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Penicillin-Binding Proteins of Multiply Antibiotic-Resistant South African Strains of *Streptococcus pneumoniae*

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Multiply drug-resistant South African pneumococci (with penicillin minimal inhibitory concentrations ranging from 0.2 to 12.5 μg/ml) showed several types of major alterations in their penicillin-binding protein (PBP) pattern compared with that of a penicillin-susceptible laboratory strain of *Streptococcus pneumoniae* (R6; penicillin minimal inhibitory concentration = 0.006 μg/ml). Genetic transformants were obtained by using South African pneumococcus (strain 8249) deoxyribonucleic acid as donor and the competent cells of strain R6 as recipient; seven classes of transformants with progressively higher penicillin resistance were isolated, and their PBPs were tested. The PBP patterns exhibited a gradual shift from a pattern similar to that of the recipient to a pattern resembling that of the donor strain as the level of penicillin resistance increased.

In 1977, a number of different strains of *Streptococcus pneumoniae* resistant to beta-lactams and other antibiotics were isolated in South African children with meningitis, pneumonia, and bacteremia or pneumonia and empyema (1, 11). Since the organisms did not produce beta-lactamases (16), they represent cases of intrinsic beta-lactam resistance in pneumococci.

The biochemical basis of intrinsic beta-lactam resistance is not well understood in any bacteria. In this study, we describe the penicillin-binding proteins (PBPs) of six penicillin-resistant South African pneumococcal strains which are also resistant to a number of other antibiotics. The PBPs of these resistant strains exhibit striking and multiple differences from the PBP pattern of a penicillin-susceptible laboratory reference strain (R6). With deoxyribonucleic acid (DNA) isolated from one of the resistant strains (8249) and penicillin-susceptible R6 cells as recipients, we found that the high level of penicillin resistance of the donor strain could only be introduced into the susceptible bacteria in a stepwise fashion by several rounds of genetic transformation, in analogy with the case of the stepwise acquisition of oxacillin resistance (21). By using these isogenic and progressively more resistant transformants, we determined that increasing resistance to penicillin was paralleled by discrete and gradual changes in the PBPs, shifting from the pattern of the susceptible bacteria toward the pattern of the resistant strains.

MATERIALS AND METHODS

Strains. Multiply antibiotic-resistant *S. pneumoniae* strains were provided by Hendrik J. Koornhof of the South African Institute of Medical Research, Johannesburg. These strains have the following minimal inhibitory concentrations (MICs) for benzylpenicillin, in micrograms per milliliter: CMR40, 0.2; A95210, 1.6; 140, 3.1; A9229, 3.1; 8249, 6.2; and D20, 12.5 *S. pneumoniae* R6 is a derivative of the Rockefeller University laboratory strain R36A, and has a benzylpenicillin MIC of 0.006 μg/ml.

Growth. All the strains were grown in a casein-based semisynthetic medium at pH 8.0 (20) supplemented with yeast extract (Difco Laboratories; final concentration, 1 mg per ml of growth medium). The medium is referred to as C+y. Culture growth was monitored by nephelometry (20), using a Coleman nephocolorimeter.

MICs were determined by the tube dilution method (10) in C+y medium. Stocks of cultures, frozen in 10% glycerol-containing C+y, were kept at −70°C.

Genetic transformation. DNA was isolated from strain 8249 by a previously described method (14). Competent, penicillin-susceptible recipient cells (R6) were treated with this DNA under conditions routinely used in transformation experiments (10). One milliliter of competent cells (7×10^7 viable units) was incubated with 0.2 μg of 8249 DNA per ml at 30°C for 15 min. After this, 2 ml of C+y containing albumin (0.8 mg/ml; Armour fraction V) and pancreatic deoxyribonuclease (1 μg/ml; Worthington Biochemicals) was added, and the suspension was incubated at 37°C for 90 min. Various dilutions were then plated into C+y agar (final agar concentration, 0.8%) containing the following concentrations (in micrograms per milliliter) of benzylpenicillin (Eli Lilly & Co., Indianapolis, Ind.): 0.012, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8. After a single round of transformation, transformants were obtained only on the plates (primary selection plates) with the two lowest concentrations of penicillin. As an illustration, the following number of transformants were isolated in a typical experiment: 6.5×10^5 colonies resistant to 0.012 μg of penicillin per ml and 6.0×10^2 colonies resistant to 0.025 μg of penicillin per ml. No colonies were observed on plates containing 0.05 μg of penicillin per ml.

