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Variation in Penicillin-Binding Protein Patterns of Penicillin-Resistant Clinical Isolates of Pneumococci

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A large number of pneumococcal isolates (over 80 strains) from a variety of geographic locales and representing a spectrum of resistance levels from a penicillin MIC of 0.003 μg/ml up to an MIC of 16 μg/ml were analyzed for their penicillin-binding protein (PBP) patterns. With a few exceptions, the great majority of strains with penicillin MICs up to about 0.05 μg/ml contained the same set of five PBPs with molecular sizes typical of those of susceptible pneumococci. In strains with penicillin MICs of about 0.1 μg/ml and up, virtually all isolates showed two common features: (i) all isolates showed loss of PBP 1A (98 kilodaltons) with or without a parallel appearance of a "new" PBP that ranged in molecular size between 96 and 97 kilodaltons; and (ii) in strains with penicillin MICs of 0.5 μg/ml or more, PBP 2B could not be detected on the fluorograms even with very high concentrations of radioactive penicillin. Beyond these two common features, resistant strains with similar penicillin MICs showed a surprising variety of PBP profiles (i.e., in the number and molecular sizes of PBPs), each characteristic of a given isolate. We suggest that in pneumococci remodeling of critical PBPs in more than one way may result in comparable levels of penicillin resistance.

Beta-lactam antibiotic resistance involving modification of the biochemical targets of penicillin—the penicillin-binding proteins (PBPs)—has by now been detected among clinical isolates of most of the major human invasive pathogens (8, 9). In the case of pneumococci, detailed analysis of the biochemical and genetic basis of high-level penicillin resistance (500- to 1,000-fold increase in the MICs of benzylpenicillin) has so far been restricted to two South African isolates: strains 8249 and D20 (16). These two resistant strains had a common PBP pattern that was completely different from the PBP pattern of the penicillin-susceptible laboratory strain in the number of PBPs detectable by the fluorographic technique as well as in the molecular sizes and penicillin affinities of the individual PBPs (10, 15). The penicillin resistance (high MIC) of the South African strains could be introduced into a susceptible laboratory strain by genetic transformation. However, acquisition of donor-level resistance was extremely difficult: it required several consecutive rounds of transformation and a stepwise selection for higher and higher levels of resistance. The series of low-, intermediate-, and high (donor)-level transformants generated in this manner exhibited a gradual shift in PBP profile from that characteristic of the penicillin-susceptible recipient strain toward the patterns typical of the DNA donors (15). Transformants with low-level resistance had PBPs like the susceptible recipient, except for lower affinity for penicillin. Transformants with intermediate resistance showed a further decrease in penicillin affinity as well as alterations in the molecular sizes of some of the PBPs. Finally, transformants with the highest resistance level had drastically altered PBPs, which resembled those of the DNA donor cells in number, molecular size, and antibiotic affinities.

The purpose of studies to be described here was to look at the PBPs of other clinical isolates of pneumococci and determine whether the characteristic PBP patterns seen in strains 8249 or D20 and in the low- and intermediate-resistance genetic transformants constructed with the DNAs of these two strains represent the unique and only PBP pattern typical of a particular resistance level (MIC). Given the complex mechanism and multigenic nature of pneumococcal penicillin resistance, one may expect this to be the case. Examination of a large number of clinical isolates yielded the unexpected result that strains with both intermediate- and high-level penicillin resistance exhibited a surprisingly large number of distinct PBP patterns, suggesting that pneumococci are capable of remodeling their PBPs in more than one way to achieve lower antibiotic affinity and penicillin resistance.

(Some of the findings described in this communication have been presented in a preliminary form at the Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 8 through 12 May 1988.)

