INFECTION AND IMMUNITY, Feb. 2004, p. 742–749 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.742–749.2004

Host-Inducible Immunogenic Sphingomyelinase-Like Protein, Lk73.5, of *Leptospira interrogans*

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Received 9 July 2003/Returned for modification 16 August 2003/Accepted 20 October 2003

Leptospira interrogans **causes a variety of clinical syndromes in animals and humans. Although much information has accumulated on the importance of leptospiral lipopolysaccharide in protective antibody responses, relatively little is known about proteins that participate in immune responses. Identification of those proteins induced only in the host is particularly difficult. Using a novel double-antibody screen designed to identify clones in a gene library of *L. interrogans* serovar Pomona expressing host-inducible proteins, we have characterized a gene (*lk75.3*) encoding a sphingomyelinase-like preprotein of 648 amino acids with cytotoxic activity for equine pulmonary endothelial cells and weak hemolytic activity for equine and rabbit erythrocytes. *lk73.5* was found as a single gene copy in all serovars of *L. interrogans* but not in other *Leptospira* spp. except *L. inadai*. The open reading frame (ORF) for Lk73.5 is followed by another partially homologous sequence containing an ORF (*sph-like 2*) for a 28.7-kDa peptide. Lk73.5 and Sph-like 2 share 95.1 and 97.7% amino acid identity with putative sphingomyelinases Sph2 and Sph1 (N terminus) from *L. interrogans* serovar Lai (S.-X. Ren, G. Fu, X.-G. Jiangk, R. Zeng, Y.-G. Miao, H. Xu, Y.-X. Zhang, H. Xiong, G. Lu, L.-F. Lu, H.-Q. Jiang, J. Jia, Y.-F. Tu, J.-X. Jiang, W.-Y. Gu, Y.-Q. Zhang, Z. Cai, H.-H. Sheng, H.-F. Yin, Y. Zhang, G.-F. Zhu, M. Wank, H.-L. Huangk, Z. Qian, S.-Y. Wang, Wei Ma, Z.-J. Yao, Y. Shen, B.-Q. Qiang, Q.-C. Xia, X.-K. Guo, A. Danchinq, I. S. Girons, R. L. Somerville, Y.-M. Wen, M.-H. Shik, Z. Chen, J.-G. Xuk, and G.-P. Zhao, Nature 422:88-893, 2003). Substantial homologies to sphingomyelinases from other leptospiras and other bacteria are also present. Lk73.5 was not detected in leptospiras cultured at 30 or 37°C. The recombinant protein reacted strongly with sera from recently infected mares but not with sera from horses vaccinated with commercial pentavalent bacterin. The host-inducible immunogenic Lk73.5 should have value in distinguishing vaccine from infection immune response.**

Leptospirosis, a worldwide disease, is caused by several different *Leptospira* spp. that are invasive for a wide range of mammalian hosts, including humans, horses, dogs, pigs, cattle, and wildlife. Symptoms of infection vary from subclinical to potentially fatal with multiorgan involvement (30, 38, 50). Immune-based uveitis leading to blindness (12, 26, 40, 51) is a devastating sequela in humans and horses. Survival of the organism in water and persistent urinary shedding by wildlife, rodents, and livestock complicate control of the disease (30, 50). Increased economic and recreational activity in pristine areas as well as flooding associated with climatic change have increased human exposure to leptospiras. Indeed, several large outbreaks have occurred in Nicaragua (49, 52), Brazil (27), the United States (8), and Malaysia (9), and leptospirosis is now considered a reemerging infectious disease (30).

Knowledge of the epizootiology of leptospirosis has been important in the design of effective preventive strategies. Control by vaccination has been less effective, and leptospira biologicals are generally unsatisfactory in terms of efficacy, spectrum of cross protection, and duration of immunity. Leptospira vaccines, in general, stimulate protective immunity which is inferior to that developed following recovery from either acute or subclinical leptospirosis (3–5). Hypothetical antigens specifically induced during in vivo growth but not expressed by in vitro cultures may play an important role in stimulating protective immune responses. Moreover, recent findings have indicated that vaccine efficacy may be related to as-yet-unknown protein antigens with the ability to elicit Th1 immune responses (34, 35).

Over the past 5 years, novel methods such as in vivo expression technology, signature tag mutagenesis, and differential fluorescence induction have been used to identify host-induced antigens (21). The lack of a methodology for genetic manipulation of the leptospira genome limits application of these approaches in the study of the antigens in the pathogenic *Leptospira* spp. However, the pioneering work of Saint Girons et al. (13) provides an opportunity to express and investigate the activity of genes of pathogenic leptospiras in saprophytic host strains and suggests that genetic manipulation of pathogenic leptospiras may soon be possible.

Our approach to the identification of leptospiral host-inducible antigens has been to use a modification of in vivo-induced antigen technology (20), in which a gene library of *Leptospira interrogans* is screened with a convalescent-phase serum pool followed by a second screen with antiserum to bacterin of in vitro-cultured leptospira to identify clones positive for genes expressed only in vivo. Using this approach, we have identified a novel, in vivo-induced immunogenic sphingomyelinase-like protein of *L. interrogans*, determined its amino acid sequence,

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and studied its lytic and cytotoxic effects on erythrocytes and pulmonary endothelial cells.

