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Altered Penicillin-Binding Proteins in Methicillin-Resistant Strains of *Staphylococcus aureus*

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The penicillin-binding proteins (PBPs) of a methicillin-resistant (MR) and a methicillin-susceptible (MS) *Staphylococcus aureus* were compared by various approaches involving the use of high-specific-activity [^{3}H]penicillin as a reagent. The MR and MS strains were found to contain PBPs of the same number and electrophoretic mobilities. However, saturation of PBPs 1, 2, and 3 by methicillin in the MR strain required the use of several thousands of micrograms of antibiotic per milliliter, whereas 0.2 to 0.4 μg of methicillin per ml was sufficient to effectively compete with [^{3}H]penicillin for the PBPs of the MS strain. Additional experiments indicate that these differences most likely reflect a greatly decreased affinity of the PBPs of the MR strain as compared to those of the MS strain. Shift of the pH of the culture medium of the MR strain from pH 7.0 to 5.2 resulted in an immediate drop in phenotypic resistance to methicillin (from a minimal inhibitory concentration value of 3,200 μg/ml at pH 7.0 to 0.8 μg/ml at pH 5.2). Examination of the methicillin affinities of PBPs in MR bacteria grown at pH 5.2 showed the presence of the same low-affinity PBPs as in bacteria grown at pH 7.0. Thus, the pH-dependent resensitization to methicillin cannot be explained by a parallel increase in the antibiotic affinities of the PBPs.

Extensive studies on the nature of methicillin resistance in *Staphylococcus aureus* have led to the recognition of several pleimorphic properties in these mutants. These include reduced susceptibility to lysostaphin (17), deficiency in protein A (23), and a change in the net surface charge of the cells (9). In addition, variation in several growth parameters was found to have profound influence on the expression of methicillin resistance. Specifically, growth of resistant mutants at low pH (16, 20) or in the presence of ethylenediaminetetraacetate (19) was shown to result in suppression of methicillin resistance. Furthermore, it was reported that, with at least some of the methicillin-resistant isolates, bacterial cultures appeared to be unable to fully express resistance if the culture was passed in drug-free medium and subsequently plated on the surface of agar plates containing methicillin. Addition of certain supplements to the agar such as high concentrations of sucrose or sodium chloride, or incubation of the plates at temperatures suboptimal for growth (30°C instead of 37°C), resulted in the recovery of an increased proportion of the population behaving as phenotypically methicillin-resistant bacteria (1, and for review 16). Serial passage in the presence of beta-lactams had the same effect (18), and the term "heterogeneity" was used to refer to this phenomenon.

Biochemical studies on amino acid, amino sugar, and teichoic acid composition (24) failed to detect significant chemical differences between the methicillin-susceptible (MS) and -resistant (MR) staphylococci. These studies have led to an extensive characterization of the physiology of MR staphylococci. Nevertheless, the biochemical basis of resistance has remained a puzzle.

In an attempt to learn more about the mechanism of this interesting phenomenon, we undertook a study of the penicillin-binding proteins (PBPs) of MS and MR staphylococci. We shall describe two major conclusions of these studies. (i) PBPs 1, 2, and 3 of the MR staphylococci appear to have greatly decreased affinities for methicillin when compared to the affinities of PBPs from an MS strain, and the degree of affinity changes is in rough proportion to the increase in the methicillin minimal inhibitory concentration (MIC) value for the bacteria. (ii) The phenotypic loss of antibiotic resistance observable in MR cultures during growth at pH 5.2 is not due to a pH-dependent increase in the methicillin affinities of PBPs.

(A preliminary description of these findings was reported at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy in September 1980 [B. J. Hartman and A.

Tomasz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, abstr. no. 713, 1980].)

MATERIALS AND METHODS

Organisms. The four strains of *S. aureus* used in this study are described in Table 1.

Growth curves. All experiments were carried out in tryptic soy broth (TSB; Difco) at the appropriate pH of 7.0 or 5.2. pH shifts were made using either concentrated HCl or NaOH. Methicillin was added at the times indicated on the figures. Growth was measured using a nephelometer (Coleman Instruments, Oak Brook, Ill.).

Membrane preparations used for in vitro labeling with ^{3}H-labeled penicillin. Each strain was grown to log phase in 1 liter of TSB at either pH 7.0 or 5.2. The organisms were centrifuged at 10,000 rpm for 15 min at 0°C, and the collected cells were suspended in 10 ml of fresh medium. These were then broken open by shaking with glass beads (100 μm diameter) in a Mickle disintegrator (Mickle Laboratory Engineering Co., Gomshall, Surrey, England) at 4°C for 90 min. Deoxyribonuclease and ribonuclease (100 μg/ml each) were added, and after 10 min of incubation at 37°C the suspension was centrifuged at 4°C, first at 3,000 rpm for 5 min (to remove glass beads) and then at 8,000 rpm for 10 min to remove cell walls and unbroken cells. The supernatant was then spun at 45,000 rpm for 45 min at 0°C. The pellet was suspended and washed with fresh medium and respun at 45,000 rpm for 45 min. The resulting pellet was suspended in 2 ml of medium at the appropriate pH, quickly frozen, and stored at −70°C until used. Protein concentrations were determined using the Lowry method (13).

Tritium-labeled benzylpenicillin. Tritium-labeled benzylpenicillin (ethylpiperidinium salt) with a specific activity of 25 Ci/mmol was supplied by Merck & Co., Inc., Rahway, N.J., and stored in acetone at −20°C. Immediately before use the acetone was evaporated and replaced by 0.01 M potassium phosphate buffer (pH 7.0).

