ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1986, p. 559–564
0066-4804/86/100559-06$02.00/0
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Vol. 30, No. 4

Antigenic Relationships among Penicillin-Binding Proteins 1 from Members of the Families *Pasteurellaceae* and *Enterobacteriaceae*

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Received 24 March 1986/Accepted 1 July 1986

Penicillin-binding proteins (PBPs) from *Haemophilus influenzae* RD purified by a combination of affinity chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroelution were used to immunize rabbits to obtain specific antisera. Antisera directed against PBP 1 (1b) of *H. influenzae* cross-reacted with representative organisms of the family *Pasteurellaceae* and with many members of the family *Enterobacteriaceae* but not with other gram-negative organisms. Immunization with purified PBP 3 of *H. influenzae* produced antisera that reacted with PBP 1 (1b) of *H. influenzae* and showed the same cross-reactive pattern with other species as the anti-PBP 1 antiserum. A 24,000-molecular-weight polypeptide of *H. influenzae*, not radiolabeled by [^{35}S]penicillin, reacted with antisera against purified PBPs 1 (1a, 1b), 2, and 3. The results suggest that antigenic epitopes are shared among similar PBPs from related species and even among different PBPs within the same species.

Penicillin-binding proteins (PBPs) are important proteins found in the cytoplasmic membranes of bacteria and are essential for peptidoglycan synthesis (16, 18). Since inactivation or loss of the low-molecular-weight PBPs is not lethal (4, 11, 14), the higher-molecular-weight PBPs appear to be the essential enzymes for cell wall synthesis (16). These high-molecular-weight PBPs are also likely the critical targets of beta-lactam antibiotics (9, 16). Thus, in addition to the well-recognized role of beta-lactamases in antibiotic resistance, an alteration of the target molecules is a possible form of resistance that has recently been recognized and appears to be found in a variety of different gram-negative and gram-positive organisms (5).

In view of the important role that these proteins play in cell wall synthesis and beta-lactam susceptibility, it is desirable to obtain more detailed structural and functional information. Although the high-molecular-weight PBPs of *Escherichia coli* have been studied extensively (2, 3, 7–9, 18), relatively little information is available for other species. It is also unclear at present what relationship exists between the PBPs of *E. coli* and other species.

As an early step in our study of high-molecular-weight PBPs from *Haemophilus influenzae*, we have prepared antisera against purified PBP 1 (PBP 1a and PBP 1b), PBP 2, and PBP 3. To assess their potential as affinity reagents and as immunological reagents for screening cloned *H. influenzae* PBPs, we determined their specificity by Western blot analysis. We report here on the reactivity of the anti-PBP antisera against PBPs from *H. influenzae* and a variety of other bacterial species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 1. Enterobacterial strains were grown on blood agar plates at 37°C, whereas all other strains were grown on chocolate agar plates supplemented with CVA enrichment (GIBCO Laboratories, Grand Island, N.Y.) in an atmosphere containing 5% CO_2. All liquid cultures were grown on supplemented BHI broth (brain heart infusion broth containing CVA enrichment and 10 μg of hemin per ml).

Purification of PBPs. *H. influenzae* RD was grown overnight on supplemented BHI broth, and cells were collected and washed three times with 50 mM potassium phosphate (pH 7.0) buffer by centrifugation at 8,000 × *g* for 20 min at 4°C. The wet cell pellet was suspended in the above buffer containing 1 mM phenylmethylsulfonyl fluoride and 100 μg each of pancreatic DNase and RNase per ml and passed twice through a French pressure cell at 16,000 lb/in^2. Cellular debris was removed by centrifugation at 5,000 × *g* for 15 min, and the crude membrane fraction was collected and washed two times by centrifugation at 38,000 × *g* for 40 min. The crude membrane pellet was suspended in 50 mM potassium phosphate buffer containing 1 mM phenylmethylsulfonyl fluoride to a protein concentration of approximately 4 mg/ml and then diluted with an equal volume of 100 mM Tris hydrochloride–1 M LiCl–2 mM beta-mercaptoethanol (pH 8.0).