Purification of transformant classes representing various levels of penicillin resistance. Clones picked from plates containing 0.012 μg of penicillin per ml were transferred to the surface of a blood agar plate (master plate) with the help of sterile platinum loops. After overnight growth, the colonies of the master plate were replica plated to four new blood agar plates containing penicillin at 0.012, 0.025, 0.05, and 0.1 μg/ml. Colonies that grew up only on 0.012 μg/ml (but not at the higher antibiotic levels) were further purified by transfer to blood agar containing 0.012 μg/ml. After overnight incubation at 37°C, a clone was picked up from this plate and used as a representative of the corresponding level (0.012 μg/ml, called pen 0.012) of penicillin resistance.

Clones representing the next level of penicillin resistance (0.025 μg/ml; pen 0.025) were isolated in a similar fashion from the primary selection plates containing penicillin at 0.025 μg/ml.

The construction of the next higher level of penicillin-resistant transformants (0.05 and 0.1 μg/ml; pen 0.05 and pen 0.1, respectively) required another transformation reaction in which competent pen 0.025 cells were used as recipients of strain 8249 DNA.

Transformants resistant to penicillin at 0.2, 0.4, and 0.8 μg/ml (pen 0.2, pen 0.4, and pen 0.8, respectively) were obtained by using strain 8249 DNA and competent pen 0.1 cells as recipients.

Construction of genetic transformants approaching more closely the higher penicillin resistance level of the donor strain (MIC = 6.2 μg/ml) is in progress.

In each case, colonies representing a penicillin resistance class were purified by the replica-plating procedure described above.

Stocks of the transformants were stored at −70°C in 10% glycerol-containing medium.

Tritiated benzylpenicillin. Radioactive benzylpenicillin (ethylpiperidinium salt) was prepared by synthetic methods and was the generous gift of E. O. Stapley, Merck, Sharp & Dohme, Rahway, N.J. manuscript in preparation; specific activity, 30 mCi/μmol.

In vivo labeling of PBPs. The PBPs were identified after treatment of whole cells with radioactive penicillin. The assay involved the addition of different concentrations of benzyl[^{3}H]-penicillin (Table 1) to 1-ml portions of exponentially growing organisms (7.0 × 10^7 viable bacteria/ml) at 37°C for 10 min. Nonradioactive benzylpenicillin was added at 1.7 mM (final concentration), and the bacteria were recovered by centrifugation at 12,000 × *g* for 2 min at 4°C. The organisms were resuspended in 50 μl of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% Sarkosyl NL-97 and incubated at 37°C for 5 min. This resulted in the complete lysis of bacteria. Samples (80 μl) were then prepared for slab polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis and detection of PBPs. Discontinuous electrophoresis in polyacrylamide slab gels containing sodium dodecyl sulfate was performed essentially as described by Laemmli and Favre (12), except that the concentrations of acrylamide and *N,N'*-methylenebisacrylamide in the stacking and separating gels were 7 and 0.117% and 3 and 0.05%, respectively.

Electrophoresis was carried out at a constant voltage of 60 until the tracking dye had just entered the separating gel and at a constant voltage of 150 thereafter. The gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (6) and destained with several changes of 30% methanol containing 10% acetic acid.

Gels were prepared for fluorography as described by Bonner and Laskey (2), and the fluorograms were produced by using Kodak RP Royal X-Omat (code XR-2) film, which had been presensitized to the appropriate extent (13) with X rays. The exposure time for the fluorograms was 2 to 4 days at −70°C. The number and intensity of bands on the fluorograms were evaluated by visual observation.

Reagents. All chemicals were reagent-grade, commercially available products. Acrylamide and *N,N'*-methylenebisacrylamide were electrophoresis grade (Bio-Rad Laboratories, Rockville, N.Y.).

RESULTS

PBPs of the South African strains of *S. pneumoniae*. The PBPs of the penicillin-resistant (South African) and the penicillin-susceptible (R6) strains are shown in Fig. 1.