Labeling of membranes (in vitro labeling). Portions (25 μl) of the membrane preparations (≈300 μg of protein) were mixed with [^{3}H]penicillin in buffer at various concentrations of the antibiotic. The suspensions were incubated in borosilicate glass tubes (10 by 75 mm) at 37°C for 15 min, the reaction was stopped by addition of excess nonradioactive penicillin (5 μl of 12.5-mg/ml cold penicillin for each 25-μl cell sample), and 25 μl of potassium phosphate buffer (0.2 M, pH 7.0) was added to each tube. Sarkosyl (NL-97; 20% solution) was added next (5 μl), and the samples were maintained at room temperature for 20 min. A 20-μl volume of sample dilution buffer and 10 μl of 2-mercaptoethanol were added, and the entire sample was boiled for 2 min and applied to polyacrylamide slab gels.

Labeling of live cells (in vivo labeling). An overnight culture of each organism was grown in TSB at either pH 7.0 or 5.2. This culture was then diluted into 75 ml of fresh medium and grown to log phase. The cells were spun at 10,000 rpm for 10 min and suspended in 750 μl of fresh prewarmed TSB at the appropriate pH (100-fold concentration). Samples (25 μl) were then used for in vivo labeling of PBPs with 4 to 5 μl of [^{3}H]penicillin as described for in vitro labeling. After the addition of nonradioactive penicillin and pH 7.0 buffer, the samples received 5 μl of lysostaphin (100 μg/ml final concentration). The addition of 0.2 M (pH 7) buffer was important to adjust the pH to that optimal for the activity of lysostaphin. Lysis was allowed to proceed at 37°C for 30 min. The lysates received Sarkosyl and were processed as in the in vitro experiments.

Competition experiments. Competition experiments (in vivo and in vitro) were done in two ways. In one type of design the cells or membranes were preincubated with various concentrations of the competing nonradioactive methicillin (5 to 8 μl) for 15 min—before the addition of [^{3}H]penicillin at a single, saturating concentration which was 1 μg/ml for strain 209-P and strain 27, and 50 μg/ml for RUCUS 1112 and strain 592 → 27$_{11}$ (sequential labeling). In the second design, the cells and membranes received both drugs simultaneously (simultaneous labeling).

Gel electrophoresis and fluorography. Discontinuous sodium dodecyl sulfate-polyacrylamide slab gels were made as described by Laemmli (11), except

TABLE 1. *Properties of S. aureus strains used*

Strain	Relevant phenotype[a]	MIC (μg/ml)				Origin of strains
		Methicillin		Penicillin G		
		7.0[b]	5.2[b]	7.0[b]	5.2[b]	
209-P	MS, L$^-$	0.4	0.2	<0.2	<0.2	ATCC 4538 P
RUCUS 1112	MR, weak L$^+$	3,200.0	0.8	50.0	50.0	Rockefeller Univ.-Cornell Univ. *Staphylococcus* (N.Y. Hospital clinical isolate)
27	MS, L$^-$	0.8	0.8	0.02	0.02	Richard Novick, Public Health Research Institute, N.Y.
592 → 27$_{11}$	MR, L$^-$	625.0	1.6	5.0	0.006	Richard Novick, Public Health Research Institute, N.Y.

[a] Abbreviations: MS, methicillin susceptible; MR, methicillin resistant; L$^-$, β-lactamase negative; L$^+$, β-lactamase positive.

[b] pH of cultivation.

that the concentrations of acrylamide and bisacrylamide were 10 and 0.13%, respectively, in the separating gel and 5 and 0.068%, respectively, in the stacking gel. Electrophoresis was performed at 75 V through the stacking gel and 120 V through the separating gel until the leading edge had run off the gel for 1 h. Gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (6) and destained with methanol-acetic acid-water (30%:5%:65%). Fluorography was done by the method of Bonner and Laskey (2) using presensitized Kodak X-Omat XR-2 film and 3 to 5 days of exposure at −70°C.

Penicillinase. Penicillinase was assayed using nitrocefin (Glaxo Research Ltd., Greenford, Middlesex, England) (15).

Antibiotics. Antibiotics were gifts kindly supplied by the following companies: methicillin (Beecham Labs, Piscataway, N.J.), benzylpenicillin, cephaloridine, and cephalothin (Eli Lilly & Co., Indianapolis, Ind.), nafcillin (Wyeth Labs, Philadelphia, Pa.), cefoxitin (Merck & Co., Inc.), carbenicillin and cephalexin (Bristol Laboratories, Syracuse, N.Y.), sulbenicillin (Takeda Chemicals, Osaka, Japan), and nitrocefin (Glaxo Research Ltd.). Lysostaphin was obtained from Schwarz-Mann of Orangeburg, N.Y. Acrylamide, *N,N'*-methylenebisacrylamide, sodium dodecyl sulfate, TRIZMA Base (TRIS), and glycine were obtained from Bio-Rad of Rockville, N.Y. Kodak X-Omat XR-2 film was used for all fluorography.

RESULTS

Table 1 summarizes some of the relevant properties of the MR and MS strains used in our studies. MR strain RUCUS 1112 which was chosen for detailed study showed some, but not all, of the pleiotrophic properties described in the literature for other MR isolates of *S. aureus* (16). Notably absent was the "heterogeneity" as defined by Sabath and others (1, 18). The MIC values for methicillin and most other beta-lactams tested showed the pH dependence which is characteristic of the MR staphylococci (16, 20). Exceptions were benzylpenicillin and cephaloridine; in these cases MIC values did not vary with the pH (Table 2). Minimal bactericidal concentrations were roughly equivalent to MICs at pH 7.0. The striking drop in the MIC values at pH 5.2 was not accompanied by a parallel decrease in minimal bactericidal concentration values.