A 150-ml sample of crude membrane preparation was mixed with 10 ml of 6-aminopenicillinic acid–carboxymethyl Sepharose prepared as described previously (7) and gently agitated at room temperature for 60 min. Triton X-100 was added to 1% final concentration, and the mixture was agitated for an additional 30 min. The mixture was transferred to a coarse fritted glass funnel (250 ml), washed four times with 10 ml of 50 mM potassium phosphate–0.1% Triton X-100–0.5 M NaCl–2 mM beta-mercaptoethanol (pH 7.0), transferred into a 30- by 2.5-cm glass column, and washed with an additional 20 ml of the above buffer.

The column was transferred to 37°C, and the PBPs were eluted with 80 ml of 0.1% Triton X-100–2 mM beta-mercaptoethanol–1 M NH_2OH–0.5 M Tris hydrochloride (pH 8.7) buffer. Four 20-ml fractions were collected and then extensively dialyzed against 50 mM Tris hydrochloride–0.1% Triton X-100–1 mM beta-mercaptoethanol (pH 7.5) buffer at 4°C. The fractions were separately applied to 0.6-ml DE52 columns, washed with 1.2 ml of wash buffer (50 mM Tris hydrochloride, 1% Triton X-100, pH 7.5), and eluted with 1.2 ml of wash buffer containing 2 M NaCl. The fractions were dialyzed against the wash buffer overnight at 4°C.

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TABLE 1. Bacterial strains

Genus and species	Strain designation[a]	Source
Haemophilus influenzae	Rd	Our collection
Haemophilus parainfluenzae	KC21, NCTC 4101	W. Albritton
Haemophilus hemoglobinophilus	KC18	W. Albritton
Haemophilus parasuis	KC20	W. Albritton
Haemophilus aphrophilus	KC19	W. Albritton
Haemophilus pleuropneumoniae	KC23	W. Albritton
Pasteurella multocida	KC287, NCTC 10322	W. Albritton
Pasteurella pneumotropica	KC 288	W. Albritton
Actinobacillus lignieresii	NCTC 4189	W. Albritton
Escherichia coli	DH1	Our collection
Enterobacter aerogenes	ATCC 13048	Our collection
Shigella flexneri	ATCC 12022	Our collection
Serratia marcescens	ATCC 8100	Our collection
Brucella abortus		Our collection
Bordetella parapertussis	ATCC 15311	Our collection
Bacillus cereus	FH 26-5	Our collection
Neisseria meningitidis	B16B6	Our collection
Neisseria lactima	FH 57-5	Our collection
Neisseria pharyngis	FH 17-7	Our collection
Hemophilus ducreyi	ATCC 27722	Our collection
Salmonella typhimurium	ATCC 14028	Our collection
Moraxella lacunata	FH 52-3	Our collection
Pseudomonas aeruginosa	PAO1	Our collection
Staphylococcus aureus	Cowan 41	Our collection

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; FH, Foothills Hospital, Calgary, Alberta Canada.

A sample of the fractions (50 μl) was labeled by the addition of 5 μl of [^{35}S]penicillin G (62 ng, 0.73 μCi) and incubation for 15 min at room temperature. The reaction was stopped by the addition of 10 μl of 120 mg of cold penicillin G per ml, 10 μl of 20% Sarkosyl, and 50 μl of electrophoresis sample buffer (20 mM beta-mercaptoethanol, 0.2 M Tris hydrochloride, 3% glycerol, 0.002% bromophenol blue, pH 6.8). The radiolabeled sample was applied to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel along with a control radiolabeled crude membrane preparation, molecular weight standards, and a preparative well of unlabeled sample.

After electrophoresis the labeled portion of the gel was stained with Coomassie blue (0.25% in methanol-acetic acid-H_2O [5:1:5]) and destained; the regions of unstained portion of the gel corresponding to PBP 1 (a and b), PBP 2, and PBP 3 were excised and placed in 5 ml of electrophoresis buffer and stored at 4°C. The labeled portion of the gel was dried and subjected to fluorography by the method of Bonner and Laskey (1). After fluorography had confirmed that the excised gel slices contained the appropriate PBP, the samples were electroeluted in a homemade electroelution apparatus at 30 mA for 5 h. The bridging buffer was removed and replaced with a platinum wire, and the proteins were electrophoresed off the dialysis membrane by reversing the current twice for 1 min. The samples were stored at 4°C. Purity of the samples was assessed by SDS-PAGE followed by silver staining (10). Only the derived immunizing polypeptides were detected when 10 μg of protein was applied per well.

