

Penicillin-Binding Proteins in *Borrelia burgdorferi*

CARL URBAN,^{1,2*} JAMES J. RAHAL,^{1,3} RAYMOND J. DATTWYLLER,⁴ PETER GOREVIC,⁴
AND BENJAMIN J. LUFT⁴

Department of Medicine, Booth Memorial Medical Center, Flushing, New York 11355¹; Department of Microbiology, New York University School of Medicine, New York, New York 10016²; Department of Medicine, Albert Einstein College of Medicine, New York, New York 10461³; and Department of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794⁴

Received 23 April 1990/Accepted 18 July 1990

Penicillin-binding proteins were identified in *Borrelia burgdorferi* membranes. A 94-kilodalton penicillin-binding protein was the first to be labeled with tritiated penicillin and was the first band to disappear in a competition experiment. Its binding ability was destroyed when membranes were preboiled. In addition, several of these penicillin-binding proteins comigrated with bands previously identified as surface proteins.

The primary therapy for infections caused by *Borrelia burgdorferi*, the etiological agent of Lyme disease, has been beta-lactam antibiotics, including penicillin and ceftriaxone (6, 15). Spratt first demonstrated that beta-lactam antibiotics inactivate enzymes involved in peptidoglycan synthesis in both gram-positive and gram-negative bacteria and named these proteins penicillin-binding proteins (PBPs) (13, 14). Penicillin is effective against *Treponema pallidum* (7) and *Borrelia hermsii* (12), and several recent reports have demonstrated PBPs in these organisms, indicating that they possess peptidoglycanlike structures (1a, 5, 11). Although penicillin is also effective against *B. burgdorferi*, penicillin concentrations as high as 1 mg/ml fail to kill 99% of *B. burgdorferi* organisms within the first 24 h of coinoculation (9). We have conducted experiments to identify PBPs in *B. burgdorferi* to explore possible mechanisms for this delayed killing.

B. burgdorferi (ATCC B31) was grown in BSK 11 medium (1) at 33°C for 4 days to a concentration of approximately 10⁹ cells per ml. The cells were harvested by centrifugation at 10,000 × g for 10 min at 10°C. The pellet was washed and centrifuged three times in cold phosphate-buffered saline (pH 7.2). Membranes were prepared by suspension of the cells in phosphate-buffered saline at a density of 10⁹ cells per ml and sonicated (Biosonic) with six 15-s pulses at 4°C or frozen-thawed two times at -70°C. Undisrupted cells and cell debris were removed by centrifugation at 4,000 × g for 20 min, and the supernatant was centrifuged at 46,000 × g for 60 min at 4°C. The pellet was washed, and membranes were suspended in phosphate-buffered saline at a concentration of 3 mg/ml and kept frozen at -70°C until ready for use. Protein was determined by the method of Bradford (4), with bovine serum albumin as a standard. Benzyl[4-³H]penicillin (72.8 mCi/mg; gift of Merck & Co., Inc., Rahway, N.J.) was dried in a 1.5-ml Eppendorf tube, 200 µg of membranes was added, and the mixture was incubated for 15 min at 37°C. The reaction was stopped by the addition of 5 µl of unlabeled benzylpenicillin G (60 mg/ml; Squibb-Marsan, Inc., Cherry Hill, N.J.). In some experiments, 20 µl of 20% Sarkosyl was added. For competition experiments, various concentrations of antibiotics were incubated with membranes for 20 min at 37°C, tritiated penicillin was added, and the mixture was incubated for 15 min at 37°C.

PBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Laemmli buffer system (8) and 10% gels. En³Hance (New England Nuclear Corp., Boston, Mass.) was used for fluorography by following manufacturer specifications. Gels were dried and exposed to Kodak X-Omat R film or preflashed film at -70°C for 3 to 14 days.

Figure 1, lane a, shows a typical Coomassie blue-stained preparation of *B. burgdorferi* membranes, and Fig. 1, lanes b to d, shows PBP profiles of *B. burgdorferi* membranes incubated with increasing amounts of radiolabeled penicillin.

