Phenotypic Suppression of Methicillin Resistance in *Staphylococcus aureus* by Mutant Noninducible Penicillinase Plasmids

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Methicillin (intrinsic) resistance of *Staphylococcus aureus* was suppressed almost completely by regulatory gene (*penI*) mutations of penicillinase plasmids that made penicillinase production strictly noninducible. Methicillin resistance was restored by secondary regulatory gene mutations that altered the noninducible phenotype or by complementation with a compatible plasmid that did not bear the noninducible mutation. No evidence was obtained for genetic linkage between a penicillinase plasmid and the gene for methicillin resistance. We suggest, therefore, that the mutant noninducible repressor acted in *trans* by binding to a site on the methicillin resistance determinant. This hypothesis would imply an appreciable degree of homology between penicillinase plasmids and methicillin resistance genes.

Two types of resistance to penicillins occur in clinical isolates of *Staphylococcus aureus*. One type is caused by formation of penicillinase (β-lactamase, EC 3.5.2.6) which catalyzes the hydrolysis of benzylpenicillin and many of its congeners. Some semisynthetic penicillins, exemplified by methicillin, and the cephalosporins are refractory to staphylococcal penicillinase and therefore retain their effectiveness against penicillinase-producing *S. aureus*. A second type of resistance, usually designated methicillin resistance or intrinsic resistance, is effective against all penicillins, including the penicillinase-resistant derivatives, and the cephalosporins (3, 6, 13, 14, 33). The mechanism of this type of resistance is as yet undetermined. Staphylococcal penicillinase inactivates methicillin slowly, but methicillin-resistant strains are not more active in this respect than many methicillin-susceptible strains (2, 11, 12, 17). Furthermore, methicillin-resistant staphylococci initiate growth in broth containing methicillin before there is appreciable inactivation of the antibiotic (4, 33). Elimination of the gene for penicillinase production from methicillin-resistant strains abolishes penicillinase formation, but methicillin resistance persists (7, 12, 13, 31, 32). Thus, there is no evidence that staphylococcal resistance to methicillin depends upon the action of a penicillinase or any other method of inactivation of the antibiotic (30).

A relation between the genetic determinants for these two types of resistance has not been clearly established. The determinant for penicillinase is often borne on a plasmid that carries, among other markers, determinants for resistance to several inorganic ions (19, 23). Penicillinase formation is inducible and is regulated negatively by a cytoplasmic repressor, the product of a regulatory gene (*penI*) closely linked to the structural gene for the enzyme (21). A similarly regulated penicillinase linkage group may occupy a chromosomal location without any other associated markers (1, 26). The genetic locus of methicillin resistance is still uncertain. Transduction experiments with our strains have shown no linkage of this determinant with penicillinase plasmids (8). However, other workers, using different strains, have reported some co-transduction of methicillin resistance and penicillinase plasmids and have suggested that methicillin resistance may be plasmid-mediated (9, 10). In any case, methicillin resistance is not functionally dependent on penicillinase formation since, as we have noted above and have con-

1 A preliminary report was presented at the 72nd Annual Meeting of American Society for Microbiology, 23-28 April 1972, Philadelphia, Pa. (Abstracts, p. 165.)
firmed in independent experiments, resistance is expressed in cells from which the penicillinase plasmid has been eliminated. However, despite the apparent genetic independence of these two types of resistance, in our strains at least, we have found evidence for functional interaction between a class of mutant penicillinase plasmids and methicillin resistance. In this paper, we report experiments showing that methicillin resistance is phenotypically suppressed in staphylococci that harbor a mutant noninducible penicillinase plasmid.

