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Expression of Methicillin Resistance in Heterogeneous Strains of *Staphylococcus aureus*

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The phenotypic expression of methicillin resistance was studied in a number of clinical isolates and laboratory strains of *Staphylococcus aureus*. The methicillin-resistant *S. aureus* strains could be divided into three classes, homogeneous, heterogeneous, and thermosensitive heterogeneous methicillin-resistant *S. aureus*, on the basis of their plating efficiencies at 30 or 37°C on methicillin-containing agar plates. Heterogeneous strains of methicillin-resistant *S. aureus* were composed of two subpopulations: a small minority of cells (10^{-5} to 10^{-3}; MIC, 600 to 1,000 μg/ml) that expressed resistance to high concentrations of methicillin at 37°C, and a majority of cells (MIC, 5 μg/ml) that remained susceptible to the drug at 37°C. Cultures of a thermosensitive heterogeneous strain were able to grow in the presence of high concentrations of methicillin, provided that the growth temperature was 30°C. Such cultures lost their phenotypic resistance within 30 min (i.e., in less than one doubling time) after the growth temperature was shifted to the nonpermissive 37°C. Shift of the temperature of the culture in the reverse direction (37 to 30°C) resulted in an equally rapid expression of phenotypic resistance. The majority of the cells in such heterogeneous strains may be considered heat (or salt) conditional in their phenotypic expression of methicillin resistance. Both heterogeneous and thermosensitive heterogeneous strains, irrespective of their temperature of cultivation and degree of phenotypic resistance, contained detectable quantities of the 78-kilodalton penicillin-binding protein 2a (PBP 2a) that previous studies have suggested is a biochemical correlate of methicillin resistance in homogeneous strains of methicillin-resistant *S. aureus*. Methicillin resistance was lost, and PBP 2a was not detectable in any of the strains grown at pH 5.2. These findings suggest that the genetic determinant(s) of PBP 2a is transcribed and translated in all cells and all classes of methicillin-resistant *S. aureus*. However, in contrast to the homogeneous strains, in heterogeneous and thermosensitive heterogeneous isolates the ability to synthesize PBP 2a is apparently not sufficient to provide a resistant phenotype. In these strains some additional, as yet undefined factor(s) is also needed for the expression of methicillin resistance.

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates frequently possess a characteristic referred to as heterogeneity (1, 10, 18, 23, 24). This term refers to the fact that within a given strain of MRSA, only a small proportion of CFU are able to express resistance to beta-lactam antibiotics at the normal incubation temperature of 37°C and in growth medium at pH 7.0. The degree of heterogeneity is variable but usually only 1 CFU of 10^4 to 10^7 CFU are phenotypically resistant. Modification of growth conditions can increase the proportion of CFU that express resistance to various degrees and can approach 100%. The modifications include a reduction in the incubation temperature from 37 to 30°C (1, 10, 18, 23) or an increase in the osmolality of the growth medium (2, 10, 23). Individual isolates are known to respond to the conditions described above to various degrees.

For example, some strains in which only 0.01% of CFU can give rise to colonies on methicillin-containing plates at 37°C can increase the proportion of CFU expressing resistance to almost 100%, provided that the temperature of incubation is lowered to 30°C (23). In some other isolates lower incubation temperature causes only a modest increase, e.g., from 0.001 to 0.01–0.1%, in the proportion of phenotypically resistant cells. In this study we examined 21 MRSA strains of geographically diverse origins for a number of properties, including the degree of heterogeneity, effect of culture conditions on heterogeneity, selection of an unstable homogeneously resistant subpopulation from heterogeneous strains, and the profile of penicillin-binding proteins (PBPs). We were surprised to find detectable quantities of the low-affinity extra PBP in all heterogeneous strains examined, irrespective of the degree of phenotypic expression of resistance. Previous studies in several laboratories with homogeneously methicillin-resistant strains have established the presence of an extra PBP with low affinity for methicillin in these bacteria, and it has been suggested that this extra PBP is a biochemical correlate of resistance (6, 13–15).

The presence of the low-affinity PBP may be a necessary but not sufficient condition for the methicillin-resistant phenotype in the heterogeneous strains of MRSA.

MATERIALS AND METHODS

Strains. The relevant characteristics of the 21 MRSA strains used in this study are shown in Table 1.

Growth media. Stocks of all strains were stored frozen at −70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol. Cultures were grown in TSB at 37 or 30°C with shaking overnight in a gyratory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) and were then diluted into fresh prewarmed TSB and grown to the middle of the exponential growth phase (about 2×10^8 to 5×10^8 CFU/ml), at which time the cultures were used for the experiments.

Plating was done on TSB agar (1.5% agar [Difco]), and the pH of TSB or TSB-agar was adjusted to the desired value by the addition of dilute HCl or NaOH with an Altex Zeromatic

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TABLE 1. Methicillin-resistant phenotypes

Resistance phenotype	No. of strains tested	Methicillin MIC at pH 7.0 (μg/ml)[a]	EOP % at 37°C[b]	EOP % at 30°C[b]
Thermosensitive heterogeneous	9	60–2,500	0.005–0.3	30–100
Heterogeneous	5	10–1,200	<0.00007–0.001	0.004–0.3
Homogeneous	7	40–2,500	5–100	40–100

[a] Determined by tube dilution with 10^5 to 10^6 CFU/ml at 24 to 48 h of incubation.

[b] EOP %, number of CFU on methicillin-containing plates/number of CFU on methicillin-free plates × 100 (tested on TSB-agar containing 50 μg of methicillin per ml).

IV pH meter with glass electrodes (Beckman Instruments, Inc., Fullerton, Calif.).

Growth was measured by determination of the turbidity, as determined by the light scattering of the cultures (Nepho-Colorimeter; Coleman Instruments, Oak Brook, Ill.). Viable counts (CFU) were determined by plating the cultures after they were serially diluted in distilled water. Heterogeneity of methicillin resistance was quantitated by determining the efficiency of plating (EOP %) on agar plates containing various concentrations of methicillin. (EOP %, the number of CFU on methicillin-containing plates/the number of CFU on methicillin-free plates × 100.) Plates were incubated for 72 to 96 h before the colonies were counted.