South African strain 8249 was selected for a more detailed study. The pattern of PBPs obtained by in vivo labeling of this strain with [^{3}H]penicillin showed several striking differences with respect to the PBPs of the antibiotic-

TABLE 1. *Concentrations of [^{3}H]penicillin used in the in vivo labeling of PBPs*

Dilution[a] of [^{3}H]penicillin reagent	μl of [^{3}H]penicillin reagent added to 1 ml of cell suspension	[^{3}H]penicillin concn (μg/ml)
Undiluted	50	22.35
Undiluted	10	4.47
Undiluted	3	1.34
1:10	10	0.447
1:10	3	0.134
1:100	10	0.0447
1:100	3	0.0134
1:1,000	10	0.00447
1:1,000	3	0.00134
1:10,000	10	0.000447
1:10,000	3	0.000134

[a] Dilutions were made in 10 mM potassium phosphate buffer, pH 6.8.

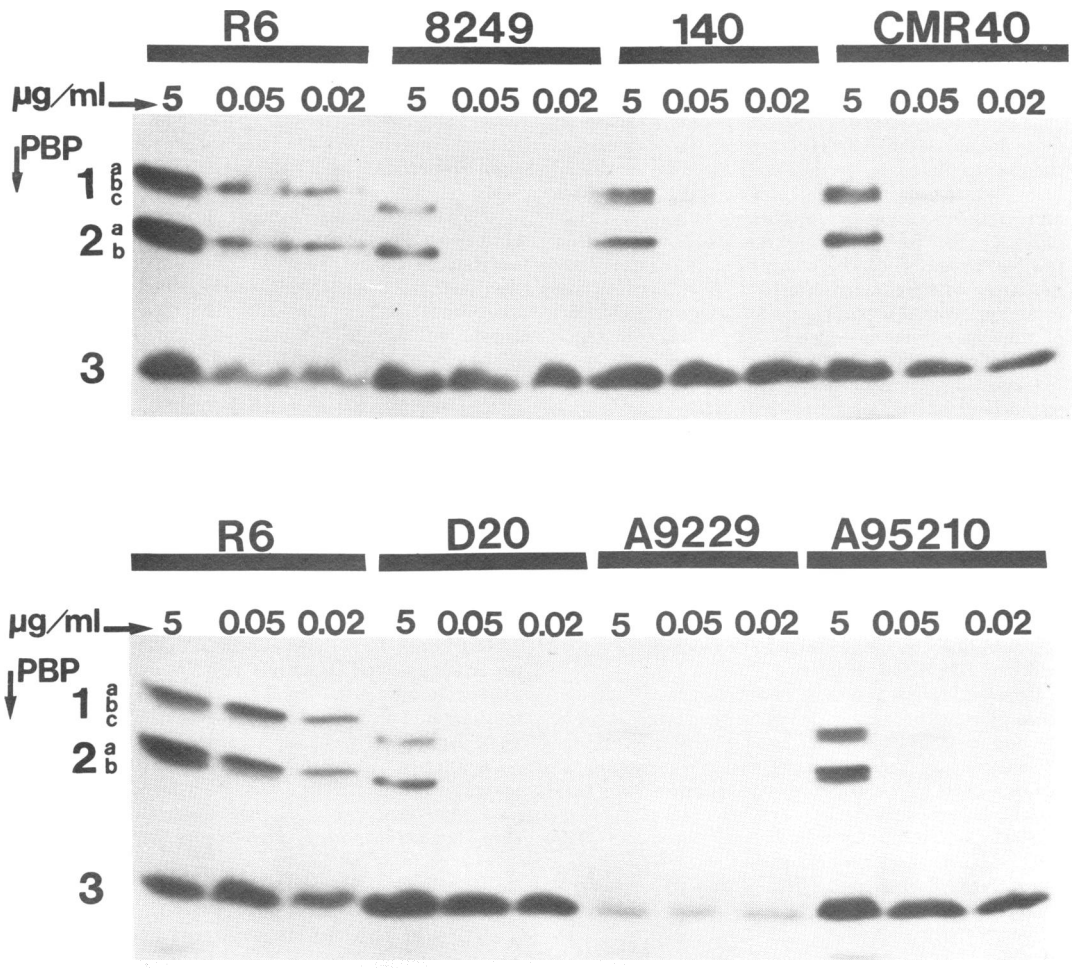

FIG. 1. *PBPs of six strains of South African pneumococci and R6. The band running ahead of PBP3 and visible at higher penicillin concentrations in several of the figures represents residual reagent.*

susceptible strain R6 (Fig. 2). These included: (i) lack of PBP 1a and 1b; (ii) appearance of a new, faster-moving protein (lower molecular weight), named PBP 1c; and (iii) an apparent decrease in the affinity of PBP 2a for [^{3}H]penicillin. Occasional gels where PBP 2b was also resolved (see Fig. 4) showed that strain 8249 also lacked this PBP.