MATERIALS AND METHODS

The pneumococcal strains used in these studies and some of their relevant properties are listed in Table 1. The clinical isolates were obtained from the collections of the following colleagues: R. Facklam (Centers for Disease Control, Atlanta, Ga.), N. Figura (University of Siena, Siena, Italy), V. Fischetti (Rockefeller University), D. Hansman (Sydney, Australia), H. Koornhof (Johannesburg, Republic of South Africa), L. Marri (Temple University, Philadelphia, Pa.), M. McCarty (Rockefeller University), M. Modde (Neuchatel, Switzerland), A. Parkinson (Centers for Disease Control Arctic Branch, Anchorage, Alaska), M. Simberkoff (Veterans Administration Hospital, New York, N.Y.), and E. Simoes (University of Colorado, Denver). The strains represent a wide variety of isolation dates (1938 through 1987) and isolation sites (South Africa, New Guinea, United States) and several clinical sources (nasopharyngeal carriage, cerebrospinal fluid, blood, middle ear); the strains also belong to a variety of serotypes.

Bacterial stocks were kept in the frozen state at −70°C in growth medium containing 10% (final concentration) glycerol. For labeling of PBPs, bacteria were grown at 37°C in C medium (7) supplemented with yeast extract (0.2%, final concentration; Difco Laboratories, Detroit, Mich.). Growth was followed by monitoring optical density at 620 nm

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TABLE 1. Clinical isolates of pneumococci and some of their relevant properties

Strain	Penicillin MIC (μg/ml)	Serotype	Isolation date	Source	Geographic site
SP-108	0.003	15 B	1939		CDC,[a] Atlanta
R6	0.006				
L7-0170	0.008	8	1986	Blood	Kotzebue, Alaska
L7-0172	0.008	14	1986	Blood	Kotzebue, Alaska
L7-1074	0.008	12 F	1987	Blood	Kotzebue, Alaska
L7-0175	0.008	8	1987	Blood	Kotzebue, Alaska
L7-0178	0.008	14	1986	Blood	Anchorage, Alaska
L7-0180	0.010	14	1986	Blood	Anchorage, Alaska
L7-0088	0.015	14	1984	Blood	Bethel, Alaska
L7-0092	0.015	14	1983	Blood	Bethel, Alaska
L7-0094	0.015	14	1983	Blood	Bethel, Alaska
L7-0101	0.015	23 F	1984	CSF[b] and blood	Bethel, Alaska
L7-0114	0.015	14	1982	Blood	Bethel, Alaska
SA-3	0.015		1979	Nasopharnyx	South Africa
SA-23	0.015	6	1979	Nasopharnyx	South Africa
L7-0171	0.016	12 F	1986	Blood	Kotzebue, Alaska
L7-0173	0.016	12 F	1987	Blood	Kotzebue, Alaska
L7-0176	0.016	8	1987	Blood	Kotzebue, Alaska
L7-0177	0.016	14	1986	Blood	Anchorage, Alaska
L7-0179	0.016	14	1986	Blood	Anchorage, Alaska
SP-148	0.025	36	1942		CDC, Atlanta
SP-99	0.025	11 A	1942		CDC, Atlanta
SP-96	0.025	10 A	1938		CDC, Atlanta
SP-95	0.025	9 V	1939		CDC, Atlanta
SA-45526	0.030				South Africa
L7-0085	0.030	10 A	1984	Blood	Bethel, Alaska
L7-0087	0.030	4	1982	Blood	Bethel, Alaska
L7-0090	0.030	14	1984	Blood	Bethel, Alaska
L7-0096	0.030	14	1983	Blood	Bethel, Alaska
L7-0102	0.030	7 F	1984	Blood	Bethel, Alaska
L7-0104	0.030	14	1983	Blood	Bethel, Alaska
L7-0106	0.030	14	1983	Blood	Bethel, Alaska
L7-0108	0.030	14	1983	Blood	Bethel, Alaska
L7-0099	0.030	33 F	1983	Blood	Bethel, Alaska
L7-0115	0.030	4	1983	Blood	Bethel, Alaska
SA-34034	0.040	4	1984	CSF	South Africa
L7-0113	0.040	4	1984	Blood	Bethel, Alaska
L7-0662	0.040	19 F	1985	Blood	Bethel, Alaska
L7-0110	0.060	19 A	1983	Blood	Bethel, Alaska
L7-0660	0.080	19 F	1985	Blood	Bethel, Alaska
L7-0661	0.080	6 B	1985	Blood	Bethel, Alaska
VA-Ben	0.080	9	1987	Blood	New York
L7-0091	0.120	19 A	1983	Blood	Bethel, Alaska
L7-0095	0.120	19	1984	Blood	Bethel, Alaska
L7-0097	0.120	19 A	1984	Midear	Bethel, Alaska
L7-0100	0.120	19 A	1983	Blood	Bethel, Alaska
L7-0103	0.120	18 C	1984	Blood	Bethel, Alaska
L7-0105	0.120	19 A	1984	Blood	Bethel, Alaska
L7-0107	0.120	19 A	1984	Blood	Bethel, Alaska
L7-0111	0.120	19 A	1983	Blood	Bethel, Alaska
L7-0112	0.120	6 B	1982	Blood	Bethel, Alaska
VA-Bri	0.200	15	1987	Blood	New York
L7-0086	0.250	23 F	1983	Blood	Bethel, Alaska
L7-0089	0.250	4	1985	Blood	Bethel, Alaska
L7-0109	0.250	14	1985	Blood	Bethel, Alaska
SA-45588	0.250	14	1985	Blood	South Africa
SA-13979	0.250	6	1985	Blood	South Africa
SA-42607	0.500	6		Pus	South Africa
SA-45443	0.500	19	1985	Blood	South Africa
P-3003	0.500	6			Papua, New Guinea
Giu	0.500	23	1987	Blood	Seattle
G-285	0.600	23	1987	Nasopharynx	New York
G-777	0.600	23	1987	Nasopharynx	New York
SA-34142	1.0	19	1984	Blood	South Africa
SA-23301	1.0	19	1985	Blood	South Africa
SA-381	1.0	19	1979	Blood	South Africa
SA-14750	1.0			Blood	South Africa