MATERIALS AND METHODS

Bacterial strains and media. *L. interrogans* serovars Pomona type kennewicki (JEN 4), Pomona (Pomona) Copenhageni (M 20), Canicola (Hond Utrech IV), Grippotyphosa (Andaman), Hardjo (Hardjoprajitno), and Bratislava (Jez Bratislava) were kindly provided by Mike Donahue (Livestock Disease Diagnostic Center, University of Kentucky, Lexington). The nonpathogenic *Leptospira biflexa* serovar Biflexa (Codice) was obtained from The National Veterinary Services Laboratories, Ames, Iowa. Leptospiras were cultivated in Johnson-Harris–bovine serum albumin–Tween 80 medium (24) (Bovuminar PLM-5 Microbiological Media; Intergen, Purchase, N.Y.) at 30°C unless otherwise indicated. *Escherichia coli* SOLR and XL1-Blue MRF′ (Stratagene, La Jolla, Calif.) were hosts for phage manipulation and plasmid excision. *E. coli* NovaBlue and BL21(DE3) (Novagen, Madison, Wis.) were used for cloning and expression of recombinant proteins and were routinely grown in Luria-Bertani broth or on Luria-Bertani agar.

Library screening. A λZAPII library containing 3- to 5-kb random fragments of *L. interrogans* serovar Pomona type kennewicki chromosomal DNA (25) was screened to identify phage that expressed gene products reactive with a pool of sera from mares that had recently aborted due to *L. interrogans* serovar Pomona type kennewicki infection. Reactive plaques were picked, replated, and rescreened until all gave a positive signal with the antiserum. A secondary screen was performed with a pool of antisera from ponies vaccinated with bacterin prepared from *L. interrogans* serovar Pomona type kennewicki cultured at 30°C (36) to identify plaques reactive with sera for mares that had aborted but not with antiserum against the bacterin. Negative clones were selected as potential producers of in vivo-induced antigens. These clones were analyzed by immunoblotting to confirm expression and to estimate sizes of proteins.

Gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in an X-Cell SureLock Mini-Cell (Invitrogen, Carlsbad, Calif.) for 2 h at 125 V in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]). Samples for electrophoresis were mixed with an equal volume of 2× gel loading buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol, and 0.1% bromophenol blue) and boiled for 5 min before loading. The gels were rinsed twice in water and stained with Simply Blue Safe Stain (Invitrogen) for visualization of protein bands. Proteins were also transferred to Protran nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell, Keene, N.H.) and blocked with 3% gelatin in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]). The membranes were incubated with antisera, followed by incubation with protein G conjugated to horseradish peroxidase (Zymed, San Francisco, Calif.). Bound conjugate was detected by using 12 mg of 4-chloro-1-naphthol (Sigma, St. Louis, Mo.), dissolved in 5 ml of methanol–25 ml of Tris-buffered saline–30 μl of 30% hydrogen peroxide, for approximately 10 min.

DNA sequencing and analysis. Plasmids excised from selected recombinant phages by using the ExAssist helper phage and *E. coli* SOLR (Stratagene) were isolated by using the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.) and sequenced by using standard T7 and M13 reverse and custom design primers. Sequencing was performed in a commercial DNA sequencing facility (Davis Sequencing LLC, Davis, Calif.), and editing was with Chromas 1.61 (Technelysium Pty Ltd., Queensland, Australia). Nucleotide sequences were aligned and connected by using DNASIS (Hitachi Software Engineering America, Ltd., San Diego, Calif.). Analyses of nucleotide sequence and deduced amino acid sequences were performed with DNASIS and the Genetics Computer Group package of programs (Wisconsin Package version 10.0; Genetics Computer Group, Madison, Wis.) Protein structure was also predicted by using PSORT (http://psort.nibb.ac.jp/), SignalP, TMHMM (http://www.cbs.dtu.dk/), and COILS (http://www.ch.embnet.org/index.html). Homologies were identified by a BLAST search with the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

Protein expression. Primers expF and expR (Table 1), including an *Xho*I restriction enzyme site, were designed by using Primer 2 (Scientific & Educational Software, 1991). The forward primer was designed so that the recombinant His tag fusion would not include the predicted signal peptide. The sequence encoding mature Lk73.5 was PCR amplified from genomic DNA of *L. interrogans* serovar Pomona type kennewicki, which was denatured at 92°C for 2 min before 25 cycles of 92°C at 1 min, 56°C at 1 min, and 72°C for 3 min followed by digestion with *Xho*I and ligation into pET-15b (Novagen). The resulting construct was transformed into *E. coli* BL21(DE3) (Novagen). Expression of Lk73.5 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when the culture reached an optical density of 0.6 at 600 nm, and cells were harvested after 3 h. Recombinant His_6-Lk73.5 was isolated by using TALON metal affinity resin (Clontech Laboratories, Inc., Palo Alto, Calif.) under denaturation conditions. Fractions containing His_6-Lk73.5 were combined and dialyzed against 20 mM Tris–50 mM NaCl buffer (pH 7.5). This preparation (recLk73.5) was used for all downstream applications.

TABLE 1. Oligonucleotide primers used in this work

Primer	Sequence (5′→3′)[a]	Location relative to *lk73.5* start codon
expF	gcg**ctcgag**TTACCCGAAAAAGAATCCTC	72
expR	gcg**ctcgag**TCCGGATTTAAGAGGCCAGG	1954
LeN3	CCATCGTGGAAGATTTGTGG	571

[a] Boldface indicates an *Xho*I site; lowercase indicates primer extension.

Sera. Ponies were inoculated subcutaneously with 500 μg of recLk73.5 adsorbed to 30% aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, N.Y.) in a volume of 2 ml and boosted 2 weeks later with the same dose. Serum was harvested by jugular venipuncture 5 weeks after the initial immunization. Sera from mares that had recently aborted due to naturally acquired infection with pathogenic *Leptospira* were kindly provided by Barbara Smith (Livestock Disease Diagnostic Center, University of Kentucky, Lexington). Sera from horses vaccinated with commercial leptospira pentavalent bacterin were obtained from horses in a riding stable in north Georgia. Sera from horses infected with *Borrelia burgdorferi* (10) and *Ehrlichia* sp. (11) were kindly provided by Yung-Fu Chang (Cornell University, Ithaca, NY). Negative control sera were obtained from ponies on the University of Kentucky North Farm with no known exposure to *Leptospira*.

