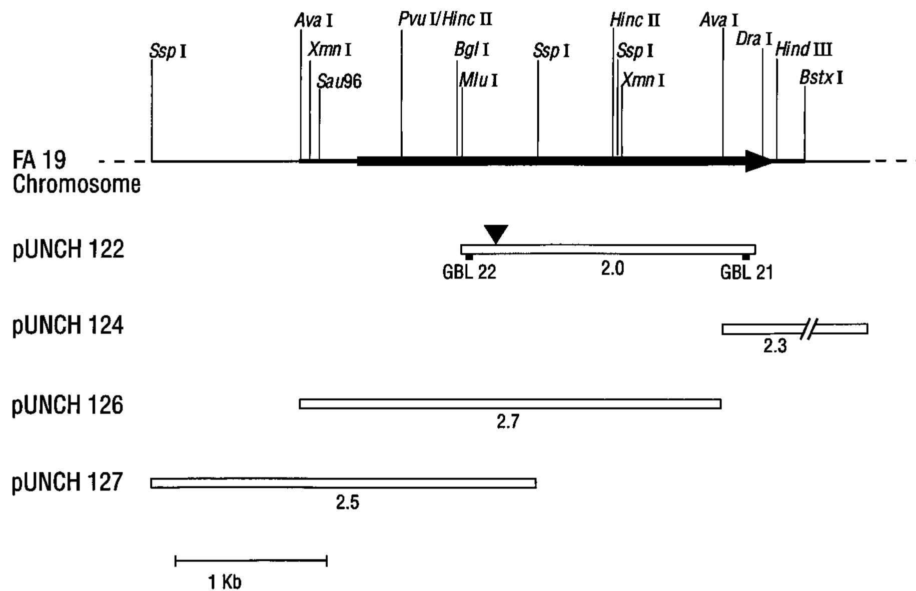

FIG. 1. Physical map of the *N. gonorrhoeae lbpA* gene. The restriction map of the cloned FA19 chromosomal fragment(s) is shown at the top. Plasmid designations are given on the left of the schematic illustration of each insert. GBL21 and GBL22 refer to the oligonucleotides used as probes in isolating the clones pUNCH124 (GBL21) and pUNCH126 and pUNCH127 (GBL22). The length of the cloned insert is shown in kilobases. The heavy and mid-heavy regions of the FA19 chromosome represent sequenced DNA as shown in Fig. 6. The *lbpA* ORF is marked with a heavy arrow. Symbol: ▼, position of the mTnCm3 insertion in pUNCH129 and strain FA6775.

in CDM supplemented with TF (Fig. 3). Growth inhibition in CDM containing LF was presumably due to sequestering of free Fe by LF. Fe uptake assays employing either [^{55}Fe]LF or [^{55}Fe]TF showed that FA6775 was unable to take up [^{55}Fe]LF but accumulated ^{55}Fe from TF normally (data not shown). These data showed that FA6775 was incapable of using LF as an Fe source.

LF binding. An indirect solid-phase dot blot assay employing HRP-labeled anti-human LF antibodies was used to evaluate LF binding. LF^{+} strain FA19 bound LF well, as expected, and binding was enhanced when the cells were Fe stressed (Fig. 4). The transposon mutant FA6775 failed to bind detectable LF by this assay (Fig. 4). The failure of FA6775 to bind LF

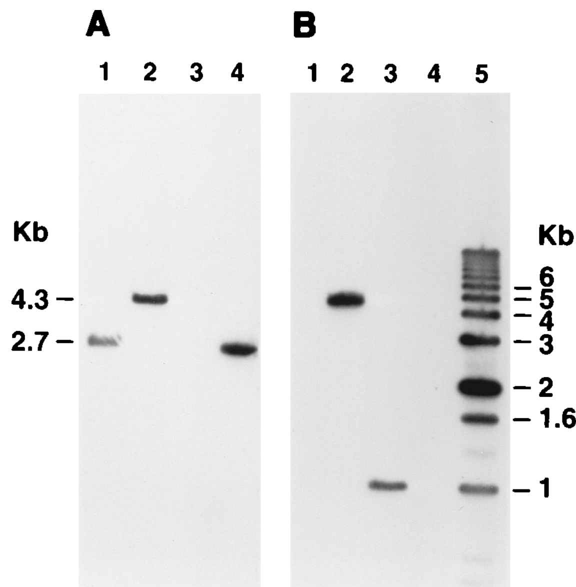

FIG. 2. Characterization of the transposon mutant FA6775. Chromosomal DNAs (5 μg each) were digested with *Ava*I and subjected to Southern blotting. The sizes (in kilobases) of the bands are shown on the left. (A) The probe was a ^{32}P-labeled 2.7-kb *Ava*I fragment from pUNCH126. (B) The probe was a mixture of ^{32}P-labeled 1-kb mTnCm3 fragment and ^{32}P-labeled 1.0-kb DNA ladder (GIBCO-BRL). Lanes: 1, wild-type strain FA19; 2, mutant strain FA6775; 3, 1.0-kb *cat* DNA (probe); 4, 2.7-kb *Ava*I insert DNA (probe); 5, 1.0-kb DNA ladder (with molecular sizes in kilobases on the right).