Continued on following page

TABLE 1—*Continued*

Strain	Penicillin MIC (μg/ml)	Serotype	Isolation date	Source	Geographic site
G-721	1.0	19	1987	Nasopharynx	New York
F-108	1.0	19	1987	Nasopharynx	New York
SA-95210	1.6	6A	1977	Nasopharynx	South Africa
SA C-07	2.0				South Africa
SA Straus	2.0	19	1985	Middle ear	South Africa
SA-49995	4.0				South Africa
SA-64147	4.0	6	1983	Blood	South Africa
SA-61382	4.0				South Africa
SA-10760	4.0	6A	1979		South Africa
G-320	5.0	23	1987	Nasopharynx	New York
SA-8249	6.0	19A	1977	CSF	South Africa
CS-029055	8.0	14	1987		CDC, Atlanta
SAD-20	12.0	19A	1977	CSF	South Africa
CS-029044	16.0	14	1987		CDC, Atlanta

[a] CDC, Centers for Disease Control.
[b] CSF, Cerebrospinal fluid.

(Sequoia-Turner Spectrophotometer; Sequoia-Turner, Mountainview, Calif.). At the middle of the logarithmic phase of growth, PBPs were labeled as described earlier (4, 14) with either 1.0 or 5.0 μg of tritiated benzylpenicillin per ml, and cultures were incubated at 37°C for 10 min.

[^{3}H]benzylpenicillin ethylpiperidinium salt (Merck Sharp & Dohme, Rahway, N.J.)-labeled cultures were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described earlier (4, 14). Determination of MICs was done by the standard tube dilution method with C medium (7) supplemented with 0.2% yeast extract and 10^4 to 10^5 bacteria per ml as inocula. Tubes were read after overnight (16 h) incubation at 37°C.