MICs were determined by either the agar dilution method with 10^6 organisms or the broth dilution method with 10^5 organisms as the initial inocula (27), and MICs were read after 48 h of incubation at 37°C. All determinations were in TSB either on agar plates or in liquid culture.

Labeling of PBP in growing bacteria. Cultures were first grown to late log phase (about 10^9 CFU/ml) in TSB at pH 7.0 or pH 5.2 and at either 37 or 30°C with shaking. Next, the cultures were diluted (1.0 ml into 100 ml of fresh, prewarmed TSB) and grown again to the middle of the exponential growth phase. The cells were collected by centrifuging 100 ml of the culture (10,000 × *g*, 0°C, 15 min) and suspending the bacteria in 1.0 ml of fresh TSB. Samples (25 μl) of this suspension were then incubated at 37°C for 10 min with various concentrations of ^{3}H-labeled benzylpenicillin (ethylpiperidinium salt; specific radioactivity, 25 Ci/mmol; kindly supplied by Merck & Co., Inc., Rahway, N.J.) (21). Immediately before use the acetone solvent of [^{3}H]penicillin was evaporated and replaced by 0.1 M potassium phosphate buffer (pH 7.6). After incubation the samples were boiled for 2 min. Lysostaphin (5 μl of a 1,000-μg/ml solution in 0.01 M potassium phosphate buffer) was added, and the samples were incubated at 37°C for another 30 min. Finally, 25 μl of sample dilution buffer (8) was added, and the entire sample was boiled again for 2 min.

In competition experiments the samples were preincubated for 10 min with various concentrations of methicillin, followed by exposure to [^{3}H]penicillin (10 μg/ml) for an additional 10 min and processed as described above.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) was carried out with 10% acrylamide–0.13% bisacrylamide in the running gel (13 by 18 cm). The stacking gel was composed of 5% acrylamide–0.07% bisacrylamide. Samples were added to the gel lanes and run through the stacking gel at 75mV and then electrophoresed through the running gel at 120 mV for 45 to 60 min past the time that the leading edge had passed the bottom of the running gel.

After the gels were stained with Coomassie brilliant blue (11) and destained overnight with methanol-acetic acid-water (30:5:65), they were processed by first removing the water by soaking in dimethyl sulfoxide (DMSO) (two times for 30 min each time) followed by soaking in a 20% (wt/vol) 2,5-diphenyloxazole (PPO)-DMSO solution for 2 h. The gels were then rehydrated in a water-glycerol solution for 45 to 60 min, dried thoroughly, and exposed to Kodak X-OMAT XR-2 film for 3 to 14 days at −70°C (4). The relative intensities of the bands were quantitated with a scanning densitometer (Quick Scan Jr.; Helena Instruments, Beaumont, Tex.).

Measurement of peptidoglycan synthesis. *N*-Acetyl-D-(1-^{3}H)glucosamine (1 μCi, 0.2 μg/ml; Amersham International, Amersham, United Kingdom) was added to 100-μl culture samples for 5 min at 30 or 37°C (28). The reaction was stopped by the addition of excess cold *N*-acetyl-D-glucosamine (1 mg) and the immediate freezing of the sample in an ethanol-dry ice slurry. When all the samples were prepared, cold 50% trichloroacetic acid (TCA) was added to each sample. The samples were filtered through 3MM filter disks (Whatman Ltd., Maidstone, England), washed several times with 10% TCA followed by washing with water and then 95% ethanol. The filter disks were dried at 100°C and the filter disks were immersed in PPO–1,4-bis(5-phenyloxazolyl)benzene (POPOP)–toluene scintillant (11.37 g of PPO, 0.38 g of POPOP, 1 gallon [ca. 3.8 liters] of toluene). The samples were counted in a Nuclear-Chicago (Chicago, Ill.) Mark II scintillation counter and expressed as counts per minute.

Assay for penicillinase. The presence of penicillinase was assayed with nitrocefin (Glaxo Research Ltd., Greenford, Middlesex, England) (17).

Curing of penicillinase plasmids. Most of the isolates showed penicillinase activity. Cultures of such strains were grown at sub-MIC concentrations of ethidium bromide (approximately 2 to 4 μg/ml) (5) at 37°C for 18 h or 44°C for 18 h. Cultures were then plated on nonselective TSB agar plates, and the colonies were replica plated onto TSB plates containing 10 μg of benzylpenicillin per ml. Clones that did not appear on the latter plates were picked from the master plate, purified, and tested for penicillinase with nitrocefin; and the MIC to benzylpenicillin and methicillin was determined. Curing of penicillinase plasmids was found to be necessary for the titration of the PBP profiles of the bacteria.

RESULTS

Most of the 21 MRSA isolates exhibited high levels of methicillin resistance, showing MIC values of over 600 to 2,500 μg of methicillin per ml. When the EOP of the isolates was tested on agar plates containing 50 μg of methicillin per ml and the plates were incubated at 30 or 37°C, the 21 isolates could be separated into three distinct classes (Table 1). Seven strains expressed methicillin resistance homogeneously in approximately 100% of the cells present and at either temperature of incubation. In a second class of isolates (five strains), the population of cells was heterogeneous with respect to the expression of resistance, and the EOP was only increased to a minor degree by lowering the temperature of incubation to 30°C. The third and largest class (nine strains) showed low EOP when exposed to methicillin at 37°C, but the EOP increased greatly, approaching a value of 100% when the incubation temperature was