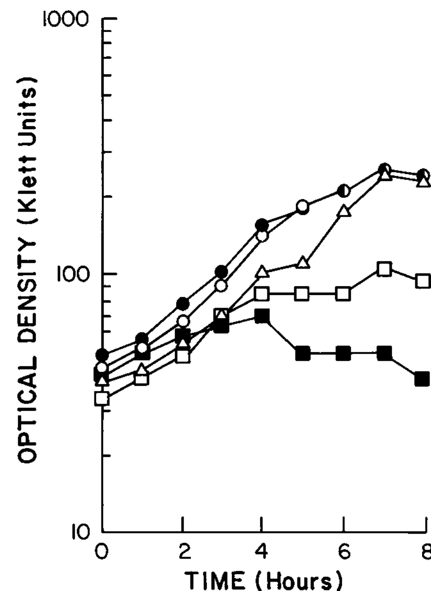

FIG. 3. Growth of the *lbpA* mutant FA6775 in CDM in the presence of LF. Cells were grown initially in CDM overnight and then reinoculated into CDM or CDM supplemented with LF (5 μM) or TF (5 μM). Symbols: ●, FA19 TF; ○, FA19 LF; □, FA6775 with no addition; △, FA6775 TF; ■, FA6775 LF. The results represent a mean of two experiments.

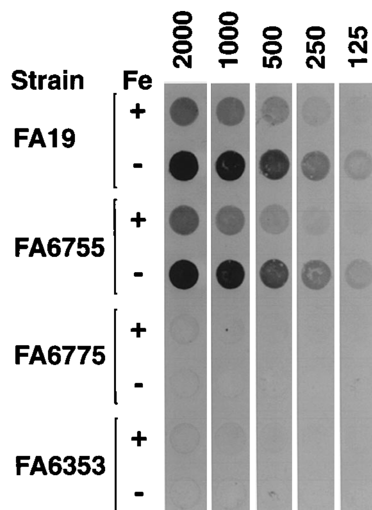

FIG. 4. Binding of LF to Fe-sufficient (Fe^+) and Fe-stressed (Fe^-) whole cells of the following strains: wild-type FA19; LF^+ transformant of FA6353, i.e., FA6755; LF^- transposon mutant FA6775; and LF^- ethylmethane sulfonate-induced mutant FA6353. Concentrations of HRP-conjugated anti-LF antibody (in nanograms per milliliter) are shown above each column.

was independent of Fe starvation, indicating loss of a functional LF receptor. An LF^+ transformant designated FA6755 obtained by transforming pUNCH122 DNA into LF^- strain FA6353 restored the ability to bind LF by this assay, confirming that pUNCH122 contained DNA essential to expression of an LF receptor.

SDS-PAGE and Western blot analysis. Outer membranes prepared from different strains were analyzed by SDS-PAGE for expression of the Fe-repressible proteins (Fig. 5). Wild-type strain FA19 (lane 4) and an isogenic LF^+ TF^- strain, FA6338 (lane 2), exhibited a protein band on a silver-stained SDS-polyacrylamide gel which was slightly larger than 100-kDa Tbp1 (Fig. 5A, lanes 4 and 6). The LF^- transposon mutant FA6775 (lane 6) and an LF^- TF^- double mutant, FA6783, obtained by transforming the TF^- FA6838 strain with LF^- FA6775 DNA (lane 8) lacked the same protein band (Fig. 5A). This band migrated at the expected position of affinity-purified Lbp (19, 34, 56) and was seen more readily in mutant FA6338 lacking 100-kDa Tbp1 (lane 2). Immunoblot assays (Fig. 5B) showed that LF^- mutant strains FA6775 and FA6783 (lanes 6 and 8) failed to react with a MAb specific for meningococcal Lbp (48, 49). We concluded that the mTnCm3 insertion in FA6775 blocked the production of Lbp1, a receptor for LF, presumably by inactivating the *lbpA* structural gene encoding Lbp1. We also noted that the $Tbp1^-$ mutants showed increased amounts of Lbp1 compared with the $Tbp1^+$ strains in both SDS-polyacrylamide gel and Western blot assays (Fig. 5A, lane 2, and Fig. 5B, lane 2). The amount of Lbp1 produced in the wild-type strain FA19 was significantly less than the amount of produced Tbp1 (Fig. 5A, lane 4).