Effect of the pH of culture medium on the methicillin susceptibility of the MR strain. Figures 1 and 2 show the growth and response to methicillin addition in the MS and MR strains when grown at either pH 7.0 or pH 5.2 in TSB medium with aeration. Inspection of the figures allows several conclusions. The MS strain 209-P grew somewhat faster at pH 7.0 than at pH 5.2 (average doubling times being 20 and 40 min, respectively). Addition of methicillin to the MS

TABLE 2. *Effect of pH on the antibacterial effects of various β-lactams in the MR strain S. aureus RUCUS 1112*

Antibiotic	MIC value (μg/ml)	
	pH 7.0	pH 5.2
Methicillin	3,200.0 (3,200)[a]	0.8 (3,200)
Benzylpenicillin	50.0 (50)	50.0 (50)
Nafcillin	400.0 (400)	0.1 (>100)
Carbenicillin	800.0 (>800)	50.0 (>800)
Sulbenicillin	400.0 (800)	0.4 (12.5)
Cephalothin	50.0 (200)	0.05 (100)
Cephaloridine	6.3 (25)	6.3 (50)
Cefoxitin	100.0 (400)	0.4 (>100)
Cephalexin	400.0 (800)	0.8 (12.5)

[a] The numbers in parentheses are minimal bactericidal concentrations in micrograms per milliliter.

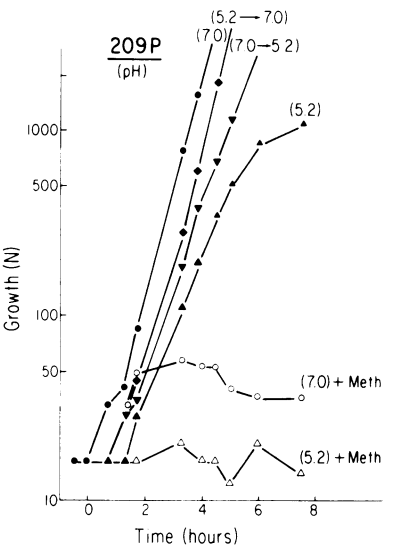

FIG. 1. *Growth and methicillin response of the MS strain 209-P grown at pH 7.0 and 5.2. Two cultures of strain 209-P (10 ml each) were grown at 37°C with aeration in TSB medium with pH adjusted either at 7.0 (culture 7.0) or at 5.2 (culture 5.2). When the cell concentrations reached ca. 5 × 10^8 colony-forming units per ml, each of these cultures was diluted back (0.1 ml into 10 ml of fresh prewarmed media) to initiate the following three subcultures: two TSB cultures at pH 7.0 plus one culture at pH 5.2 (from culture 7.0) and two pH 5.2 plus one pH 7.0 TSB cultures (from culture 5.2). One of the pH 7.0 subcultures derived from culture 7.0 received 1 μg of methicillin per ml [(7.0) + Meth]; the other served as control. Similarly, one of the pH 5.2 subcultures derived from culture 5.2 received 1 μg of methicillin per ml [(5.2) + Meth]; the other [(5.2)] received no drug. Subcultures labeled (7.0 → 5.2) and (5.2 → 7.0) represent bacteria backdiluted into media with the alternative pH value (culture 7.0 to pH 5.2 or culture 5.2 into pH 7.0, respectively). Growth was monitored by a nephelometer.*

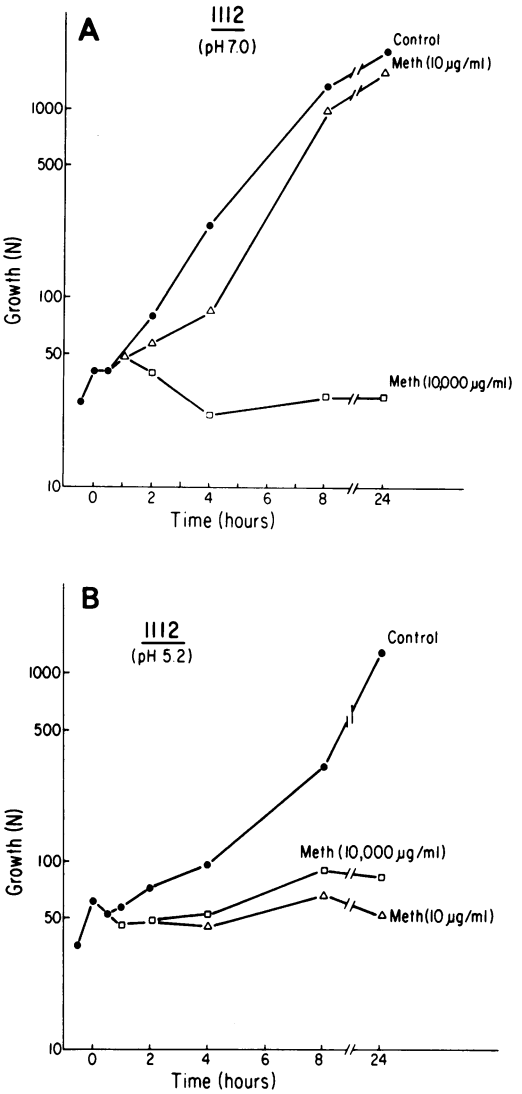

FIG. 2. *Growth and methicillin response of the MR strain 1112 grown at pH 7.0 and 5.2. Cultures of the MR strain were grown as described in the legend for Fig. 1, either at pH 7.0 (A) or at pH 5.2 (B). Methicillin (10 or 10,000 μg/ml) was added at zero time.*

