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Penicillin-Binding Proteins in *Clostridium perfringens*

TIMOTHY F. MURPHY,† MICHAEL BARZA, AND JAMES T. PARK

Division of Infectious Disease, Tufts New England Medical Center, and the Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

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The penicillin-binding proteins (PBPs) of *Clostridium perfringens* were studied. Six PBPs ranging in molecular weight from approximately 42,000 to 100,000 were detected in the cytoplasmic membrane. The relative affinities of the PBPs for 16 beta-lactam antibiotics were determined. Most of the drugs saturated PBP 3 and 4 at concentrations equal to their minimal inhibitory concentrations, suggesting that these PBPs are the killing targets for beta-lactams. Mecillinam showed unique properties; it had a higher affinity for PBP 5 than for other PBPs, and it was the only agent tested which caused inhibition of growth without saturating PBP 4. Interestingly, all beta-lactam antibiotics tested induced filament formation despite having different binding patterns to the PBPs of *C. perfringens*.

Bacteria contain multiple penicillin-binding proteins (PBPs) in their cytoplasmic membranes. Many of these proteins have enzymatic activity, which is important for peptidoglycan synthesis, and perform specific functions related to elongation, septation, and maintenance of cell shape (13, 15, 18). PBPs are also the "targets" of beta-lactam antibiotics. Studies of the interaction between these drugs and receptors have provided insight into both the mechanism of action of beta-lactam antibiotics and some basic principles of bacterial growth and division.

PBPs have been investigated in various species of bacteria, but they have not yet been studied in obligate anaerobes. In this study, we report the presence of six PBPs in *Clostridium perfringens*, determine the relative affinities of each PBP for a variety of beta-lactam antibiotics, and draw preliminary conclusions regarding which PBPs are primary targets for beta-lactam antibiotics. We chose to study this particular species of anaerobe because it is a well-established cause of serious infection for which beta-lactam antibiotics are usually drugs of choice (5-7).

MATERIALS AND METHODS

Bacterial cultures. Strains designated 607, 2696, 2499, and 2533 are clinical isolates of *C. perfringens* which were cultured in the Anaerobic Research Laboratories of Tufts New England Medical Center, Boston, Mass. Colonies from blood agar plates were inoculated into 50 ml of brain heart infusion broth supplemented with yeast extract and hemin (BHIS) in a Coy anaerobic chamber. After overnight incubation at 37°C, the total 50-ml culture was added to 1 liter of prereduced BHIS. After 2 to 4 h in the chamber, the cells were harvested in the late exponential phase of growth by centrifugation in a Sorvall RC-2 centrifuge at 8,000 × *g* for 5 min.

Membrane preparation. Once harvested, the cells were kept on ice, and all centrifugations were performed at 4°C. The cells were washed and suspended in 0.05 M KPO_4 (pH 7.0) buffer. They were then disrupted by intermittent sonication for 3 min with a Branson Sonifier (Branson Sonic Power Co.). After unbroken cells were removed by centrifugation at 1,500 × *g* for 10 min, the membrane fraction was collected by centrifugation at 140,000 × *g* for 10 min, washed, and suspended in 250 μl of buffer. This fraction, consisting of cytoplasmic membrane and cell walls, will be referred to as "membranes." One liter of culture yielded about 500 μl of membrane suspension.

Binding of [^{125}I]furazlocillin. [^{125}I]furazlocillin was used to assay PBPs. Its principal advantage over [^{14}C]benzylpenicillin is that autoradiographs with [^{125}I]furazlocillin are exposed overnight, whereas fluorographs with [^{14}C]benzylpenicillin require several weeks of exposure. To determine if these two radiolabeled agents produced similar patterns, PBPs were assayed by using both agents (see Results). The preparation and properties of [^{125}I]furazlocillin will be published elsewhere.