Immunization. New Zealand White rabbits (1.5 to 2.0 kg) were immunized initially with 0.6 ml of purified PBP (approximately 150 μg) in electrophoresis buffer injected into the following three sites: intramuscularly in the thigh, subcutaneously in the flank, and intradermally in the back. The first injection was accompanied by intraperitoneal injection of 500 μg of muramyl dipeptide in 1 ml of sterile saline. Subsequent injections of 50 to 150 μg of protein in 300 to 500 μl of buffer were given intravenously at 2-week intervals for a total of at least six injections. Sera from test bleeds were analyzed by Western blot analysis of radiolabeled crude membrane preparations followed by autoradiography of the immunoblot paper to determine the specificity of the restriction. Terminal bleeding was performed by cardiac puncture under anesthesia, and the resultant sera were aliquoted and stored at −70°C.

Radiolabeling of PBPs. Radiolabeling of PBPs in whole cells and membranes was essentially as described previously (15).

Western blot analysis. Whole cell preparations were prepared by diluting overnight cultures 1:20 with fresh medium and incubating at 37°C until the absorbance at 600 nm reached 0.3 to 0.5, centrifuging, and suspending in 20 mM Tris hydrochloride (pH 7.0). Strains that did not grow well in supplemented BHI broth (*Haemophilus ducreyi*, *Moraxella lacunata*, etc.) were scraped from supplemented chocolate plates and suspended in 20 mM Tris hydrochloride (pH 7.0). The cell suspensions were aliquoted and stored at −70°C until protein determinations were performed. Crude membrane preparations were prepared essentially as described above for the purification of PBPs, suspended in a small volume of 50 mM Tris hydrochloride (pH 7.5) containing 1.0 μM phenylmethylsulfonyl fluoride, aliquoted, and stored frozen at −70°C.

Samples containing 120 μg of protein for whole cells or 60 μg of protein for crude membranes were added to the above buffer to a final volume of 80 μl, and 20 μl of electrophoresis sample buffer was added. The samples were subjected to SDS-PAGE with the Laemmli system (12) and then electrophoresed onto nitrocellulose paper overnight at 30 V by the method of Towbin et al. (17).

The nitrocellulose paper was then blocked in a solution of TBS (50 mM Tris hydrochloride, 150 mM NaCl, pH 7.5) containing 1.3% (wt/vol) fish gelatin (Hipure Liquid Gelatin; Norland Products Inc., New Brunswick, N.J.) for 1 h at 37°C. The blocking solution was removed, a dilution of

rabbit antiserum in blocking solution was added, and incubation was continued for 1.5 h at 37°C. The solution was removed, the filter was washed three times in TBS without fish gelatin, blocking solution containing 400 ng of peroxidase-conjugated protein A per ml was added, and incubation was continued for an additional 1.5 h at 37°C. The solution was removed, and the paper was washed three times with TBS lacking fish gelatin and then developed with Bio-Rad HRP color development reagent according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Specificity of hyperimmune sera. To obtain PBP-specific sera, rabbits were immunized with extensively purified PBPs. The combination of affinity chromatography and SDS-PAGE followed by electroelution allowed us to obtain a limited amount of highly purified material for immunization. Initially, affinity chromatography was performed on detergent-solubilized membranes (7), but this resulted in the presumed PBP 3 polypeptide not being radiolabeled by [^{35}S]penicillin G. Altering the procedure by binding the membrane preparation directly to the affinity resin before detergent solubilization resulted in the isolation of a PBP 3 polypeptide that was radiolabeled with penicillin. This purification procedure was adopted because it allowed us to confirm the identity of the polypeptide species utilized for immunization.

