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Molecular Cloning, Nucleotide Sequence, and Characterization of *lppB*, Encoding an Antigenic 40-Kilodalton Lipoprotein of *Haemophilus somnus*†

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***Haemophilus somnus* is a facultative intracellular pathogen which causes a wide range of diseases in cattle. To identify putative virulence determinants, a genomic library of *H. somnus* in *Escherichia coli* was screened for Congo red binding, a property associated with virulence in pathogenic bacteria, and subsequently with bovine hyperimmune sera raised against *H. somnus* HS25. A Congo red-binding clone carrying a 1.8-kb DNA insert was found to encode a strongly seroreactive LppB protein with an apparent molecular weight of 40,000. The nucleotide sequence of the entire DNA insert was determined. Two open reading frames coding for polypeptides with calculated molecular weights of 21,893 and 30,721 were identified. The larger open reading frame encoded LppB, while the smaller reading frame encoded a nonseroreactive protein with a relative molecular mass of approximately 18 kDa. The 16 amino-terminal amino acids of the deduced LppB polypeptide showed strong sequence homology to the signal peptide of secreted bacterial proteins, and the sequence Leu-Ala-Ala-Cys at the putative cleavage site corresponds to the consensus cleavage sequence of bacterial lipoproteins. Synthesis of the mature LppB lipoprotein in *H. somnus* was inhibited by globomycin, a specific inhibitor of signal peptidase II. LppB was localized to the outer membrane of *H. somnus*.**

In recent years, there has been an increasing interest in the biology of the gram-negative bacterium *Haemophilus somnus*, a pathogenic organism associated with hemophilosis in cattle (3, 15, 16). Possible virulence mechanisms of *H. somnus* include interference with phagocyte function (29) and binding activities for the Fc region of immunoglobulin G (4, 34) and for transferrin (22). Using hyperimmune serum against the virulent *H. somnus* HS25, we have recently cloned and characterized the genes coding for two antigens (32, 33). These antigens were identified as a ribosomal protein similar to *Escherichia coli* S9 and a 40-kDa lipoprotein, LppA.

The putative virulence factor which has received most attention so far is a 40-kDa outer membrane protein (OMP). Using monospecific bovine polyclonal antibody against this antigen, Corbeil and coworkers have shown that the 40-kDa OMP is present in all *H. somnus* isolates tested (5). They have also demonstrated, in a passive protection experiment, that the same monospecific antiserum efficiently prevents *H. somnus*-induced pneumonia (12). A 39-kDa OMP antigenically distinct from the 40-kDa OMP has also been identified. This protein reacts with convalescent-phase serum and is also conserved among all *H. somnus* isolates tested (5).

The abilities of various pathogenic bacteria to adsorb the aromatic dye Congo red have been strongly correlated with virulence (6, 17, 23, 25, 31). In *Shigella flexneri*, Congo red binding (Crb) has been associated with increased infectivity in the HeLa cell model (6). Virulence genes encoding the Crb phenotype are present on the chromosome of *Yersinia pestis* (24) and on the large virulence plasmids found in *Yersinia enterocolitica* and in the enteroinvasive *E. coli* and *S. flexneri* (25, 36). Competition experiments have indicated that the Crb sites on the surface of *Aeromonas salmonicida* and *S. flexneri* also recognize structurally similar porphyrin compounds such as hemin (6, 17). Deneer and Potter (7) have found that *Actinobacillus pleuropneumoniae* strains expressing iron-regulated OMPs show a marked increase in Congo red adsorption and hemin binding compared with those in cells which were grown under iron-replete conditions. Prebinding of Congo red essentially eliminated the ability of the cells to bind hemin, suggesting that they bind to the same sites on the cell surface.

H. somnus HS25 binds Congo red on agar plates and in liquid medium. In this report, we describe the molecular cloning and sequence analysis of a gene (*lppB*) coding for an antigenic 40-kDa Congo red-binding lipoprotein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* DH5α F'IQ [ϕ80 *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *gyrA96 relA1* Δ(*lacZYA-argF*)*U169*/F' *lacI*^{q} *proAB*$^+$ *lacZ*ΔM15 Tn*5* (Km^r)], BD1854 (*minA minB thi rpsL his lac mtl man mal xyl tonA*), and JM105 (28) are from the laboratory collection. *E. coli* strains were grown in Luria-Bertani or M63 medium (20). Congo red agar consists of Luria-Bertani agar containing 0.05% Congo red. Ampicillin and kanamycin were used at 100 and 40 μg/ml, respectively. *H. somnus* HS25 has been used in earlier experiments to induce experimental hemophilosis in calves (14). Growth conditions for strain HS25 and the construction of the genomic *H. somnus* library in plasmid pGH433 have been described elsewhere (32). Plasmid pAA507 is a derivative of pGH433 (32) with a multiple cloning region consisting of *Nco*I, *Bam*HI, and *Sma*I sites downstream from the *tac* promoter. For iron-restricted growth, brain heart infusion

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broth (Difco Laboratories, Detroit, Mich.) containing 0.1% Tris base and 0.001% thiamine monophosphate (BHI-TT) was supplemented with the iron chelator 2,2′-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 300 μM. Iron-replete bacteria were grown in BHI-TT containing 50 μM $Fe(NO_3)_3$.