culture (at 1-μg/ml concentration) resulted in the cessation of growth (Fig. 1) and loss of viability (not shown). The doubling times of the MR culture RUCUS 1112 were about 60 min at pH 7.0 and 90 to 120 min at pH 5.2. Addition of methicillin (10 μg/ml) to the MR culture grown at pH 7.0 allowed continued growth; inhibition of these cultures required the addition of very high concentrations (in the milligram per milliliter range) of the antibiotic (Fig. 2A). The striking drop in methicillin resistance in MR cultures grown at pH 5.2 is shown in Fig. 2B. Shifting the pH value of MR cultures from pH 7.0 to 5.2 (and vice versa) resulted in a rapid adjustment of both the growth rates and the methicillin susceptibilities: within 30 min of the pH adjustment to pH 5.2 (i.e., in less than a generation time), the bacteria exhibited high susceptibility to methicillin addition (Fig. 3A). Similarly, the reverse pH shift (from pH 5.2 to 7.0) was accompanied by the rapid expression of methicillin resistance (Fig. 3B).

Two additional experiments were designed to better characterize the nature of this pH-dependent antibiotic resistance. In the first, methicillin (10 μg/ml) was added to a culture growing at pH 7.0. As expected, at this particular pH, this concentration of antibiotic did not inhibit growth (see curve D in Fig. 4). However, shift of the culture pH, at various times after methicillin addition, resulted in a virtually instant cessation of growth (Fig. 4).

Figure 5 shows that preexposure of the MR culture to methicillin (10 μg/ml) while growing at pH 5.2 which normally inhibits growth would still allow resumption of culture growth provided that the pH was shifted up to 7.0 immediately or within an hour after the addition of the antibiotic (see Fig. 5, cultures A and B). This presumably reflects the relatively slow irreversible inactivation (killing) of the bacteria when they were exposed to methicillin at pH 5.2. Nevertheless, the growth rate of culture B remained somewhat slower than that of either the drug-free control culture or culture A. Upshift of pH after more than 1 h of antibiotic addition (culture C) produced a long lag before visible turbidity increase would commence (presumably representing the outgrowth of small subpopulations of surviving bacteria).

PBPs of MS and MR staphylococci grown and assayed at pH 7.0. Figure 6 shows a titration of the PBPs of MS and MR staphylococci each determined in two different assay systems: (i) by exposing exponentially growing bacteria to the radioactive antibiotic (in vivo labeling); and (ii) by using membranes (in vitro labeling). In the former method, a direct comparison of the MIC values and PBP saturation values is possible.

The results allow several conclusions. (i) MS and MR staphylococci seem to have the same PBPs as far as number and electrophoretic mobilities are concerned, with the three high-molecular-weight PBPs shown considered to be the major PBPs (10). PBP-4 is not apparent on the gels, presumably because of its known rapid turnover (10). (ii) Comparison of the in vivo and in vitro titration profiles shows no signs of a

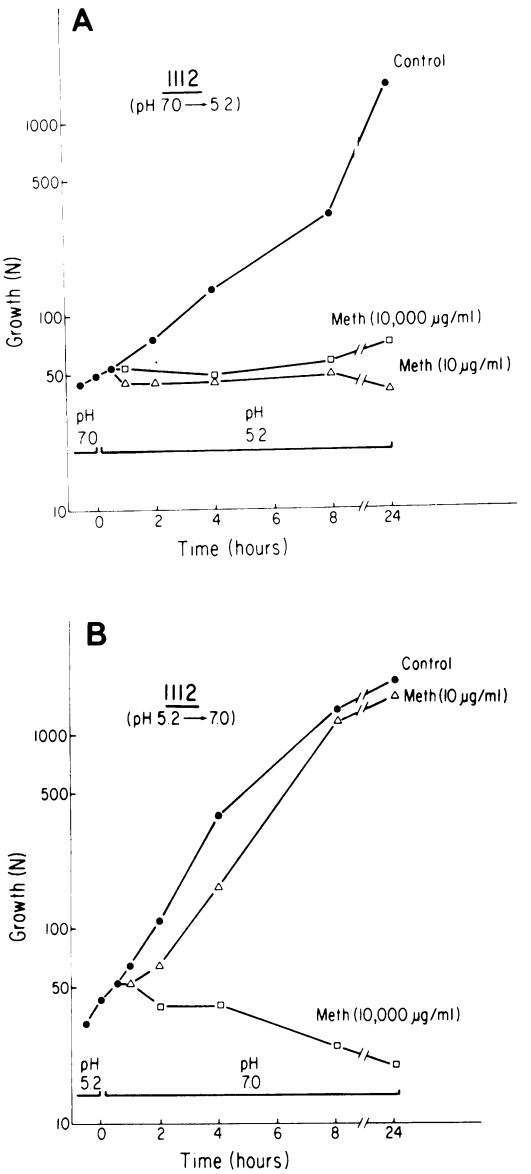

FIG. 3. *Effect of shift in the pH of growth medium on the methicillin response of strain 1112. (A) The pH of an exponential-phase culture of the MR strain 1112 (2 × 10⁷ colony-forming units per ml, grown at 37°C with aeration in TSB, pH 7.0) was rapidly adjusted to pH 5.2 by the addition of 12 N HCl. The culture was then divided into three parts, two of which received methicillin at a 10- or 10,000-μg/ml concentration. Incubation at 37°C was continued, and growth was monitored by a nephelometer. (B) The pH of another culture of MR strain 1112 grown at pH 5.2 was adjusted to pH 7.0 by the addition of 10 N NaOH in a manner similar to that described for (A). The culture was again divided into a control and two methicillin-treated subcultures.*