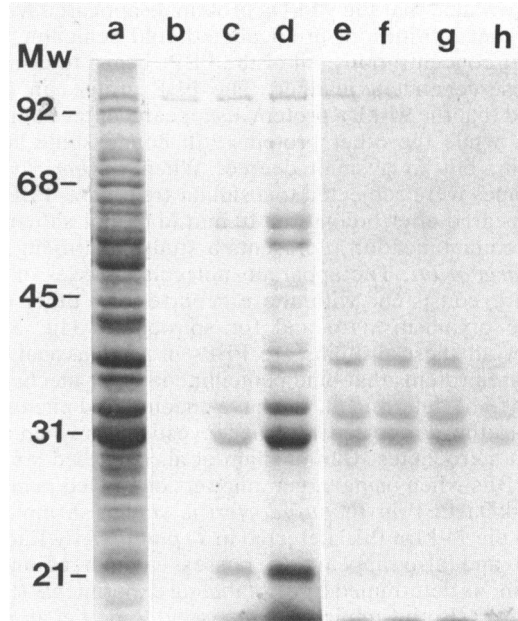


FIG. 1. PBPs of *B. burgdorferi*. Membrane preparations of *B. burgdorferi* were coinoculated with various concentrations of penicillin, separated on 10% SDS-PAGE slab gels, and fluorographed. Lanes: a, Coomassie blue-stained gel of *B. burgdorferi* membranes (200 µg); b to d, fluorograms obtained when penicillin was used at 0.32 µg/ml (lane b), 3.2 µg/ml (lane c), and 6.4 µg/ml (lane d) in the coinoculation. In competition experiments, membrane preparations were preincubated with no penicillin (lane e) or with 0.24 µg (lane f), 2.4 µg (lane g), or 24 µg (lane h) of cold penicillin per ml prior to being coinoculated with 3.2 µg of tritiated penicillin per ml. Molecular weights (Mw) are given in thousands.

* Corresponding author.

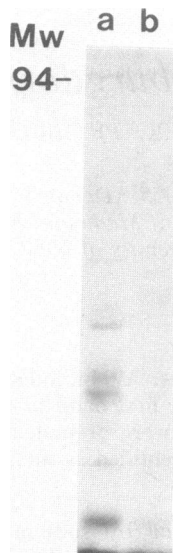


FIG. 2. Effect of heat denaturation on the PBPs. Membrane preparations (200 μ g) were either preboiled (lane b) or not (lane a) prior to coincubation with tritiated penicillin. Proteins were separated on 10% SDS-PAGE slab gels and fluorographed. The molecular weight (Mw) is given in thousands.

PBPs with apparent molecular masses of 94 and 57 kilodaltons (kDa) were labeled with the smallest amounts of penicillin, followed by PBPs of 64, 59, 39, 37, 34, 31, 22, and 18 kDa. The results of a competition experiment (Fig. 1, lanes e to h) revealed that the 94-kDa protein disappeared with the lowest concentration of preincubated cold penicillin, while at higher concentrations, all of the PBPs began to disappear in a dose-dependent manner. The PBP profiles in Fig. 2 revealed that the 94-kDa protein disappeared after boiling for 10 min, while the other proteins still bound some labeled penicillin, but to a small degree. When *Escherichia coli* membranes were subjected to a similar treatment, PBPs 1 to 6 disappeared after boiling for 10 min (data not shown).

This communication represents a study identifying PBPs in *B. burgdorferi*. The apparent molecular masses of these PBPs are consistent with those reported for other gram-negative organisms (16) and for spirochetes (1a, 5, 11). Whether all these proteins are PBPs in the classical sense and/or are proteins that bind penicillin via other mechanisms remains to be determined. We have encountered phenomena similar to those reported by other investigators when working with spirochetes. Cunningham et al. identified six additional PBPs when using larger amounts of labeled penicillin. The 94-kDa PBP in *B. burgdorferi* is similar in molecular mass to the 94-kDa PBP detected in *T. pallidum* by Radolf et al. (11) and also appears to possess a high affinity for penicillin, as determined by our labeling experiments (Fig. 1, lanes b to f). Both Cunningham et al. and Radolf et al. raised questions about proteins that are labeled with penicillin but are not true PBPs (5, 11). *E. coli* membranes were also run as controls in our experiments. The tritiated penicillin that was used in all of our experiments labeled the six classical PBPs reported by Spratt (13), and boiling the membranes before labeling totally obliterated any binding.