**MATERIALS AND METHODS**

**Organisms.** The nomenclature and the designations of most of the organisms employed have been described (8, 25). The methicillin-resistant S. aureus strain Dumas, originally described by Dornbusch et al. (10), was obtained from S. J. Seligman. In transduction experiments, the donor of methicillin resistance was strain C5, originally isolated as strain Vililazu (8). The usual recipient was NCTC 8325, bearing an appropriate transduced penicillinase plasmid. Strain 8325 was susceptible to all antibiotics. Penicillinase plasmids are designated by the prefix P, followed by the Roman numeral I or II to designate the compatibility group and then a subscript to indicate the strain in which the plasmid was first detected, e.g., PII, (25). The compatibility types of plasmids PII, PII, and PII, are taken from Peyru et al. (25). We determined that the plasmid native to strain C5 was type I by preparing stable heterodiploids with it and PII.

**Transduction.** The transductional vectors were either phage 80, propagated by the soft agar method, or the temperate phage PII, liberated by strain 8325 after induction by ultraviolet irradiation (20). The procedures for transduction of methicillin resistance and other markers have been described (8).

**Susceptibility tests to antibiotics and Cd**

The disc diffusion method with paper discs containing the antibiotic and an inoculum swabbed from a turbid broth culture on plates of heart infusion (HI) agar (Difco) was used for qualitative assessment. Incubation was at 30 C for 24 hr. The following antibiotic discs were used: methicillin (5 µg), oxacillin (1 µg), cephalothin (30 µg), benzylpenicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), or streptomycin (10 µg). Methicillin-resistant strains gave no zone of inhibition by this method with any of the aforementioned penicillins or with cephalothin. Susceptible strains, e.g., strain 8325, gave large zones of inhibition. A quantitative assessment of antibiotic resistance was obtained by measuring the number of colonies formed by a small inoculum, approximately 50 to 100 colony-forming units, on brain heart infusion (BHI) agar (Difco) plates containing serial twofold dilutions of antibiotic incubated at 32 C (8). The minimal inhibitory concentration (MIC) of methicillin was defined as the concentration that reduced colony formation to 50% of the control value. In the current series of experiments the MIC of methicillin for strain C5 was 200 µg/ml, slightly higher than that previously reported (141 µg/ml) (8).

Resistance to cadmium ion (cad) was detected by ability to grow on BHI agar plates containing 2.5 × 10⁻⁴ M Cd(NO₃)₂.

**Elimination of plasmids.** The procedure for elimination of plasmids has been described (8).

**Mutagen treatment.** For mutagenic treatment with ICR-191, strain C5 was grown in HI broth (Difco) to a density of 5 × 10⁶ colony-forming units. Two ml of a solution of ICR-191 was added to 2-ml samples of culture to give final concentrations of the mutagen ranging from 6 to 150 µg/ml. The cultures were incubated for 60 min at 37 C and centrifuged. The pellet was resuspended in 5 ml of broth, incubated overnight, plated on BHI agar, and replicated either to BHI-methicillin agar (12.5 µg/ml) or to penicillinase indicator plates. Colonies that appeared to be methicillin-susceptible or noninducible for penicillinase were picked from the master plate for further testing.

The procedure for mutagenic treatment with ethylmethane sulfonate has been described (19). For detection of penicillinase regulatory mutations, treated cells were incubated for 3 hr in BHI broth and then plated on penicillinase indicator agar containing 0.5 µg of methicillin/ml. Presumably noninducible colonies were subcultured twice for purification and tested for methicillin resistance and penicillinase production. For detection of cadmium-susceptible mutants, the cells were plated on BHI agar containing 7.5 × 10⁻³ M sodium arsenate (to eliminate plasmid-loss mutants) and later replicated to BHI plates containing 2.5 × 10⁻⁴ M Cd(NO₃)₂. Colonies that failed to replicate were purified. The plasmid location of the cad mutation was confirmed by transduction to 8325.

**Penicillinase induction and assay.** Cultures for induction and assay were started by inoculating 10 ml of BHI broth with several colonies picked from a BHI plate. After 1.5 hr of incubation at 37 C, 10 ml of fresh broth was added. The culture was then divided into two flasks. A gratuitous inducer, 2,2'-carboxyphenyl)benzoyl-6-amino penicillanic acid (Imperial Chemical Industries, Ltd.), 7.5 µg/ml, was added to one flask (16). Incubation with shaking was continued for 4 hr. Samples (3 ml) were then taken for assay into tubes containing 0.3 mg of chloramphenicol in 0.3 ml of water.