DNA techniques. Restriction enzymes, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, T4 polynucleotide kinase, and exonuclease III were used as recommended by the suppliers (Pharmacia [Canada] Inc., Baie d'Urfe, Canada; and New England Biolabs Ltd., Mississauga, Canada). DNA sequencing was accomplished by the chain termination method, essentially as described by Messing (19). All oligonucleotides were made with a Gene Assembler Plus (Pharmacia LKB Biotechnology, Uppsala, Sweden) DNA synthesizer.

Screening of *H. somnus* genomic library. Recombinant plasmids were transformed into *E. coli* JM105 and plated on Congo red agar. After 2 days of incubation at 37°C, the Congo red-binding (Crb^+) colonies were picked and purified to single colonies on identical plates. One colony from each original Crb^+ transformant was then tested for the expression of *H. somnus* antigens by the colony blot method (10).

Preparation of recombinant proteins and specific antiserum. The recombinant proteins produced by JM105/pMS11 were obtained after induction of the *tac* promoter with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as insoluble aggregates (11). The aggregated proteins were solubilized in 4 M guanidine hydrochloride, and the concentration of LppB was estimated by Coomassie staining of a sodium dodecyl sulfate (SDS)-polyacrylamide gel with serial dilutions of the recombinant LppB protein and a bovine serum albumin standard (Pierce Chemical Co., Rockford, Ill.). Specific serum against the recombinant proteins was raised in a calf by two intramuscular injections (at a 3-week interval) of solubilized aggregates containing 100 μg of recombinant LppB in the adjuvant Emulsigen Plus (MVP Laboratories, Ralston, Nebr.) at 33% (vol/vol).

PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) of *H. somnus* and *E. coli* proteins was performed in the Laemmli system (18). Transfer of proteins onto nitrocellulose membranes and reactions on the blots were performed as previously described (33). The primary antiserum was bovine hyperimmune serum raised against live *H. somnus* HS25 (32) or bovine serum raised against the recombinant proteins from JM105/pMS11.

Analysis of plasmid-encoded proteins. Purification and labelling of minicells has been described previously (32).

Hybridization techniques. Northern (RNA) blotting was performed as described by Sambrook et al. (28). RNA was extracted from *H. somnus* and *E. coli* by standard techniques (33) and electrophoresed through 1.4% agarose gels containing formaldehyde. Three micrograms of RNA was used per lane. A 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used for molecular weight standards. The RNA was blotted onto a nitrocellulose membrane and hybridized to a DNA probe labelled at the 5′ end with ^{32}P by using T4 polynucleotide kinase. The probe was a synthetic oligonucleotide complementary to the 5′ end of the coding strand of *lppB*. After hybridization, blots were washed twice for 2 h at 50°C in 0.5% SDS–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Cell fractionation. Exponentially growing cells of *H. somnus* HS25 were broken by passage through a French pressure cell, and the various cellular fractions, including Sarkosyl-insoluble outer membranes (9), were obtained by differential centrifugation as previously described (26). Outer membranes were also prepared by sucrose gradient centrifugation of total membranes in a two-step gradient (13). The proteins from cell lysates and various fractions were precipitated at 10% (wt/vol) trichloroacetic acid for 40 min on ice, pelleted by centrifugation at 15,000 × *g* for 20 min, and washed twice with methanol to remove lipids before analysis by SDS-PAGE.

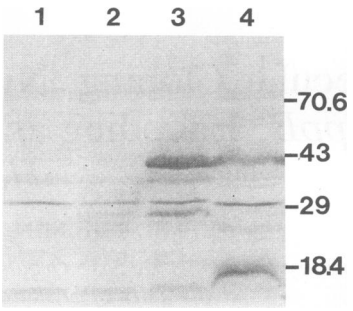

FIG. 1. Western blot of *H. somnus* antigens and whole cells of JM105 harboring the indicated plasmids with bovine hyperimmune serum. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and reacted with the serum. Lane 1, pGH433-IPTG; lane 2, pMS11; lane 3, pMS11-IPTG; lane 4, HS25 whole cells. Where indicated, cultures were induced with 2 mM IPTG for 2 h before the cells were harvested. The equivalent of 0.4 ml of the cultures was applied to each lane. Numbers to the right indicate the positions of the molecular weight markers (in thousands).