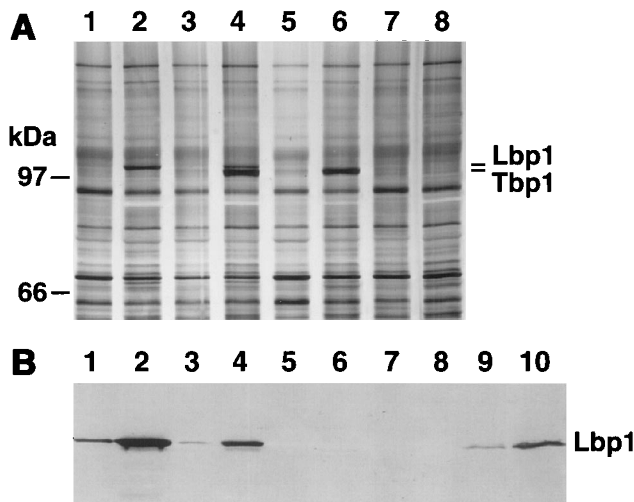

FIG. 5. Lbp1 expression by gonococcal and meningococcal strains. (A) Silver-stained SDS–7.5% polyacrylamide gel; (B) Western blot probed with MAb A4FA7. Lanes 1 to 8 contain outer membranes from gonococcal strains; lanes 9 and 10 contain total membranes from a meningococcal strain. Lanes 1, 3, 5, 7, and 9 contain membranes prepared from Fe-sufficient cells; lanes 2, 4, 6, 8, and 10 contain membranes prepared from Fe-stressed cells. Lanes: 1 and 2, LF^+ TF^- FA6338; 3 and 4, wild-type FA19; 5 and 6, LF^- TF^+ FA6775; 7 and 8, LF^- TF^- FA6783; 9 and 10, wild-type *N. meningitidis*, strain FAM20 (66).

Nucleotide sequence analysis. The DNA sequence obtained from pUNCH122, pUNCH124, pUNCH126, and pUNCH127 (Fig. 1) is presented in Fig. 6. This 3.3-kb region revealed an open reading frame (ORF) of 2,832 nucleotides starting with ATG at position 278 and ending with TAA at position 3110. Putative regulatory sites were identified on the basis of their similarity to *E. coli* consensus sequences. An AGGTGG that resembles the ribosome binding site sequence AGGAGG (59) was located 5 bases upstream from the putative translational start site. A sequence GAT ATT GAA AAT GAA GTT G which matched the consensus *E. coli* Fur box sequence gaT aAT gat aAt cAT Tat c (20) at four of seven conserved positions was located 88 bases upstream of the ribosome binding site. Unambiguous −10 and −35 sequences (29) were not clearly identified by inspection of the DNA sequence. The 10-base sequence TTCAGACGGC located 549 bases downstream from the translational start site was identical with the gonococcal DNA uptake sequence (22, 25). Eight bases downstream from the termination codon TAA, there was a sequence, GCCGTCTGGA, that shares 9 of 10 bases of the gonococcal DNA uptake sequence (22, 25). Further downstream was a string of 7 Ts, suggesting the existence of a transcription terminator, but no inverted repeat typical of a rho-independent transcription terminator, was found. We designated this ORF as *lbpA*, because it clearly encoded a protein (Lbp1) involved in the function or expression of the LF receptor.

Translation of the *lbpA* coding region predicted a protein of 943 amino acids. A typical signal sequence of 24 amino acids was located at the N terminus. Limited amino-terminal sequence of the mature Lbp protein confirmed that the mature protein started at amino acid 25 of the ORF shown in Fig. 6 (data not shown). The 919-amino-acid mature polypeptide had a calculated molecular mass of 103 kDa and a pI of 10.16. This predicted size corresponded well with the observed size of Lbp of 101 to 105 kDa on SDS-PAGE (18, 35, 56).