Penicillinase content of whole cultures was assayed by an iodometric method (17). With strains producing small amounts of enzyme (<0.5 unit/mg) we used a micro method (18, 19). This method employs suspensions of staphylococci washed at the centrifuge and then resuspended in 0.9% NaCl to eliminate the relatively high nonspecific uptake of iodine by complex broth media. Therefore the extracellular penicillinase, which amounts to 10 to 20% of the total enzyme in wild-type PI plasmids, was excluded from the assay.

Penicillinase indicator plates were prepared as described by Novick and Richmond (22) with the addition of 0.5 µg of methicillin per ml to induce penicillinase formation. On this medium, wild-type
or constitutive penicillinase-producing colonies darkened promptly when flooded with 1% benzylpenicillin solution. Noninducible colonies gave no color with 1% penicillin but usually stained feebly with 10% penicillin solution. Penicillinase-negative colonies and most mutants that produced very small amounts of the enzyme ("micro" mutants [28]) gave no stain with either concentration of penicillin. Presumably noninducible colonies were purified by sub streaking. The penicillinase phenotype of each mutant was confirmed by quantitative assay.

RESULTS

Methicillin-susceptible mutants of strain C5. In a search for methicillin-susceptible mutants, we treated strain C5 with ICR-191. Five colonies were detected that failed to grow after replication to medium containing 12.5 \( \mu \text{g} \) of methicillin per ml. Two mutants, strains C5-75 and C5-100, obtained after treatment with 75 and 100 \( \mu \text{g} \) of ICR-191/ml, respectively, were examined further. Data will be presented from experiments with strain C5-75, although all essential points were confirmed with C5-100.

Table 1 indicates that strain C5-75 was much more susceptible to methicillin than its parental strain and was almost as susceptible as strain 8325, a penicillinase-negative, methicillin-susceptible strain. In disc susceptibility tests strain C5-75 gave zones of inhibition 15 mm in diameter with the methicillin disc, 32 mm with cephalothin, 12 mm with oxacillin, and an ill-defined zone of thin growth with benzylpenicillin. The parental C5 strain gave no zones of inhibition with any of these agents.

Strain C5-75 produced a nearly normal basal level of penicillinase, but, in contrast to its parental strain, gave no increase in enzyme when grown with inducer (Table 1). Noninducibility for penicillinase in C5-75 was a plasmid function for it was transduced with the plasmid. After its transduction into strain 8325 bearing a constitutive mutant of a chromosomal penicillinase determinant, \( \text{PL}_{C5-75} \) repressed penicillinase to wild-type levels. Thus, \( \text{PL}_{C5-75} \) determined a functional repressor. \( \text{PL}_{C5-75} \) was transduced by selection for cadmium resistance into methicillin-resistant strains. The penicillinase plasmids had first been eliminated from these recipient strains by treatment with ethidium bromide. Of 400 to 500 transductant colonies tested by replication to penicillinase indicator plates containing 0.5 \( \mu \text{g} \) of methicillin per ml, all had acquired noninducible penicillinase production. After replication to medium containing 12.5 \( \mu \text{g} \) of methicillin per ml, the transductants gave tiny colonies, obviously smaller than those produced by the similarly replicated penicillinase-negative recipient strain. The methicillin MIC values of representative transductant clones are given in Table 2.

Further evidence was obtained that the plasmid mutation in C5-75 suppressed methicillin resistance phenotypically. Phages grown on C5 and on C5-75 transduced methicillin resistance to strain 8325 (\( \text{PI}_{240} \)) at equal rates (2 \( \times 10^{-8} \) plaques-forming unit). Elimination of the plasmid from C5-75 by treatment with ethidium bromide restored methicillin resistance. The plasmid-cured host strain remained methicillin-resistant when \( \text{PL}_{C5} \) was transduced into it but acquired the susceptible phenotype coordinately with acquisition of \( \text{PI}_{C5-75} \).