Globomycin treatment of *H. somnus*. The treatment of exponentially growing cells of *H. somnus* HS25 with globomycin, a specific inhibitor of signal peptidase II (8), has been described previously (33).

Nucleotide sequence accession number. The 1,815-nucleotide sequence of *H. somnus*, which includes the coding sequence for an 18-kDa polypeptide and LppB, can be retrieved from GenBank by using accession number L10653.

RESULTS

Cloning of the gene for LppB. A genomic *H. somnus* library was transformed into JM105, and among several thousand ampicillin-resistant transformants, approximately 0.1% were found to bind Congo red (Crb^+) on Congo red agar plates. The *E. coli* strain JM105 had a very low ability to bind Congo red on these plates. Twenty Crb^+ transformants were subsequently screened with hyperimmune serum in a colony blot assay, and five were found to be seroreactive. Western blots (immunoblots) of whole-cell lysate proteins separated by SDS-PAGE showed that three transformants contained plasmids (designated pMS11, pMS14, and pMS15) which each had the coding capacity for seroreactive proteins migrating at 28 kDa and in the 35- to 40-kDa range (Fig. 1, lane 3 shows data for pMS11). The 40-kDa seroreactive protein encoded by pMS11 comigrated with a strong seroreactive 40-kDa protein of *H. somnus* (Fig. 1, lanes 3 and 4). The addition of 2 mM IPTG to the growth medium of JM105/pMS11 greatly increased the expression of the 40-kDa protein (Fig. 1, lanes 2 and 3), indicating that the corresponding gene is in the proper orientation to be positively expressed by the *tac* promoter in pMS11 (Fig. 2A). The radiolabelled DNA insert from pMS11 was found to hybridize to pMS14, pMS15, and *H. somnus* DNA but not to the plasmids isolated from the other two seroreactive Crb^+