A comparison of the nucleotide sequence of gonococcal *lbpA* with that of meningococcal *lbpA* (49) revealed that they were 94% identical. The upstream sequence of the gonococcal *lbpA* lacked the 6-base repeat of either GAT GTT or GAT GCT that is repeated eight times in the meningococcal *lbpA* gene sequence (Fig. 6) (49). The deduced amino acid sequence of Lbp1 from the gonococcus was 94% identical to that of the meningococcal Lbp (49) (Fig. 6). Lbp1 also was 46% identical and 65% similar to Tbp1 (18) of the same gonococcal strain (data not shown) but only 18% identical and 42% similar to Tbp2 (2). Alignment of Lbp1 to TonB-dependent outer mem-

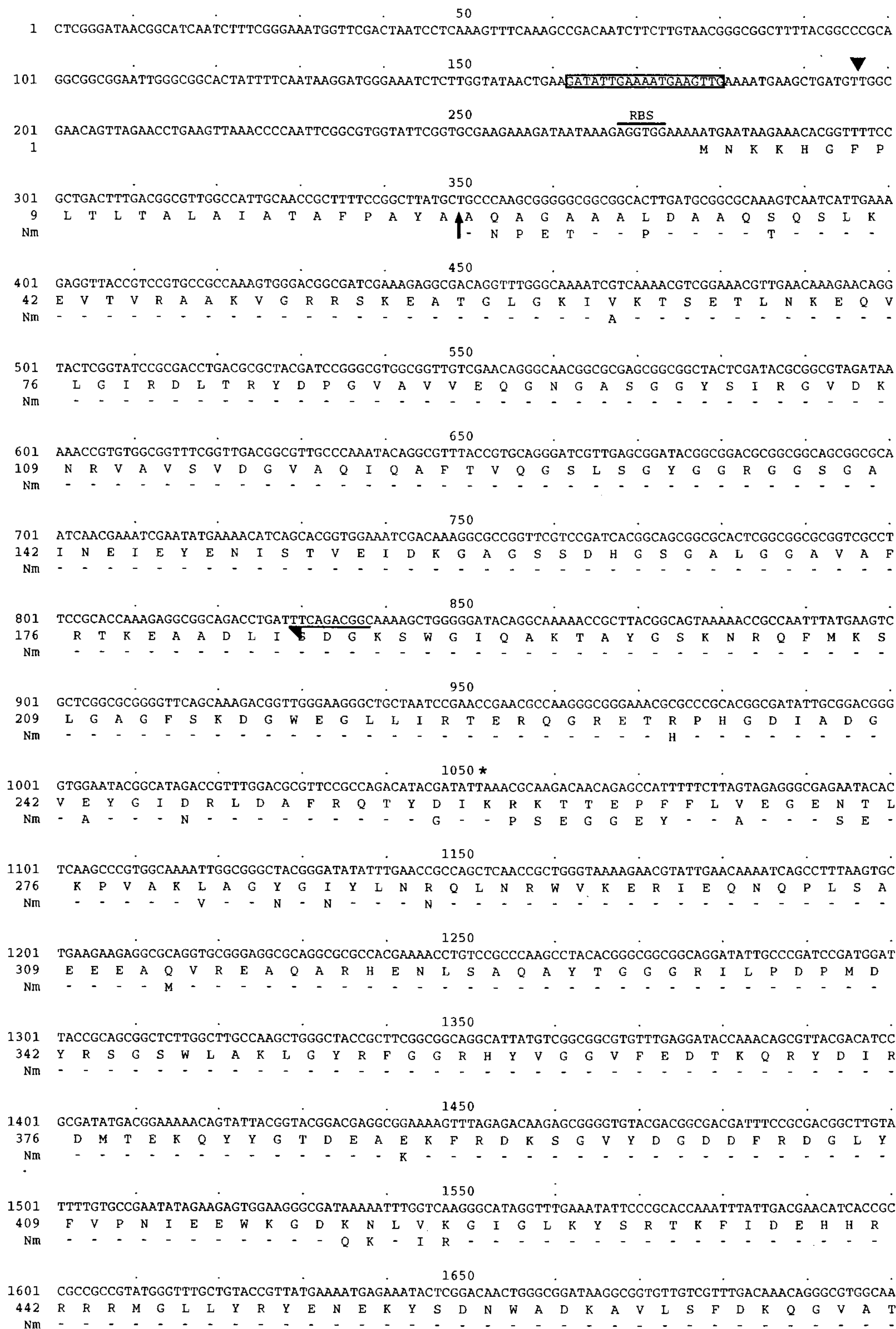

FIG. 6. DNA sequence of the *N. gonorrhoeae lbpA* gene and comparison of the predicted amino acid sequences of *N. gonorrhoeae* and *N. meningitidis* (Nm). The nucleotide sequence of the *lbpA* region starts from the left-most *Ava*I site shown in Fig. 1. The putative ribosome binding site (RBS) is marked, the possible Fur binding site is boxed, and the possible DNA uptake sequences are indicated by horizontal arrows. A typical leader sequence cleavage site (vertical arrow) is proposed. Symbol: ▼, site of the missing 6-bp repeat present in *N. meningitidis lbpA*. Residues of *N. meningitidis* that are identical to Lbp1 of *N. gonorrhoeae* are indicated by dashes. The asterisk at nucleotide position 1052 indicates the site of 3-bp region (AAA) which is deleted in *N. gonorrhoeae* but which is present in *N. meningitidis*. The double asterisk at nucleotide position 1988 shows the site of a 3-bp region (AAT) which is deleted in *N. meningitidis* but which is present in *N. gonorrhoeae.*