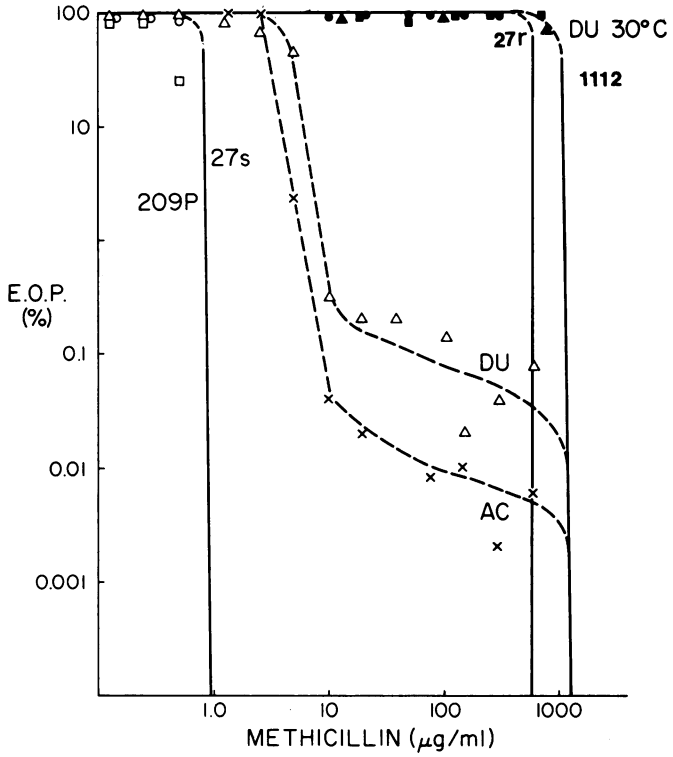

FIG. 1. EOP of several strains of *S. aureus* on increasing concentrations of methicillin-containing agar plates at 37°C. Strains 209P (○) and 27s (□) are methicillin susceptible. Strain 1112 (●) is a homogeneous MRSA, and strain 27r (■) is an isogenic, homogeneous methicillin-resistant transductant from strain 27s. Strains DU (△) and AC (×) are heterogeneous MRSA. Strain DU 30°C (▲) is strain DU grown at an incubation temperature of 30°C.

30°C. We suggest that these be referred to as thermosensitive heterogeneous MRSA.

The classification of the 21 strains was done on the basis of the determination of the EOP at a single concentration (50 μg/ml) of methicillin. Figure 1 shows the response of populations of homogeneous and heterogeneous strains of MRSA and two methicillin-susceptible strains of staphylococci against a broad range of concentrations of methicillin. It may be seen that both susceptible strains (27s and 209P) as well as the homogeneous strains 27r and 1112 showed a sharp all or nothing response with all bacteria capable of growing below, but virtually none above, the MIC. This indicates the presence of a single population of bacteria with uniform antibiotic susceptibility. The plots of the two heterogeneous MRSA strains (DU and AC) showed curves with less steep slopes, suggesting heterogeneity, i.e., the presence of several cell populations with differing drug susceptibilities: (i) a majority (over 98% of the CFU in the case of the AC strain) of phenotypically susceptible cells with an MIC of ≤5 μg/ml for methicillin; (ii) a small minority (1 in 10^5 CFU in strain AC) of cells with an MIC of >625 μg per ml, and, possibly (iii) several small populations of cells (frequency, 10^{-3} to 10^{-5}) with intermediate methicillin susceptibilities. Also shown on Fig. 1 is the homogeneous expression of resistance in the thermosensitive heterogeneous strain DU when grown at 30°C.

Production of transiently homogeneous resistant cell populations (HOM*) from heterogeneous MRSA. The MIC of the heterogeneous strains for methicillin, as measured by the tube dilution method, was that of the most resistant subpopulation (625 to 1,250 μg per ml in the case of strain AC) provided that the bacterial inoculum used was large enough (at least 10^6 in the case of strain AC) to include at least one of the highly resistant cells in the MIC tubes. The culture of strain AC that grew in the MIC tube containing 100 μg of methicillin per ml (AC100) was found to consist of a homogeneously resistant population (HOM*) when tested by EOP on TSB agar containing a wide range of concentrations of methicillin. HOM*, therefore, refers to a homogeneous population of cells selected by growth of a heterogeneous strain at 37°C in a given concentration of methicillin above the MIC for the susceptible majority of cells in the strain. Figure 2 compares the antibiotic response of the original AC strain with the response of this AC100 HOM* selected by preexposure of strain AC to 100 μg of methicillin per ml.

When methicillin concentrations substantially lower than 100 μg/ml were used for selection (e.g., 10 or 50 μg/ml), the culture that grew up (AC10 or AC50) was still found to have a high EOP (about 100%) even on plates containing 600 μg of methicillin per ml. No colonies grew on the plates containing 1,200 μg of antibiotic per ml. It seems, therefore, that

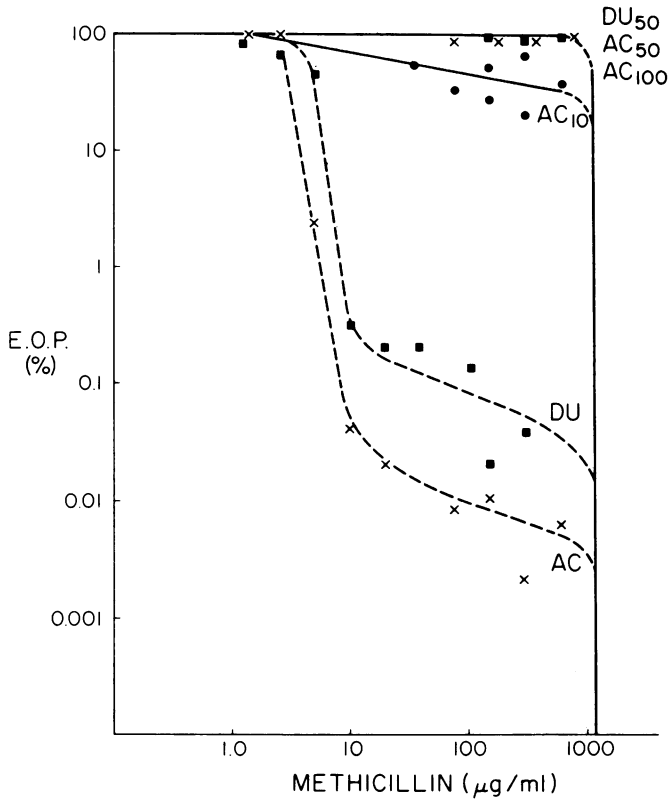

FIG. 2. Two major subpopulations of heterogeneous strains of MRSA determined by EOP. Strain DU (■) was grown at 37°C in drug-free medium before plating. Strain DU 50 (■) is strain DU grown in the presence of 50 μg of methicillin per ml overnight at 37°C and then plated for the determination of EOP at 37°C. Strain AC (×) was grown at 37°C in a drug-free medium before plating; strain AC10 (●) is strain AC grown in the presence of 10 μg of methicillin per ml overnight at 37°C and then plated for the determination of EOP at 37°C; strains AC50 and AC100 (×) are strain AC grown in the presence of 50 and 100 μg of methicillin per ml, respectively, overnight at 37°C and then plated for the determination of EOP AT 37°C.