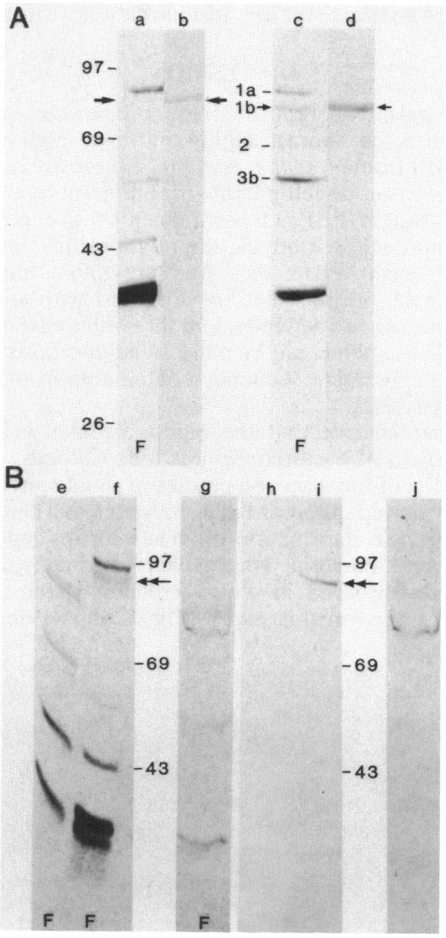

FIG. 1. Specificity of anti-PBP 1 antiserum. Crude membrane preparations were labeled with [^{35}S]penicillin G as described in Materials and Methods and then subjected to SDS-PAGE on 10% (A) or 7% (B) acrylamide gels. The gels were electroblotted onto nitrocellulose paper overnight and developed by an immunoassay with a 1:200 (lanes b and d) or a 1:10 (lanes h, i, and j) dilution of anti-PBP 1 antiserum. Autoradiographs, indicated by an F, were prepared by exposing X-Omat XARS film to dried immunoblots for 7 days at −70°C before development. Lanes: a and b, *E. coli* DH1; c and d, *H. influenzae* RD; e, *P. aeruginosa* PAO1; f, *E. coli* DH1; g, *H. influenzae* RD whole cells; h, *P. aeruginosa* PAO1; i, *E. coli* DH1; j, *H. influenzae* RD whole cells. Numbers refer to molecular weights (10^3) of standard proteins, and arrows indicate overlapping bands on the immunoblot and autoradiograph. 1a, 1b, 2, and 3b refer to *H. influenzae* PBPs.

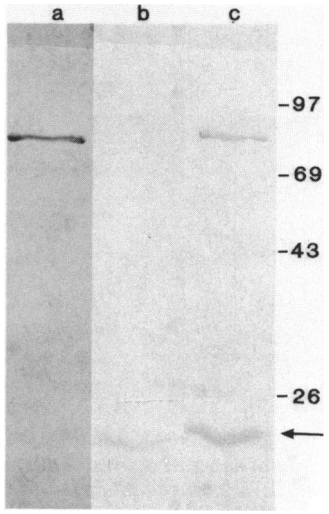

FIG. 2. Comparison of reactivity of antisera prepared against PBP 1, PBP 2, and PBP 3. Crude membrane preparations from *H. influenzae* RD were subjected to SDS-PAGE with 10% acrylamide gels and electroblotted overnight onto nitrocellulose paper. Strips of nitrocellulose representing individual lanes were developed separately by immunoassay by using the indicated titers of primary antisera. Lanes: a, anti-PBP 1 antiserum, 1:150; b, anti-PBP 2 antiserum, 1:20; c, anti-PBP 3 antiserum, 1:20. Numbers refer to molecular weights (10^3) of standard proteins.

Immunization with extensively purified PBP 1 (a and b) resulted in the production of PBP 1-specific antisera. The antisera reacted strongly with an 80,000-molecular-weight band in *H. influenzae* (Fig. 1A, lane d, small arrow) which corresponds with PBP 1b on the autoradiograph (lane c, small arrow). The antisera reacted against a 90,000-dalton polypeptide in *E. coli* (Fig. 1A, lane b, large arrow) which corresponds to the PBP 1b band on the autoradiograph (lane a, large arrow). To minimize the possibility that the immunological reactivity was against a non-PBP polypeptide migrating close to PBP 1b, the experiment was repeated several times with differing electrophoretic conditions. Lanes e through j in Fig. 1B show the results of electrophoresis for a longer time in gels containing 7% acrylamide, which achieves more effective separation of high-molecular-weight polypeptides. The results confirm the assignment of the immunologically reactive polypeptide in *E. coli* (Fig. 1B, lane i, double arrow) to PBP 1b (lane f, double arrow). It is also noteworthy to mention that a 20-fold difference in titer of antiserum was used in the immunoblots shown in Fig. 1A and B, indicating that the antiserum retains its specificity even at relatively low dilution.