PBPs of genetic transformants resistant to different levels of penicillin. Figure 3 shows the PBP pattern of a series of low-level penicillin-resistant transformants (pen 0.012, pen 0.025, pen 0.05, and pen 0.1). A series of successive changes could be readily observed. (i) The lowest-level transformant, pen 0.012, showed a PBP pattern similar to that of the susceptible R6 strain, except for the fact that PBP 2a had an affinity for [^{3}H]penicillin which was lower than that of strain R6 but higher than that of strain 8249. (ii) As the level of resistance increased to 0.025 μg of penicillin per ml (pen 0.025), an additional change occurred as a new protein, called PBP 2a′, appeared. (iii) The next alteration occurred when PBP 2b disappeared (Fig. 4) as the level of resistance increased to 0.05 μg of penicillin per ml (pen 0.05). (iv) The pen 0.1 transformant exhibited still an additional variation: a decreased affinity of PBP 1a for [^{3}H]penicillin compared with R6.

Figure 4 includes the PBP patterns not only of the transformants with low resistance levels (pen 0.012, pen 0.025, pen 0.05, and pen 0.1), but also of strains with higher levels of resistance (pen 0.2, pen 0.4, and pen 0.8). The pen 0.2 transformant had a PBP pattern similar to that of pen 0.1. However, as the level of resistance

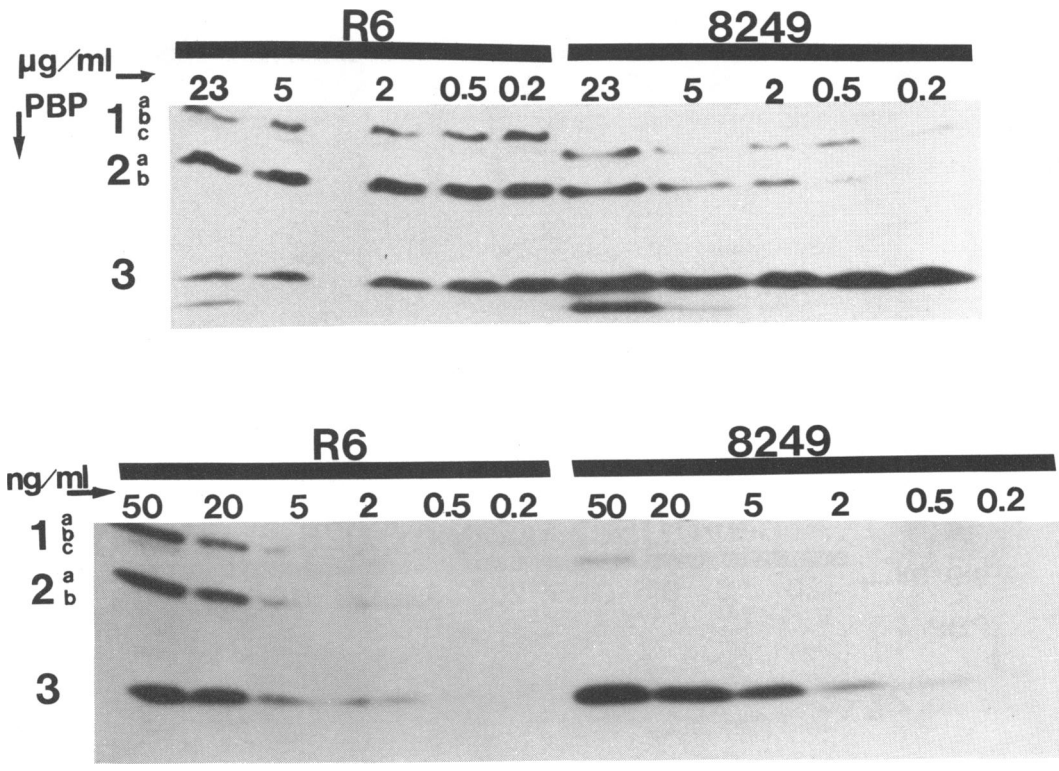

FIG. 2. *Affinity of PBPs for [^{3}H]penicillin in the South African pneumococcus (strain 8249) and the susceptible strain R6.*

increased to 0.4 μg of penicillin per ml (pen 0.4), the pattern of PBPs began to resemble closely the pattern seen in the South African strain 8249 in several respects: affinity of PBP 2a for radioactive penicillin continued to decrease; PBP 1a and PBP 1b disappeared; and a faster-moving protein (PBP 1c) appeared. However, this protein showed a decreased affinity for the [^{3}H]-penicillin when compared with strain 8249.