An amount of [^{125}I]furazlocillin (4,000 μCi/μmol; ~400 μCi/ml) sufficient to saturate all of the PBPs was added to 20 μl of membrane suspension (final concentration of furazlocillin, ~20 μg/ml) and incubated at 30°C for 10 min (1). Binding was stopped by adding 1 ml of a solution of nonradioactive penicillin G (2 mg/ml) and placing the samples on ice. The bound membranes were collected by centrifugation, and the pellets were gently rinsed with buffer. Solubilization of the cytoplasmic membrane proteins (including PBPs) was accomplished by suspending the pellet in 20 μl of 2% sodium lauryl sarcosinate (Sarco-

† Present address: School of Medicine, State University of New York at Buffalo, Buffalo, NY 14215.

syl) and incubating at room temperature for 20 min (3). After centrifugation at 140,000 × *g* for 10 min, 20 μl of supernatant containing bound PBPs was added to 10 μl of gel sample buffer containing 3% sodium dodecyl sulfate, 0.2M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8), 30% glycerol, and 0.003% pyronine Y. The sample was then heated in a boiling water bath for 3 min. The Sarcosyl-insoluble pellet was also boiled with sample buffer and subjected to electrophoresis to determine whether any PBPs were present in this fraction.

Electrophoresis and autoradiography. Each sample was loaded into a gel slot of a sodium dodecyl sulfate-polyacrylamide slab gel which was pre-run at 60 V for 15 min. The stacking gel consisted of 5% acrylamide and 0.07% *N,N'*-methylene-bis-acrylamide; the separating gel consisted of 9% acrylamide and 0.12% *N,N'*-methylene-bis-acrylamide. Electrophoresis was performed at 60 V initially and was increased to 120 V when the tracking dye entered the separating gel (14). When the electrophoresis was completed (2.5 h), the gel was removed from the apparatus, placed on a filter paper, and dried under vacuum. The dried gel was then placed with Kodak X-Omat RP-5 X-ray film and a Dupont intensifying screen and exposed at −70°C for about 18 h. PBPs were detected by inspection of the autoradiograph after it was developed.

Release of bound beta-lactam from PBPs. To measure the rate of release of the products of bound [^{125}I]furazlocillin from PBPs, membranes were incubated with [^{125}I]furazlocillin at 30°C for 10 min. A 100-fold excess of nonradioactive penicillin G was added. Samples of 20 μl were removed from the water bath immediately and after 30, 60, 90, and 120 min and placed on ice (14). Subsequent steps for detecting PBPs were the same as those described above.

Binding of beta-lactam antibiotics. The binding of nonradioactive beta-lactams by PBPs was determined by measuring the amount of each PBP remaining available to react with [^{125}I]furazlocillin after preincubation of membranes with the test antibiotic (1, 14).

Two microliters of a solution of beta-lactam was incubated with 20 μl of membrane suspension at 30°C for 10 min, followed by the addition of [^{125}I]furazlocillin and incubation for an additional 15 min. Binding was terminated by adding 1 ml of non-radioactive penicillin G (2 mg/ml) and placing the sample on ice. Subsequent steps for detection of PBPs were identical to those described above.

The binding patterns of 16 beta-lactams were determined in this manner. Antibiotics were laboratory standard powders which were provided by the companies which produced them. Minimal inhibitory concentrations (MICs) for the various antibiotics were determined by a broth dilution method. Tubes containing BHIS plus varying concentrations of the test antibiotic in twofold dilutions were inoculated with bacteria to yield a concentration of approximately 10^5 organisms per ml. After incubation for 18 h in an anaerobic chamber, the tubes were examined. The MIC was defined as the lowest concentration of antibiotic at which turbidity was absent by visual inspection. Fourfold dilutions above and below the MICs of each agent were assayed, i.e., concentrations equal to 1/16 MIC, 1/4 MIC, 1 MIC, 4 MIC, and 16 MIC. The amount of binding to PBPs of each agent was estimated by visual inspection of the bands. Comparison of these bands with those resulting from binding of [^{125}I]furazlocillin without a competitor allowed an estimation of the amount of binding of the beta-lactams at various concentrations to each PBP. This was expressed as one of three results. Absence of binding was defined as no difference in the intensity of a corresponding band compared with the control sample without a competitor. Saturation of a protein (>90% binding) was defined as complete or almost complete disappearance of a band compared with the control. Partial binding was defined as a diminished intensity of a band compared to the control sample.