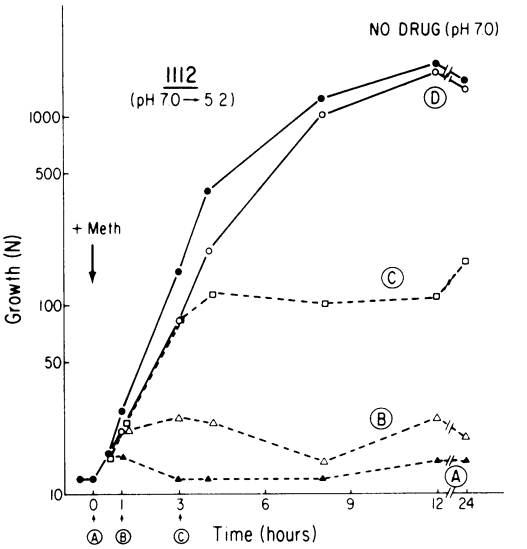

FIG. 4. *Sensitization of MR strain 1112 to methicillin by shifting the culture pH from 7.0 to 5.2. An exponentially growing culture of 50 ml of strain 1112 in pH 7.0 TSB was divided into three subcultures at time 0. One of these (10 ml) received methicillin at 10 μg/ml (Ⓓ) and was allowed to continue growth in the pH 7.0 medium. Another subculture (10 ml) was allowed to grow at pH 7.0 without antibiotic [no drug (pH 7.0).] The rest of the pH 7.0 culture (30 ml) received methicillin (10 μg/ml), and incubation was continued. At various times after antibiotic addition, 10-ml portions of this latter culture were removed, pH was adjusted to 5.2, and the incubation was continued. This shiftdown in pH was done immediately after the addition of methicillin (Ⓐ), and 1 h (Ⓑ) and 3 h (Ⓒ) after methicillin addition. The effect of methicillin in combination with the pH shift on culture growth was followed by nephelometry. Dashed lines represent cultures with pH shift.*

significant penetration barrier in the MR strain. (iii) A striking difference was apparent between the PBPs of the MR and MS strains, namely, substantially higher concentrations of [^{3}H]penicillin were needed for saturation labeling of PBPs of the MR strain than those of the MS strain. Furthermore, the concentrations needed to achieve saturation roughly corresponded to the penicillin MIC values of the respective strains (50 versus <0.2 μg/ml).

An exactly analogous situation was observed when methicillin was used in competition with simultaneously added [^{3}H]penicillin (administered at a constant concentration corresponding to the appropriate saturating concentrations of the respective organisms, i.e., 1.0 μg of [^{3}H]pen-

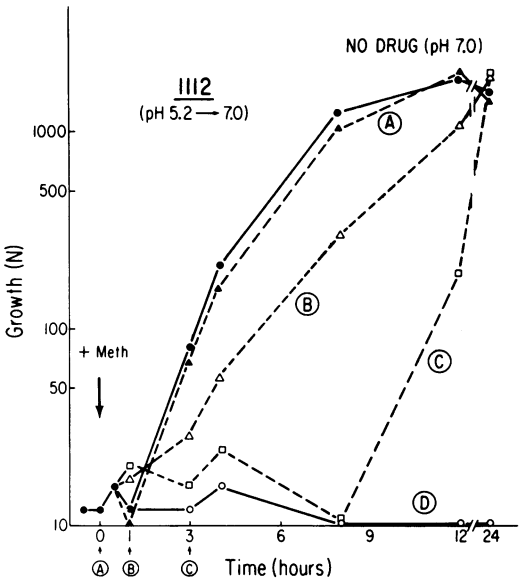

FIG. 5. *Effect of shift in culture pH from pH 5.2 to pH 7.0 during treatment of the MR strain 1112 with methicillin. An exponentially growing culture of strain 1112 in pH 5.2 TSB was divided into subcultures at time 0. One culture (10 ml) received methicillin (10 μg/ml) and was allowed to further incubate in the pH 5.2 medium (Ⓓ). Another 10-ml subculture was adjusted to pH 7.0 and allowed to grow [no drug (pH 7.0)]. The rest of the 30-ml bacterial culture received methicillin (10 μg/ml), and incubation was continued. At three different times after antibiotic addition (0 min, Ⓐ; 1 h, Ⓑ; and 3 h Ⓒ), 10-ml portions were removed, pH was adjusted to 7.0, and incubation was continued. The response of these cultures to methicillin was followed by nephelometry.*

icillin per ml for the MS and 50.0 μg of [^{3}H]-penicillin per ml for the MR strain) (Fig. 7). Again, it may be seen that substantially higher concentrations of methicillin were needed to effectively compete with penicillin in the MR strain (about 1,000 to 4,000 μg of methicillin per ml) as compared to the MS strain (about 0.2 to 0.4 μg of methicillin per ml). These saturation values correspond roughly to the MIC values of the respective strains. Identical results were obtained when the methicillin-resistant transductant (592 → 27_{11}) was used instead of strain RUCUS 1112 (Fig. 8, pH 7.0).

PBPs of MR and MS staphylococci grown and assayed at pH 5.2. Membranes were isolated from MR and MS staphylococci grown in TSB adjusted to pH 5.2, and the PBPs were titrated by incubating such preparations at pH 7.0 and at pH 5.2 with various concentrations of [^{3}H]penicillin or with various concentrations of methicillin plus a constant (saturating) concentration of [^{3}H]penicillin. The same experiments were repeated by using in vivo labeling of MR and MS cells growing at pH 5.2.