The highest level of penicillin resistance tested (pen 0.8) showed a pattern similar to that of pen 0.4 except that PBP 2a' was absent (as it was in strain 8249). Figure 5 compares the penicillin affinity of the PBPs of this transformant with those of the susceptible R6 strain. The affinity changes can be clearly seen by simple visual observation of the fluorograms.

Table 2 is a descriptive summary of the sequential PBP changes observed in these series of transformants; also included are the PBP patterns of the parental South African strain 8249 and the susceptible R6 strain.

DISCUSSION

Penicillin resistance in South African *S. pneumoniae* strains is accompanied by several observable changes in their PBPs compared with the pattern seen in a wild-type susceptible laboratory strain (R6). The multiplicity of changes observed in the PBPs of these strains suggests that high-level resistance to penicillin involves a number of sequential biochemical alterations. This conclusion is supported by findings with genetic transformants, in which a gradual acquisition of increasing levels of penicillin resistance is paralleled by gradual changes in the PBP pattern, which shifts from a pattern resembling that of the susceptible strain (in the low-level penicillin-resistant transformants) to a pattern closer to that of the South African parental strain (in the higher-level penicillin-resistant transformants).

High levels of penicillin resistance can not be transferred in a single step from the highly resistant South African pneumococcus, strain 8249 (penicillin MIC = 6.2 μg/ml), to the penicillin-susceptible wild-type strain R6. Acquisition of resistance to penicillin occurs in a stepwise process, similar to the previously described acquisition of oxacillin resistance (21).

Acquisition of resistance to penicillin in naturally occurring strains (South African pneumo-

