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Characterization of *Moraxella* (*Branhamella*) *catarrhalis lbpB*, *lbpA*, and Lactoferrin Receptor *orf3* Isogenic Mutants

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Pathogenic members of the family *Neisseriaceae* produce specific receptors to acquire iron from their host's lactoferrin and transferrin. Recently, putative *Moraxella catarrhalis* lactoferrin receptor genes and a third open reading frame (*lbpB*, *lbpA*, and *orf3*) were cloned and sequenced. We describe the preliminary characterization of isogenic mutants deficient in LbpB, LbpA, or Orf3 protein.

The concentration of free iron in the mammalian host is below levels which permit proliferation of invading microbes. To acquire this essential element, the strict human pathogens *Moraxella* (*Branhamella*) *catarrhalis*, *Moraxella lacunata*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* and the strict bovine pathogen *Moraxella bovis* produce host-specific transferrin (Tf) and lactoferrin (Lf) receptors to circumvent the host's iron sequestration (see reference 15 for a review).

This receptor-mediated mechanism has at least two outer membrane components, designated (Tf binding protein [Tbp] or Lf binding protein [Lbp]) A and B, to facilitate iron capture from host Tf or Lf. The essential TonB-dependent A component spans the outer membrane and contains amphipathic beta sheets that are proposed to ultimately form the channel across the outer membrane (19). The predominately hydrophilic B constituent is attached to the outer membrane via a lipid tail. Naturally occurring B^- mutants have not been reported, and genetically engineered B^- mutants are either impaired (2, 6, 14) or are unable (16) to utilize the parent iron source in vitro, implying that B provides a selective advantage in vivo. A significant humoral immune response to B occurs during a natural infection, (8, 9), and antibodies to the B component are bactericidal, (10, 11, 18), suggesting B may be a useful vaccine target.

A unique feature of the iron acquisition process in *M. catarrhalis* is that inactivation of the gene encoding the CopB outer membrane protein abrogates iron acquisition from both human Tf (hTf) and human lactoferrin (hLf) (1, 8). This phenotype is similar to that of insertional mutants in the periplasmic iron binding protein (17) or TonB protein (5) in *Neisseria* species and infers a role for CopB in the iron acquisition process. Although CopB displays some binding activity toward hLf (7, 8), it may not be related to the iron acquisition process. Insertional inactivation of the CopB homolog, FrpB, in pathogenic *Neisseria* spp. does not alter iron acquisition from Tf and Lf (4, 20), and there does not appear to be a CopB homolog in other species with a Tf receptor-mediated iron acquisition pathway, such as *Haemophilus influenzae* (13). This suggests either that the iron acquisition pathway in *M. catarrhalis* is unique or that the phenotype of the *copB* mutants is due to indirect effects on the iron acquisition pathway.

The genes encoding the Tf and Lf receptor proteins are normally found in an operon consisting of the gene encoding the B component immediately preceding the A structural gene (6, 15). The arrangement of the receptor genes differs in *M. catarrhalis*. The *tbpA* gene precedes the *tbpB* gene and is separated by an intervening open reading frame (ORF) (18). Although the order of the Lf receptor genes conforms to the standard arrangement, there is a third unique ORF immediately downstream of *lbpA* with putative independent promoters preceding the *lbpA* and *orf3* genes (11).

To examine the role of the *M. catarrhalis* LbpB, LbpA, and Orf3 proteins in iron acquisition from Lf and whether the genes coding for these proteins are in an operon, we created defined mutants in which the native coding sequences had been replaced with insertionally inactivated derivatives. The individual *lbpB*, *lbpA*, and *orf3* genes were PCR amplified (see Table 1 for primers) from *M. catarrhalis* and subcloned into pT7-7 (22). The ORFs were interrupted with the *kan* cassette from pUC4K (Fig. 1), and the linearized plasmid DNA was used for natural transformation of *M. catarrhalis* N141 (8), selecting for Kan^r isolates. The *lbpB* gene was also inactivated by insertion of a *kan*Ω cassette from pHP45 (12) and then replacement of the pHP45 Kan^r marker with the *kan* cassette from pUC4K, since the original *kan* marker does not appear to be functional in *M. catarrhalis*. Southern hybridization and PCR analysis were performed to confirm gene replacement with the insertionally inactivated derivatives (data not shown).