Luft et al. described several proteins labeled with two different techniques used to detect surface polypeptides (10). It is interesting to note that the molecular masses of some of these are similar to those of the PBPs in our study, especially

the 31-kDa major outer surface protein (OSP A). In addition, Bergstrom et al. reported sequence homology between the β -lactamase from *Staphylococcus aureus* and the OSP A outer membrane protein from *B. burgdorferi* (3). In previous studies, Luft et al. also demonstrated significant homology between the first 20 amino acid residues of the 22-kDa protein and the deduced amino acid sequence of the OSP A protein (10). Also, immunologic cross-reactivity exists among the highly basic borrelian proteins with apparent molecular masses of 66, 34, 31, 22, and 20 kDa (7a). More recently, Zhu and co-workers identified a protein in *Bacillus licheniformis* with a region that binds beta-lactams (17). This protein has significant sequence homology to class D β -lactamases and no detectable hydrolyzing activity. The results of our studies, taken together with the previously mentioned data, suggest that many of the proteins in *B. burgdorferi* may be functionally related in their ability to bind penicillin. Furthermore, while the ultrastructure of *B. burgdorferi* is not well defined, recent evidence suggests that the PBPs of *E. coli* may not be exclusively located in the cytoplasmic membrane (2). Experiments using immunological approaches to determine the topology of the various borrelian proteins as well as PBP assays with cloned OSP A and OSP B are currently under way. A novel mechanism of penicillin binding to a number of membrane proteins of *B. burgdorferi* may explain the limited lethal effect of penicillin on this organism.

LITERATURE CITED

1. Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* 57:71-75.
- 1a. Barbour, A. G., W. T. Todd, and H. G. Stoenner. 1982. Action of penicillin on *Borrelia hermsii*. *Antimicrob. Agents Chemother.* 21:823-829.
2. Bayer, M. H., W. Keck, and M. E. Bayer. 1990. Localization of penicillin-binding protein 1b in *Escherichia coli*: immunohistochemical microscopy and immunotransfer studies. *J. Bacteriol.* 172:115-135.
3. Bergstrom, S. V., G. Bundoc, and A. G. Barbour. 1989. Molecular analysis of linear plasmid encoded major surface proteins (OSP A and OSP B) of the Lyme disease spirochete, *Borrelia burgdorferi*. *Mol. Microbiol.* 3:479-486.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
5. Cunningham, T. M., J. N. Miller, and M. A. Lovett. 1987. Identification of *Treponema pallidum* penicillin-binding proteins. *J. Bacteriol.* 169:5298-5300.
6. Dattwyler, R. J., J. J. Halperin, D. J. Volkman, and B. J. Luft. 1988. Treatment of late Lyme borreliosis: randomized comparison of ceftriaxone and penicillin. *Lancet* ii:1191-1194.
7. Idsoe, O., T. Guthe, and R. R. Wilcox. 1972. Penicillin in the treatment of syphilis. The experience of three decades. *Bull. W.H.O.* 47(Suppl. 1):1-68.
- 7a. Jiang, W., B. J. Luft, P. Munoz, R. J. Dattwyler, and P. Gorevic. 1990. Cross-antigenicity between the major surface proteins (OSP A and OSP B) and other proteins of *Borrelia burgdorferi*. *J. Immunol.* 144:284-289.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
9. Luft, B. J., J. J. Halperin, D. J. Volkman, and R. J. Dattwyler. 1988. New chemotherapeutic approaches in the treatment of Lyme disease. *Ann. N.Y. Acad. Sci.* 539:93-102.
10. Luft, B. J., W. Jiang, P. Munoz, R. J. Dattwyler, and P. D. Gorevic. 1989. Biochemical and immunohistochemical characterization of the surface proteins of *Borrelia burgdorferi*. *Infect. Immun.* 57:3637-3645.
11. Radolf, J. D., C. Moomaw, C. A. Slaughter, and M. V. Norgard. 1989. Penicillin-binding proteins and peptidoglycan of *Trepo-*

- nema pallidum* subsp. *pallidum*. Infect. Immun. 57:1248-1254.
12. **Schuhardt, V. T.** 1952. Treatment of relapsing fever with antibiotics. Ann. N.Y. Acad. Sci. 55:1209-1221.
 13. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA 72:2999-3003.
 14. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72:341-352.
 15. **Steere, A. C., J. Green, R. T. Schoen, E. Taylor, G. J. Hutchinson, D. W. Rahn, and S. E. Malawista.** 1985. Successful parenteral penicillin therapy of established Lyme disease. N. Engl. J. Med. 312:869-874.
 16. **Tomasz, A.** 1986. Penicillin binding proteins and the antibacterial effectiveness of beta-lactam antibiotics. Rev. Infect. Dis. 8(Suppl. 3):S260-S278.
 17. **Zhu, Y. F., I. H. A. Curran, B. Jones, J. M. Ghuysen, and J. O. Lampen.** 1990. Identification of BlaR, the signal transducer for beta-lactamase production in *Bacillus licheniformis*, as a penicillin-binding protein with strong homology to the OXA-2 beta-lactamase (class D) of *Salmonella typhimurium*. J. Bacteriol. 172:1137-1141.