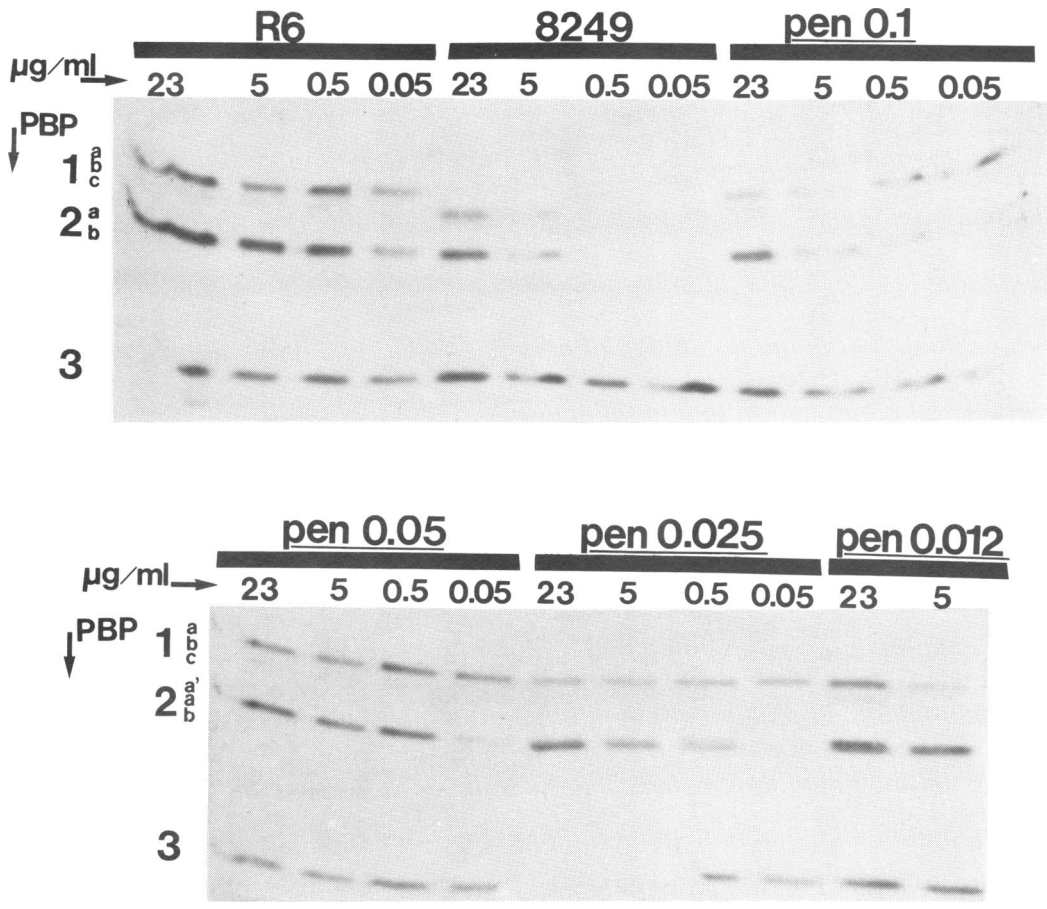

FIG. 3. *PBPs of R6, South African strain 8249, and a series of transformants resistant to low levels of penicillin.*

cocci) may occur in a stepwise process similar to the way in which highly penicillin-resistant mutants are obtained in the laboratory, where the isolation of such mutants requires a stepwise selection since single-step penicillin-resistant mutants show only small incremental increases in beta-lactam resistance (4, 5, 8, 17). It seems likely that the evolution of highly resistant South African pneumococci may have occurred in nature by a similar process, where the widespread use of antimicrobial agents has provided a strong selective environment for these resistant strains. Indiscriminate use of oral antibiotics for long periods of time may mimic in nature the conditions used in the laboratory for selecting mutants with slight increases in antibiotic resistance.

Using DNA from one South African strain (8249) as donor in a series of transformation experiments, several strains differing only in their level of resistance to penicillin were constructed. The availability of such isogenic and progressively more penicillin-resistant bacteria allowed the study of the pattern of PBPs as resistance to penicillin increases; a series of sequential and cumulative changes was observed in the following order: (i) decrease in affinity of PBP 2a (pen 0.012, pen 0.025, pen 0.05, pen 0.1, pen 0.2, pen 0.4, and pen 0.8); (ii) appearance of PBP 2a' (pen 0.025, pen 0.05, pen 0.1, pen 0.2, and pen 0.4); (iii) loss of PBP 2b (pen 0.05, pen 0.1, pen 0.2, pen 0.4, and pen 0.8); (iv) decrease in affinity of PBP 1a (pen 0.1 and pen 0.2); and (v) disappearance of PBP 1a and PBP 1b as PBP 1c appears (pen 0.4 and pen 0.8).

The naturally occurring strains of South African pneumococci examined in this study, with penicillin MICs ranging from 0.2 to 12.5 μg/ml, exhibit PBP alterations predictable from the findings observed with the genetic transformants.