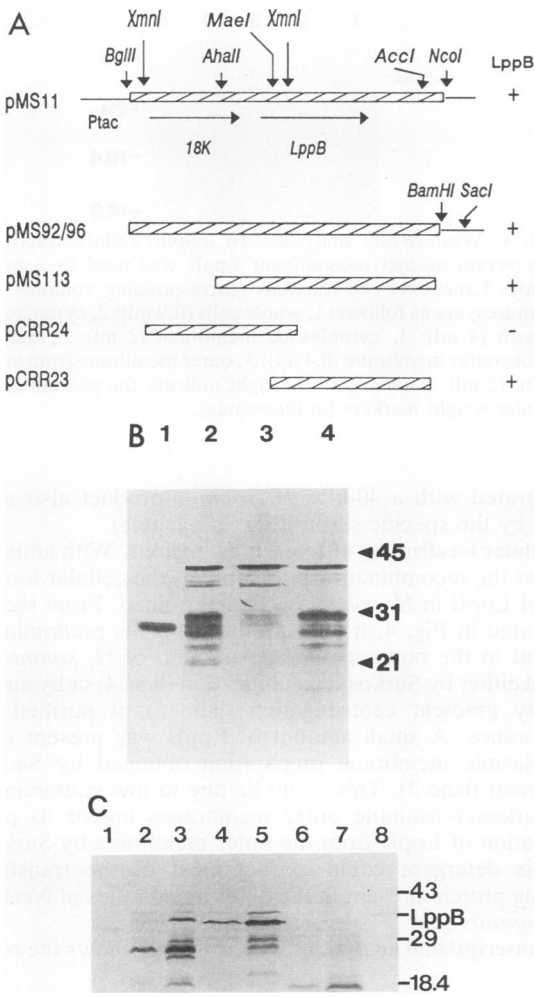

FIG. 2. (A) Structure and properties of specific plasmids. Open boxes designate *H. somnus* DNA cloned into pGH433 (pMS11, pCRR23, and pCRR24) or pTZ18R (pMS92/96, and pMS113). Thin lines represent vector DNA, and the arrows indicate the location and direction of transcription of *lppB* and the 18-kDa protein gene. Restriction sites used in the construction of the various plasmids are indicated. Where two names are separated by a slash, the fragment was cloned in both orientations. P*tac* is the *tac* promoter located on pGH433. The abilities of the plasmids to direct the synthesis of LppB in JM105 are indicated (+ and −). (B) Autoradiogram of an SDS-polyacrylamide gel electropherogram of labelled polypeptides from minicells (BD1854) harboring various plasmids. Lane 1, pTZ18R; lane 2, pMS113; lane 3, pMS92; lane 4, pMS11-IPTG. IPTG indicates that 2 mM IPTG was added to the cells during labelling. Numbers to the right indicate the positions of the molecular weight markers (in thousands). (C) Western blot of *H. somnus* antigens and of *E. coli* JM105 harboring various plasmids. A bovine serum raised against recombinant proteins produced by JM105/pMS11 was used as a primary antibody. Lane 1, pGH433; lane 2, pMS11; lane 3, pMS11-IPTG; lane 4, pCRR23; lane 5, pCRR23-IPTG; lane 6, pCRR24; lane 7, pCRR24-IPTG; lane 8, HS25 whole cells. IPTG indicates that the *E. coli* transformants were induced for 2 h with 2 mM IPTG before harvesting of the cells. The equivalent of 0.7 ml of the cultures was applied to each lane. Numbers to the right indicate the positions of the molecular weight markers (in thousands).

transformants, suggesting that the same 40-kDa protein is encoded by these three plasmids (data not shown). Also, the same insert did not hybridize to plasmid pMS22, encoding LppA (33), showing that pMS11 encodes a novel 40-kDa protein called LppB (data not shown).

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TATTGGATTGGACCAATAGGACTGGCAGAAAATGAATCGGAAGGAACGGACTTCCATGCCGTTAAAAACGGCTAT   75
GTGTCAATTACACCCATTCAAACAGATATGACGGCATATCATTCAATGACAGCTTTACAACAATGGTTAGATAAG   150

GAATAACGATAATCTTTTCATCGAAGGAATAAAACATGAAAATTTTCGGTACGCTATATGATAAAACTATGCAAT   225
                                  M  K  I  F  G  T  L  Y  D  K  T  M  Q

GGGCAAATCACCGTTTTGCTACATTTTGGCTAACTTTTGTTAGTTTTATTGAGGCTATTTTCTTCCCAATACCAC   300
W  A  N  H  R  F  A  T  F  W  L  T  F  V  S  F  I  E  A  I  F  F  P  I  P

CTGATGTCATGCTTATTCCGATGTCAATAAATAAACCTAAATGTGCTACTAAAATTTGCATTTTATGCAGCAATGG   375
P  D  V  M  L  I  P  M  S  I  N  K  P  K  C  A  T  K  F  A  F  Y  A  A  M

CTTCAGCCATTGGTGGGGCAATTGGTTATGGATTAGGTTATTACGCTTTTGATTTCATACAAAGTTATATTCAAC   450
A  S  A  I  G  G  A  I  G  Y  G  L  G  Y  Y  A  F  D  F  I  Q  S  Y  I  Q

AATGGGGTTATCAACAACATTGGGAAACTGCTCTTTCTTGGTTCAAAGAATGGGGTATTTGGGTAGTTTTCGTTG   525
Q  W  G  Y  Q  Q  H  W  E  T  A  L  S  W  F  K  E  W  G  I  W  V  V  F  V
                                    AhaII
CAGGTTTTTCACCTATTCCTTATAAAAATTTTTACGATTTGTGCAGGCGTCATGCAAATGGCATTTTTGCCTTTCT   600
A  G  F  S  P  I  P  Y  K  I  F  T  I  C  A  G  V  M  Q  M  A  F  L  P  F

TACTTACTGCCTTTATTTCTCGTATTGCAAGATTTTTGCTCGTTACCCATTTAGCGGCTTGGAGCGGAAAAAAAT   675
L  L  T  A  F  I  S  R  I  A  R  F  L  L  V  T  H  L  A  A  W  S  G  K  K

TTGCTGCGAAATTACGTCAATCTATTGAATTTATCGGTTGGTCAGTTGTCATTATTGCTATAGTTGTATATCTTG   750
F  A  A  K  L  R  Q  S  I  E  F  I  G  W  S  V  V  I  I  A  I  V  V  Y  L

TCTTGAAATAATCTAAGATAAAAAATGAATATAAAGTAACGGAGAATTTACATGAAAAAATTTTTACCTTTATCT   825
V  L  K  END                                    M  K  K  F  L  P  L  S

ATTAGTATCACTGTACTAGCTGCTTGTAGTTCACACACTCCGGCTCCGGTAGAAAATGCTAAGGATTTAGCACCA   900
 I  S  I  T  V  L  A  A  C  S  S  H  T  P  A  P  V  E  N  A  K  D  L  A  P

AGTATTATCAAACCGATTAATGGTACAAACTCAACCGCTTGGGAACCTCAAGTTATTCAACAAAAGATGCCCGAA   975
 S  I  I  K  P  I  N  G  T  N  S  T  A  W  E  P  Q  V  I  Q  Q  K  M  P  E