Cytotoxicity of recLk73.5 for EEC. Cytotoxicity of recLk73.5 was determined by measuring lactate dehydrogenase (LDH) released from equine pulmonary endothelial cells (EEC) (22, 33) (a gift of James MacLachlan, University of California, Davis), using a CytoTox96 cytotoxity kit (Promega, Madison, Wis.). Cells from a subconfluent culture in modified Eagle's medium were plated in triplicate on a 96-well plate (Nalge Nunc International, Rochester, N.Y.) and incubated for 12 to 15 h at 37°C in 5% CO_2. A confluent monolayer of EEC was washed twice with phosphate-buffered saline, and serial dilutions of recLk73.5 from 0.16 to 50 μg/ml were added. Phospholipase C from *Clostridium perfringens* (25 μg/ml) and recQ1p42 (50 μg/ml) (36) were used as positive and negative controls, respectively. Following incubation for 1 h at 37°C in 5% CO_2, the plate was centrifuged at 800 × *g* for 5 min, and 50 μl of supernatant from each well was transferred to another plate containing substrate solution and incubated at room temperature for 30 min. The absorbance was measured at 490 nm after addition of 50 μl of stop solution. Cytotoxicity was calculated by using the formula % cytotoxicity = $100 \times (A_{sample} - A_{background})/(A_{total} - A_{background})$, where A_{sample} was the absorbance at 490 nm of reaction mixture from treated cells and $A_{background}$ and A_{total} were the absorbances of reaction mixtures from untreated cells and from cells lysed with lytic buffer, respectively. The experiment was repeated three times.

Hemolytic activity of recLk73.5. Different concentrations of recLk73.5 and recQ1p42 (80 μg/ml), as a negative control, were added to a 10% suspension of rabbit erythrocytes in 10 mM Tris–100 mM NaCl (pH 7.5), followed by incubation at 37°C for 1 h. The suspensions were then immediately cooled in ice and centrifuged, and the absorbance of the supernatants was measured at 440 nm. Complete lysis was obtained by resuspension of erythrocytes in water.

Detection of *lk73.3* in genomes of other leptospiras. Genomic DNAs of *L. interrogans* serovars Bratislava, Canicola, Hardjo, Grippotyphosa, Copenhageni, and Pomona and of *L. biflexa* were isolated from 10 ml of stationary-phase cultures. Cells were resuspended in 400 μl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) containing 0.5% SDS and 100 μg of proteinase K per ml and incubated for 1 h at 37°C. NaCl was added to a final concentration of 1 M. DNA was precipitated from the aqueous phase with 2 volumes of 95% ethanol following protein extraction with phenol-chloroform and chloroform. Chromosomal DNA of *B. burgdorferi* was kindly provided by Yung-Fu Chang (Cornell University). DNAs of *Leptospira borgpetersenii* serovar Hardjo, *Leptospira santarosiae* serovar Tropica, *Leptospira inadai* serovar Malaya, *Leptospira weilii* serovar Coxi, *Leptospira noguchi* serovar Fortbragg, and *Leptospira kirschneri* serovar Gryppotyphosa were kindly provided by Richard Zuerner (National Animal Disease Center, Ames, Iowa). Leptospiral DNA was digested to completion with *Hin*dIII, and separated on a 0.8% agarose gel, and transferred to a Hybond-N nylon