RESULTS

Each pneumococcal strain was grown to mid-log phase and exposed to a standard saturating dose of radioactive penicillin (1 to 5 μg [^{3}H]penicillin per ml for 10 min), and PBP patterns were determined by the fluorographic technique. Each PBP pattern determination was repeated at least two or three times with freshly grown cultures. Thirty-five penicillin-susceptible strains (MIC, 0.003 to 0.03 μg/ml) examined with this technique showed a remarkable degree of similarity to one another in PBP patterns. Figure 1 shows the PBP patterns of 14 of these isolates; in this particular group we included all of the relatively few strains showing variation in the basically common PBP pattern typical of penicillin susceptible strains. Three types of variation in pattern were observed. (i) One strain, SP108 (isolated in 1939; serotype 15B), showed no detectable PBP 2B. (ii) Three strains, 34034, A661, and A175, had PBP 3 with a higher molecular size (58 kilodaltons [kDa]) than that seen in the standard laboratory strain (52 kDa). (iii) In several (11 of 36) strains PBP 1 appeared to have a slightly lower molecular size than the usual PBP 1A of the laboratory strain (about 97 instead of 98 kDa). Examples of these may be seen in Fig. 1 (strains 99, 88, 96, 175, and 171). Upon repeated cultivation each strain reproduced its unique PBP pattern.

The second group of clinical isolates (46 strains) represented a range of MICs from 0.04 to 16.0 μg/ml. For 18 of these strains, penicillin MICs were equal to or higher than 1.0 μg/ml. Figure 2 shows the PBP patterns of a select group of 10 such strains, along with 3 intermediately resistant strains (Fig. 2B). In several lanes preparations of the penicillin-susceptible laboratory strain R6 were also run.

Even a superficial comparison of Fig. 1 and 2 reveals the striking variability in the PBP patterns of resistant strains as contrasted with the basic similarity of pattern characteristic of the susceptible isolates. The PBP patterns of resistant strains were stable and reproducible upon repeated culturing of the bacteria. Figure 3 is a sketch constructed on the basis of the fluorogram in Fig. 2; it summarizes in a simplified way the PBP patterns in 11 resistant strains relative to the PBP pattern of the susceptible laboratory strain R6.

Although the particular PBP patterns seen in resistant cells showed no clear correlation with serotype or with the year or country of isolation, there was a relationship appar-

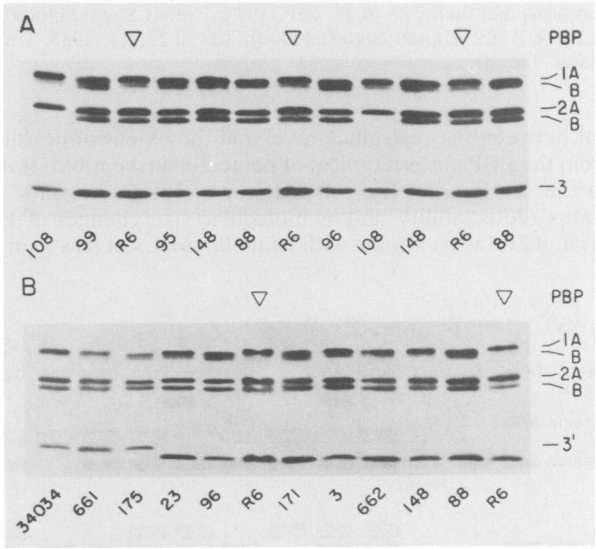

FIG. 1. PBP pattern of penicillin-susceptible clinical isolates of pneumococci. Open arrows (▽) indicate PBPs of the penicillin-susceptible laboratory strain R6. All strains had penicillin MICs of <0.05 μg/ml, except for strain A-661 (penicillin MIC, 0.08 μg/ml). Serotypes, dates, and sites of isolation were as follows: SP-108 (15B, 1939, United States), SP-99 (11A, 1942, United States), SP-95 (9V, 1939, United States), SP-148 (36, 1942, United States), A-88 (14, 1984, United States, blood), SP-96 (10A, 1938, United States), SA-34034 (4, 1984, South Africa, cerebrospinal fluid), A-661 (6B, 1985, United States, blood), A-175 (8, 1987, United States, blood), SA-23 (6, 1979, South Africa, nasopharynx), A-96 (14, 1984, United States, blood), A-171 (12F, 1986, United States, blood), SA-3 (-, 1979, South Africa), A-662 (19F, 1985, United States).