Specificity of suppression of antibiotic resistance by the C5-75 plasmid. To determine the specificity of suppression of methi-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Penicillinase (units/ml)</th>
<th>Methicillin MIC (( \mu \text{g}/\text{ml} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{PL}_{C5} )</td>
<td>1.5</td>
<td>202</td>
</tr>
<tr>
<td>( \text{PL}_{C5-75} )</td>
<td>1.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* The minimal inhibitory concentration (MIC) of penicillinase-negative, methicillin-susceptible strains by this method was 2 \( \mu \text{g}/\text{ml} \).

The penicillinase plasmid was eliminated from C5 by growth with ethidium bromide.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Penicillinase (units/mg)</th>
<th>Methicillin MIC (( \mu \text{g}/\text{ml} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325*</td>
<td>( \text{PI}_{C5} )</td>
<td>2.1</td>
<td>240</td>
</tr>
<tr>
<td>8325*</td>
<td>( \text{PI}_{C5-75} )</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>8325*</td>
<td>( \text{PI}_{C5-75} )</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C6*</td>
<td>( \text{PI}_{C5} )</td>
<td>5.3</td>
<td>325</td>
</tr>
<tr>
<td>C6*</td>
<td>( \text{PI}_{C5-75} )</td>
<td>4.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Dumas*</td>
<td>( \text{PI}_{C5} )</td>
<td>2.5</td>
<td>250</td>
</tr>
<tr>
<td>Dumas*</td>
<td>( \text{PI}_{C5-75} )</td>
<td>2.8</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* This strain had acquired methicillin resistance by transduction from strain C5. It harbored a \( \text{PI}_{240} \) penicillinase plasmid that was eliminated prior to introduction of \( \text{PI}_{C5} \) or \( \text{PI}_{C5-75} \).

* These strains were natively methicillin-resistant. Their penicillinase plasmids were eliminated prior to introduction of \( \text{PI}_{C5} \) or \( \text{PI}_{C5-75} \).
cillin resistance by Plc5-75, we prepared a derivative of strain C5 bearing separate plasmid determinants for resistance to tetracycline and chloramphenicol and chromosomal determinants for resistance to streptomycin and novobiocin. In this host strain, Plc5-75 suppressed only methicillin resistance (Table 3).

**Methicillin-resistant "revertants" of strain C5-75.** To test the stability of the mutation in strain C5-75, we plated 9 × 10⁶ colony-forming units of C5-75 at 30°C on BHI agar plates containing 12.5 μg of methicillin/ml. Under these conditions all cells of C5 formed colonies, but only 1/20,000 cells of C5-75 gave rise to colonies, which proved to be stably resistant on further subculture and testing. Table 4 shows that all resistant clones had an altered penicillinase phenotype owing to elimination of the plasmid or to secondary mutations involving the penicillinase plasmid. Of the plasmid mutants, over 80% were penI7 constitutive mutants. They produced large amounts of penicillinase without inducer but were repressed when transduced into cells containing Plc15, a wild-type compatible plasmid. The clones containing an apparently wild-type inducible plasmid were not investigated further. Nine clones had the properties of inducible "micro" mutants (28), for they produced minute amounts of penicillinase that were increased 50- to 150-fold by exposure to inducer. Penicillinase regulatory mutants of this phenotype were shown by Richmond to arise from a mutation at a site, penI4, very close to penI7. Thus, "reversion" of strain C5-75 to methicillin resistance was associated coordinately either with plasmid loss or with secondary regulatory mutations of the C5-75 plasmid that abolished its non-inducibility. Although the "micro" mutants were phenotypically methicillin-resistant by the disc test, one of them had a relatively low MIC (Table 4), appreciably lower than strain C5 even after elimination of Plc5.