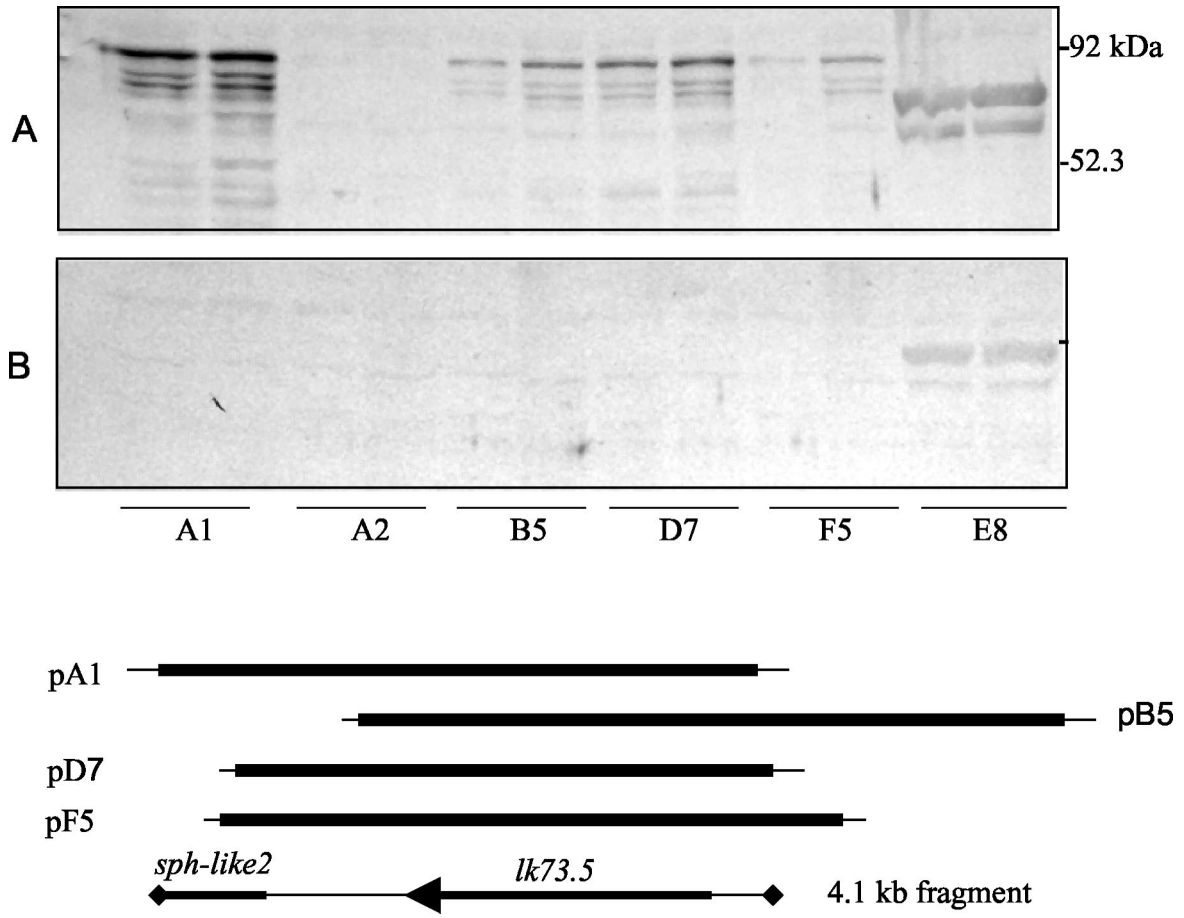


FIG. 1. Leptospiral proteins expressed by *E. coli* SOLR that contain plasmids rescued from phage clones. Mid-log-phase cultures were subjected to SDS-PAGE and immunoblotted with pools of sera from mares that had aborted (A) and from horses immunized with cultured leptospiras (B). Clones A1, B5, D7, and F5 produced an 80-kDa that was antigen reactive only with sera from mares that had aborted. A2 was an *E. coli* clone that did not express the antigen. Clone E8 expressed a leptospira protein that was reactive with both pools of sera. Proteins from each clone were loaded in duplicate. The lines represent alignment of the inserts from clones A1, B5, D7, and F5 and the 4.1-kb nucleotide sequence submitted to GenBank. *lk73.5* and *sph-like 2* are shown on the lower line. The positions of inserts of clones 2 and 8 were not determined.

membrane (Amersham, Piscataway, N.J.) according to the manufacturer's protocol. A hybridization probe was produced by amplifying the 5′ end of *lk73.5* with the expF and LeN3 primers and randomly labeling the amplicon with biotin by using the NEBlot phototype kit (New England BioLabs, Inc., Beverly, Mass.). DNA-DNA hybridization was performed overnight at 42°C in buffer containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 5× Denhardt solution, 10% dextran sulfate, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml. The membrane was washed twice at room temperature in 2× SSC with 0.1% SDS and twice at 60°C in 0.1× SSC with 0.1% SDS. Hybridization was detected with the Phototope-Star detection kit (New England BioLabs) according to the manufacturer's instructions.

Nucleotide sequence accession number. The nucleotide sequence has been deposited in the GenBank database under accession number AF320511.

RESULTS

Isolation and characterization of the gene encoding Lk73.5. Eleven clones reactive with sera of mares that had aborted but not with antiserum to cultured leptospiras were identified. Six of these clones expressed an 80-kDa protein. Figure 1 shows the reactivities of sera used to screen the library with *E. coli* containing plasmids rescued from four of these recombinant phages. A series of lower-molecular-mass bands reactive only with sera from mares that had aborted were evident in each clone expressing the 80-kDa protein but not in an *E. coli* clone that did not express this protein. It was therefore concluded that the multiple bands are the result of proteolytic digestion. Restriction mapping of plasmids indicated that the inserts overlapped. Sequence analysis confirmed this and revealed two open reading frames in a 4.1-kb overlapping fragment (Fig. 1). The first encodes a protein with a calculated molecular mass of 73.5 kDa that was designated Lk73.5 (Fig. 2). The probable Shine-Dalgarno sequence (GGAGA) was located 9 nucleotides upstream of the ATG start codon. Several potential *E. coli* σ^{70} promoters were predicted for *lk73.5*. One consisted of the sequences TTcAtt and TAaaAT in the −35 and −10 regions (matches to the consensus sequence is indicated by uppercase) separated by 19 nucleotides. Three pairs of direct repeats, O1, O2, and O3, separated by 94, 126, and 26 nucleotides, respectively, flanked the potential promoter area. Immediately downstream of *lk73.5*, a stem-and-loop structure ($\Delta G = -22.3$ kcal/mol) followed by five thymines resembled a ρ-independent transcriptional terminator. The amino terminus of Lk73.5 appears to consist of a 23-amino-acid signal peptide