In order to demonstrate that the insertional inactivation resulted in loss of the appropriate protein, we performed Western blot analysis with the isogenic mutants. Equal wet volumes of pelleted cells (avoids optical density estimation errors due to clumping) grown under iron-limiting conditions were boiled in Laemmli sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted in triplicate. We had previously generated a rabbit polyclonal antiserum against Lbp isolated from *M. catarrhalis* (8); this serum was preabsorbed with LbpB-maltose binding protein (Mbp) (see below) and used to develop one blot (Fig. 2A). Compared to the parental strain (Fig. 2A, lane 1), LbpA production was not altered in the *orf3::kan* mutant (Fig. 2A, lane 5) or the *copB::kan* mutant (Fig. 2A, lane 6). In contrast, LbpA production was abolished in both the *lbpA::kan* (lane 4) and *lbpB::kan*Ω (lane 3) mutants, and was

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TABLE 1. Oligonucleotide primers used for PCR

Gene or region[a]	Primer sequence (5′→3′)	Restriction site added
lbpB 5′	CATATGAGTACTGTCAAAACCCCCCATA	*Nde*I
lbpB 3′	AAGCTTATTTATCTTTAACAGCCCCAAAGAC	*Hin*dIII
lbpA 5′	CATATGACCACGCACCGCTTAAACCTTGCC	*Nde*I
lbpA 3′	AAGCTTAAAACTTCATTTCAAGACTGGC	*Hin*dIII
orf3 5′	CATATGACCTGTTTACCAAAGACC	*Nde*I
orf3 3′	AAGCTTAAAACGACCGACCAAGCT	*Hin*dIII
lbpB mature[b] 5′	TCTAGACGCTCTGATGACATCAGCGTC	*Xba*I
lbpA mature[b] 5′	AGAAGGGGTTCTGGTGTGGCAGTTTTACCCCTA	*Xmn*I
lbpB internal (717)	TTACATCATGACAAAGGCGATGGC	N/A
lbpA internal (727)	TTTGGTATGAAACGCCACCGAACC	N/A
lbpA internal (738)	TTATATAATGCCACCATCAAAGGGG	N/A
orf3 internal (732)	AGATGACAGCTCATGCGTGGTG	N/A
orf3 internal (728)	AAGGCGTTGATGGGTAAAGGGG	N/A
tbpA 5′ (223)	GCGCAA(TC)GA(AG)GTNACNGGNNTNGGNAA[c]	N/A
tbpA 3′ (424)	CCCGGGAGATCTTTAAAACTTCATTTCAAGTGC	N/A

[a] Numbers in parentheses represent oligonucleotides described in the legend to Fig. 4.
[b] Primer used for preparing in-frame fusion with the *malE* gene in the pMalC vector.
[c] N represents an equimolar mixture of the four nucleotides.

diminished following insertion of the *kan* cassette into the *lbpB* gene (lane 2), suggestive of an operonic organization.

A duplicate blot was probed with antiserum prepared against purified recombinant LbpB-Mbp fusion protein for detection of LbpB production (Fig. 2B). Compared to the parental strain (Fig. 2B, lane 1), insertional inactivation of the *lbpB* gene (Fig. 2B, lanes 2 and 3) resulted in the loss of LbpB, whereas LbpB expression was unaffected in the *lbpA::kan*, *orf3::kan*, and *copB::kan* mutants (Fig. 2B, lanes 4, 5, and 6, respectively). We were unable to produce a fusion protein of Orf3 with Mbp, and thus could not prepare antiserum for assessment of Orf3 production. As a control, we probed a replicate blot with antiserum prepared against native Tf receptor complex (Fig. 2C) (7). Neither TbpA expression (solid box) nor TbpB expression (solid circle) was altered by any of the *kan* insertions.