The reciprocal experiment was also performed. About 150,000 colony-forming units were spread on penicillinase indicator plates containing 0.5 μg of methicillin per ml. Eight clones were identified that produced relatively large amounts of penicillinase. Of these, seven were constitutive and one was inducible. All were methicillin-resistant by the disc tests.

**Noninducible mutants of other plasmids.** The foregoing experiments suggested that the noninducible penicillinase repressor in strain C5-75 suppressed methicillin resistance. It was obviously desirable to determine whether other mutagens and other plasmids would produce mutations that suppressed methicillin resistance. After ethylmethane sulfonate treatment of strain C5(Plc15) and of methicillin-resistant transductants of strains 8325(Plc1524) and 8325(Plc154), we detected presumptively noninducible mutants on penicillinase indicator plates and assayed their penicillinase production and methicillin resistance. Mutants were obtained from each of two experiments with each strain, but no further precautions were taken to exclude sibs. Table 5 indicates that all mutants that were noninducible and produced 0.5 to 5.4 units of penicillinase per mg suppressed methicillin resistance. Each of these mutants gave a zone of inhibition (9 to 14 mm) in the methicillin disc susceptibility test. The mutations in 10 strains were shown to be plasmidic by transduction. One mutant of 8325(Plc15a) with a methicillin MIC of 3 to 6 μg/ml was exceptional in that it had the prop-

**Table 3. Specificity of suppression of antibiotic resistance by Plc5-75.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methicillin</td>
</tr>
<tr>
<td>C5 (Plc5)</td>
<td>200</td>
</tr>
<tr>
<td>C5 (Plc5)a</td>
<td>5</td>
</tr>
</tbody>
</table>

* Strain C5 was natively chromosomally resistant to streptomycin. A penicillinase plasmid-free derivative was spread on agar containing 5 μg of novobiocin/ml. A clone that grew on this plate was used as recipient for the successive transduction of plasmids mediating resistance to tetracycline and to chloramphenicol. The tetracycline resistance determinant originated in strain 55C1. It was transduced to 8325 and from it to C5. Tetracycline resistance was not eliminated from 8325 by ethidium bromide, but the frequency of its transduction was progressively reduced by increasing dosages of ultraviolet irradiation of its transducing phage (19). Strain 8325 bearing this determinant contained circular, covalently closed DNA molecules with a molecular weight of about 2.8 million (unpublished observations of P. Stiffler and H. M. Sweeney). The chloramphenicol resistance determinant was the C22.1 plasmid furnished by W. V. Shaw who obtained it from R.P. Novick (21).
penicillinase derivative cured basal g/ml. zone tests. susceptibility true to plasmid of strain test. produced with mutants zones gave after resistance treatment, nate 100 to 100 100-200 for wild-type strains.

This designates the ratio of induced penicillinase activity to the uninduced level. It was approximately 100 to 200 for wild-type strains.

These strains had lost cadmium resistance and were negative on penicillinase indicator plates.

Numbers in parentheses are numbers of "revertants" analyzed.

This designates the ratio of the induced penicillinase activity to the basal level. It was approximately 100 to 200 for wild-type plasmids.

The host strain was a methicillin-resistant transductant of strain 8325. Without any penicillinase plasmid this strain had a methicillin MIC of 400 μg/ml.

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Penicillirnase repressor in C5-75 suppressed methicillin resistance, we investigated the dominance relations of PIc5.75 in the presence of another compatible plasmid bearing a wild-type or mutant penI5 gene. We prepared cadmium-susceptible mutants of PIc5 and PIc5.75, and transduced them into a plasmid-cured derivative of C5. The cadmium-susceptible mutant of PIc5.75 still suppressed methicillin resistance in C5. We then transduced PII167 and its penI5- derivative into each of the foregoing strains by using selection for cadmium resistance. We confirmed that the progeny were stable plasmid heterodiploids by transduction to a penicillinase-negative recipient with the separate recovery of each plasmid phenotype. Table 6 shows that PIc5.75 cad1 repressed a penI5- mutant of PII167, as was expected. The heterodiploid strains containing PIc5.75 cad1 and either wild-type PII167 or its constitutive mutant were inducible for penicillinase and were resistant to methicillin, although to a somewhat lesser degree than the corresponding heterodiploid containing PIc5.