Morphological effects of beta-lactam antibiotics. To determine whether beta-lactams with differing affinities for PBPs cause different morphological changes in *C. perfringens*, varying concentrations of penicillin G, mecillinam, cephalothin, cefoperazone, furazlocillin, and ampicillin were added to exponentially growing cultures of strain 607 under anaerobic conditions. Cells were examined at 30-min intervals with a phase-contrast microscope. Observations were continued for 5 h or until the cells lysed.

RESULTS

PBPs. Multiple PBPs were present in each of the strains of *C. perfringens*. Figure 1, lane A shows those of strain 607. There were six PBPs ranging in molecular weight from approximately 100,000 to 42,000, as estimated by comparisons with PBPs of *Escherichia coli*. PBP 5 and 6 were the major bands. The Sarcosyl-insoluble fraction was also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography; no PBPs were detected in this material, confirming that the PBPs were fully soluble in Sarcosyl. PBPs of strain 607 were also assayed by using saturating concentrations of [^{14}C]benzylpenicillin, and the pattern was identical to that obtained by using [^{125}I]furazlocillin.

To assess the possibility that the binding patterns of whole cells might differ from those of membrane preparations, washed cells of strain 607 were incubated with [^{125}I]furazlocillin at 30°C for 10 min. The PBPs were then isolated and subjected to electrophoresis and autoradiography as described above. The resulting PBPs were identical to those obtained with isolated membrane preparations.

To determine whether there might be interstrain differences in the binding patterns, we compared the PBPs of strains 2696, 2499, and 2533 with those of strain 607. The resulting profiles were similar, with a few minor exceptions. Strain 2696 exhibited two additional faint bands, one above and one below PBP 5. In strain 2499, PBP 3 was weaker and PBP 4 was stronger than the corresponding bands in strain 607. The PBPs of strain 2533 appeared to be identical to those of strain 607.

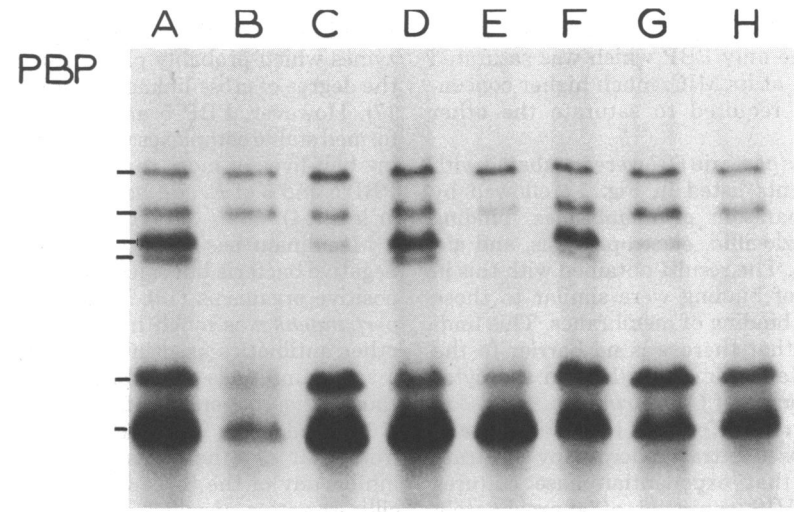

FIG. 1. *Autoradiograph showing PBPs of C. perfringens. Lane A shows PBPs of membranes bound directly with [^{125}I]furazlocillin. Lanes B through H show PBPs of membranes preincubated with non-radioactive beta-lactum antibiotics at their MICs, followed by binding with [^{125}I]furazlocillin. Lane B, penicillin G; lane C, cefoperazone; lane D, mecillinam; lane E, cephalothin; lane F, furazlocillin; lane G, ampicillin; lane H, cefamandole.*

Release of bound beta-lactam from PBPs. We studied the lability of the bonds between antibiotic and PBPs. Membranes of strain 607 bound with [^{125}I]furazlocillin were incubated with excess non-radioactive penicillin G at 30°C for intervals of up to 2 h and subjected to electrophoresis and autoradiography. All six PBPs appeared to be identical to those of membranes bound with [^{125}I]furazlocillin but not incubated with excess penicillin. This finding indicates that the half-life of the PBP-[^{125}I]furazlocillin complex is considerably more than 2 h for all six PBPs of *C. perfringens*.