The specificity of the antiserum that was attained could be

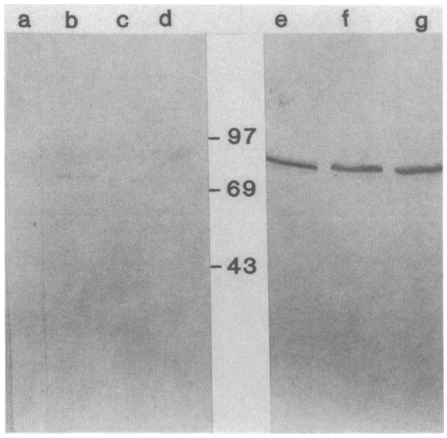

FIG. 3. Reactivity of anti-PBP 1 antiserum against species of the family *Pasteurellaceae*. Whole cell preparations (120 μg of protein) were subjected to SDS-PAGE with 10% acrylamide gels, electroblotted overnight onto nitrocellulose paper, and developed by immunoassay with a 1:50 dilution of anti-PBP 1 (*H. influenzae*) antiserum. Lanes: a, *P. aeruginosa*; b, *H. parasuis*; c, *P. pneumotropica*; d, *H. pleuropneumoniae*; e, *P. multocida*; f, *H. parainfluenzae*; g, *H. influenzae* RD. Numbers refer to molecular weights (10^3) of standard proteins.

attributed to the purity of the immunizing antigen but could also be partially due to the choice of immunoadjuvant. In an earlier experiment when Freund adjuvant was utilized in lieu of muramyl dipeptide, a longer immunization schedule (6 months) was necessary to obtain significant anti-PBP 1 reactivity. The reactivity to PBP 1 was accompanied by reactivity to a number of non-PBP polypeptides not recognized by the second antiserum.

Immunization with PBP 2 and PBP 3 did not result in antisera with evident activity against the immunizing proteins. After extended immunization with PBP 3 (4 months), however, reactivity against PBP 1b was observed (Fig. 2, lane c). In addition, the anti-PBP 3 antisera had a pattern of reactivity toward other species of bacteria that was similar to that of the anti-PBP 1 antisera. After immunization with PBP 2 the only activity that was detected was against a 24,000-dalton polypeptide that was also weakly recognized by the anti-PBP 1 and anti-PBP 3 antisera (Fig. 2, arrow) but was not detected by normal rabbit serum. This polypeptide was not radiolabeled with [^{35}S]penicillin G in our experiments.

Reactivity of antisera against other bacterial species. The antiserum developed against PBP 1 (a and b) from *H. influenzae* Rd also reacted with high-molecular-weight polypeptides from species in the family *Pasteurellaceae* (Fig. 3). The reactivity was conclusively demonstrated to be against PBP 1b in both *H. influenzae* and *E. coli* (Fig. 1) and occurred in a position of penicillin-binding activity in the other species. Figure 3 illustrates that the anti-PBP 1 antiserum reacted strongly with a high-molecular-weight polypeptide in *H. influenzae* (lane g), *Haemophilus parainfluenzae* (lane f), and *Pasteurella multocida* (lane e), reacted weakly with *Haemophilus pleuropneumoniae* (lane d), *Pasteurella pneumotropica* (lane c), *Haemophilus parasuis* (lane b), and was undetectable in reaction with *Pseudomonas aeruginosa* (lane a; Fig. 1, lane e). Additional experiments have shown weak reactivity against *Actinobacillus lignieresii*, *Haemophilus hemoglobinophilus*, and *Haemophilus aphrophilus* and undetectable reactivity against *Haemophilus ducreyi*, *Neisseria* species, and representative gram-positive organisms (*Bacillus cereus* and *Staphylococcus aureus*).