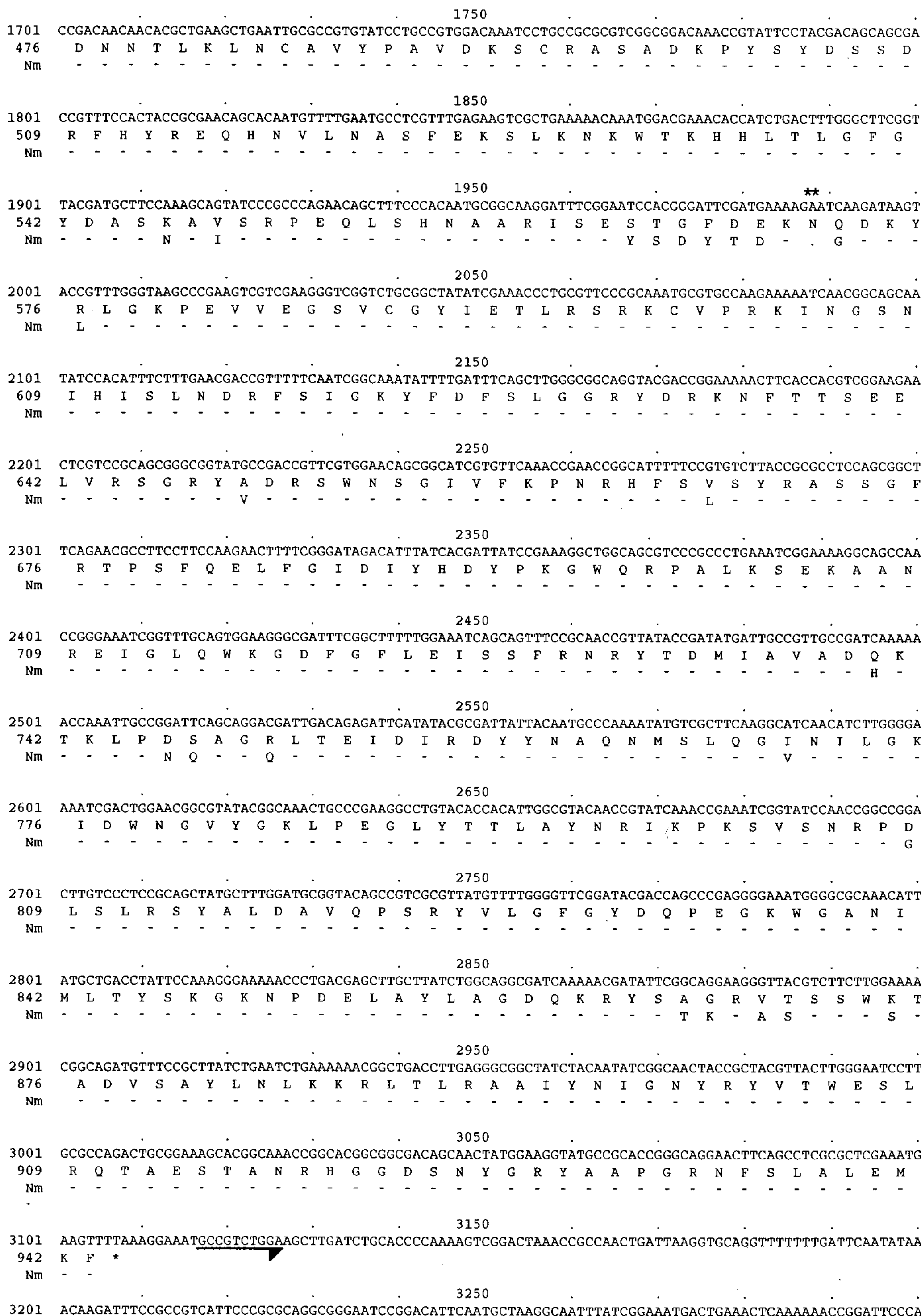

FIG. 6—*Continued.*

brane receptors (data not shown) showed that Lbp1 was homologous to the TonB-dependent family of receptors, as was true for Tbp1 (18) but not for Tbp2 (2). Sequences upstream or downstream of the *lbpA* ORF did not show significant amino acid sequence similarity with other known proteins currently in GenBank.

