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Cloning of a Hemolysin Gene from *Leptospira interrogans* Serovar hardjo

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A DNA fragment encoding both hemolysin and sphingomyelinase C activity was cloned from the pathogenic bacterium *Leptospira interrogans* serovar hardjo. Initial clones were obtained by screening a genomic library in EMBL3 for hemolytic activity. Both hemolytic and sphingomyelinase C activities were coded for by a 3.9-kilobase *Bam*HI fragment. The hemolysin was expressed from its own promoter in *Escherichia coli* K-12. Similar DNA sequences were also present in the serovars tarassovi and ballum.

The genus *Leptospira* comprises two species. One, *L. biflexa*, is nonpathogenic and lives free in soil, water, or marine habitats (15, 16). The other *L. interrogans*, is the etiologic agent of leptospirosis, a worldwide infection affecting both humans and wild and domestic animals (16). In spite of the importance of leptospirosis, the mechanism of pathogenicity is still unknown. Virulent strains of *Leptospira* exhibit higher hemolysin activity than avirulent strains, and strains cultured for long periods under laboratory conditions lose virulence and hemolysin production simultaneously (25). Hemolysin, protease (e.g., collagenase), hyaluronidase, and phospholipase production have been claimed as possible virulence factors (2, 23–25). Here we report the cloning of a DNA fragment coding for one of these potential virulence factors, the hemolysin.

Leptospiral strains were grown in EMJH medium (24) to a density of 5×10^8 cells per ml. Chromosomal DNA was extracted from 100-ml cultures as described previously (13). A genomic library of *L. interrogans* (serovar hardjo, strain Sponselee) DNA in EMBL3 containing 4.3×10^5 recombinant phages was constructed. All cloning procedures were performed as described by Maniatis et al. (20). A total of 8,000 recombinant phages were plated onto LB agar, and after overnight incubation they were overlaid with washed sheep erythrocytes in top agar. After 4 to 6 h at 37°C, five plaques (HL1 to HL5) showed β-hemolytic zones. Isolation of DNA from these recombinant phages and restriction enzyme analysis showed that all shared some DNA fragments. This was confirmed by Southern hybridization analysis. A 3.9-kilobase (kb) *Bam*HI fragment (pHL2-B3), which was derived from the 13.2-kb insert of HL2, cloned into the plasmid vector Bluescript (Stratagene) still conferred hemolytic properties to the transformed cells. Hemolysis could be detected independently of the orientation of the insert; the hemolysin was therefore probably transcribed from its own promoter in *Escherichia coli*. This is in accordance with the observations of Charon and co-workers (26, 27), who cloned the *trpE* and *argE* genes from the apathogenic *L. biflexa* in *E. coli* and found that these genes are transcribed in *E. coli*. The nucleotide sequence of the *argE* gene has been determined (27), and a sequence resembling the *E. coli* promoter consensus sequence has been identified (27). Since transcription of leptospiral genes in *E. coli* has now been observed in two independent cases, leptospiral promoters seem to be recognized by *E. coli* RNA polymerase.

Protein synthesis encoded by pHL2-B3 was determined in minicells by the method of Gaastra and Klemm (11). The plasmid codes for a protein with a molecular mass of 39.2 kilodaltons (kDa). In the presence of 10% ethanol (which inhibits the signal peptidase I of *E. coli*), a protein of 41.6 kDa was expressed (Fig. 1).

Genomic DNA from 16 different pathogenic and nonpathogenic serovars belonging to 13 serogroups of *Leptospira* species were tested for the presence of the hemolytic determinant. The 3.9-kb *Bam*HI fragment of pHL2-B2 was radiolabeled by random priming (10) and was used as a probe. The genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, blotted, and hybridized at 42°C as described previously (5). The filters were washed three times in 0.3 M sodium chloride–0.3 M sodium citrate (pH 7.0) containing 0.1% sodium dodecyl sulfate at room temperature and twice in 0.015 M sodium chloride–0.0015 M sodium citrate (pH 7.0) containing 0.1% sodium dodecyl sulfate at 65°C. The washed blots were dried and subjected to autoradiography. Only DNA of three strains, all of which were pathogenic, were found to possess DNA sequences homologous to the probe (Fig. 2). Two hybridizing bands of 3.4 and 1.8 kb appeared in lane 6 of Fig. 2 (the serovar hardjo strain used for cloning). The other bands were caused by the partial digestion of the genomic DNA. Two bands of 3.4 and 1.9 kb hybridized with the probe in lane 4 of Fig. 2 (serovar tarassovi) and of 9.0 and 1.9 kb in lane 9 of Fig. 2 (serovar ballum).