Since the multiplicity of changes involves all

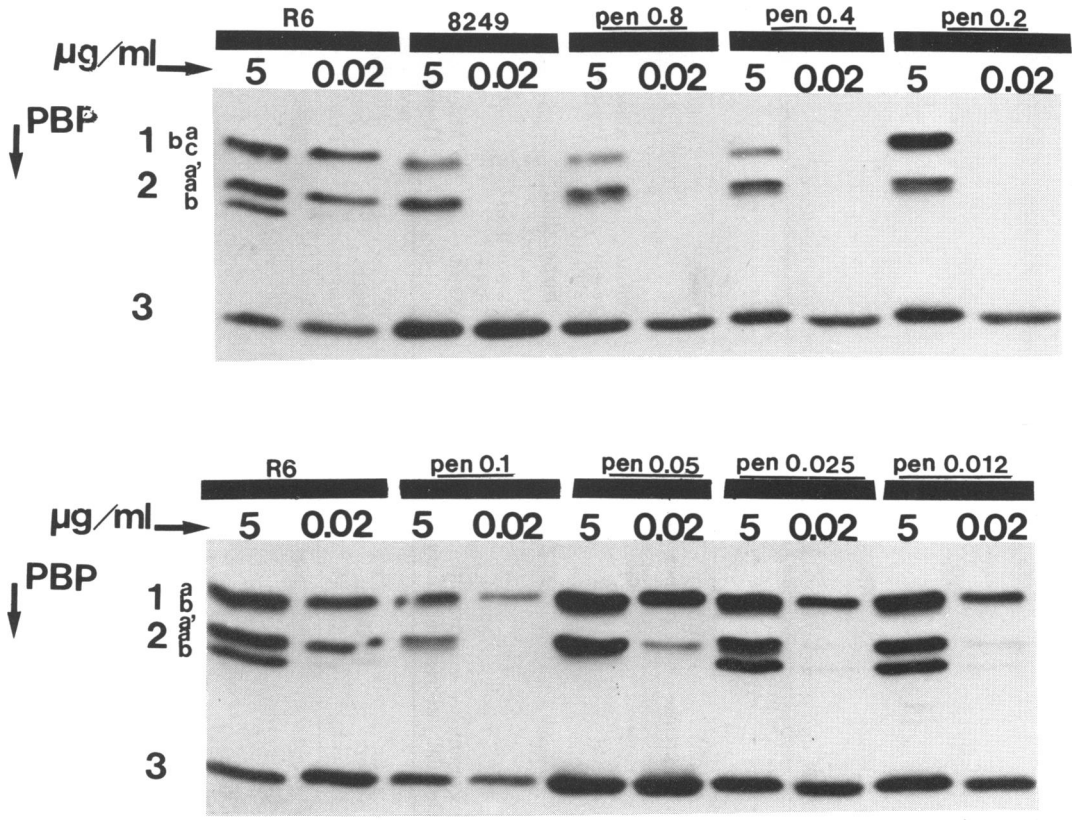

FIG. 4. *PBPs of R6, South African strain 8249, and a series of transformants resistant to different levels of penicillin.*

but one (PBP 3) of the pneumococcal PBPs, it is likely that PBP groups 1 and 2 each perform physiologically important functions in pneumococci, indicating the presence of multiple targets for penicillin action. No changes in the labeling pattern (affinity and/or quantity) of PBP 3 could be detected either in the South African strains (penicillin MICs ranging from 0.2 to 12.5 μg/ml) or in the transformants (penicillin MICs ranging from 0.012 to 0.8 μg/ml); therefore, the physiological function of this binding protein remains to be determined.

The introduction of radioactive penicillin for the identification of PBPs in various strains of bacteria has allowed the design of experimental approaches for clarifying the mechanisms of penicillin's antibacterial action. On the basis of the selective binding of mecillinam to PBP 2 of *Escherichia coli,* this binding protein has been identified as the target of this beta-lactam in *E. coli* (18). In another approach, a series of cloxacillin-resistant mutants of *Bacillus subtilis* was isolated, and it was determined that the relative affinity of PBP 2 for cloxacillin showed parallel changes with increasing drug resistance; also, it has been proposed that PBP 2 may be the lethal target of cloxacillin in *B. subtilis* (3). Using a similar approach, Reynolds has shown that PBP 1 of *B. megaterium* may be the killing target of penicillin action (7); the physiological importance of the same binding protein was also indicated by the finding that PBP 1 of *B. megaterium* was the only binding protein detectable in bacteria exposed to penicillin at the MIC (15).

The genetic transformants used in the studies reported here allowed us to resolve the high levels of penicillin resistance of the South African pneumococci into discrete steps, which are paralleled by discrete biochemical changes. The surprising number (five types) of detectable changes in binding proteins (see above) that accompany the stepwise acquisition of penicillin resistance in the isogenic transformants suggests that penicillin resistance in pneumococci involves sequential and cumulative alterations in a number of penicillin-susceptible enzymes. Similar PBP patterns could be observed during in vitro labeling of membranes prepared from pen-

TABLE 2. *Comparison of PBPs of R6, South African strain 8249, and transformants differing in levels of penicillin resistance*

PBP	Strain or transformant								
	R6	pen 0.012	pen 0.025	pen 0.05	pen 0.1	pen 0.2	pen 0.4	pen 0.8	8249
1a	Present	Present	Present	Present	Affinity decreases	Affinity further decreases	Absent	Absent	Absent
1b	Present	Present	Present	Present	?[a]	?	?	Absent	Absent
1c	Absent	Absent	Absent	Absent	Absent	Absent	Present, Affinity lower than in 8249	Present Affinity higher than pen 0.4, lower than 8249	Present
2a′	Absent	Absent	Present[b]	Present	Present	Present	Present	Absent	Absent
2a	Present	→ Gradually decreasing affinity →							Present, low affinity
2b	Present	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent
3	Present	Present	Present	Present	Present	Present	Present	Present	Present

[a] PBP 1b was not resolved.
[b] Possible differences in affinities could not be detected by visual observation.