The antiserum did react strongly with PBP 1b from *E. coli*, and this reactivity was further investigated by testing other members of the *Enterobacteriaceae*. The anti-PBP 1 antiserum reacted strongly with a high-molecular-weight polypeptide in *E. coli* (Fig. 4, lane g), *Enterobacter aerogenes* (lane f), *Salmonella typhimurium* (lane e), *Serratia marcescens* (lane d), and *Shigella flexneri* (lane c) and was undetectable in reactivity against *P. aeruginosa* (lane a) and *Neisseria meningitidis* (lane b). Further experiments revealed a lack of reactivity to high-molecular-weight polypeptides in species of other gram-negative genera such as *Bordetella*, *Brucella*, or *Moraxella*. Although not extensively tested, the anti-PBP 3 antisera had a similar pattern of reactivity against high-molecular-weight polypeptides from the various bacterial species tested (data not shown).

DISCUSSION

In this paper we report on the preparation of specific antisera directed against highly purified, high-molecular-weight PBPs from *H. influenzae*. Since there are no previous reports on the development of antisera against high-molecular-weight PBPs, these reagents have provided us with an approach to studying the relationships among high-molecular-weight PBPs that was previously unavailable. Antigenic relationships that are observed with an immunological approach are dependent on three-dimensional conformation and as such could be a useful adjunct to information on linear polypeptide sequences obtainable from DNA sequence analysis.

The essential role that the high-molecular-weight PBPs play in growth of bacteria requires a considerable extent of conservation of function and predicts a significant functional homology among different bacterial species. There is, however, very little information on relationships among PBPs from different bacterial species, except that similar PBP profiles are observed in closely related species (8). The results presented in this paper (Fig. 3 and 4) demonstrate

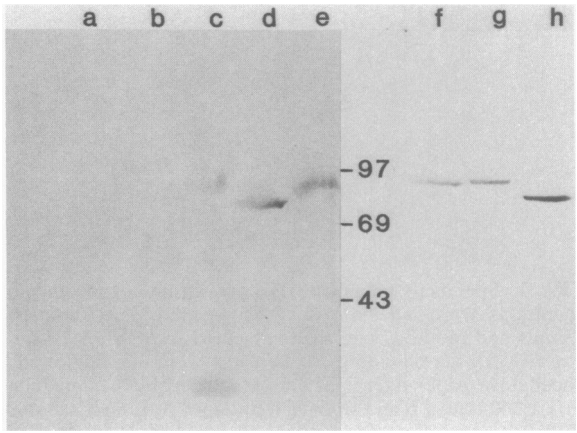

FIG. 4. Reactivity of anti-PBP 1 antiserum against species of the family *Enterobacteriaceae*. Experimental conditions were as in Fig. 3, except that nitrocellulose paper was developed with 1:100 (lanes f through h) or 1:20 (lanes a through e) dilutions of anti-PBP 1 antiserum. Lanes: a, *P. aeruginosa*; b, *N. meningitidis*; c, *S. flexneri*; d, *S. marcescens*; e, *S. typhimurium*; f, *E. aerogenes*; g, *E. coli*; h, *H. influenzae*. Numbers refer to molecular weights (10^3) of standard proteins.

that some antigenic sites of high-molecular-weight PBPs are fairly well conserved throughout members of the family *Pasteurellaceae* and even among members of the *Enterobacteriaceae*. The potential role of immunological reagents against PBPs to assist in determining taxonomic relationships is supported by the observation that the anti-PBP 1 antisera did not react against *H. ducreyi* but reacted against virtually all other *Haemophilus*, *Pasteurella*, and *Actinobacillus* species tested. This is consistent with the observation that *H. ducreyi* does not show significant DNA homology with other *Haemophilus* species (6). Furthermore, although the antiserum reacted with a wide variety of species within the families *Pasteurellaceae* and *Enterobacteriaceae*, it did not react with representative gram-negative organisms from the genera *Neisseria*, *Brucella*, *Moraxella*, and *Pseudomonas*. In this context it is interesting to note that antisera developed against purified PBP 1 from *P. aeruginosa* cross-reacted with PBPs from other *Pseudomonas* species, but not with *E. coli* or *H. influenzae* (A. J. Godfrey, B. Beckthold, and L. E. Bryan, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 696, 1985). The presence of more than one antibody species directed against possibly a number of PBP-associated antigenic epitopes makes any comment on degree of relatedness by strength of immunological reaction controversial. If monoclonal antibody reagents were developed against specific epitopes, however, more definitive conclusions could be reached.