To study the nature of the hemolysin, 50 ml of liquid phage lysates from the five hemolytic clones was prepared by superinfection of *E. coli* LE392 in LB medium with a multiplicity of infection of 2. The lysates were spun at 10,000 × *g* for 15 min, and the supernatants were brought to 70% saturation with solid ammonium sulfate and placed overnight at 4°C. The precipitate was collected and was then suspended in 4 ml of 10 mM Tris hydrochloride (pH 7.2). The solutions were dialyzed at 4°C against several changes of Tris hydrochloride (pH 7.2). Partially purified hemolysins were assayed for sphingomyelinase C and phospholipase C activities by using chromogenic substrate analogs as described previously (12, 19), with the modifications of Bernheimer and Bey (4). All five clones gave a positive reaction in the sphingomyelinase C assay and were negative in the

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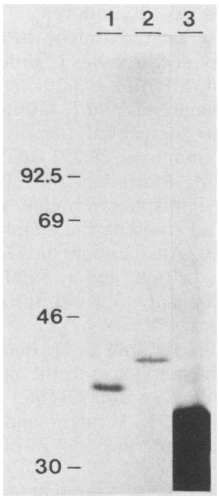

FIG. 1. Autoradiograph of ^{35}S-radiolabeled proteins expressed by plasmid pHL2-B3 in *E. coli* minicells. The proteins were analyzed by electrophoresis in a 15% polyacrylamide gel. The apparent molecular mass is indicated in kilodaltons on the left. Lane 1, pHL2-B3; lane 2, pHL2-B3 (10% ethanol added); lane 3, Bluescript vector without insert.

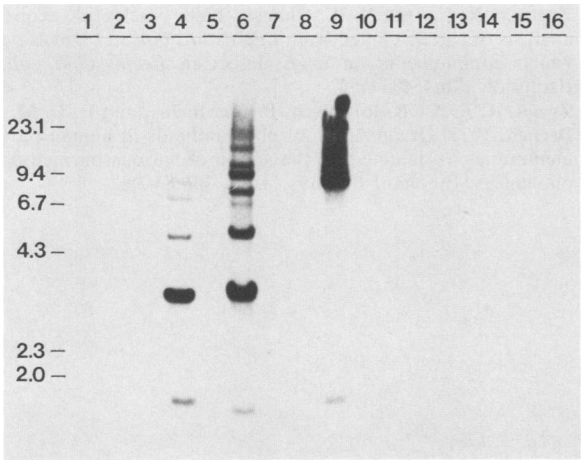

FIG. 2. Presence of the cloned hemolysin-sphingomyelinase C gene among various *Leptospira* strains. Genomic DNA from different strains was digested with *Hin*dIII, electrophoresed in an 1% agarose gel, and blotted onto nitrocellulose. The blot was hybridized with the ^{32}P-radiolabeled, 3.9-kb insert DNA fragment from pHL2-B3. Size markers (in kilobases) are indicated on the left. The following *Leptospira* strains were tested (species, serogroup, serovar, and strain names are indicated, respectively) in the following lanes: 1, *L. interrogans*, Canicola, canicola, Hond Utrecht IV; 2, *L. biflexa*, Sermaranga, patoc, Patoc1; 3, *L. interrogans*, Javanica, poi, Poi; 4, *L. interrogans*, Tarassovi, tarassovi, Mitis Johnson; 5, *L. interrogans*, Icterohaemorrhagiae, copenhageni, Wijnberg; 6, *L. interrogans*, Sejroe, hardjo, Sponselee; 7, *L. interrogans*, Australis, bratislava, Jež-bratislava; 8, *L. interrogans*, Pomona, pomona, Pomona; 9, *L. interrogans*, Ballum, ballum, Mus 127; 10, *L. interrogans*, Icterohaemorrhagiae, copenhageni, RGA; 11, *L. interrogans*, Pyrogenes, pyrogenes, Salinem; 12, *L. interrogans*, Hebdomadis, hebdomadis, Hebdomadis; 13, *L. biflexa*, Semaranga, sao paulo, Sao Paulo; 14, *L. interrogans*, Sejroe, hardjo, Hardjoprajitno; 15, *L. biflexa*, Andamana, andamana, Ch11; 16, *L. interrogans*, Autumnalis, rachmati, Rachmat.