The incubation of cells or membranes at pH 5.2 during the assay of PBPs led to relatively minor changes in the labeling pattern, and these were virtually identical in both MS and MR cells and independent of the pH of cultivation and whether or not the assays were performed in vivo (Fig. 9) or in vitro (not shown). Surprisingly, the saturation of the PBPs of MR cells grown at pH 5.2 (a condition that suppresses the genetic resistance to methicillin) required virtually the same high concentration of [^{3}H]penicillin as in the case of pH 7.0-grown bacteria. Similarly, effective competition for the PBPs required the same high concentration of methicillin (1,000 to 4,000 μg/ml) as in the pH 7.0-grown MR cells. Both in vitro and in vivo labeling gave identical results (Fig. 8 and 10, in vitro not shown).

Effect of preincubation of the MR cells or MR membranes with methicillin before the addition of [^{3}H]penicillin. In experiments exactly analogous to those described earlier, MR cells or membranes of MR cells (grown at pH 7.0) were preincubated with various concentrations of methicillin for 15 min, and the reagent [^{3}H]penicillin was added afterwards at a constant (saturating) concentration. In this modified experimental design, methicillin was shown to effectively prevent binding of [^{3}H]penicillin to the PBPs of MR staphylococci at low concentrations (0.1 to 1.0 μg/ml) both in the in vivo (Fig. 11) and in the in vitro assays (not shown). The pH of the growth and incubation media did not affect this finding. Thus, in contrast to the results of the true competition experiments (i.e., simultaneous exposure to methicillin and [^{3}H]penicillin), preexposure to methicillin did not allow the detection of the striking differences in labeling between the PBPs of MR and MS staphylococci.

DISCUSSION

The major finding reported here is the dramatic decrease in the affinity of the PBPs of MR staphylococci for methicillin as compared to MS strains. Since radioactively labeled methicillin is unfortunately not available, the affinity change of the PBPs could only be characterized by competition experiments using varying concentrations of nonradioactive methicillin with [^{3}H]benzylpenicillin present at a constant concentration corresponding to that needed to saturate the PBPs of the respective microorganisms. Significantly, this penicillin concentration

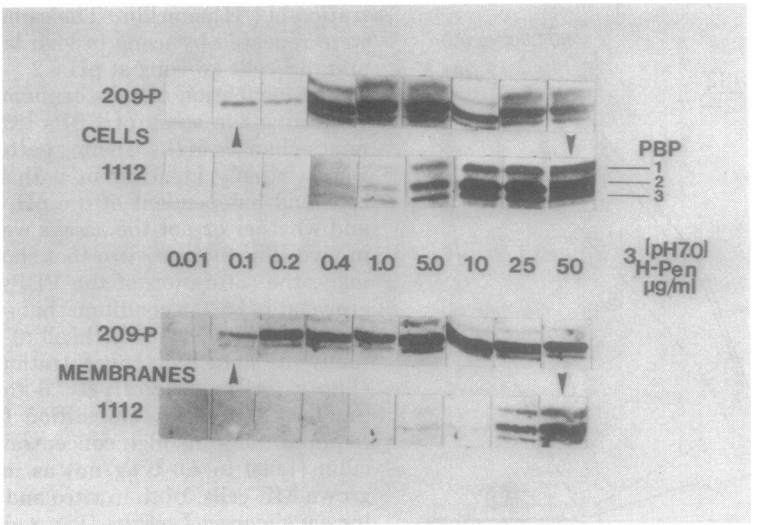

FIG. 6. *Titration of the PBPs of MS (strain 209-P) and MR (strain 1112) staphylococci in live cells and membrane preparations. Bacteria were grown at pH 7.0, and membranes were also prepared from pH 7.0-grown cells. Labeling of PBPs was performed as described in the text. Arrows indicate MIC of the organisms to benzylpenicillin.*

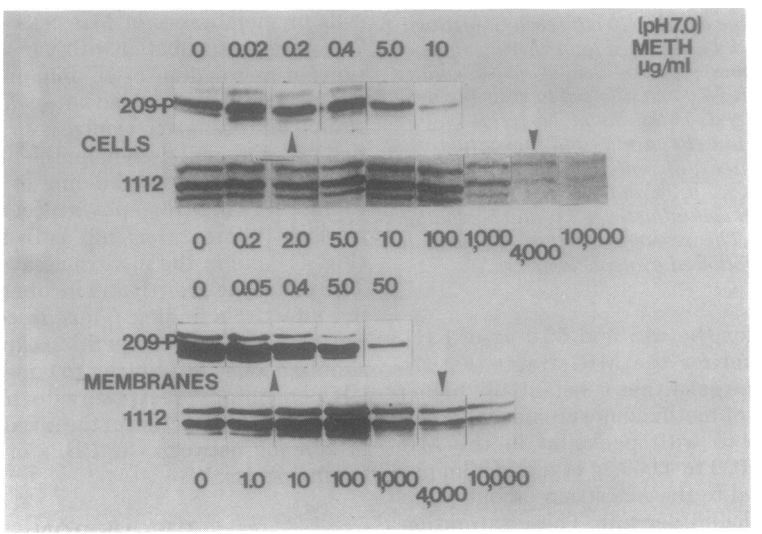

FIG. 7. *Competition between methicillin and [^{3}H]penicillin for the labeling of PBPs of MS (strain 209-P) and MR (strain 1112) staphylococci. Labeling was performed as described in the text. Arrows indicate the MIC of methicillin.*

was different for the MS strain (0.2 μg/ml) and the MR strain RUCUS 1112 (50 μg/ml), and these penicillin concentrations corresponded closely to the MIC values for benzylpenicillin in the MS versus MR strains. As long as the cells or membranes were exposed to the methicillin and [H^3]penicillin simultaneously (i.e., true competition experiments), the PBPs of MR cells required very high concentrations of methicillin (1,000 to 4,000 μg/ml) to compete with penicillin for binding. However, if methicillin was added 15 min before the [^{3}H]penicillin, the same low concentrations of methicillin (0.1 to 1.0 μg/ml) were sufficient to block labeling of the PBPs of both MS and MR cells. Our interpretation of these findings is that the PBPs of MR cells have a greatly reduced affinity for methicillin relative to their affinities for benzylpenicillin. On the