Binding patterns of beta-lactam antibiotics. Figure 1, lanes B through H, shows the binding patterns of seven different agents to the PBPs of strain 607 at MICs. Table 1 shows a summary of competition experiments with 16 beta-lactams at concentrations equal to their MICs. Each result listed in Table 1 was determined by performing approximately four separate experiments with each agent. Mecillinam had a unique binding pattern and will be discussed separately below. Several observations can be made regarding the binding properties of the remaining 15 agents. (i) All 15 beta-lactams completely saturated PBP 4 at their MICs. Most agents showed partial binding to PBP 4 at lower concentrations. (ii) All agents except furazlocillin and carbenicillin saturated PBP 3 at their MICs. These two agents saturated PBP 3 at four times the MICs. (iii) None of the antibiotics saturated PBP 1 or 2 at or below their MICs. However, cephalothin, cephaloridine, and cefoxitin showed partial binding of PBP 2 at their MICs. These were the only agents tested which contain a thiophene ring on the side chain of the beta-lactam ring. (iv) The concentrations at which different beta-lactams bind PBP 5 and 6 were quite variable.

In contrast to all other beta-lactams assayed, mecillinam failed to saturate PBP 4 even at

TABLE 1. *Binding of beta-lactam antibiotics to PBPs of C. perfringens at their MICs.*

Beta-lactam	MIC (μg/ml)	Binding to PBP:[a]					
		1	2	3	4	5	6
Amoxicillin	0.25	0	0	2+	2+	+	+
Ampicillin	0.031	0	0	2+	2+	0	+
Carbenicillin	0.5	0	0	+	2+	+	+
Cloxacillin	0.25	0	0	2+	2+	+	+
Penicillin G	0.031	0	0	2+	2+	2+	+
Furazlocillin	0.125	0	0	+	2+	+	+
Cefamandole	0.5	0	0	2+	2+	+	+
Cefoperazone	0.016	0	0	2+	2+	0	0
Cefoxitin	1.0	+	+	2+	2+	2+	2+
Cephradine	8.0	+	0	2+	2+	2+	+
Cefuroxime	2.0	+	0	2+	2+	+	+
Cephalexin	1.0	0	0	2+	2+	+	+
Cephaloridine	1.0	0	+	2+	2+	2+	0
Cephalothin	1.0	0	+	2+	2+	2+	0
Moxalactam	0.25	0	0	2+	2+	0	+
Mecillinam	64	0	+	+	+	2+	0

[a] 0, No binding, i.e., no difference in intensity of band as compared to control sample with no competitor; +, binding detected, i.e., diminished intensity of band compared to control; 2+, >90% binding, i.e., almost complete disappearance of band.

concentrations 16 times the MIC of the agent. PBP 5 was the only PBP which was saturated by mecillinam at its MIC; much higher concentrations were required to saturate the other PBPs.

Washed cells of strain 607 were incubated with the seven agents listed in Fig. 1, followed by washing, preparation of membranes, binding with [^{125}I]furazlocillin, electrophoresis, and autoradiography. The results obtained with this in vivo method of binding were similar to those obtained with binding of membranes. This finding indicated that there was no barrier to the binding of beta-lactams by PBPs in the cytoplasmic membrane of *C. perfringens*.

Morphological effects of beta-lactam antibiotics. Phase-contrast microscopy of strain 607 revealed that exponential-phase cultures growing in BHIS consisted of approximately equal proportions of short filaments (about 2 to 6 times the length of a bacillus) and bacilli. The other strains of *C. perfringens* showed varying proportions of these two morphological forms. However, in stationary-phase cultures, all cells were bacilli. The addition of penicillin G, mecillinam, cephalothin, cefoperazone, furazlocillin, and ampicillin to logarithmic-phase cultures of strain 607 all induced long filament formation; the average length of these filaments was approximately 10 to 15 times the length of a bacillus, and some were much longer.