In order to provide alternate evidence for the operonic arrangement of *lbpB* and *lbpA* and to determine whether *orf3* was part of the *lbp* operon, we initiated reverse transcription-PCR (RT-PCR) experiments. The RT-PCR experiments were performed with SuperScript II RNase H^{-} reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. Total RNA was isolated with the Qiagen RNeasy mini kit (Qiagen, Inc.) and subjected to amplification-grade DNase I (Gibco BRL) treatment in the presence of RNasin (Promega Corporation). cDNA was generated by using oligonucleotide 728 (Fig. 1) and treated with RNase H (Gibco BRL), prior to being used as a template for subsequent PCR. RT-PCR products were obtained from intergenic regions between *lbpB*::*lbpA* and *lbpA*::*orf3* (see Fig. 4, lanes 2 and 4, respectively). The same reactions did not yield the corresponding bands when RNA from the *lbpB::kan*Ω strain was used (data not shown). As a control to exclude the possibility of chromosomal contamination, oligonucleotides specific for the *M. catarrhalis tbpA*

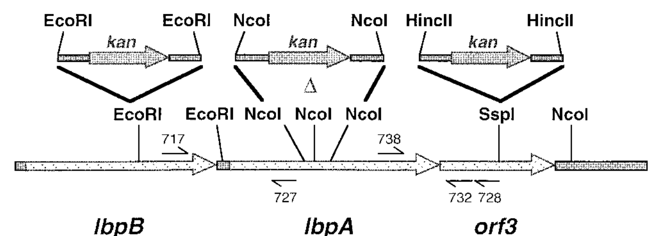

FIG. 1. Construction of *M. catarrhalis* N141 isogenic mutants. A kanamycin resistance marker (*kan*) derived from pUC4K with either flanking *Eco*RI sites, *Nco*I sites, or *Hin*cII sites was ligated into either the unique *Eco*RI site of the *lbpB* gene, between the *Nco*I sites of the *lbpA* gene (intervening DNA removed), or the unique *Ssp*I site of the Lfr *orf3* gene, respectively. The DNA was linearized with restriction sites that flanked the DNA inserts and then used for natural transformation into *M. catarrhalis* N141, and mutants were selected on BHI medium containing 20 μg of kanamycin sulfate per ml.

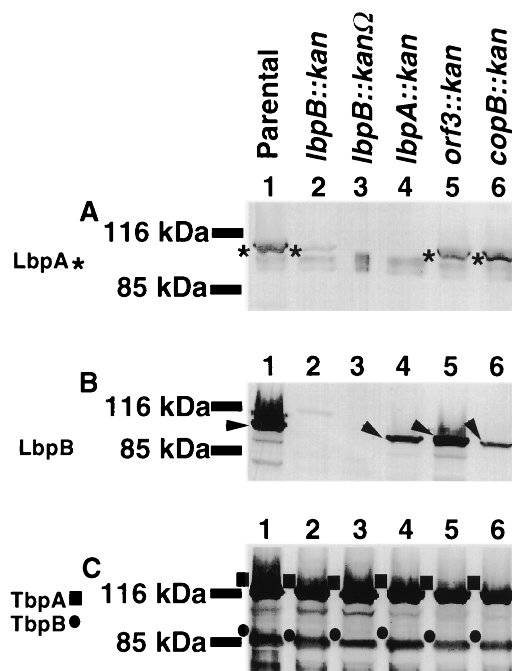

FIG. 2. Western blot analysis of *M. catarrhalis* mutants. Equal amounts of *M. catarrhalis* cells grown under iron-limiting conditions were resuspended and boiled in Laemmli sample buffer prior to SDS-PAGE and Western blotting. The remaining binding sites on the blots were blocked with Tris-buffered saline (pH 7.5) containing 0.5% Bio-Rad nonfat dry milk blocker and incubated with rabbit polyclonal antisera (A) predominantly specific for LbpA (B), Mbp::LbpB, or (C) TbpA and TbpB. The blots were washed in Tris-buffered saline and then incubated with HRP-labeled antirabbit sera and developed.