Mutation in natural LF$^-$ isolates. Genetic linkage and LF receptor functional studies reported earlier suggested that the *lrf* mutations in LF$^-$ clinical isolates affect LF receptor gene expression (13). Transforming DNAs from pUNCH126 (containing all but the 3′ end of *lbpA*) and pUNCH124 (containing the 3′ end of the *lbpA*) (Fig. 1) were tested for their ability to transform seven LF$^-$ clinical isolates to LF$^+$. These strains included F62, FA1012, FA1092, FA1095, FA3002, and FA6748, which

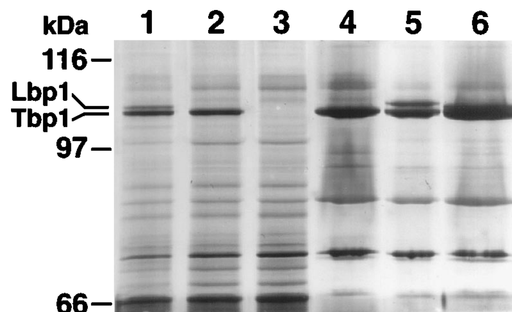

FIG. 7. Lack of Lbp1 expression due to *lrf-4*, a natural *lbpA* mutation. A silver-stained SDS–7.5% polyacrylamide gel containing outer membranes prepared from Fe-starved cells is shown. The differences in protein profiles (except for Tbp1 and Lbp1) between lanes 1 to 3 and 4 to 6 are due to the use of two different batches of outer membrane preparations. Lanes: 1, LF^+ FA19; 2, LF^- FA6775; 3, LF^- TF^- FA6783; 4, LF^- FA6748; 5, LF^+ FA6854 (a transformant of FA6748); 6, LF^+ FA6859 (a revertant of FA6748).

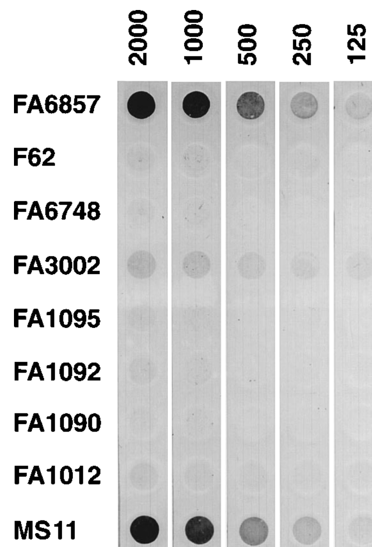

FIG. 8. Inability of Fe-starved whole cells of LF^- natural gonococcal isolates to bind LF. Concentrations of HRP-conjugated anti-LF antibody (in nanograms per milliliter) are shown above each column. Strains are shown on the left.

were previously shown to contain genetically linked *lrf* mutations (13), and an additional strain, FA1090 (16). All but FA1090 and FA3002 were transformed to LF^+ by pUNCH126 DNA, but none was transformed to LF^+ by pUNCH124 DNA (data not shown). The presence of restriction and modification systems active against transforming DNA isolated from a foreign host may account for the failure in transformation of strains FA1090 and FA3002 (64). The *lrf-4* mutation in strain FA3000 could not be transformed to LF^+ with FA19 $lbpA^+$ DNA isolated from *E. coli* cells, but when the FA3000 *lrf-4* mutation was introduced (12) into FA19, creating LF^- FA6748 (*lrf-4*), it was transformed to LF^+ by either pUNCH122 or pUNCH126 DNA at a frequency of about 10^{-4}/CFU (data not shown). Similar experiments with the *lrf* alleles in FA1090 and FA3002 were not undertaken. Thus, the lesions in five of seven tested *lrf* loci were located entirely within the 2.7-kb *Ava*I fragment containing *lbpA*. Since pUNCH126 included an additional 277 bp upstream of the *lbpA* ORF (Fig. 6), it is possible that some *lrf* mutations may be located just outside *lbpA*. The location of the five different *lrf* lesions in the *lbpA* locus is consistent with our previous observation (13) that the *lrf* loci are closely linked.

To further characterize the nature of the clinical *lbpA* mutations, we tested the ability of the LF^- strains to revert spontaneously to LF^+. Strain F62 reverted to LF^+ at a frequency of 2×10^{-8}, while the FA19 derivative strains FA6353 and FA6748 reverted to LF^+ at a frequency of 1×10^{-7} and 2×10^{-6}, respectively. These frequencies suggested that the *lbpA* mutations in these strains were point mutations.

