Stability of Penicillinase Plasmids in *Staphylococcus aureus*

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The isolation of mutants of *Staphylococcus aureus* that are affected in the stability of penicillinase plasmids is described. One mutation is plasmid borne and results in nonreplication of the plasmid at 42 C. A second type of mutation is host-borne and gives rise to instability of both mcr1 and mcr11 penicillinase plasmids but not a tetracycline-resistant plasmid.

The genes responsible for penicillinase production in *Staphylococcus aureus* are usually carried on an extrachromosomal piece of deoxyribonucleic acid (DNA)—the penicillinase plasmid (9). In addition to structural and control genes for penicillinase, some plasmids carry genes conferring resistance to a series of inorganic ions (13, 16) and in one case resistance to erythromycin (8).

Penicillinase plasmids can be divided into two groups, mcr1 and mcr11 (11), on the basis of compatibility. A possible molecular basis for such compatibility was first suggested by Jacob, Brenner, and Cuzin (7), who postulated that for each replicon in the bacterial cell there is a membrane attachment site with distributive and possibly replicative functions. The compatibility groups may reflect the existence of a discrete membrane attachment site for each group. Each plasmid would have a type-specific mcr1 or mcr11 region which could recognize this site. Incompatible plasmids would then be those competing for the same attachment site, whereas compatible plasmids would be maintained at different sites. One way of studying the attachment site hypothesis is to study mutants altered in plasmid replication or stability.

Mutants of *S. aureus* have been isolated in which only one of the two plasmid types is highly unstable (11, 15). By using one such mutant, Novick (11) was unable to separate the compatibility and stability functions by recombination. These results and those of Richmond (15) suggest that the regions governing stability and compatibility are very near to one another or are the same.

Novick (10) isolated 24 mutants that were temperature-sensitive for penicillinase production as a result of failure of plasmid replication at 42 C. Thirteen of these provided to be host-determined mutations and 11 were plasmid determined. By using the host-determined mutants, Novick further investigated the behavior of wild-type plasmids having the compatibility type opposite to that present in the original strains. Some of these mutants could not maintain plasmids of either compatibility group at 42 C, whereas in others plasmids of opposite mcr type were maintained at 42 C, but were less stable than in a wild-type host strain.

In *S. aureus* PS80, the penicillinase genes are chromosomal (1). This strain is capable of maintaining plasmids of either compatibility group, as are other strains. For these two reasons, we thought that this strain would be a good one in which to study the interaction of chromosomal and plasmid genes. As a preliminary to such an investigation, we studied the stability of a penicillinase plasmid in *S. aureus* PS80 in an attempt to clarify the molecular basis of this stability. Novick's work with temperature-sensitive mutants is confirmed with this strain; we also report the isolation of a host-borne mutation which renders members of both penicillinase compatibility groups unstable but which does not affect the stability of a plasmid conferring resistance to tetracycline.

**MATERIALS AND METHODS**

**Media.** The media used, CY and 0.3CY, were described previously (9). For liquid culture of organisms, Oxoid Tryptone Soy Broth (TSB) was used in all cases.

**Strains: nomenclature.** The convention of Peyru, Wexler, and Novick (14) has been used. The symbol penZ refers to the structural gene for penicillinase, and penT refers to the gene controlling inducible synthesis of penicillinase. *asa, cad,* and *mer* refer to the loci determining resistance to arsenate, cadmium, and mercury ions, respectively. *tet* and *ero* refer to loci determining resistance to the antibiotics tetracycline and erythromycin.

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Notations without parentheses after the strain designations refer to chromosomal genes; plasmid genes are enclosed in round parentheses.

Square parentheses are used to indicate that the genes enclosed within the parentheses have been deleted from the genome.

Wild-type strain PS80 (NCTC 9789) is the propagating strain for phage 80 of the International Phage Typing Set. It is an inducible penicillinase producer (1). The wild type harbors a mcrA plasmid, and its genotype is defined: PS80(P1₉₇₈₉) (see Table 1). When the strain is maintained on nutrient agar, a change occurs in its genetic constitution in that these distinct genetic elements become linked and cotransducible. The change is due to duplication of the penicillinase genes, one copy remaining on the chromosome and one becoming incorporated into the plasmid (3). The plasmid now carries the penI and penZ genes in addition to the ion-resistance genes and is the parent plasmid of mutants described in this paper, and is designated (P1₉₇₈₉ penI penZ).