AGTATGAGAGTGCCGAAAGCAACAAACTCCACTTATCAACCTGAAATCATTCAACAAAATCAACAAAAAACAGAA   1050
 S  M  R  V  P  K  A  T  N  S  T  Y  Q  P  E  I  I  Q  Q  N  Q  Q  K  T  E

TCGATAGCAAAAAAACAGGCTCTACAAAATTTTGAAATTCCAAGAGATCCTAAAACTAATGTGCCTGTTTATAGC   1125
 S  I  A  K  K  Q  A  L  Q  N  F  E  I  P  R  D  P  K  T  N  V  P  V  Y  S

AAAATTGATAAGGGTTTTTACAAAGGTGATACTTACAAAGTACGCAAAGGCGATACCATGTTTCTTATTGCTTAT   1200
 K  I  D  K  G  F  Y  K  G  D  T  Y  K  V  R  K  G  D  T  M  F  L  I  A  Y

ATTTCAGGCATGGATATAAAAGAATTGGCCACACTAAATAATATGTCTGAGCCATATCATCTGAGTATTGGACAA   1275
 I  S  G  M  D  I  K  E  L  A  T  L  N  N  M  S  E  P  Y  H  L  S  I  G  Q

GTATTGAAAATTGCAAATAATATTCCCGATAGCAATATGATACCAACACAGACAATAAATGAATCAGAGGTGACA   1350
 V  L  K  I  A  N  N  I  P  D  S  N  M  I  P  T  Q  T  I  N  E  S  E  V  T

CAAAATACAGTCAATGAGACATGGAATGCTAATAAACCAACAAATGAACAAATGAAACCCGTTGCTACACCAACA   1425
 Q  N  T  V  N  E  T  W  N  A  N  K  P  T  N  E  Q  M  K  P  V  A  T  P  T

CATTCAACAATGCCAATCAATAAAACACCTCCAGCCACCTCAAATATAGCTTGGATTTGGCCAACAAATGGAAAA   1500
 H  S  T  M  P  I  N  K  T  P  P  A  T  S  N  I  A  W  I  W  P  T  N  G  K

ATTATTCAAGGATTTCCAGTGCTGATGGAGGCAATAAAGGTATTGATATTAGCGGTTCTCGTGGACAAGCTGTT   1575
 I  I  Q  G  F  S  S  A  D  G  G  N  K  G  I  D  I  S  G  S  R  G  Q  A  V

AATGCAGCAGCTGCATGGACGCAGTTGTATATGCCGGAGACGCTTTACGTGGATATGGTAATTTAATTATTATTA   1650
 N  A  A  A  A  W  T  Q  L  Y  M  P  E  T  L  Y  V  D  M  V  I  END

AACATAATGACAGTTATTTAAGTGCTTATGCACATAATGAAAGTATACTCGTCAAAGATCAGCAAGAAGTTAAAG   1725
CGGGTCAACAAATTGCTAAAATGGGAAGTTCTGGAACAAACACAATCAAACTCCATTTTAAATTCGTTATTTTGG   1800
TCAATCAGTAGATCC                                                               1815
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FIG. 3. Nucleotide sequence of the *H. somnus lppB* region. The sequence of the antisense strand is shown with numbering starting from the 5′ end. The location of the *Aha*II cleavage site is indicated. Putative Shine-Dalgarno sequences (30) are underlined, and putative start and stop codons are overlined. The predicted polypeptide sequences of the two ORFs are shown in one-letter code below the DNA sequence.

Nucleotide sequencing and analysis. The 1.8-kb insert from pMS11 was subcloned into the *Hin*cII site of pTZ18R by using *E. coli* JM105 as the host strain. Two plasmids, pMS92 and pMS96, carrying the insert in opposite orientations were obtained (Fig. 2A). To generate a series of nested deletions for DNA sequencing, plasmid pMS92 was cut at the unique *Sac*I and *Bam*HI sites (Fig. 2A) and subjected to exonuclease III degradation, removal of the overhangs by S1 nuclease, and religation. The DNA sequence deduced from the single-stranded DNA templates of the deletion subclones generated from pMS92 was used to design complementary oligonucleotide primers for the complete sequencing of the single-stranded DNA template produced from pMS96. Figure 3 shows the sequence of the entire insert. Two large open reading frames (ORFs) were identified. The first ORF (ORF1) starts with an ATG codon at nucleotide 186 and ends with a TAA codon at nucleotide 759. ORF1 could code for a 191-amino-acid polypeptide with a calculated molecular weight of 21,893. A second ORF (ORF2), located immediately downstream of ORF1, begins with an ATG codon at position 802 and ends with a TAA codon at position 1639. The molecular weight of the deduced 279-amino-acid polypeptide was calculated to be 30,721. The predicted polypeptide has a positively charged amino-terminal region followed by a hydrophobic region (from amino acids 4 to 13)