To determine whether the *lbpA* gene was present in naturally occurring LF^- strains, chromosomal DNA from strains F62, FA1012, FA1090, and FA6748 was digested with *Ava*I and tested in a Southern blot with pUNCH126 as the probe. One hybridizing band was detected in all strains tested, although the sizes of the band differed (data not shown). This showed that the *lbpA* gene was present in clinical strains defective in LF utilization.

To determine the effect of clinical *lbpA* mutations on the expression of the 103-kDa protein, we studied the SDS-PAGE membrane profile of LF^- strain FA6748 which contains the *lrf-4* allele (Fig. 7). There was no apparent Lbp1 protein in FA6748. Each of two LF^+ strains, FA6854 (lane 5) and FA6859 (lane 6), obtained by transformation of strain FA6748 with $lbpA^+$ DNA (pUNCH126) and spontaneous reversion of FA6748 to LF^+, respectively, expressed Lbp1. These results showed that *lrf-4* contains a mutation in *lbpA*, although we did not attempt to identify the mutation by sequencing *lrf-4.*

We showed previously that two of the genetically characterized *lrf* mutants lacked LF receptor activity (13). We tested an additional 10 natural (clinical) LF^- strains, including FA1012, FA1090, FA1092, FA1095, FA3002, FA6748, and F62, for LF binding in a solid-phase dot blot assay and obtained similar results (Fig. 8 and data not shown). Conversely, all tested LF^+ strains, including 16 clinical isolates obtained from Ian Maclean, three LF^+ transformants, namely, FA6857, FA6854, and FA6755, obtained by transforming F62 and FA6748 with pUNCH126 and FA6353 with pUNCH122, and three spontaneous revertants, namely, FA6858, FA6859, and FA6860, derived from LF^- strains F62, FA6748, and FA6353, respectively, exhibited LF receptor activity (Fig. 4 and 8 and data not shown). This further supported the conclusion that strains unable to use LF as an Fe source are uniformly lacking in LF receptor activity.

Western blots were used to test whether the LF^- isolates expressed detectable 103-kDa Lbp1 (Fig. 5 and 9). None of 10 tested LF^- clinical isolates expressed detectable levels of Lbp1, whereas all 18 LF^+ strains tested did express Lbp1, as detected by binding of MAb A4FA7 (Fig. 9 and data not shown). LF^+ transformants or revertants of LF^- strains (FA6755, FA6854, FA6857, FA6858, FA6859, and FA6860) were restored in their abilities to bind MAb A4FA7 (Fig. 9 and data not shown). We concluded that natural LF^- isolates expressed no Lbp1, bound no detectable LF by solid-phase dot

FIG. 9. Lack of Lbp1 expression by LF^- natural isolates of gonococci. A Western blot containing total membranes prepared from Fe-starved cells probed with MAb A4FA7 is shown. Lanes: 1, LF^+ strain MS11; 2 through 9, LF^- strains FA1012, FA1090, FA1092, FA1095, FA3002, FA6353, FA6748, and F62, respectively; 10, LF^+ transformant of F62, FA6857.

blot, and could be repaired by a wild-type *lbpA* gene or a closely linked upstream locus.

DISCUSSION

A number of human pathogens can bind LF to their cell surface, including *N. gonorrhoeae* (13, 35, 39, 56), *N. meningitidis* (56, 57), *Branhamella catarrhalis* (56), *Mycoplasma pneumoniae* (68), *Trichomonas vaginalis* (47), *Treponema pallidum* (1), *Treponema denticola* (63), *Bordetella pertussis* (52), and *Aeromonas hydrophila* (6). However, the mechanism of Fe uptake from LF has not been well characterized. A putative 105-kDa receptor for LF utilization was identified in pathogenic *Neisseria* spp. by affinity isolation (18, 34, 56). Further evidence for a specific functional LF receptor was provided by competitive solid-phase binding assays (35) and isolation of LF receptor-deficient gonococci (13). We have isolated the gonococcal gene *lbpA*, encoding the 103-kDa gonococcal protein Lbp1, and have proven that Lbp1 is essential to LF receptor function and uptake of Fe from LF. While this work was in progress, an *lbpA* gene in *N. meningitidis* was cloned, sequenced, and expressed in *E. coli*, with similar overall conclusions (48, 49).