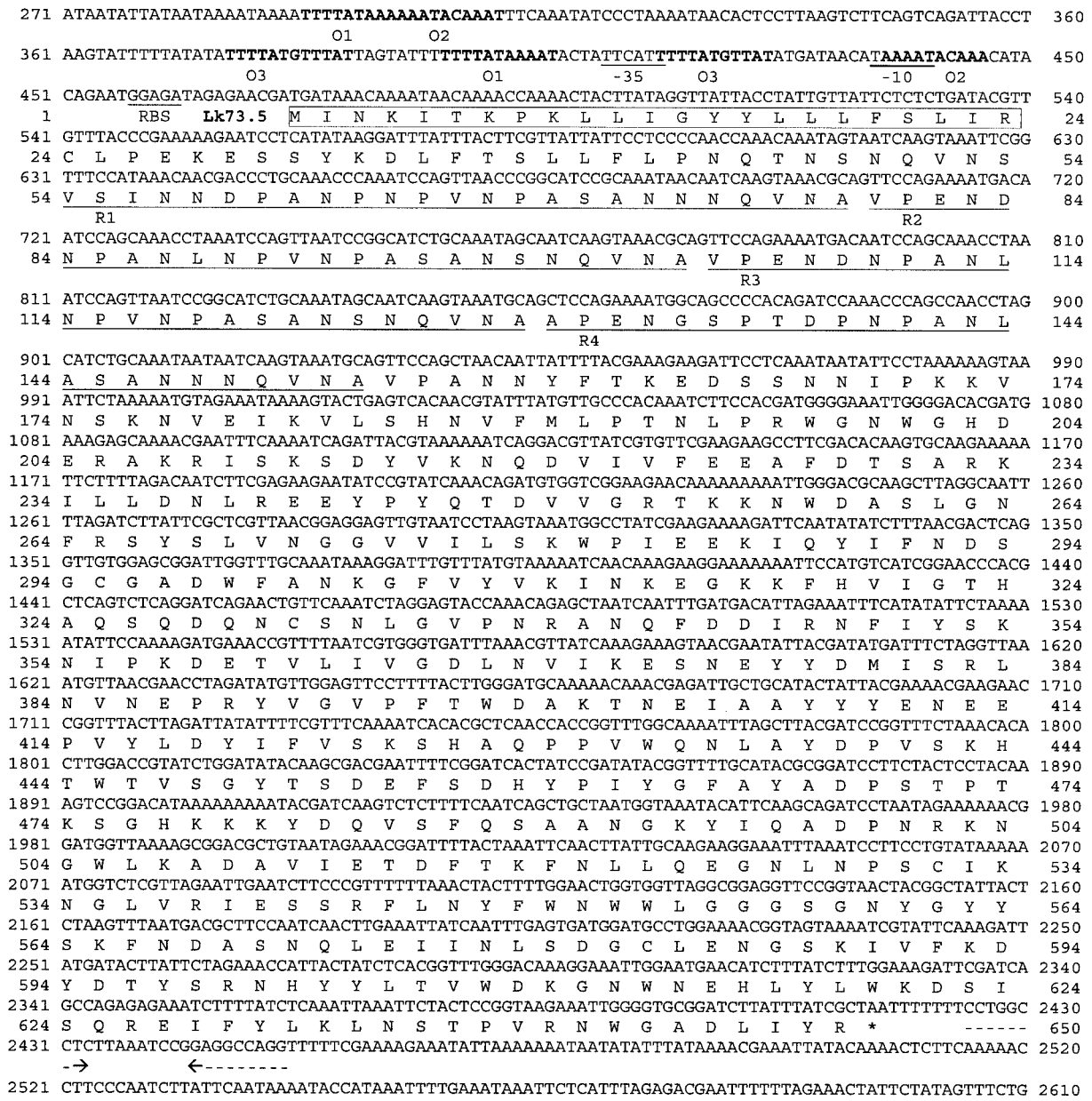


FIG. 2. Nucleotide sequence of the leptospiral gene encoding Lk73.5. The sequence of the 4.1-kb fragment is shown from position 271 through 2610. Probable −10 and −35 promoter sequences, ribosomal binding sites (RBS), and amino acid repeats R1 to R4 are underlined. The putative signal sequences are boxed, and a putative ρ-independent transcriptional terminator is indicated by dashed arrows. The direct nucleic acid repeats are shown in boldface.

sequence with a lipobox-like (LIR ↓ C) structure at its carboxy terminus. The proceeding amino acid sequence contained four 25-mer repeats spanning residues 54 to 154. Two of these repeats, R2 and R3, are perfectly identical; the others are partially divergent. A second open reading frame located downstream of *lk73.5* (Fig. 1) was designated *sph-like 2* and encoded a 28.7-kDa peptide with partial homology to the amino terminus of Lk73.5.

Homology to other proteins. The Lk73.5 protein showed 95.1% amino acid identity with a putative sphingomyelinase, Sph2, from *L. interrogans* serovar Lai (41). Excluding the amino-terminal repetitive sequence, 63.9 and 54.9% homology was shared with sphingomyelinase C from *L. borgpetersenii* serovar Hardjo (42) and with hemolytic protein of *L. interrogans* serovar Lai (28), respectively. The central part of Lk73.5 (180 to 462 amino acid residues) corresponding to the exo/endo/phos conservative domain (pfam03372.5) also demonstrated substantial homology to phospholipases from *Staphylococcus aureus* (49.8%) (39), *Bacillus cereus* (47.6%) (23), *Listeria ivanovii* (46.9%) (14), and *Pseudomonas spp*. (41.9%) (46).

Expression of Lk73.5 by cultured leptospiras. Western blot analysis of whole-cell lysates of *L. interrogans* serovar Pomona type kennewicki cultured at 30°C, at 37°C, and after a temperature shift from 30 to 37°C (Fig. 3) did not show detectable

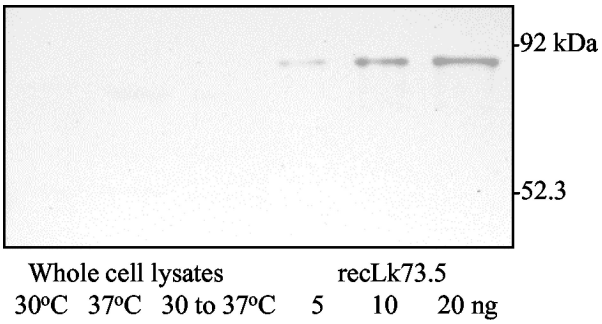


FIG. 3. Immunoblot of *L. interrogans* serovar Pomona type kennewicki cultured at 30°C, cultured at 30°C and shifted to 37°C, and cultured at 37°C. Whole-cell lysates containing 20 μg of total protein and 5, 10 and 20 ng of recLk73.5 were separated by SDS-PAGE, transferred to nitrocellulose, and probed with horse antiserum against recLk73.5.

levels of Lk73.5 expression. Lk73.5 was not detected by Western blotting and enzyme-linked immunosorbent assay in supernatants of all leptospira cultures (data not shown).