and a lipoprotein box, Leu-Ala-Ala-Cys, at the predicted signal peptidase II cleavage site. This peptide sequence strongly resembles the signal peptide of procaryotic lipoproteins (35), including the recently characterized lipoprotein LppA from *H. somnus* (33). Upstream from both ORFs, there is a putative ribosome binding site (underlined in Fig. 3) and a 7-bp AT-rich spacer followed by the potential ATG start codon. Both reading frames have been confirmed by sequencing the fusion junction of the in-frame fusions made between ORF1 or ORF2 and the leader peptide on pAA507, in pCRR24 and pCRR23, respectively (Fig. 2A), which expressed the fusion proteins described below.

Analysis of plasmid-encoded proteins. ORF2 contained in the DNA sequence between the *Aha*II site (at position 570 in Fig. 3) and the *Nco*I site (from pGH433) in pMS11 was subcloned into the *Sma*I site of pTZ18R (Fig. 2A). JM105 harboring the resulting plasmid pMS113 reacted with *H. somnus* hyperimmune serum in a colony blot assay (data not shown), suggesting that *lppB* is located downstream of the *Aha*II site.

Plasmids pMS11, pMS92, and pMS113 were introduced into the minicell-producing strain BD1854, and plasmid-encoded polypeptides were analyzed in minicells by being labelled with [^{35}S]methionine and subsequent SDS-PAGE. From the data presented in Fig. 2B, it appears that these plasmids encode polypeptides in the 35- to 40-kDa range, with an abundant 40-kDa protein, which are absent in cells containing the vector pTZ18R (lane 1). Thus, *lppB* must be located downstream of the *Aha*II site and should correspond to ORF2 in Fig. 3. A number of smaller polypeptides in the 25- to 30-kDa range are also present in minicells expressing the 40-kDa protein. Since JM105 cells harboring pMS113 had a strong Crb$^+$ phenotype, it indicates that LppB binds Congo red.

To further study the polypeptides encoded by plasmid pMS11, two fusion plasmids were constructed. Plasmid pCRR23 was obtained by end filling the 855-bp *Mae*I-*Acc*I fragment of pMS11 (Fig. 2A) with the Klenow fragment of DNA polymerase I and subsequent ligation into pAA507 digested with *Sma*I. Thus, pCRR23 encodes a fusion protein between the leader peptide of pAA507 and the 14th amino acid from the *lppB* coding region (Fig. 3). pCRR24 was made by subcloning the 830-bp *Xmn*I fragment of pMS11 (Fig. 2A) into *Sma*I-cut pAA507, resulting in an in-frame fusion between the leader peptide on the vector and the third amino acid from ORF1 (Fig. 3). Figure 2C shows a Western blot of whole-cell lysates of *E. coli* DH5α F′IQ containing plasmid pGH433, pMS11, pCRR23, or pCRR24 (induced or not with IPTG) and of *H. somnus* HS25 reacted with a specific serum raised against the recombinant proteins synthesized by JM105/pMS11. As did hyperimmune serum, the specific serum recognized polypeptides in the 35- to 40-kDa and 25- to 30-kDa ranges in cells containing pMS11 (Fig. 2C, lane 3). In addition, the specific serum recognized an 18-kDa protein not previously detected with hyperimmune serum (Fig. 2C, lane 3). Plasmid pCRR23 directed the synthesis of polypeptides in the 35- to 40-kDa and the 25- to 30-kDa ranges (Fig. 2C, lane 5) which were all detected by hyperimmune serum (data not shown). Plasmid pCRR24 encoded the 18-kDa protein (Fig. 2C, lane 7). The insert-specified polypeptide products of pMS11, pCRR24, and pCRR23 were all synthesized in larger amounts upon induction of the transformants with IPTG. Thus, the ORF1 in Fig. 3 encodes an 18-kDa protein, while *lppB* encodes products migrating in the 35- to 40-kDa and the 25- to 30-kDa ranges. A large recombinant LppB product (present in each of the lanes 2 to 5 [Fig. 2C]) comigrated with a 40-kDa *H. somnus* product also recognized by the specific serum (Fig. 2C, lane 8).