The results presented in this paper provide evidence that the high-molecular-weight PBPs from *H. influenzae* have some similarity in antigenic epitopes. Immunization with purified PBP 1 or PBP 3 from *H. influenzae* resulted in antisera that reacted with PBP 1b from *H. influenzae* and *E. coli*, indicating an antigenic epitope that is shared by the immunizing antigens. The results with radiolabeled PBPs shown in this paper and additional experiments performed with unlabeled penicillin (data not shown) show that penicillin does not significantly decrease the immunological reaction, indicating that the penicillin binding site is not the immunodominant site responsible for the observed cross-reactivity.

Immunization with purified PBP 2 resulted in reaction against a 24,000-dalton polypeptide that was also recognized by the anti-PBP 1 and anti-PBP 3 antisera (Fig. 2, arrow), but not by normal rabbit serum. Reativity against this polypeptide could be attributed to the immunizing polypeptides and not to a nonspecific adjuvant effect. Sera from rabbits with a similar exposure to adjuvant but immunized with *P. aeruginosa* PBPs had no reactivity to this polypeptide even at very high titers (data not shown). This indicates that the low-molecular-weight polypeptide contains an antigenic epitope shared with the high-molecular-weight PBPs. Whether the low-molecular-weight polypeptide is a specific degradation product of one of the higher-molecular-weight PBPs or an ancestrally related polypeptide is at present unclear. It is noteworthy to mention that low-molecular-weight polypeptides that reacted with high titers of both anti-PBP 1 and anti-PBP 3 antisera were also observed in other species in the family *Pasteurellaceae* and *Enterobacteriaceae*.

The inability of the anti-PBP 2 and anti-PBP 3 antisera to detect the immunizing antigen in whole cell or membrane preparations is not fully understood but may be a reflection of the relatively small quantities of these two proteins in the cell. The PBP 1 polypeptides are readily detected in Coomassie blue-stained gels of inner membrane preparations from *H. influenzae*, whereas PBP 2 and PBP 3 are only evident after radiolabeling and in autoradiographs are labeled much more weakly than PBP 1. This may explain why the anti-PBP 1 and anti-PBP 3 antisera detect the PBP 1b polypeptide and not the PBP 3 polypeptide. An additional possibility is that the PBP 1b polypeptide can more effectively restore the native antigenic site after SDS-PAGE and electroblotting than can the PBP 2 and PBP 3 polypeptides. This phenomenon has been elegantly demonstrated with the class 2 outer membrane proteins from *Neisseria meningitidis*, which show a differential capacity to restore native antigenic determinants after SDS-PAGE and electroblotting (13).

The anti-PBP 2 antisera might be expected to have reactivity against PBP 1b if PBP 2 shares antigenic epitopes with PBP 1 and PBP 3. The rabbit immunized with purified PBP 1 (a and b) developed detectable titers against PBP 1b after four injections, whereas the PBP 3-immunized rabbit did not develop detectable reactivity against PBP 1b until the eighth injection. Since the PBP 2-immunized rabbit was sacrificed after only six injections, it is possible that extending the immunization schedule might have achieved anti-PBP 1b activity.

The results in this paper have demonstrated the efficacy of utilizing hyperimmune serum prepared against purified PBPs to study the antigenic relationships among PBPs from various bacteria. The observed relationships were unexpected and may not have been recognized even if complete sequence data had been available for these polypeptides. We feel that further antigenic analysis with monoclonal antibody reagents is warranted to provide more conclusive evidence for similar antigenic epitopes being present on the polypeptides presented in this paper.

ACKNOWLEDGMENTS

This work was supported by grant MT4350 from the Medical Research Council. A.B.S. was supported by a Medical Research Council fellowship.

We thank W. L. Albritton for generously supplying us with bacterial strains from the family *Pasteurellaceae*.

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