Reactivity of recLk73.5 with horse sera. All sera from mares that had recently aborted showed strong reactivity with recLk73.5 (Fig. 4). Negative control sera included sera from horses infected with *B. burgdorferi* or human granulocytic ehrlichiosis agent or from horses with no exposure to these agents or leptospiras. Sera from horses immunized with *L. interrogans* serovar Pomona type kennewicki grown at 30 or 37°C or vaccinated with commercial pentavalent leptospira vaccine were unreactive.

Hemolytic and cytotoxic activities of recLk73.5. EEC exposed to serial dilutions of Lk73.5 released LDH in a concentration-dependent manner that reached a plateau at 25 μg/ml. The effect was less than but comparable to that for phospholipase C from *C. perfringens*, which was used as a positive control. Leptospira outer membrane lipoprotein Q1p42 (36), expressed and purified under the same conditions as Lk73.5, had almost no effect on release of LDH (Fig. 5A). recLk73.3 was only mildly hemolytic for rabbit and horse erythrocytes (Fig. 5B), and this activity was not increased in the presence of well-known phospholipase activators, such as Ca, Mg, and Mn (28, 46), at concentrations of 2 to 10 mM (data not shown).

***lk73.5* in other leptospiras.** Southern blot hybridization with a probe consisting of the specific 5′ end of *lk73.5* revealed only a single gene copy in all *L. interrogans* serovars. With the exception of *L. inadai*, other *Leptospira* spp. did not contain the gene. Bands that hybridized were of similar size (2.1 kb) for *L. interrogans* serovar Pomona type kennewicki and *L. interrogans* serovar Pomona, whereas for other leptospiras they varied from 1.7 to 2.5 kb, indicating differences in upstream sequences (Fig. 6). PCR amplification with primers expF and expR, specific for sequence encoding mature Lk73.5, produced bands that were of the predicted size for *L. interrogans* serovars Pomona and Canicola but slightly smaller for other leptospiras. No signals were detected for *L. borgpetersenii*, *L. santarosiae*, *L. weilii*, *L. noguchi*, or *L. kirschneri* (data not shown). Subsequent sequencing of the 5′ ends of amplicons showed that the differences are due to deletion of 75 nucleotides from the sequence encoding repeat R3, one of the two perfect repeats located in the N terminus of Lk73.5 (Fig. 7).

DISCUSSION

Currently used leptospiral vaccines provide protection by inducing production of antibodies against lipopolysaccharides, serogroup-specific antigens found on the bacterial surface. These vaccines induce high levels of lipopolysaccharide-specific antibodies that confer short-term protection against acute infection by the corresponding serovars. A growing body of experimental data suggests that an effective cross-protective vaccine based on one or more leptospiral proteins is feasible (6, 19, 45). Because of their accessibility to host antibody, proteins located in the outer membrane are promising candidates for inclusion in future vaccines. Several such proteins, including LipL32 (18), LipL41 (44), and OmpL1 (17), for which an outer membrane location has been demonstrated experimentally, have been identified. This list might also include immunoreactive proteins such as hemolysin (28), GroEL (2), DnaK (1, 15), and LipL45 (32), the cellular location of which is not well defined. These proteins were identified either by screening chromosomal DNA libraries with rabbit antisera to in vitro-grown leptospira (LipL45) or by hybridization with oligonucleotide probes based on N-terminal amino acid sequences of particular proteins isolated from cultured leptospira (OmpL1, LipL41, and LipL32) and by PCR with degenerative primers (hemolysin). Thus, proteins involved in the infectious process and absent or poorly expressed on cultured leptospira might a priori have been excluded from further consideration. Our approach assumed that potential protective antigens, represented in the gene library with the same probability as other proteins, might be identified by sequential screening with convalescent-phase antibody and antibody specific for the antigens of in vitro (30°C)-cultured bacteria (bacterin).

In previous work, a similar double-antibody screen of a chromosomal DNA library of *L. interrogans* serovar Pomona type kennewicki to detect proteins upregulated at 37°C led to discovery of the immunogenic lipoprotein Qlp42, expression of

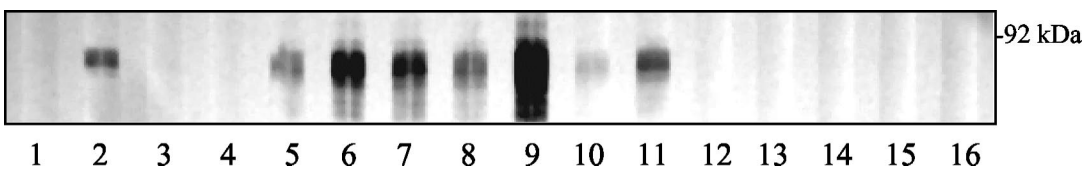


FIG. 4. Reactivity of recLk73.5 with horse sera. The protein was purified by metal affinity chromatography, subjected to SDS-PAGE separation, and probed with normal horse serum (lane 1), horse serum specific for recLk73.5 (lane 2), equine Lyme disease-positive serum (lane 3*), Ehrlichia* sp.-positive serum (lane 4), post-leptospiral abortion sera (lanes 5 to 11), horse antisera to bacterins prepared from *L. interrogans* serovar Pomona type kennewicki cultured at 30 and 37°C (lanes 12 and 13, respectively), and sera from horses immunized with pentavalent commercial bacterin (lanes 14 to 16).