FIG. 3. Analysis of binding of various mutants to HRP-hLf conjugates. Equal amounts of resuspended *M. catarrhalis* cells grown under iron-limiting conditions were spotted onto the cellulose acetate-nitrocellulose membranes. The blot was placed in blocking solution and then incubated in low-stringency Lf binding buffer (50 mM Tris buffer containing 0.1 M NaCl [pH 6.0]), containing 1:1,000 dilutions of peroxidase-conjugated hLf (HRP-hLf). The blots were repeatedly washed in Tris-buffered saline and then developed with the HRP-substrate mixture.

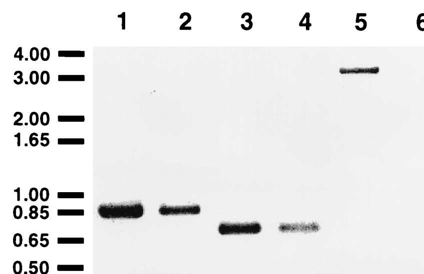

FIG. 4. RT-PCR analysis of the *lbp* operon. *M. catarrhalis* N141 was grown under iron-limiting conditions were used in the Qiagen RNeasy mini kit for total RNA isolation. Purified RNA was subjected to amplification-grade DNase I (Gibco BRL) in the presence of RNasin (Promega Corporation). RT was performed with SuperScript II RNase H^- reverse transcriptase (Gibco BRL) by using oligonucleotide 728. The resulting cDNA was subjected to RNase H (Gibco BRL) prior to use in subsequent PCR analysis. The negative image of the agarose gel is illustrated with the molecular sizes indicated in kilobases on the left. Lanes 1, 3, and 5, PCR with chromosomal DNA. Lanes 2, 4, and 6, PCR with reverse-transcribed cDNA. Lanes 1 and 2, PCR with oligonucleotides 717 and 727. Lanes 3 and 4, PCR with oligonucleotides 738 and 732. Lanes 5 and 6, PCR with oligonucleotides 224 and 424, which amplify *M. catarrhalis tbpA*.

gene were used. The *tbpA* gene was amplified only from the chromosomal preparation (Fig. 4, lane 5), but not the cDNA preparations (lane 6).

A solid-phase binding assay under low-stringency buffer conditions, which enhance detection of Lf-Lbp interactions (6, 8), was performed to evaluate the ligand-binding properties of the isogenic mutant strains. In comparison to the parental strain, the *lbpB::kan* and *lbpA::kan* mutants displayed only weak Lf binding activity (Fig. 3). Residual Lf binding activity was detected with the *lbpB::kan*Ω mutant, possibly due to nonspecific interactions between the cationic portion of Lf (3) with other components present, perhaps CopB (8). In contrast, the Lf binding activity of the *orf3::kan* and *copB::kan* mutants was comparable to that of the parental strain. All isolates displayed similar binding activity for horseradish (HRP) peroxidase-conjugated hTf (data not shown).

The defined mutants were then examined for their ability to utilize various iron sources by using a previously described plate growth assay (7). Iron-starved organisms were spread onto brain heart infusion (BHI) medium (Difco, Detroit, Mich.) containing the iron chelator etheylenediaminedi(*o*-hydroxyphenylacetic) acid (EDDHA [100 μM]) and sterile disks containing the individual iron sources were placed onto the medium to allow localized diffusion. The bovine pathogen *M. bovis*, which utilizes only bovine Lf and Tf, and an *M. catarrhalis* N141 *copB* mutant, which binds hLf and hTf normally, but cannot utilize these iron sources (1, 8), served as controls. Growth of *M. bovis* was observed only when bovine Lf or Tf, and not hLf or hTf, was provided as the iron source (Table 2).

TABLE 2. Growth characteristics of *M. catarrhalis* mutants

Strain description	Growth on sole iron source[a]:			
	hTf	hLf	bLf	bTf
M. catarrhalis wild type	++++	++++	−	−
lbpB::kan	++++	+	−	−
*lbpB::kan*Ω	++++	−	−	−
lbpA::kan	++++	−	−	−
orf3::kan	++++	++++	−	−
copB::kan	−	−	−	−
M. bovis	−	−	++++	++++

[a] ++++, zone of growth surrounding disk greater than 4 mm; +, zone of growth surrounding disk less than 1 mm; −, no observable growth.