incubation with methicillin at any concentration above the MIC value of the methicillin-susceptible majority population (approximately 5.0 μg/ml) of the AC strain selected for the small subpopulation of the most resistant bacteria. Identical findings were obtained with the thermosensitive heterogeneous strain DU, in which cultivation in the presence of 50 μg of methicillin per ml at 37°C led to the selection of a HOM* strain, DU50. These findings suggest that these particular heterogeneous strains are composed of only two types of cell populations: a drug-susceptible majority and a highly drug-resistant minority. The possibility of the existence of additional subpopulations with intermediate levels of resistance cannot be excluded for all the MRSA strains.

Instability of HOM* strains. The HOM* strains obtained by drug selection were unstable in the absence of antibiotic in the medium. A culture of the HOM* strain DU50 was diluted into drug-free TSB and grown with aeration at 37°C, and the EOP on 50 μg of methicillin per ml was tested at intervals. Figure 3 shows that during such cultivation the homogeneity of the HOM* strain was lost with rates that were characteristic of the isolate and also depended on the detailed conditions used in the original selection. Eventually, such cultures acquired the same degree of heterogeneity that was characteristic of the original heterogeneous isolates (23).

Response of a thermosensitive heterogeneous strain to methicillin treatment. Figure 4 shows the effects of methicillin on growth of strain DU at 37 and 30°C. As expected, cultures of strain DU were not significantly inhibited by methicillin concentrations up to 1,000 μg/ml at 30°C. However, even 10 μg/ml was inhibitory for cultures grown at 37°C. A temperature shift in either direction quickly caused the expected change in the phenotype of the culture. Bacteria grown at 30°C and shifted to 37°C lost the phenotypic methicillin resistance within 30 min after the shift. Similarly, a shift from 37 to 30°C resulted in a rapid expression of phenotypic resistance (Fig. 5).

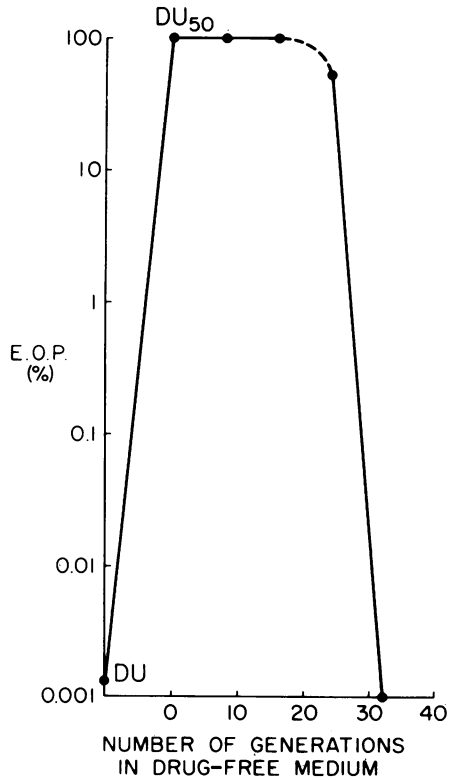

FIG. 3. Instability of a homogeneous subpopulation selected from a heterogeneous MRSA. Strain DU was grown in the presence of 50 μg of methicillin per ml at 37°C overnight, and a homogeneously resistant population (DU50) was selected as determined by EOP. DU50 was then grown for multiple generations in drug-free medium at 37°C, and the degree of heterogeneity was determined by EOP measured on agar plates containing 50 μg of methicillin per ml.

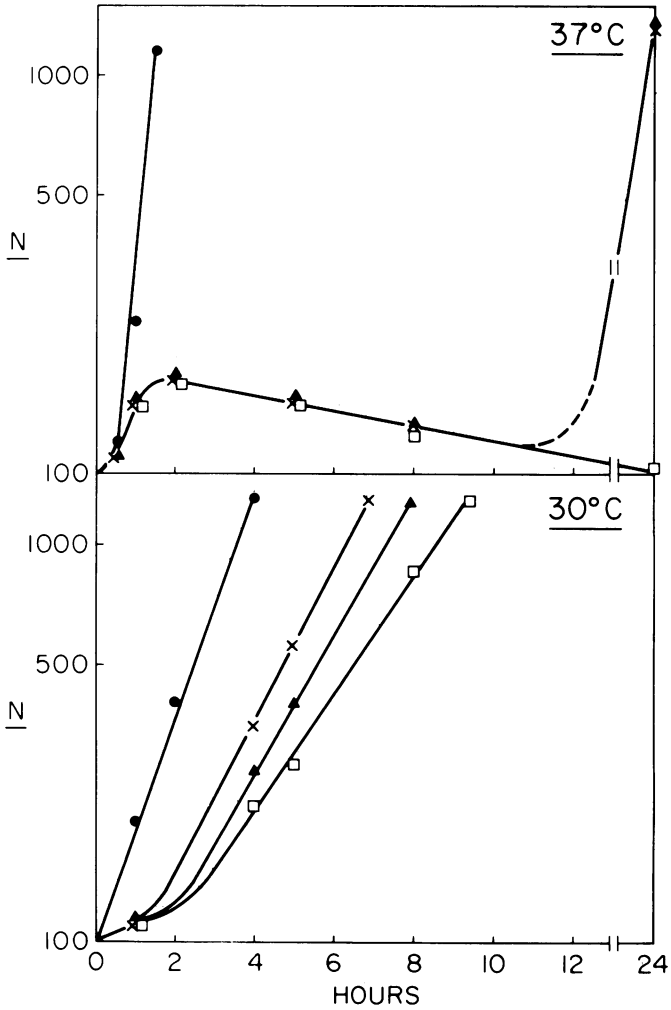

FIG. 4. Strain DU grown at 37 and 30°C in the presence of various concentrations of methicillin in liquid culture medium. Symbols: N, nephelos units (turbidity); ●, control (no drug); ×, 10 μg of methicillin per ml; ▲, 50 μg of methicillin per ml; □, 1,000 μg of methicillin per ml. The MIC for DU at 37 and 30°C was 625 and 1,250 μg/ml, respectively.