Thus, it appears that complementation of PIc5.75 cad1 by either a wild-type or a constitutive mutant PII167 concomitantly restored
penicillinase inducibility and methicillin resistance to the host strain.

Strain Dumas had an unusually high methicillin MIC that was reduced to 30 μg/ml by the elimination of its native plasmid and the introduction of PIc5-75 (Table 2). The residual resistance in this strain offered an opportunity to determine whether a dual set of plasmids noninducible for penicillinase would enhance suppression of methicillin resistance. Table 7 indicates that the combined presence of noninducible mutants of PIc5-75 and PIi14, had an additive effect on suppression of methicillin resistance.

Test for linkage between PIc5 and methicillin resistance. In view of the functional relation between the noninducible mutant plasmids and the expression of methicillin resistance, we repeated our earlier experiments for evidence of genetic linkage between these determinants. Phage 80 grown on strain C5 was used to transduce either methicillin resistance or PIc5 to a plasmid-free strain of 8325. This strain had previously borne PI124, and was an effective transductional recipient of methicillin resistance (8). Among 476 methicillin-resistant transductants, none replicated on plates containing 2.5 × 10^{-4} M Cd(NO₃)₂. Among more than 5,000 cadmium-resistant transductants, none replicated to plates containing 12.5 μg of methicillin/ml. Thus, these experiments failed to give any evidence of genetic linkage between PIc5 and the determinant for methicillin resistance.

**DISCUSSION**

Although mapping data are not available, the properties of the noninducible mutants, namely their lack of response to inducer and their spontaneous transition to constitutive or other regulatory mutants, leave little doubt that they arise from lesions in penI₁, the classical regulatory gene. Similar noninducible mutants were investigated by Richmond (29). He described their phenotypic repair in heterodiploid cells containing a noninducible plasmid and a second wild-type or constitutive plasmid. Since each plasmid determined a serologically distinct penicillinase, he was able to show that penicillinase production in these diploid cells was equally divided between the two plasmids. We assume, but have not proven, a similar equality of enzyme production upon induction of our heterodiploid strains.

Richmond recognized the similarity of the noninducible penicillinase plasmid mutants to the iₙ mutants of the lac operon in *Escherichia coli* (34). However, he suggested that the staphylococcal mutants might arise in a region different from that associated with constitutive penI₁⁻ mutants because the noninducible mutation was fully complemented by either a wild-type or a penI₁⁻ plasmid; that is, either heterodiploid had the inducible phenotype. By contrast, the lac iₙ mutants are partially or fully dominant even against an i⁻ gene in *trans* (5). The lac iₙ mutants give rise to lac⁺ "revertants" by secondary regulatory mutations, usually i⁻ or iₚ. The similarity with the restoration of methicillin resistance by the secondary regulatory mutations in the noninducible plasmids is apparent. Richmond suggested that the noninducible mutation might involve a "second regulatory region" which had in the normal state an inductive function, in contrast to the repressive function of the other region which gave rise to penI₁⁻ mutants. The products of the two regions presumably associated to form a multimeric regulatory molecule. An alternate hypothesis, based upon current knowledge that lac repressor is a tetramer composed of identical subunits, might be that the penI⁻ and the noninducible penicillinase mutations occurred in different sites of the same cistron and that the repair of the noninducible mutations in heterodiploids was an extreme example of intragenic complementation. Admittedly, complete phenotypic repair by intragenic complementation would be unusual. We have no additional evidence bearing on either model.