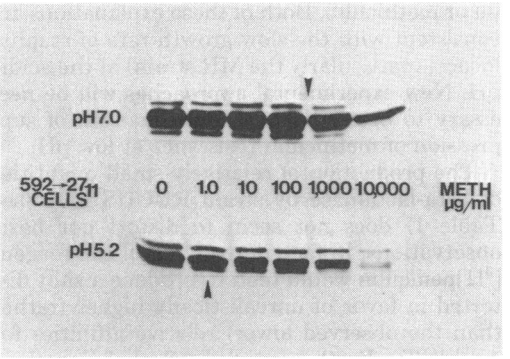

FIG. 8. *Competition between methicillin and [^{3}H]penicillin for the labeling of PBPs of MR (transductant 592 → 27$_{11}$) staphylococci grown either at pH 7.0 or at pH 5.2. Arrows indicate the MIC of methicillin.*

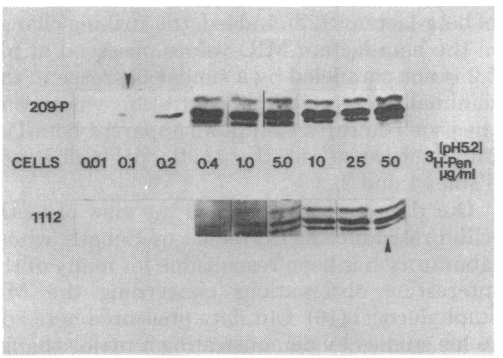

FIG. 9. *Labeling of PBPs with [^{3}H]penicillin in MS (strain 209-P) and MR (strain 1112) staphylococci grown at pH 5.2. Arrows indicate the MIC of benzylpenicillin.*

other hand, if methicillin molecules had a chance to attach to the PBPs of MR cells (in the absence of radioactive penicillin), the methicillin-PBP complexes were stable enough (presumably because of low deacylation rates [7]) to effectively prevent the labeling of PBPs by the [^{3}H]penicillin subsequently added.

Analogous findings have been reported in the literature in the case of *Escherichia coli* and *Pseudomonas* PBPs in which the demonstration of selective affinities of certain beta-lactams for specific PBPs required preincubation with the nonradioactive antibiotic before the assay with the radioactive benzylpenicillin (14). Clearly, a superior way of performing these experiments would be to use radioactively labeled methicillin. While our findings only indicate a dramatic decrease in the relative affinities of the MR PBPs for methicillin over that for benzylpenicillin, it seems reasonable to propose that when growing MR cells encounter methicillin in their environment, these same binding proteins will have a similar relative decrease in affinity for methicillin over that for the endogenous natural substrates of the physiological reaction(s) (cell wall synthesis) catalyzed by these proteins. This interpretation is supported by the fact that the concentration of methicillin needed to saturate the PBPs of the MR cells is close to the MIC concentration of the drug. Furthermore, the excess concentration of [^{3}H]benzylpenicillin needed to saturate the PBPs of MR cells is again in the vicinity of the increased MIC value that these bacteria have for benzylpenicillin.

The dramatic pH-dependent drop of the MIC values of MR cells from the value of 3,200 µg/ml (at pH 7.0) to 0.8 µg/ml (at pH 5.2) may be taken as a sign for some change in the interaction between the methicillin molecules and their cellular targets (PBPs) at the acidic pH. Indeed, a pH-dependent change in the access of methicil-

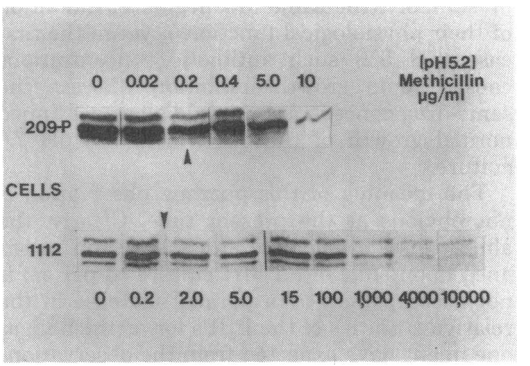

FIG. 10. *Competition between methicillin and [^{3}H]penicillin for the labeling of PBPs in MS (strain 209-P) and MR (strain 1112) staphylococci grown at pH 5.2. Arrows indicate the MIC of methicillin.*

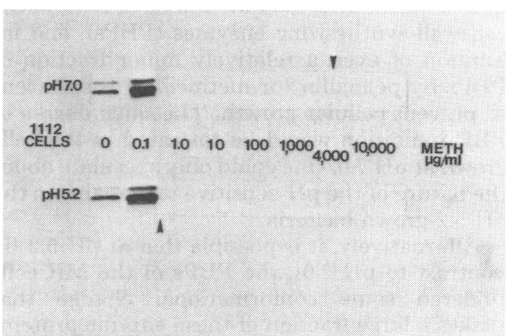

FIG. 11. *Effect of methicillin pretreatment on the labeling of the PBPs of MR (strain 1112) staphylococci grown at pH 7.0 or pH 5.2. Arrows indicate MIC of methicillin.*

lin to some beta-lactam-sensitive target enzyme has been suggested as a plausible mechanism for this peculiar characteristic of the MR staphylococci (20). However, as far as affinity of the PBPs for methicillin is concerned, this was not the case. We were surprised to find that the relative affinities of the PBPs of MR cells to methicillin versus [^{3}H]penicillin were equally low in the pH 5.2-grown (phenotypically antibiotic susceptible) and pH 7.0-grown (phenotypically resistant) MR cells in spite of the tremendous differences in sensitivity to growth inhibition by the antibiotic. Similar results were obtained when the concentrations of [^{3}H]penicillin needed to saturate the PBPs of MR cells grown at pH 5.2 and pH 7.0 were evaluated.