Effect of methicillin on the subpopulations of bacteria present in strain DU. Our results indicate that cultures of strain DU are composed of a majority of phenotypically methicillin-sensitive cells plus a small minority (10^{-5}) of resistant bacteria. These latter have been selected in the form of a homogeneous resistant population (HOM*). In the experiments described below, we tried to evaluate the methicillin susceptibilities of the phenotypically susceptible majority versus the resistant minority cells by comparing the response to methicillin of cultures of the thermosensitive

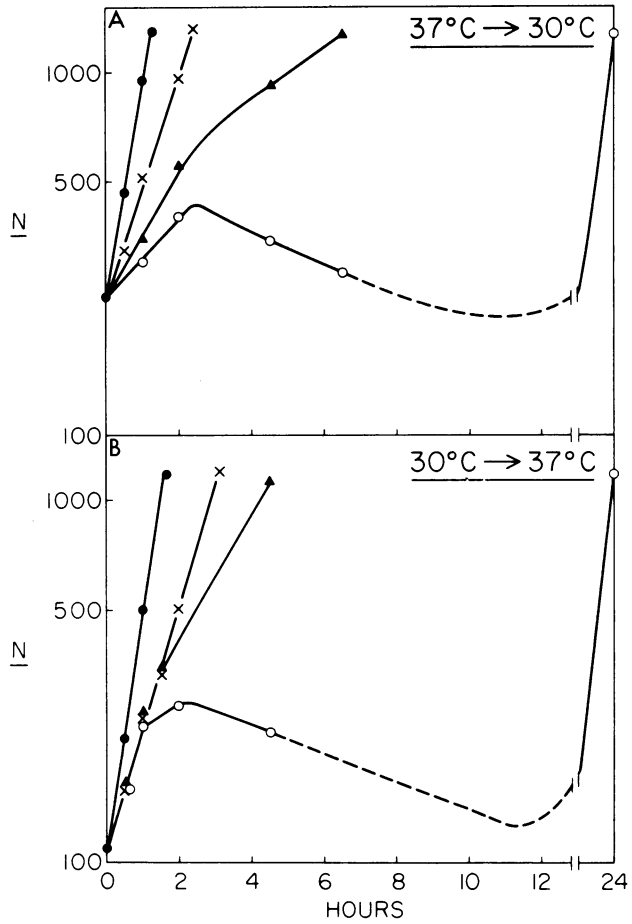

FIG. 5. Temperature shift experiments with strain DU from 37 to 30°C and from 30 to 37°C. In each case, strain DU was grown to early log phase at the first temperature and then shifted to the second temperature with or without methicillin at time zero. (A) One drug-free (control) culture was kept at 37°C (●), while another one was shifted to 30°C (×). (B) One control was kept at 30°C (×), while another was shifted to 37°C (●). Symbols: N, Nephelos units (turbidity); ●, control culture at 37°C; ×, control culture at 30°C; ○, grown in the presence of 50 μg of methicillin per ml at 37°C; ▲, grown in the presence of 50 μg of methicillin per ml at 30°C.

heterogeneous strain DU4 (strain DU cured of the penicillinase plasmid) and the derived HOM* strain (DU4*). Methicillin (50 μg/ml) was added to cultures of DU4 and DU4* growing in TSB at 30°C (Fig. 6). At this temperature both cultures had the methicillin-resistant phenotype, as indicated by continued growth (turbidity), increase in viable titer, and increase in the rates of peptidoglycan synthesis in both cultures, in spite of the presence of methicillin in the medium. At time zero of the experiment, the incubation temperature was shifted to 37°C, i.e., a temperature at which the culture DU4 (made up of a majority of thermosensitive, methicillin-susceptible cells) was expected to lose phenotypic resistance. Figure 6 shows that within about 30 min after the temperature shift, the increase in the turbidity and viable titer of the DU4 culture came to an abrupt halt, and the rate of peptidoglycan synthesis declined. On the other hand, the DU4* culture (representing a homogeneous highly methicillin-resistant cell population) was uninhibited after the temperature shift and continued to grow and synthesize peptidoglycan. Both cultures exhibited a similar initial acceleration in growth rate and cell wall synthesis after they were shifted to the higher temperature.

The experiments described contrast two sets of conditions that affect the heterogeneously resistant staphylococci in a qualitatively different manner. It is apparent that in the thermosensitive heterogeneous strain DU, cultivation at the lower temperature permits growth of all the bacteria. However, shift to the higher temperature results in the immediate loss of resistance, indicating that the lower temperature acted only as a protective environment in which phenotypic expression of the resistance trait was possible in all the bacteria. In contrast, exposure of strain DU to methicillin at 37°C results in a qualitatively different effect: the killing of the susceptible cells (the majority) and the selection of the resistant population (the minority) in the form of the unstable HOM* strain. Table 2 summarizes some experimental results that illustrate this point.

Cultures of DU grown for a prolonged time at 30°C in the presence of 50 μg of methicillin per ml did not give rise to