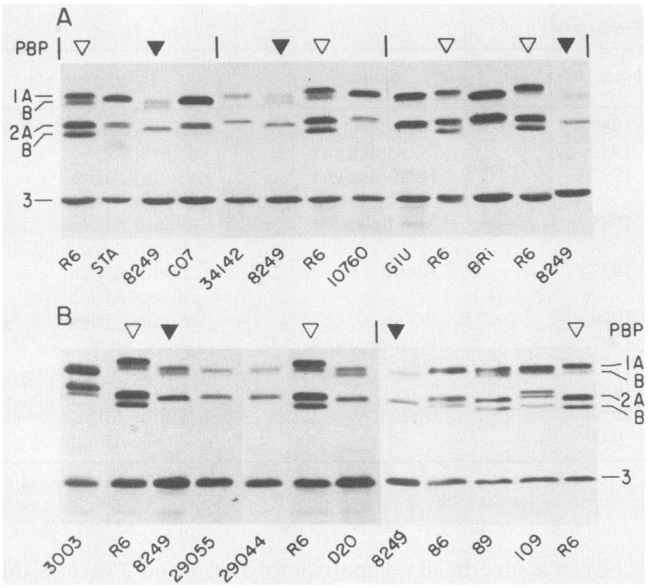

FIG. 2. PBP pattern of penicillin-resistant clinical isolates of pneumococci. Arrows indicate PBPs of the penicillin-susceptible strain (R6 (▽) and the penicillin-resistant strain 8249 (▼) reference strains. The penicillin MICs (micrograms per milliliter), serotypes, isolation dates, and sites of the various strains were as follows: R6 (0.006, rough, 1930, United States), STA (2.0, 19, 1985, South Africa, middle ear), 8249 (6.0, 19A, 1977, South Africa, cerebrospinal fluid), CO-7 (2.0, unknown, unknown, South Africa, unknown), 34142 (1.0, 19, 1984, South Africa, blood), 10760 (4.0, 6A, 1979, South Africa), Giu (0.5, 23, 1987, United States, blood), VA-Bri (0.2, 15, 1987, United States, blood), 3003 (0.5, 6, unknown, Papua-New Guinea), 29055 (8.0, 14, 1987, Czechoslovakia), 29044 (16.0, 14, 1987, Czechoslovakia), D-20 (10, 19A, 1977, South Africa, cerebrospinal fluid), 86 (0.25, 23F, 1983, United States, blood), 89 (0.25, 4, 1985, United States, blood), 109 (0.25, 14, 1985, United States, blood).

ent between the resistance level and the extent of deviation from the PBP pattern typical of penicillin-susceptible strains. In the scattergram (Fig. 4) MICs were plotted against the status (detectability and/or molecular size change) of PBP 1A and 2B. Most strains with penicillin MICs of less than 0.1

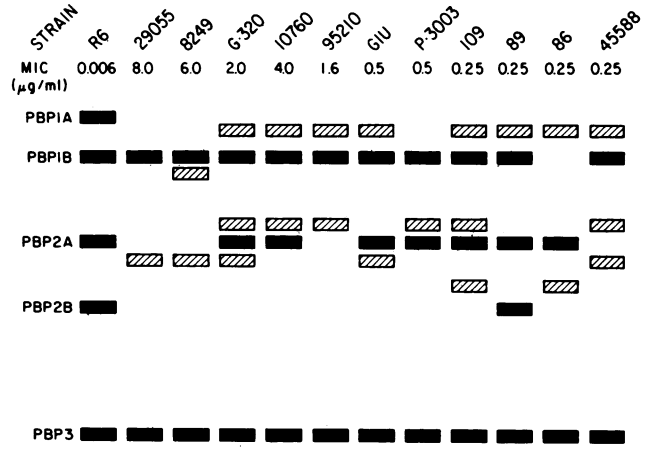

FIG. 3. Variation in the PBP patterns of penicillin-resistant isolates. Cross-hatched bars indicate PBPs of anomalous size detected in the particular resistant strain. Solid bars represent PBPs typical of the penicillin-susceptible laboratory reference strain R6.