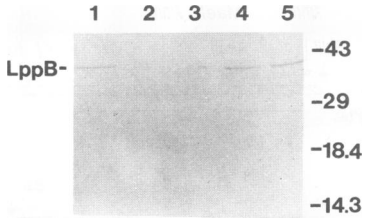

FIG. 4. Western blot analysis of *H. somnus* cellular fractions. A bovine serum against recombinant LppB was used as a primary antibody. Lanes and cell fractions (corresponding volumes of the cell cultures) are as follows: 1, whole cells (0.9 ml); 2, cytoplasm and periplasm (4 ml); 3, cytoplasmic membrane (2 ml); 4, Sarkosyl-insoluble outer membrane (0.4 ml); 5, outer membrane from sucrose gradient (2 ml). Numbers to the right indicate the positions of the molecular weight markers (in thousands).

Cellular localization of LppB in *H. somnus*. With antiserum against the recombinant LppB protein, the cellular localization of LppB in *H. somnus* was determined. From the data presented in Fig. 4, it appears that LppB is predominantly present in the outer membrane fraction of *H. somnus* obtained either by Sarkosyl solubilization (lane 4) or by sucrose density gradient centrifugation (lane 5) of purified total membranes. A small amount of LppB was present in the cytoplasmic membrane preparation obtained by Sarkosyl treatment (lane 3). This could be due to low contamination by Sarkosyl-insoluble outer membranes and/or to partial separation of LppB from the outer membrane by Sarkosyl, as this detergent could extract most of the transferrin-binding protein present in the outer membranes of *Neisseria meningitidis* (1).

Transcriptional analysis of *lppB*. Figure 5 shows the results

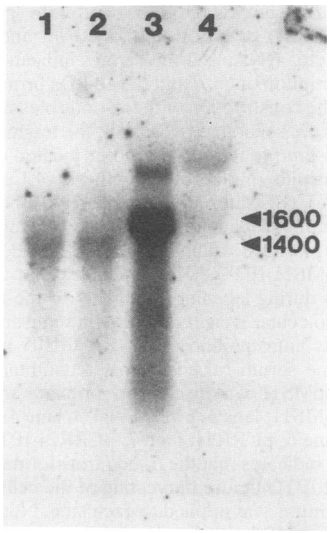

FIG. 5. Northern blot analysis of RNA. The probe, a ^{32}P-labelled synthetic oligonucleotide complementary to noncoding sequences in the 5′ end of *lppB* (nucleotides 860 through 874 [Fig. 3]), was hybridized to the blot. Following a high-stringency wash, the blot was exposed to X-ray film for several days. Lane 1, HS25/iron replete; lane 2, HS25/2,2′-dipyridyl; lane 3, JM105/pMS11; lane 4, JM105/pGH433. The sizes in nucleotides of the putative *lppB* transcripts are indicated.

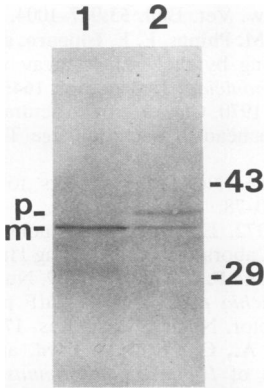

FIG. 6. Lipid modification and processing of the LppB protein. Shown is an immunoblot of whole-cell proteins from HS25 incubated for 2 h in the absence (lane 1) or presence (lane 2) of globomycin. Whole cells were precipitated at 10% (wt/vol) trichloroacetic acid. The equivalent of 0.8 ml of the cultures was applied to each lane. The locations of the mature (m) and precursor (p) forms of the LppB protein are shown. Numbers to the right indicate the positions of the molecular weight markers (in thousands).

of a Northern blot in which an oligonucleotide complementary to bases 860 to 874 at the 5′ end of *lppB* (Fig. 3) was hybridized to total RNA isolated from *H. somnus* HS25 grown with iron (lane 1) or with 2,2′-dipyridyl (lane 2) and *E. coli* strain JM105 containing pMS11 (lane 3) or pGH433 (lane 4). A single transcript of 1,400 nucleotides was detected in RNA obtained from HS25 grown under both iron-replete and iron-deficient growth conditions. The intensities of the bands are comparable, indicating that *lppB* is not regulated by iron at the transcriptional level. A transcript of 1,600 nucleotides was detected in RNA isolated from *E. coli* JM105/pMS11. Since it was absent in RNA isolated from JM105/pGH433, this transcript was specified by the DNA insert in pMS11.