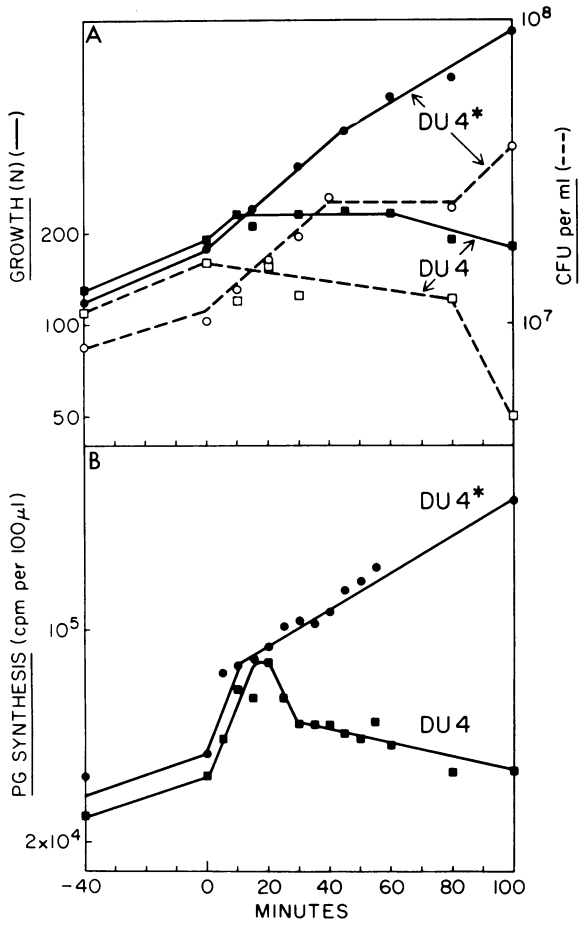

FIG. 6. Turbidity, viability, and peptidoglycan synthesis for a heterogeneous MRSA (strain DU4) and its homogeneously resistant subpopulation (DU4*). Cultures were initially grown at 30°C. Methicillin (50 μg/ml) was added at −40 min, and the temperature was shifted to 37°C at 0 min. (A) Symbols: ■, DU4 growth curve in nephelos (N) units; □, DU4 viability curve; ●, DU4* growth curve in nephelos units; ○, DU4* viability curve. (B) Peptidoglycan (PG) synthesis using 5-min pulse labeling of growing cells with *N*-acetyl-D-[1-^{3}H]glucosamine. Symbols: ■, incorporation of label into total PG in DU4; ●, incorporation of label into total PG in DU4*.

TABLE 2. Growth and plating conditions that affect EOP

Strain	Growth condition	Plating condition		EOP %
		Temp (°C)	NaCl	
DU	TSB, 37°C	37	−	0.01
		37	+	<0.01
		30	−	100.0
		30	+	100.0
	TSB-methicillin, 30°C[a]	37	−	0.12
	TSB-methicillin, 37°C	37	−	100.0
AC	TSB, 37°C	37	−	0.05
		37	+	0.1
		30	−	0.7
		30	+	100.0
	TSB-salt-methicillin, 30°C[b]	37	−	0.02
	TSB-methicillin, 37°C	37	−	100.0

[a] Methicillin, 50 μg/ml.
[b] Salt, 3% NaCl.

transiently homogeneous strains analogous to the HOM* strains. After growth of strain DU at 30°C in TSB containing 50 μg of methicillin per ml for as long as 16 generations, the EOP at 37°C (0.12%) remained close to the original value (0.1%). The low temperature of cultivation, while necessary for the phenotypic expression of resistance, nevertheless did not provide the conditions needed for the selection of HOM* strains. In contrast, exposure of cultures of DU to 50 μg of methicillin per ml at 37°C resulted in the eventual selection of the relatively unstable HOM* strains. Similar results were obtained with another heterogeneous strain (AC), but this strain required both low temperatures and the presence of salt for full expression of resistance in the majority of cells. Again, salt and methicillin at 30°C did not result in selection of a HOM* strain, whereas growth in the presence of methicillin at 37°C did result in the selection of a HOM* strain (Table 2).

PBPs in heterogeneous and thermosensitive heterogeneous strains. Several heterogeneous strains (AC, DU4, K1279, 13137, 67) were analyzed for their PBP patterns. In each case, labeling of both live cells as well as membrane preparations showed the presence of a PBP pattern typical of the homogeneously MRSA strains. A characteristic feature of the latter is the presence of an extra 78-kilodalton PBP running slightly ahead of PBP 2, a major binding protein in both susceptible and resistant staphylococci (14). Surprisingly, PBP 2a was detectable in heterogeneous strains in spite of the fact that under the conditions of cultivation only a small fraction of the bacteria present had the resistant phenotype. PBP 2a was present in detectable amounts in

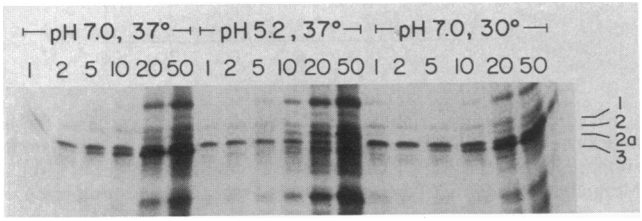

FIG. 7. Fluorograph of the PBPs of strain DU4. PBPs of strain DU4 at 37°C and pH 7.0, 37°C and pH 5.2, and 30°C and pH 7.0 in a direct titration procedure with increasing concentrations of ^{3}H-labeled benzylpenicillin, from 1 to 50 μg/ml, as indicated over each lane. Numbers to the left of the fluorograph are PBP designations.

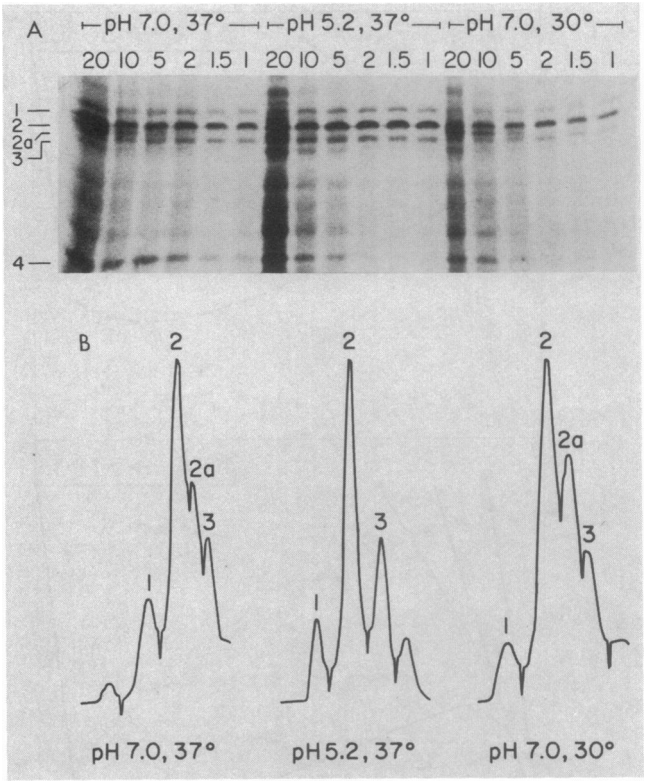

FIG. 8. Fluorograph and densitometer tracing of the PBPs of strain AC. (A) PBPs of strain AC at 37°C and pH 7.0, 37°C and pH 5.2, and 30°C and pH 7.0 in a direct titration procedure with increasing concentrations of ^{3}H-labeled benzylpenicillin, from 1 to 20 μg/ml, as indicated over each lane. (B) Densitometer tracings of the PBPs under the various growth conditions showing relative amounts of PBPs 1, 2, 2a, and 3. The appearance of a number of extra radioactive bands at high [^{3}H]penicillin concentrations makes the identification of PBPs in these lanes difficult. PBPs 1, 2, and 3 can be identified in the lanes with 1 through 5 μg of [^{3}H]penicillin per ml.