The product of the lac iₙ gene in *E. coli* is a repressor with reduced affinity for inducer and normal or increased affinity for the operator site (5, 15). Noninducibility is caused by the

**Table 7. Suppression of methicillin resistance by dual uninducible penicillinase plasmids**

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Penicillinase (units/mg)</th>
<th>Methicillin MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>PIc5-75 cadl</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td>PIi14 penI₂⁷</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>PIc5-75 cadl + PIi14, penI₂⁷</td>
<td>2.7</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*The host strain in each case was a plasmid-cured derivative of strain Dumas (Table 2).

*This mutant plasmid was obtained by ethylmethane sulfonate treatment of its parent strain 147(PIi14). The plasmid was transduced into strain Dumas containing PIc5-75 cadl by the temperate phage obtained by ultraviolet irradiation of its host strain.
failure of inducer to combine with the repressor and thereby mediate the normal allosteric reaction that releases the repressor from the operator. An analogous interpretation of our findings is that the mutant repressor, similarly refractory to inducer, binds to some genetic determinant of the methicillin resistance gene and thereby blocks its expression. Presumably the wild-type repressor also binds but is inactivated by the antibiotic necessarily used in tests for methicillin resistance. This hypothesis would imply considerable homology between the binding site on the methicillin resistance gene and the operator site on the penicillinase linkage group, and this in turn would suggest some evolutionary relation between the two resistance determinants. In addition, the repressibility of methicillin resistance implies that it may be mediated by an enzymatic mechanism, possibly related to penicillinase. If that is the case, the enzymatic reaction involved must be subtle for, as we have already indicated, methicillin-resistant staphylococci do not grossly inactivate the antibiotic.

One phenotypically "micro" mutant plasmid derived from PI14.75 and another from PI1255 suppressed methicillin resistance to a high degree. Therefore, suppression is not restricted entirely to repressors with the noninducible phenotype. It should be noted, however, that the "micro" mutant of PI14.75 probably was doubly mutant since it appeared as a partially resistant revertant of PI14.75, and the "micro" PI1255 could, conceivably, have also been a double mutant. Further studies are needed to determine whether such partially suppressing plasmids as these also bear a lesion in the region determining noninducibility for penicillinase.

If penicillinase plasmids established genetic linkage with methicillin resistance determinants present in the same host cell, then it is possible that the methicillin resistance determinant might come under the control of the penicillinase repressor as, for example, the lac operon may come under the control of the trp repressor in mutants of E. coli containing a fusion of the trp and lac operons (27). We have indicated that we found no genetic linkage between penicillinase and methicillin resistance genes. Dornbusch and colleagues, on the other hand, working with different strains and using a different transductional procedure found evidence of close linkage (9, 10). In their strain 4916 a penicillinase plasmid was regularly co-transduced with methicillin resistance (9). Furthermore, Dornbusch observed loss of methicillin resistance coordinately with loss of penicillinase plasmid markers in some strains after growth in acridines (9, 10). In comparable experiments with strain C5 we have not obtained convincing evidence of elimination of methicillin resistance by acriflavine or ethidium bromide. Furthermore, we have detected no co-transduction of a penicillinase plasmid and methicillin resistance. Thus, we have no supportive evidence for a hypothesis based upon genetic linkage of penicillinase plasmids and methicillin resistance determinants.

An additional argument against a hypothesis of suppression by direct linkage of mutant plasmids and a methicillin resistance gene is furnished by the experiments with plasmid heterodiploid cells. The gene dosage effect of dual noninducible plasmids and the reduced suppression in cells containing a noninducible and a wild-type plasmid are incompatible with a direct linkage hypothesis but are expected if suppression is mediated by a diffusible cytoplasmic repressor.

One might speculate that the noninducible mutation had its effect by allowing production of a critically small amount of penicillinase by its host. This hypothesis is not tenable since some "micro" mutants that permitted the induced production of amounts of penicillinase comparable to those in the noninducible mutants allowed substantial expression of methicillin resistance, as was also the case in cells devoid of penicillinase.

ACKNOWLEDGMENTS

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LITERATURE CITED