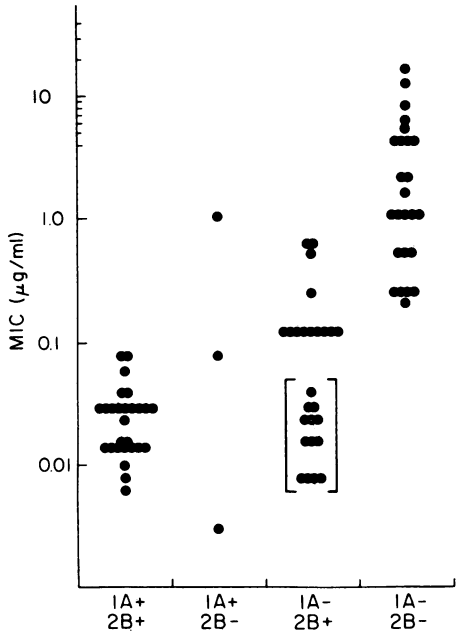

FIG. 4. Correlation between PBP pattern and MIC. Superscripts refer to the absence (−) or presence (+) of PBP 1A or 2B. Each dot represents a clinical isolate. Dots within brackets indicate strains that have lost PBP 1A, which was replaced by another PBP with slightly smaller molecular size but normal (high) penicillin affinity.

μg/ml contained PBP 2B and PBP 1A with a normal size ($1A^+$ $2B^+$) (i.e., with a size identical to that seen in strain R6). On the basis of comparison with molecular size standards, we estimate this size to be about 98 kDa. In contrast, none of the strains with penicillin MICs of >1.0 μg/ml contained detectable PBP 2B or 1A of normal size ($1A^-$ $2B^-$); this group also included a number of strains with penicillin MICs of 0.25 to 1.0 μg/ml. In these strains PBP 1A was either not detectable at all or "replaced" by a PBP of somewhat lower molecular size (96 to 97 kDa). Most strains with intermediate penicillin MICs (MIC, 0.1 to 0.5 μg/ml) contained detectable PBP 2B but no PBP 1A with the normal size ($1A^-$ $2B^+$). This latter class of PBP types also included a substantial number of strains with low penicillin MICs (0.01 to 0.05 μg/ml; see dots in brackets on Fig. 4). Finally, 3 of the 85 isolates examined contained no detectable PBP 2B and yet appeared to have normal-sized PBP 1A ($1A^+$ $2B^-$). These three odd strains had widely different MICs of benzylpenicillin (0.008, 0.1, and 1.0 μg/ml, respectively).

DISCUSSION

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis system that has been in use in our laboratory (4, 5) resolves the PBPs of the penicillin-susceptible pneumococcal laboratory strain R6 into five components: PBPs 1A (98 to 100 kDa), 1B (95 kDa), 2A (80 kDa), 2B (77 to 78 kDa), and 3 (52 kDa) (3, 4, 14). Although the presence of additional PBPs resolvable by variation in the conditions of the assay obviously cannot be excluded, our method was sufficient to establish several important and novel facts concerning PBPs of pneumococcal clinical isolates. First, the great majority of penicillin-susceptible pneumococcal strains appears to share a common, characteristic PBP pattern. A similar conservation of PBP pattern has already been noted in other bacterial species as well (2, 12, 13), and it has been proposed that PBP

patterns may be used for purposes of bacterial taxonomy (13). Nevertheless, several penicillin-susceptible strains showed deviation from this shared, typical pattern. The significance and interpretation of these anomalous PBP patterns are not clear. We have examined the cell wall stem peptide patterns of strains SP-108 (lacking PBP 2B) and A-175 (unusual, higher-molecular-size PBP 3) with a newly developed high-resolution method based on high-performance liquid chromatography (1). The peptide patterns were identical to those of other susceptible strains containing the full set of "normal" PBPs. We also examined the penicillin affinities of the anomalous (somewhat lower in molecular size) PBP 1A component seen in several susceptible strains (see strains represented by data points within brackets in Fig. 4). High affinity for benzylpenicillin, indistinguishable from the affinity of PBP 1A of susceptible strains, was seen (undocumented observations).