At first glance, these results appear to be perplexing. The exposure of two cultures of MR cells—one growing at pH 5.2 and the other at pH 7.0—to the same 10-μg/ml concentration of methicillin would produce the same low degree of binding of the antibiotics to the PBPs (and, presumably, the same low degree of inhibition of their physiological functions), yet in the first case (pH 5.2) such antibiotic concentrations caused rapid growth inhibition whereas the same drug concentration would allow continued normal growth of the bacteria in the pH 7.0 culture.

The meaning of this puzzling observation is not obvious at the present time. Clearly, the almost instantaneous loss of methicillin resistance upon shift of an MR culture to pH 5.2 is not accompanied by a parallel increase in the relative affinities of the PBPs for methicillin, as one might have expected from the observations concerning the PBPs of staphylococci grown at pH 7.0.

One can envisage at least two alternative explanations for these findings. It is conceivable that continued cellular growth (at pH 5.2) requires the unhindered functioning of 100% of the cell wall-synthesizing enzymes (PBPs), and inhibition of even a relatively minor fraction of PBPs by penicillin (or methicillin) is sufficient to prevent cellular growth. The same degree of PBP inhibition would be tolerated in the cells grown at pH 7.0. One could only speculate about the nature of the pH-sensitive process(es) in the pH 5.2-grown bacteria.

Alternatively, it is possible that at pH 5.2 (in contrast to pH 7.0), the PBPs of the MR cells undergo some conformational change that makes a large fraction of these enzyme proteins inefficient as catalysts of cell wall synthesis with their natural endogenous substrates, but this pH-dependent change is not reflected in the relative affinities of the PBPs for either penicillin or methicillin. Both of these explanations are consistent with the slow growth rate of staphylococci (particularly the MR strain) at the acidic pH. New experimental approaches will be necessary to better define the mechanism of suppression of methicillin resistance at low pH.

The production of relatively small quantities of beta-lactamase by strain RUCUS 1112 (see Table 1) does not seem to distort our basic observations. In fact, destruction of the reagent [^{3}H]penicillin would tend to produce a shift distorted in favor of unrealistically higher (rather than the observed lower) relative affinities for methicillin. Furthermore, identical observations were made using the MR transductant strain that showed no detectable penicillinase activity.

The substantially slower growth rate of the MR cells (and MS cells as well) at pH 5.2 would be expected to slow down the bactericidal effect of beta-lactams (22). Indeed, the striking change in the beta-lactam MIC values observed at pH 5.2 is not paralleled by a similar decrease in the minimal bactericidal concentration values and thus such cultures exhibit an apparent beta-lactam "tolerance" at the acidic pH value (see Tables 1 and 2).

Our data basically confirm the view of methicillin resistance as expressed by Sabath, whose laboratory has been responsible for many of the interesting observations concerning the MR staphylococci (16). Our data presented here add to his studies by demonstrating a major change in the methicillin affinities of the PBPs of MR staphylococci. In addition, our membrane studies failed to show a significant permeability barrier accounting for this resistance.

Our results are in full agreement with the observation of Bruns and Keppeler (4) that membranes prepared from MR staphylococci bound radioactive penicillin with a strikingly lower affinity than membranes of susceptible strains, whereas the maximum capacity for binding appeared to be similar in the susceptible and resistant bacteria. A recent report by Brown and Reynolds (3) has provided confirmation of our basic observation that methicillin resistance in staphylococci is accompanied by decreased beta-lactam affinity of PBPs. However, in contrast to the findings with our strains, the decreased penicillin affinity was limited to PBP 3. In addition, these authors also noted an apparent increase in the amounts of PBP 3 in the resistant strains. The reasons for these differences in the observations are not clear. They may be related to strain differences or, possibly, to differences in the degree of methicillin resistance. Multiple PBP alterations have only been seen at relatively high levels of resistance as reported for

intrinsically penicillin-resistant pneumococci (25) and gonococci (5).

PBPs are, presumably, proteins with catalytic and regulatory roles in various steps in murein biosynthesis. The drastic alterations observed in affinity to beta-lactams may also involve changes in the way such proteins handle their natural substrates (which are supposed to have chemical structures analogous to the beta-lactam antibiotics [21]). Thus, such altered PBPs may perform their synthetic functions in a somewhat "abnormal" manner, yielding a cell wall that is not identical in some fine structural feature (e.g., degree of cross-linking) to those of the MS cells. One might postulate that such change in murein structure may, in turn, cause additional abnormalities such as in the attachment of teichoic acids, penetration of the murein layers by lipoteichoic acids, or functioning of autolytic enzymes. Thus, at least in principle, one could generate the pleiotropic surface properties of the MR cells from the same fundamental changes in the PBPs. The number and type of mutations responsible for the observed PBP alterations is not known at the present time.

Alteration of PBPs appears to be a general strategy of bacteria to cope with beta-lactams in the growth medium. In addition to the case of staphylococci described here, PBP alterations seem to accompany penicillin resistance in a number of other bacterial species such as the multiply antibiotic-resistant South African strains of pneumococci (25), penicillin-resistant pneumococcal isolates from the United States (8), and intrinsically penicillin-resistant gonococci (5), and in *Pseudomonas* and *Serratia* (D. Mirelman, Y. Nuchamowitz, and E. Rubenstein, 20th ICAAC, abstr. no 353).

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