DISCUSSION

The cytoplasmic membrane of *C. perfringens* contained multiple PBPs much like those found in aerobic bacteria. The pattern of PBPs of *C. perfringens* was typical of that seen with other bacilli in that the lower-molecular-weight PBPs constitute the major bands (4).

PBP 3 and 4 were saturated by beta-lactams at their MICs with striking predictability, suggesting that these proteins are the killing targets for beta-lactams. This constancy of the killing target resembles the situation in *Bacillus subtilis*, in which PBP 2 seems to be the lethal receptor (2). By contrast, the killing of *E. coli* cells results from binding of different PBPs by different agents (13).

The variability of the concentrations at which different beta-lactams bind PBP 5 and 6 of *C. perfringens* suggests that the lower-molecular-weight PBPs are not important targets for the lethal action of these agents. For example, cefoperazone completely saturated PBP 3 and 4 and caused cell death at concentrations which resulted in no detectable binding to PBP 5 and 6. In this regard, the latter proteins are similar to the low-molecular-weight PBPs of *E. coli*, which have been identified as carboxypeptidases, enzymes which probably play a role in regulating the degree of cross-linkage of peptidoglycan (10, 17). However, PBP 5 and 6 of *C. perfringens* formed stable complexes with beta-lactams, having half-lives of more than 2 h; by comparison, PBP 5 and 6 of *E. coli* have half-lives of 20 min or less (14).

Mecillinam has activity against many gram-negative bacteria but is less active against gram-positive organisms (19). Indeed, its MIC for *C. perfringens* was much higher than that of the other antibiotics used in this study. In *E. coli*, mecillinam binds specifically to PBP 2 and causes the conversion of rods into ovoid cells (16). In addition, its mode of action is different from that of other beta-lactams; it does not inhibit any of the three known classes of penicillin-sensitive enzymes of *E. coli* (transpeptidases, endopeptidases, and carboxypeptidases) (9, 12). The binding properties of mecillinam to the PBPs of *C. perfringens* were unique. Mecillinam had a higher affinity for PBP 5 than for other PBPs, and it was the only beta-lactam tested which caused inhibition of growth without saturating PBP 4. This property may enable the use of mecillinam as a probe for studying the role of PBP 5 of *C. perfringens* in cell growth and division in future investigations.

The morphological effects on *C. perfringens* of six beta-lactams (penicillin G, mecillinam, cephalothin, cefoperazone, furazlocillin, and ampicillin) were observed. All six agents induced marked filament formation, suggesting interference with septum formation. This effect of mecillinam is somewhat surprising since this agent usually causes formation of ovoid cells. However, mecillinam has recently been reported to cause filamentation in *Fusobacterium nucleatum*, an anaerobic, gram-negative bacillus (11).

One might speculate that PBP 3 or 4 or both are important for septum formation because all agents showed some degree of binding to these PBPs at concentrations which induced filaments. Furthermore, binding experiments suggested that PBP 3 and 4 are the lethal targets for beta-lactam antibiotics. Since one mechanism by which beta-lactams exert their lethal effect is by inhibiting a transpeptidase involved in the terminal stages of peptidoglycan synthesis (20), an intriguing hypothesis is that PBP 3 or 4 or both are vital transpeptidases involved in septum formation. However, further study is needed to identify the specific functions and enzyme activities of these proteins.

In summary, the cytoplasmic membrane of *C. perfringens* contains multiple PBPs. The concentrations at which beta-lactams saturate PBP 3 and 4 correlate well with the MICs of the

agents, suggesting that these proteins are the lethal targets. Further investigation of the PBPs of *C. perfringens* will be interesting to identify specific functions of individual PBPs and to assess the role of PBPs in the recent observation of decreasing susceptibility of this species to penicillin (8).

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