The DNA sequence of *lbpA* and the predicted amino acid sequence of the Lbp1 in the gonococcus and the meningococcus are highly conserved. There are 2,832 bases in the ORFs of both gonococcal and meningococcal *lbpA* genes. Both genes contain relatively well-conserved Fur boxes. Recently, *fur* homologs were cloned and characterized in both gonococci (9) and meningococci (66). Whether the 17-kDa Fur protein is able to bind to the putative Fur box and regulates expression of the *lbpA* gene(s) has yet to be determined. Identification of the transcriptional start site of *lbpA* and the regulation of expression of Lbp1 require further work. The striking 94% identity between gonococcal and meningococcal Lbp1 suggests that the two proteins have the same function. A MAb directed against meningococcal Lbp recognized gonococcal Lbp1 specifically in all 18 LF^+ gonococcal strains tested, suggesting conservation of that epitope in all gonococci. Curiously, the same MAb does not bind to all meningococcal Lbp1s (49). The striking conservation of the predicted proteins in gonococci and meningococci (94% identity) strongly suggests that Lbp1 does not undergo much antigenic variation in vivo, perhaps indicating that Lbp1 is masked or not highly surface exposed.

Lbp1 shares features with the functionally related gonococcal TF receptor protein Tbp1 (18). Each protein has a 24-amino-acid signal sequence, similar patterns of alternating hydrophobic and hydrophilic residues in the last 10 amino acids, a C-terminal phenylalanine (65), and extensive homology with the TonB-dependent receptors (31, 51), and each is highly basic. These characteristics are consistent with the possibility that Lbp1 is a TonB-dependent outer membrane receptor protein. The strong similarity between the LF receptor protein Lbp1 and the TF receptor protein Tbp1 suggests that the binding of LF to gonococcal cells might be similar to TF binding. The TF receptor is composed of two proteins, Tbp1 and Tbp2, that arise from transcription of a single *tbpB-tbpA* operon (2). A *tbpB* probe hybridized only to the expected *tbpB* restriction fragments in low-stringency Southern blots (data not shown), suggesting that if there is an *lbpB* gene analogous to *tbpB*, the two genes are not highly conserved. The very sticky nature of LF has made it technically difficult to perform LF Western blots analogous to the TF Western blots that identified Tbp2 (2). Further experiments are required to address the possibility that there could be a second LF-binding protein analogous to Tbp2 (2).

The mechanisms of Fe removal from LF after binding of LF to the receptor and the subsequent transport of Fe across the cell membrane are unknown. Removal of Fe from the Fe-binding proteins LF and TF might involve a common protein. The presence of such a protein is suggested by a *tlu* mutation that does not interfere with LF or TF receptor function but that prevents Fe uptake from either LF or TF (13). Further characterization of the *tlu* locus may elucidate the mechanism of Fe removal from receptor proteins.

Using genetic studies, we showed previously that TF receptor mutations designated *trf* were clustered (13). Subsequently, we showed that all known *trf* mutations lay within a 2.8-kb *tbpA* structural gene (18). LF receptor (*lrf*) mutants are similar in that most of the identified *lrf* mutations are now known to be located in or immediately adjacent to *lbpA*. Naturally occurring *trf* mutations have not been described, but most of the studied *lrf* mutations were identified in clinical isolates. This clearly suggests that Lbp1 is not essential for gonococcal survival on mucosal surfaces.

Mickelsen et al. concluded that many clinical isolates of gonococci were unable to utilize LF-bound Fe on the basis of their inability to support growth in the presence of LF-bound Fe and their failure to take up radioactive Fe from LF (40). This finding was challenged by Schryvers and Lee, who found that all gonococcal isolates exhibited LF receptor activity by a solid-phase binding assay (56). In this study, we confirm that many gonococci are LF receptor deficient and show that they cannot use LF as a sole source of Fe because they are mutant in or near *lbpA* and fail to express Lbp1. Other unpublished surveys showing that at least 30% of over 800 tested gonococcal isolates do not grow on CDM supplemented with LF have been conducted (38a, 72). Therefore, it is unlikely that the discrepancies in our work and that of Schryvers and Lee (56) are due to the use of different collections of strains. We conclude that many gonococci do not express a functional LF receptor. Of course, many other mucosal pathogens, including *Haemophilus influenzae* (30, 50) as well as other bacteria that colonize mucosal surfaces (26, 45), are not able to use LF as an Fe source, suggesting that other sources are available in vivo. Some serum TF probably exudes onto inflamed surfaces (24), which could supply the Fe needs of all gonococci. The advantage of expressing a functional LF receptor remains enigmatic.

LF is bactericidal to many microorganisms (5, 15, 54). The mucosal surface and polymorphonuclear leukocytes are rich in LF (37, 38). It is possible, but untested, that an LF receptor might protect against binding of LF to another site, thereby avoiding the bactericidal effects of LF. It is also possible that a functional LF receptor aids infection in vivo by providing an additional but nonessential means to scavenge Fe from the environment. Further work is required to understand the functional implications of genetic variability in the LF receptor.

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