Posttranslational modification of the LppB protein. To test whether LppB is lipid modified in *H. somnus*, strain HS25 was treated with globomycin to produce precursor lipoproteins (8). Proteins from whole-cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The LppB protein was identified by immunoblotting with antiserum against the recombinant LppB protein. This antiserum did not cross-react with recombinant LppA (data not shown), a 40-kDa lipoprotein of *H. somnus* (33). That LppB is a lipoprotein is indicated by the presence of both a mature and a larger precursor form of this protein in *H. somnus* cells treated with globomycin (Fig. 6, lane 2) while untreated cells contain only the mature LppB (Fig. 6, lane 1).

DISCUSSION

In this study, we report the cloning and nucleotide sequence of a gene encoding an antigenic 40-kDa Congo red-binding lipoprotein, called LppB, of *H. somnus*. The gene was isolated on a 1.8-kb *Sau*3AI fragment cloned into the expression vector pGH433. The analysis of the nucleotide sequence of the entire insert revealed the presence of ORF1 and ORF2 encoding polypeptides with calculated molecular weights of 21,893 and 30,721, respectively. ORF2, which is located downstream of the *Aha*II site in the clone (Fig. 3), corresponds to the *lppB* gene. In *E. coli* minicells, plasmid pMS113, which carries the entire ORF2 and only the 3′ one-third of ORF1, directed the synthesis of polypeptides in the 35- to 40-kDa and 25- to 30-kDa ranges. Polypeptides of similar size were also synthesized by *E. coli* cells harboring the original library plasmid (pMS11) or pCRR23 which codes for a fusion protein between the 7-amino-acid leader peptide of the expression vector pAA507 and LppB. These recombinant products were recognized both by bovine hyperimmune serum raised against *H. somnus* and by specific serum raised against the products of plasmid pMS11. We believe that the smaller polypeptides are *E. coli* artifacts, since only the 40-kDa species was present in *H. somnus* (Fig. 4). Because a single *lppB* transcript was observed in *E. coli*, the polypeptides may be due to incomplete translation of the mRNA and/or to protease cleavage of the 40-kDa protein. There is a discrepancy of approximately 9,000 between the observed and calculated molecular weights of LppB. A similar difference has been observed for other lipoproteins, including the recently characterized LppA of *H. somnus* (33).

ORF1 would correspond to an 18-kDa protein gene, since a plasmid, pCRR24, which carries a protein fusion between this ORF and the leader peptide on pAA507, expressed a protein of approximately 18 kDa. This protein was not recognized by bovine hyperimmune serum raised against *H. somnus*.

No significant homology was found between the deduced amino acid sequence of LppB or the primary translation product of the 18-kDa protein gene and protein sequences compiled in GenBank release 70. However, DNA sequences from position 1590 to the end of the cloned DNA in Fig. 3 showed 65.5% identity with the *katF* promoter region from *E. coli* (21). The *katF* gene product is a putative sigma factor which positively regulates the expression of catalase hydroperoxidase II (*katE*) and exonuclease III (*xthA*) involved, respectively, in the breakdown of hydrogen peroxide (H_2O_2) and in the repair of DNA damage due to H_2O_2 (27). The presence of a DNA sequence homologous to the *katF* gene in *H. somnus* which is catalase negative (29) has yet to be explained.

In *H. somnus*, LppB processing was inhibited by globomycin (Fig. 6). This result indicates that LppB is a lipoprotein. The predicted signal peptidase II cleavage site, Leu-Ala-Ala-Cys, is followed by two serine residues, suggesting that LppB is sorted to the periplasmic face of the outer membrane (2). In agreement with this, our data show that LppB is predominantly present in the outer membrane fraction of *H. somnus* (Fig. 4).

The region between the 18-kDa protein gene and *lppB* is 40 nucleotides long and lacks any homology to the rho-independent transcriptional terminator. The close proximity of the two genes and the absence of a putative transcriptional terminator suggest that *lppB* is a distal gene in a polycistronic operon. In a Northern blot of *H. somnus* RNA, an oligonucleotide complementary to sequences in the promoter-proximal part of *lppB* hybridized to an RNA molecule of approximately 1,400 nucleotides. If transcription terminates immediately downstream of *lppB*, this transcript could be the common product of both genes.

Corbeil and coworkers have shown that all *H. somnus* isolates tested contain immunodominant surface antigens of 40 and 39 kDa (5). Of these, the 40-kDa protein could be an important vaccine candidate, since antibodies against this protein prevented experimental *H. somnus*-induced pneumonia in a passive protection experiment (12). We have previously cloned *lppA*, which codes for a 40-kDa lipoprotein of *H. somnus* (33). Comparison of the DNA sequences and the predicted polypeptides of *lppA* and *lppB* showed similarity only for the amino acid sequence Leu-Ala-Ala-

Cys, which constitutes their lipoprotein box. Whether LppB or LppA is similar to the potentially protective 40-kDa OMP remains to be investigated.

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