phospholipase C assay. Sonicates of *E. coli* cells transformed with plasmid pHL2-B3 were also sphingomyelinase C positive, when tested with the chromogenic analog and with sphingomyelin from bovine erythrocytes as the substrate (28). It is not known whether the hemolysis of sheep erythrocytes and the sphingomyelinase C activity can be copurified (4), and in strains of other serovars the presence of sphingomyelinase C activity is consistently associated with the hemolysis of sheep erythrocytes (3). Since it was found that incubation times of longer than 48 h are necessary to see a hemolysis zone surrounding colonies transformed with pHL2-B3, the protein is probably only released from the *E. coli* cells when they lyse in the late stationary phase. It is not known whether the hemolysin is only transported to the periplasm, inactivated during transport, or not transported because of incorrect processing in *E. coli* or whether additional polypeptides, which are not present or not functional in *E. coli*, are involved in transport across the bacterial membranes.

The cloning of a hemolysin from another pathogenic strain, *L. interrogans* serovar pomona, has been reported previously (8). This hemolysin is also synthesized as a precursor in *E. coli* and probably differs from the one described here. DNA from our serovar pomona strain, which also had sphingomyelinase C activity (data not shown), did not hybridize with our hemolysin probe; moreover, the molecular mass of a serovar pomona hemolysin is 67 kDa (A. A. Dain, M. N. Rozinov, and Y. G. Chernukha. VI Joint Meeting of European Leptospira workers, abstr. no. 7.7, 1988), versus 39.2 kDa for the serovar hardjo hemolysin. It is likely, in view of the reports on the different hemolytic activities (phospholipase A and sphingomyelinase C) of other leptospiral strains (1–3, 6, 7, 14, 17) and with respect to the assumed role of hemolysin production as a virulence factor (21–25), that several hemolysin genes are present in members of the genus *Leptospira*.

Leptospires do not grow in medium without iron (9). Therefore, hemolysin may be important for the release of iron from host erythrocytes, since free iron is not easily available in living organisms. Another possible role for the hemolysin is the generation of free fatty acids, the main carbon and energy source of leptospires (which they are not able to synthesize themselves) (16), by degradation of sphingomyelin. Sphingomyelin is a major lipid component of the cellular membranes of mammals (up to 50% in ruminant erythrocytes) (18). Further analysis of the cloned sphingomyelinase C gene will contribute to the understanding of the molecular biology of *Leptospira* species and give more insight in the role of sphingomyelinase C production in pathogenesis.

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LITERATURE CITED

1. **Alexander, A. D., G. Wood, F. Yancey, R. J. Byrne, and R. H. Yager.** 1971. Cross-neutralization of leptospiral hemolysins from different serotypes. Infect. Immun. **4**:154–159.
2. **Bauer, D. C., L. N. Eames, S. D. Sleight, and L. C. Ferguson.** 1961. The significance of leptospiral hemolysin in the pathogenesis of *Leptospira pomona* infections. J. Infect. Dis. **108**: 229–236.
3. **Bazovska, S.** 1978. Spingomyelinase activity of leptospira cultures. Czech. Epidemiol. Microbiol. Immunol. **27**:137–143.
4. **Bernheimer, A. W., and R. F. Bey.** 1984. Copurification of *Leptospira interrogans* serovar *pomona* hemolysin and sphingomyelinase C. Infect. Immun. **54**:262–264.
5. **Brendle, J. J., M. Rogul, and A. D. Alexander.** 1974. Deoxyri-