A second interesting finding was the surprising degree of variation in the number and molecular sizes of PBPs detectable in clinical isolates with elevated penicillin MICs. In the two highly resistant strains studied in some detail so far, the biochemical and genetic mechanism of resistance was extremely complex, and genetic transformation of high levels of resistance occurred with very low frequencies only (15). Similar difficulties were encountered during selection of even relatively low-level spontaneously penicillin resistant mutants in the laboratory (5). On the basis of these facts, one would anticipate that resistant mutants among clinical isolates would belong to a relatively few or, possibly, even a single class of mutants identifiable by a few PBP patterns predictable from the MIC. Yet, this was not the case. Penicillin-resistant clinical isolates showed a surprising degree of variation in number and molecular size of PBPs; this was in sharp contrast with the relatively monotonous and predictable PBP profile of penicillin-susceptible strains. This variation in PBP pattern was already observable among strains with intermediate resistance (MIC, 0.1 to 0.25 μg/ml) and it became frequent among strains with penicillin MICs of >1.0 μg/ml. Of the 18 strains in this last class, only one strain shared the PBP pattern of strains 8249 and D20, i.e., the two highly resistant South African strains studied in most detail so far. Examples of variation in PBP profile among strains of intermediate resistance level are shown in Fig. 2B and are reproduced in Fig. 3. Four strains, 86, 89, 109, and 45588, each with a penicillin MIC of 0.25 μg/ml, had four distinct PBP patterns. The first three isolates were from Alaska (serotypes 23F, 4, and 14); the last one (45588) was a South African isolate (serotype 14).

The methods used in the studies (short exposure to a single, high concentration of radioactive penicillin) only allowed determination of molecular sizes but not the evaluation of differences in antibiotic "affinities," which undoubtedly are also present. It is most likely that the disappearance of PBP 2B from the fluorograms of virtually all resistant strains is, in fact, a consequence of greatly decreased antibiotic affinity (rather than the lack of production of this protein), as has already been demonstrated in the case of PBP 2B of strain 8249 (3). In the schematic representation of some of the pattern differences in Fig. 3, we have assumed that if a PBP in a clinical strain had the molecular size that was exactly the same as the size of one of the five PBPs in the penicillin-susceptible standard strain R6 then, in fact, these PBPs were identical (solid bars in Fig. 3). This, of course, does not exclude major differences in antibiotic affinity. On the other hand, the PBPs detected in clinical isolates that clearly did not correspond to any of the five PBPs of strain R6 in molecular size are simply indicated by the cross-hatched bars in Fig. 3. Our data did not allow us to suggest which, if any, of the five normal PBPs those anomalous binding proteins were related to, and it is for this reason that we did not assign any numbers and names to these PBPs. We suggest that the anomalous-size PBPs may represent mutant derivatives of one or the other normal PBPs with amino acid replacements in molecular domains that cause conformational changes or alter the detergent-binding properties of the proteins. There are numerous examples in the literature describing single amino acid replacements that cause disproportionately large changes in apparent molecular size during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6, 11). Whatever the origin of these PBPs may be, each individual strain has reproduced its characteristic PBP upon repeated cultivation; therefore these patterns may be considered as heritable fingerprints of a particular resistant mutant strain. It follows, then, that penicillin resistance has emerged among clinical strains a number of times. It is also apparent that more than one kind of molecular restructuring of these proteins may be compatible with low antibiotic affinity and increased penicillin resistance, suggesting a surprising degree of plasticity in the genetic determinants of pneumococcal PBPs.

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