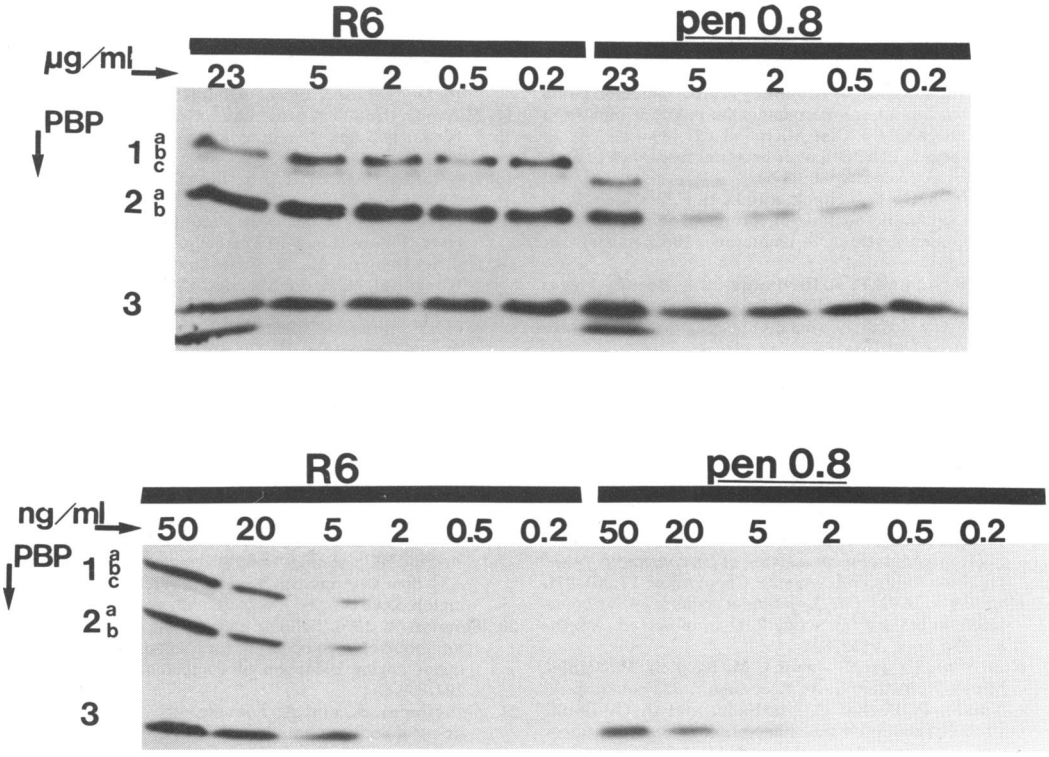

FIG. 5. *Affinity of PBPs for [^{3}H]penicillin in the susceptible R6 strain and in a high-level penicillin-resistant transformant (pen 0.8).*

icillin-susceptible and -resistant pneumococci (9a). Therefore, the PBP changes observed in the in vivo experiments reported here do not reflect selective changes in permeability. Rather, they seem to involve alterations in the properties of PBPs such as: affinity for penicillin, quantity, and turnover rate; or appearance of new proteins. However, transformants with penicillin resistance levels approaching that of the DNA donor strain (MIC = 6.2 μg/ml) have not been examined yet; it is conceivable that at this range of penicillin resistance, changes in cellular permeability would appear. Complex biochemical alterations accompanying penicillin resistance are not without precedent: in gonococci, high levels of penicillin resistance were shown to be the result of cumulative changes in a number of cellular properties, including alterations in permeability and changes in the murein structure (9).

The enzymatic reactions catalyzed by pneumococcal binding proteins are not known. The availability of isogenic strains obtained by genetic transformation should be of considerable help in identifying the biochemical alterations that correspond to the different levels of penicillin resistance, and also the normal physiological functions of the PBPs. This approach would circumvent obvious problems inherent in the direct comparison of biochemical properties of natural isolates that differ in antibiotic resistance levels.

Changes in PBPs have been observed recently in membranes prepared from strains with low levels of penicillin resistance (P. B. Percheson and L. E. Bryan, Program Abstr. Intersci. Confer. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 488, 1979).

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