cultures of strain DU4 grown either at 30°C (at which most cells are phenotypically resistant) or at 37°C (at which only 1 in 10^4 cells is resistant) (Fig. 7).

Figure 8 shows a fluorogram of the PBPs of strain AC grown at 37 or 30°C. The densitometric tracing in Fig. 8 indicates that roughly equal amounts of PBP 2a were present per unit of total protein in the bacteria at either temperature of cultivation. The PBP 2a visible in the fluorograms of strains DU and AC was similar to the one seen in homogeneously resistant cultures by an additional criterion: it was not detectable in cultures grown at pH 5.2.

DISCUSSION

MRSA have become a major cause of nosocomial disease in numerous locations around the world (12, 18, 19). The resistance of these strains to high concentrations of beta-lactam antibiotics appears to be a stable property compatible with the presence of virulence traits in the bacteria, as indicated by the fact that MRSA now make up a significant portion of staphylococcal clinical isolates and these organisms are capable of causing serious human disease (20). The 21 isolates we examined in this report originated from sources in the United States, Europe, and Australia. Some of these strains (7 of 21) contained bacterial populations that were capable of expressing homogeneous methicillin resist-

ance. All of these homogeneous strains were from the United States. The majority of isolates, however, showed a peculiar property usually referred to in the literature as heterogeneity. In these isolates only a small fraction of all the bacteria present in a culture could actually grow when challenged with methicillin during growth at 37°C (22). In a major subgroup of the heterogeneous strains, heterogeneity was a thermosensitive property. The cells were heterogeneous at one temperature (37°C) and homogeneous, or close to homogeneous, at another (30°C). Studies in several laboratories, particularly that of Sabath (22), have clarified a number of the physiological characteristics of heterogeneously MRSA. Nevertheless, the mechanism of the phenomenon remains unknown.

The degree of heterogeneity of MRSA strains, as expressed quantitatively by the EOP assay, appeared to be a stable and characteristic feature of each strain. This was tested for strains AC and DU in the following manner. A loopful of bacteria was spread on the surface of TSB-agar without drug. Single colonies were picked at random and respread on fresh agar. This process was repeated five times. Finally, several single colonies were picked, dispersed in water, and diluted into tubes containing 1 ml of TSB in such a way that most tubes received less than one bacterium as an inoculum. On incubation of 36 such tubes at 37°C, 8 gave rise to turbid cultures. Each one of these cultures contained highly methicillin-resistant cells at frequencies (EOP) corresponding to that of the original heterogeneous culture (10^{-5} to 10^{-6}). It is not likely that such heterogeneous strains represent methicillin-susceptible staphylococci that have an unusually high intrinsic rate of mutation to methicillin resistance. In our hands, the spontaneous rate of mutation to high-level methicillin resistance in penicillin-susceptible *S. aureus* is less than 1 in 10^{10}. Alternatively, a more plausible explanation of heterogeneity is that all cells in the heterogeneous culture carry the genetic marker(s) of methicillin resistance, but for some reason the phenotypic expression of resistance only occurs in a very small fraction of the population. The existence of thermosensitive heterogeneous strains (e.g., strain DU used in our studies) strongly supports this interpretation, since all cells of these strains can express resistance at the lower temperature.

We interpret the presence of PBP 2a in the heterogeneous and thermosensitive heterogeneous cultures (grown at the higher temperature) as an indication that these strains not only carry the structural gene of PBP 2a but they also transcribe and translate this protein. Therefore, in the heterogeneous strains some factor(s) other than PBP 2a must control the expression of phenotypic antibiotic resistance. We refer to this hypothetical regulatory factor(s), for the sake of discussion, as factor X. In this interpretation the basic difference between homogeneous and heterogeneous strains would be the activity of this regulatory factor and thermosensitivity of resistance in thermosensitive heterogeneous strains (e.g., strain DU), or the combined thermosensitivity plus salt requirement for the homogeneous expression of resistance (e.g., strain AC) would be related to factor X and not to PBP 2a. Results summarized in Table 2 and Fig. 5 show that the low temperature (or presence of salt) was needed for the growth of the heterogeneous cultures in the presence of high concentrations of methicillin. On the other hand, even prolonged growth under these conditions in the presence of methicillin did not induce a permanent change in the majority cells from susceptibility to resistance since shift in the temperature from 30 to 37°C caused a rapid return of susceptibility to methicillin in the culture as a whole, as indicated by a halt in growth (Fig. 5), inhibition of cell wall synthesis, and loss of viability (Fig. 6). In addition, growth at 30°C in the presence of methicillin did not select for the highly resistant minority population. Such a selection required exposure of the heterogeneous culture to methicillin at 37°C, i.e., under the condition which led to the killing of susceptible bacteria (the majority) (Table 2). We did not see any dramatic difference in the amounts of PBP 2a detectable in such cultures grown at 30 or 37°C. In contrast, the loss of phenotypic resistance at low pH is a universal property of all MRSA (25), and it seems to be due to the lack of synthesis or instability of PBP 2a at the low pH (14).