With the exception of the *copB* mutant, which was unable to utilize either the human or bovine form of Lf or Tf (1, 8), growth on hTf by the various mutants was indistinguishable from that of the parent. When we tested the *M. catarrhalis lbpA::kan* mutant, no growth surrounding the hLf-impregnated disk was observed (Table 2), affirming that LbpA is essential for utilization of iron bound to Lf. Similarly, no detectable growth was observed by the *lbpB::kan*Ω mutant when hLf was supplied as the sole iron source, consistent with the lack of LbpA produced in this strain. A small zone of growth surrounding the Lf-impregnated disk (approximately 0.5 mm in diameter) was observed with the *lbpB::kan* mutant ($LbpB^-$), suggesting this mutant retains the capacity to utilize Lf, provided the iron source was present in a sufficiently high concentration. In contrast, the zones of growth surrounding the Lf-impregnated disks for the *M. catarrhalis* parental strain and *orf3::kan* mutant appeared as dispersed areas of growth (approximately 4 to 6 mm in diameter), presumably indicative of the ability to use Lf at lower concentrations.

The *lbpB*, *lbpA*, and *orf3* genes were PCR amplified and subcloned into the pT7-7 expression vector, and expression of the recombinant proteins was induced by the addition of the CE6 bacteriophage encoding the T7 RNA polymerase (21). Substantial expression of recombinant LbpA and LbpB was observed, but there was no detectable expression of Orf3. Due to difficulties encountered in reproducibly demonstrating hLf binding activity in bacterial extracts containing the recombinant Lbps, we prepared fusions with Mbp (see Table 1) so that the recombinant proteins could be obtained in purified preparations by amylose affinity chromatography. The purified LbpB-Mbp fusion protein readily demonstrated Lf binding properties in solid-phase binding assays (6) and after SDS-PAGE (without boiling samples) and Western blotting. In contrast, no binding activity was detectable with the LbpA-Mbp fusion protein. These findings are consistent with data obtained from meningococcal and *M. catarrhalis* Lbps (6, 8, 11).

In this article, we have demonstrated that *lbpB*, *lbpA*, and *orf3* have an operonic organization in *M. catarrhalis*. RT-PCR reveals that all three genes compose a polycistronic message (Fig. 4), and insertional inactivation of *lbpB* with a *kan*Ω cassette eliminated LbpA expression (Fig. 2 and 3). Although *orf3* belongs to the same operon as *lbpB* and *lbpA*, insertional inactivation of this gene did not alter LbpA or LbpB produc-

tion (Fig. 2), hLf binding activity (Fig. 3), or acquisition of iron from hLf or hTf (Table 2). Thus, the function of Orf3 remains unknown. The rather novel organization of the *tbp* genes in *M. catarrhalis*, with the *tbpA* gene preceding an intervening ORF which in turn precedes *tbpB* (18), is certainly unlike the operonic organization in other species and warrants further study.

Insertional inactivation of the *lbpA* gene in *M. catarrhalis* (Fig. 1 and 2) clearly demonstrated that LbpA is essential for iron acquisition from hLf (Table 2), consistent with the proposal that it serves as the transmembrane channel for transport of iron across the outer membrane (6, 11). The marked impairment of iron acquisition from hLf in vitro by the isogenic mutant deficient in LbpB (Table 2) suggests that, similar to TbpB (15), it plays an important facilitory role in the iron acquisition process. Since LbpB is present in all clinical isolates examined to date (8), it may be essential in vivo and may serve as a potential vaccine candidate. The pathway for iron acquisition from hLf in *Neisseria* requires the involvement of TonB (5) for energization and a periplasmic iron binding protein (17) to mediate transport to the cytoplasmic membrane. The pathway in *M. catarrhalis* likely also involves these components, although there is currently no direct evidence to support this proposal. The current model for the iron acquisition pathway (15) cannot account for the loss of iron acquisition from Lf and Tf in strains devoid of CopB. This suggests that either the pathway model needs to be modified to incorporate an essential role for CopB or that the loss of function is indirect, perhaps by truncated CopB interfering with one of the other pathway components. Further studies are clearly needed to resolve this issue.

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