bonucleic acid hybridization among selected leptospiral serotypes. Int. J. Syst. Bacteriol. **24**:205–214.
6. **Chorváth, B.** 1974. Some properties of leptospiral phospholipase A and hemolysin. Folia Fac. Med. Univ. Comenianae, Bratislava **12**(Suppl.):43–56.
7. **Chorváth, B.** 1975. Hemolysis of human and sheep erythrocytes by pathogenic and saprophytic leptospiral strains. Biologia (Bratislava) **30**:723–726.
8. **Dain, A. A., M. N. Rozinov, T. A. Holzmayer, V. N. Gershanovich, and Y. G. Chernukha.** 1985. Cloning and the expression of the hemolysin gene of *Leptospira pomona pomona* in *Escherichia coli*. Zh. Microbiol. Epidemiol. Immunobiol. **6**:33–35.
9. **Faine, S.** 1959. Iron as growth requirement for pathogenic leptospira. J. Gen. Microbiol. **20**:246–251.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132**:6–13.
11. **Gaastra, W., and P. Klemm.** 1984. The identification of gene products in minicells and maxicells, p. 333–342. *In* J. M. Walker (ed.), Methods in molecular biology, vol. 2. Humana Press, Clifton, N.J.
12. **Gal, A. E., R. O. Brady, J. A. Barranger, and P. G. Pentchev.** 1980. The diagnosis of type A and type B Niemann-Pick disease and detection of carriers using leukocytes and a chromogenic analogue of sphingomyelin. Clin. Chim. Acta **104**:129–132.
13. **Garcia, E., H. E. N. Bergmans, J. F. van den Bosch, I. Ørskov, B. A. M. van der Zeijst, and W. Gaastra.** 1988. Isolation and characterization of dog uropathogenic *Escherichia coli* strains and their fimbriae. Antonie van Leeuwenhoek J. Microbiol. Serol. **54**:149–163.
14. **Hathaway, S. C., and R. B. Marshall.** 1980. Haemolysis as a means of distinguishing between *Leptospira interrogans* serovars *balcanica* and *hardjo*. J. Med. Microbiol. **13**:477–481.
15. **Johnson, R. C.** 1981. Aerobic spirochetes; the genus *Leptospira*. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schegel (ed.), The prokaryotes, a handbook on habitats, isolation and identification of bacteria, p. 582–591. Springer-Verlag, New York.
16. **Johnson, R. C., and S. Faine.** 1984. Genus I. Leptospira Noguchi 1917, 755, p. 62–67. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
17. **Kasarov, L. B.** 1970. Degradation of the erythrocyte phopholipids and hemolysis of erythrocytes of different animal species by leptospirae. J. Med. Microbiol. **3**:29–37.
18. **Kojima, T., Y. Yanagihara, and I. Mifuchi.** 1984. Characterization of inhibitor to leptospiral hemolysin present in bovine serum. Microbiol. Immunol. **28**:291–302.
19. **Kurioka, S., and M. Matsuda.** 1976. Phospholipase C assay using p-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75**:281–289.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Rogols, S., W. B. Fizette, and E. H. Bohl.** 1959. Studies on the relationship of a phospholipase to the hemolysin of *Leptospira pomona*. Am. J. Vet. Res. **20**:592–596.
22. **Stalheim, O. H. V.** 1971. Virulent and avirulent leptospires: biochemical activities and survival in blood. Am. J. Vet. Res. **32**:843–849.
23. **Thompson, J. C., and R. B. Marshall.** 1986. In vitro studies of haemolysis by *Leptospira interrogans* serovars *pomona* and *ballum*. Vet. Microbiol. **11**:285–292.
24. **Trowbridge, A. A., J. B. Green III, J. D. Bonnett, S. B. Shohet, B. D. Ponnappa, and W. B. McCombs III.** 1981. Hemolytic anemia associated with leptospirosis. Morphologic and lipid studies. Am. J. Clin. Pathol. **76**:493–498.
25. **Volina, E. G., L. F. Levina, and G. L. Soboleva.** 1986. Phospholipase activity and virulence of pathogenic leptospira. J. Hyg. Epidemiol. Microbiol. Immunol. **30**:163–169.
26. **Yelton, D. B., and N. W. Charon.** 1984. Cloning of a gene required for tryptophan biosynthesis from *Leptospira biflexa* serovar *patoc* into *Escherichia coli*. Gene **28**:147–152.
27. **Zuerner, R. L., and N. W. Charon.** 1988. Nucleotide sequence analysis of a gene cloned from *Leptospira biflexa* serovar *patoc* which complements an *argE* defect in *Escherichia coli*. J. Bacteriol. **170**:4548–4554.
28. **Zwaal, R. F. A., B. Roelofsen, P. Comfurius, and L. L. M. van Deenen.** 1975. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. Biochim. Biophys. Acta. **406**:83–96.