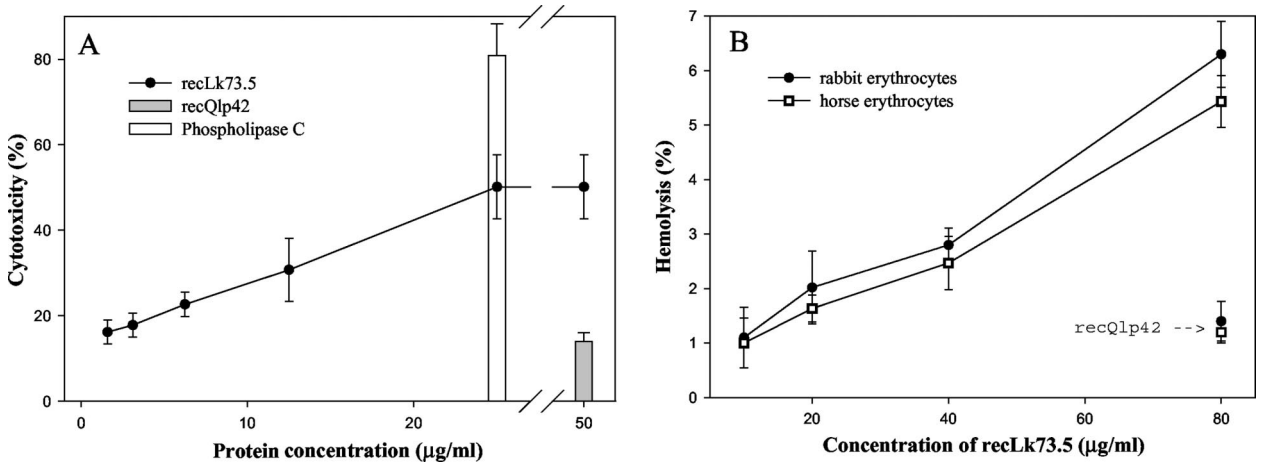


FIG. 5. Cytotoxic (A) and hemolytic (B) activities of recLk73.5. Cytotoxicity was assayed by measuring LDH released from EEC after 1 h of incubation with different concentrations of recLk73.5. Phospholipase C from *C. perfringens* (25 μg/ml) and recQ1p42 (50 μg/ml) (36), expressed and purified under the same conditions as Lk73.5, were used as positive and negative controls, respectively. Hemolytic assays were performed on a 10% suspension of rabbit or horse erythrocytes in Tris-buffered saline at 37°C for 1 h with different concentrations of recLk73.5. RecQ1p42 (80 μg/ml) was used as a negative control. Hemolysis was expressed as the percentage of hemoglobin released from the same volume of erythrocytes in distilled water minus background values. Results represent the means from three experiments ± standard deviations.

which is apparently induced by temperature shift during host invasion. Subsequently, LipL45, a homolog of this protein in *L. kirschneri* with 98% amino acid sequence identity, was described by Matsunaga et al. (32). Double-antibody screening potentially provides a means to detect leptospiral antigens that are expressed in response to cues produced during infection. Although several clones reactive with convalescent-phase but not with bacterin-specific antibody were isolated, the present study focused on Lk73.5, which was expressed by 6 of 11 clones remaining following the double-antibody screening. Lk73.5

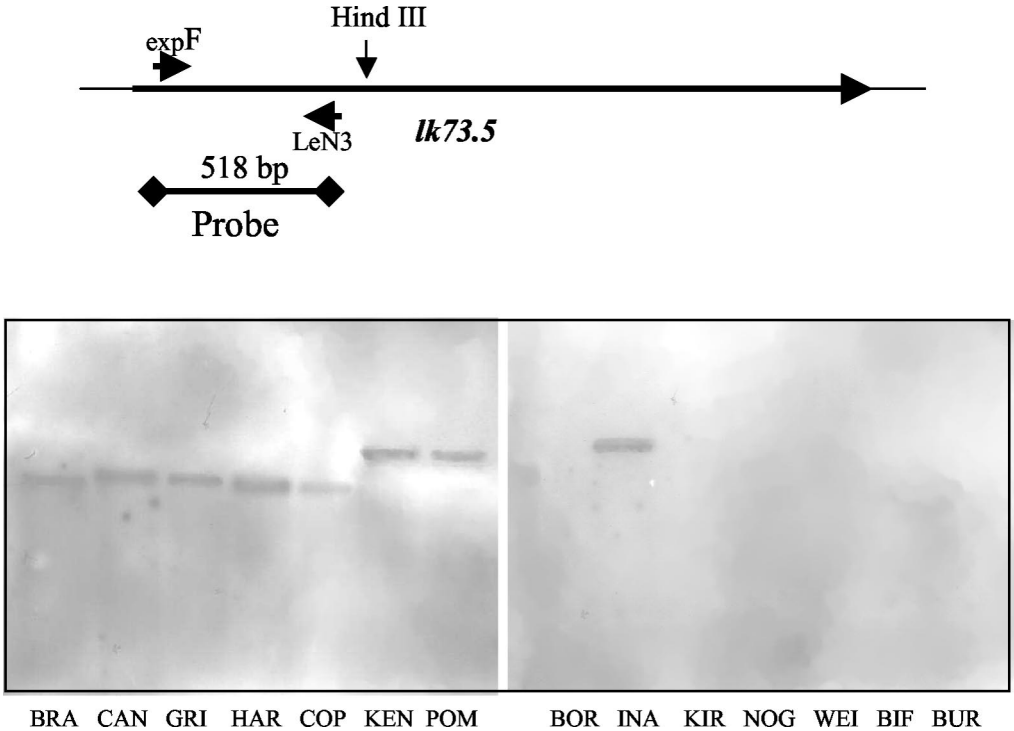
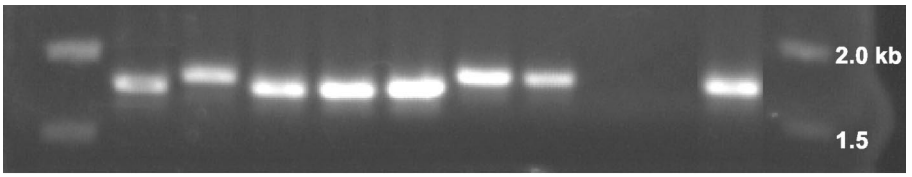