The involvement of two distinct genetic loci in the expression of methicillin resistance has been reported previously (3). It is tempting to suggest that one of these loci represents the structural gene for PBP 2a, while the other regulatory locus may be involved with factor X. Expression of resistance may require a critical number of copies of PBP 2a per cell, and factor X may represent an aspect of the regulation of gene expression that limits the rate of transcription of the structural gene for PBP 2a. Production of PBP 2a was reported to be inducible by beta-lactam antibiotics in some heterogeneous strains, and this is compatible with the model described in this study (9). Alternatively, factor X may represent some difference between the homogeneous versus heterogeneous backgrounds of resistance with respect to the regulation of autolytic activity or wall synthesis. It is conceivable, for instance, that in the heterogeneous background partial inhibition of one of the normal, beta-lactam-sensitive PBPs invokes rapid triggering of some autolysin or other irreversible activity before the low-affinity PBP 2a can take over the synthesis of cell wall material. In the homogeneous background this coupling between PBPs and autolysis may be more sluggish.

Another possibility is that PBP 2a, a protein performing a necessary role in the cell wall synthesis of methicillin-resistant organisms, is present in each of the bacteria of heterogeneous strains in a form detectable by the PBP labeling technique but is enzymatically nonfunctional. One may postulate that some secondary modification is still needed before this protein becomes functional and provides the cells with a mechanism to grow in the presence of elevated levels of methicillin. In this model factor X may be a catalyst for such secondary modification. This may involve some conformational change or rearrangement within the topography of the plasma membrane (7). A possible analogy may be the previously reported presence of enzymatically inactive PBPs of *E. coli* which are, nevertheless, detectable by the PBP fluorographic method (26).

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LITERATURE CITED

1. **Annear, D. I.** 1968. The effect of temperature on resistance of *Staphylococcus aureus* to methicillin and some other antibiotics. Med. J. Aust. **1**:444–446.
2. **Barber, M.** 1964. Naturally occurring methicillin resistant staphylococci. J. Gen. Microbiol. **35**:183–190.
3. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. J. Bacteriol. **154**:479–487.
4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
5. **Bouanchaud, D. H., M. R. Scavizzi, and Y. A. Chabbert.** 1969.

Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. J. Gen. Microbiol. **54:** 417–425.

6. **Brown, D. F. J., and P. E. Reynolds.** 1980. Intrinsic resistance to β-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122:**275–278.
7. **Bruns, W., and H. Keppeler.** 1980. Mechanism of intrinsic penicillin resistance in *Staphylococcus aureus*. Arzneim. Forsch. Drug Res. **30:**1469–1475.
8. **Buchanan, C. E., and J. L. Strominger.** 1976. Altered penicillin binding components in penicillin-resistant mutants of *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **73:**1816–1820.
9. **Chambers, H. F., B. J. Hartman, and A. Tomasz.** 1985. Increased amounts of a novel penicillin binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. J. Clin. Invest. **76:**325–331.
10. **Dyke, K. G. H.** 1969. Penicillinase production and intrinsic resistance to penicillins in methicillin-resistant cultures of *Staphylococcus aureus*. J. Med. Microbiol. **2:**261–278.
11. **Fairbanks, G. T., T. L. Steck, and D. H. F. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10:**2606–2617.
12. **Haley, R. W., A. W. Hightower, R. F. Khabbaz, C. Thornsberry, W. J. Martone, J. R. Allen, and J. M. Hughes.** 1982. The emergence of methicillin-resistant *Staphylococcus aureus* infections in United States hospitals. Ann. Intern. Med. **97:**297–308.
13. **Hartman, B. J., and A. Tomasz.** 1981. Altered penicillin binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **19:**726–735.
14. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with β-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:**513–516.
15. **Hayes, M. V., N. A. C. Curtis, A. W. Wyke, and J. B. Ward.** 1981. Decreased affinity of a penicillin-binding protein for β-lactam antibiotics in a clinical isolate of *Staphylococcus aureus* resistant to methicillin. FEMS Microbiol. Lett. **10:**119–122.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
18. **Parker, M. T., and J. H. Hewett.** 1970. Methicillin resistance in *Staphylococcus aureus*. Lancet **i:**800–804.
19. **Pavillard, R., K. Harvey, D. Douglas, A. Hewstone, J. Andrew, B. Collopy, V. Asche, P. Carson, A. Davidson, G. Gilbert, J. Spicer, and F. Tosolini.** 1982. Epidemic of hospital-acquired infections due to methicillin-resistant *Staphylococcus aureus* in a major Victorian hospital. Med. J. Aust. **1:**451–454.
20. **Peacock, J. E., Jr., D. R. Moorman, R. P. Wenzel, and G. L. Mandell.** 1981. Methicillin-resistant *Staphylococcus aureus*: microbiologic characteristics, antimicrobial susceptibilities and assessment of virulence of an epidemic strain. J. Infect. Dis. **144:**575–582.
21. **Rosegay, A.** 1981. High specific activity (phenyl-^{3}H) benzylpenicillin N-ethylpiperidium salt. J. Labelled Compd. Radiopharm. **18:**1337–1340.
22. **Sabath, L. D.** 1977. Chemical and physical factors influencing methicillin resistance of *Staphylococcus aureus* and *Staphylococcus epidermis*. J. Antimicrob. Chemother. **3**(Supp. C):47–51.
23. **Sabath, L. D., and S. J. Wallace.** 1971. Factors influencing methicillin resistance in staphylococci. Ann. N.Y. Acad. Sci. **182:**258–266.
24. **Sabath, L. D., S. J. Wallace, K. Byers, and I. Toftegaard.** 1974. Resistance of *Staphylococcus aureus* to penicillins and cephalosporins: reversal of intrinsic resistance with some chelating agents. Ann. N.Y. Acad. Sci. **236:**435–443.
25. **Sabath, L. D., S. J. Wallace, and D. A. Gerstein.** 1972. Suppression of intrinsic resistance to methicillin and other penicillins in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **2:** 350–355.
26. **Spratt, B. G.** 1977. Properties of penicillin-binding proteins of *Escherichia coli* K-12. Eur. J. Biochem. **72:**341–352.
27. **Washington, J. A., II, and A. L. Barry.** 1974. Dilution test procedures, p. 410. *In* E. H. Lenette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
28. **Wong, W., A. N. Chatterjee, and F. E. Young.** 1978. Regulation of bacterial cell walls: correlation between autolytic activity and cell wall turnover in *Staphylococcus aureus*. J. Bacteriol. **134:**555–561.