FIG. 6. Southern blot analysis of genomic DNAs from selected strains of *Leptospira* spp. DNA of *B. burgdorferi*, the genomic DNA of which has no sequence homologous to *lk73.5*, was used as a negative control. Genomic DNAs were digested with *Hin*dIII and probed with a biotin-labeled fragment of the 5′ terminus of *lk73.5*, which has less than 40% overall homology with other leptospiral sphingomyelinase genes. Lanes: BRA, *L. interrogans* serovar Bratislava; CAN, *L. interrogans* serovar Canicola; GRI, *L. interrogans* serovar Grippotyphosa; HAR, *L. interrogans* serovar Hardjo; COP, *L. interrogans* serovar Copenhageni; KEN, *L. interrogans* serovar Pomona type kennewicki; POM, *L. interrogans* serovar Pomona; BOR, *L. borgpetersenii*; INA, *L. inadai*; KIR, *L. kirschneri*; NOG, *L. noguchi*; WEI, *L. weilii*; BIF, *L. biflexa*; BUR, *B. burgdorferi*. The schematic on the top shows the positions of the single *Hin*dIII site and of the sequence used as a probe.




```
      92   R2              R3                    R4    141
KEN   NPASANSNQVNAVPENDNPANLNPVNPASANSNQVNAAPENGSPTDPNPA
BRA   NPASANSNQVNA----------------------APXNGSPADPNPT
CAN   NPASANSNQVNAVPENDNPANLNPVNPASANSNQVNAAPENGSPTDPNPA
COP   NPASANSNQVNA----------------------APXNGSSAXPNPA
GRI   NPASANSNQVNA----------------------APENGSSAXPNPA
HAR   NPASANSNQVNA----------------------APENGSPADPNPA
POM   NPASANSNQVNAVPENDNPANLNPVNPASANSNQVNAAPENGSPTDPNPA
INA   NPASANSNQVNA----------------------APENGSPADPNPA
LAI*  NPASANSNQVNA----------------------APENGSPADPNPA
```

***LAI, *Leptospira interrogans* serovar Lai (accession number NC 004342).**

FIG. 7. PCR amplification of DNAs from different leptospiras and *B. burgdorferi* with primers expF and expR and comparison of the amino acid sequences predicted for the 5′ ends of amplicons. Lanes: BRA, *L. interrogans* serovar Bratislava: CAN, *L. interrogans* serovar Canicola; GRI, *L. interrogans* serovar Grippotyphosa; HAR, *L. interrogans* serovar Hardjo; COP, *L. interrogans* serovar Copenhageni; POM, *L. interrogans* serovar Pomona; KEN, *L. interrogans* serovar Pomona type kennewicki; BIF, *L. biflexa*; IN, *L. inadai*; BUR, *B. burgdorferi*; M, 1-kb DNA ladder.

consists of 648 amino acid residues, the first 23 of which are followed by cysteine, a possible lipoprotein signal sequence. Although arginine in the −1 position is quite unusual as a lipoprotein signal peptidase cleavage site (7, 16, 47, 48), limited data on the spirochetal lipobox suggest a less conservative definition. Experimental evidence will be required to confirm whether Lk73.5 has a lipoprotein mode of membrane anchoring.

Recombinant Lk73.5 was strongly reactive with sera from mares following leptospiral abortion but showed no reactivity with sera from horses immunized with bacterins prepared from *L. interrogans* serovar Pomona type kennewicki grown at 30 and 37°C or from horses vaccinated with a commercial bacterin containing inactivated cultures of *L. interrogans* serovars Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona. In addition, no reactive band corresponding to Lk73.5 was detected by Western blot analysis of whole-cell lysates of *L. interrogans* serovar Pomona type kennewicki cultured at 30 or 37°C. These observations together suggest that Lk73.5 is produced only during infection. Repeats flanking the putative *lk73.5* promoter may function in regulation of this gene activity in a manner similar to that described for the Gal repressosome of *E. coli* (31, 43).

The moderate cytotoxicity of Lk73.5 for primary EEC as shown by release of LDH supports a role of this protein in virulence. The vascular endothelium is an important site of pathology during leptospira infection, and resulting damage to blood vessels is responsible for the characteristic hemorrhages seen in the acute disease. The mechanism of action of Lk73.5 may involve enzymatic degradation of membrane components or pore formation as described for SphH of *L. interrogans* serovar Lai (29). It is possible that the typical vascular damage requires the cooperative effect of Lk73.5 and other sphingomyelinases.

In conclusion, our studies suggest that Lk73.5, as recently described for LigA, a protein of unknown significance in virulence (37), is a host-inducible antigen which elicits strong antibody responses in infected animals. Membrane damage to endothelial cells and erythrocytes indicates its potential as a virulence factor. Although its production by other leptospiras during host invasion has not yet been demonstrated, the presence of *lk73.5* in all investigated *L. interrogans* strains and in at least one other species supports such a possibility. Thus, a body of direct and circumstantial evidence supports consideration of Lk73.5 as a component for inclusion in a subunit vaccine against leptospirosis. The protein should also have value in differentiation of antibody responses to vaccination with in vitro-grown leptospiras from those stimulated by natural infection.

ACKNOWLEDGMENTS

This work was funded by the Keeneland Association. J. E. Nally was funded by a Maxwell and Muriel Gluck Fellowship in Equine Studies.

We thank Richard Zuerner for providing leptospiral DNAs and Mike Donahue, Barbara Smith, and Yung Fu Chang for antisera.

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Editor: W. A. Petri, Jr.