Strain PS80 was derived by the growth of strain PS80(P1₉₇₈₉) at 43 C in TSB for 8 hr. [A property of wild-type penicillinase plasmids is that they are lost from the cell at low rates during growth at 43 C (2).] Cells were plated on CY agar, incubated at 37 C, and then replicated to CY agar containing 13.3 μg of cadmium acetate/ml. A cadmium-sensitive colony was selected, and this was also found to be sensitive to arsenate and mercury ions.

Strain PS80.d. was derived during the course of another series of experiments (Dyke, unpublished data). A (P1₉₇₈₉) plasmid was transduced into strain PS80(P1₉₇₈₉ penI penZ) by selecting on erythromycin. Twenty percent of transductants were resistant to cadmium and mercury salts and to erythromycin but produced no detectable penicillinase either in the presence or absence of inducer. One of these transductants was grown in CY medium at 42 C, and a cadmium-sensitive variant was obtained by replica plating. This variant is PS80.d.; it produces no penicillinase and is sensitive to cadmium and mercury salts and to erythromycin. We believe it arose by a process analogous to superinfection curing.

The mcrA plasmid used in these experiments was derived from S. aureus strain 147 (17). Phage 53 of the International Phage Typing Set was propagated in liquid medium on this strain and used to transduce cadmium resistance to strain PS80.d. One transductant was obtained and checked for compatibility with known mcrA plasmids to ensure that it was mcrA.

The strain of PS80 harboring the plasmid conferring resistance to tetracycline was obtained from E. H. Asheshov (1).

Strain 1054 is a derived penicillinase-negative variant of one of the "hospital staphylococci" referred to in a survey by Dyke and Richmond (5). Its phage typing pattern is: 80/6/7/42E/47/53/54/75/83A. Phages. Typing phage 80 (NCTC 9788) was used as the transducing phage in all experiments, apart from the transduction of the plasmid (P11₉₇₈₉) to PS80.d. described above. Phage was propagated in liquid medium on the donor strains (9).

**Mutagenesis.** Ethyl methane sulphonate (EMS) was used as mutagen. The method was that of Novick (9).

**Plate assays for penicillinase.** Two methods were used to detect the penicillinase activity of colonies on a plate. A modification of the starch-iodine technique of Perret was used most commonly (4). For the isolation of the temperature-sensitive and unstable mutants, however, the N-phenyl-1-naphthylamine-azo-O-carboxybenzene (PNCB) stain was used (12).

**Isolation of mutants.** Mutants temperature-sensitive for penicillinase production were isolated as follows: A culture of PS80.d.(P1₉₇₈₉ penI penZ) was treated with EMS and plated on 0.3CY to give about 250 colonies per plate. These were incubated at 30 C for about 24 hr, by which time the colonies were about 1 mm in diameter and then transferred to 42 C for 15 hr. Plates were stained with PNCB and inspected under a plate microscope. By following this procedure, colony centers will have grown at 30 C and the peripheral parts at 42 C; colonies that are temperature-sensitive for penicillinase production are those with dark (purple) centers, surrounded by light (orange) areas (Fig. 1). In this way large numbers of colonies could be very rapidly

**TABLE 1. Strains used and their nomenclature.**

<table>
<thead>
<tr>
<th>Full nomenclature of strain</th>
<th>Abbreviated nomenclature</th>
<th>Chromosomal genes</th>
<th>Plasmid genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS80</td>
<td></td>
<td>penI penZ</td>
<td>penI penZ</td>
</tr>
<tr>
<td>PS80(P1₉₇₈₉)</td>
<td></td>
<td>+ +</td>
<td>penI penZ</td>
</tr>
<tr>
<td>PS80(P1₉₇₈₉ penI penZ)</td>
<td></td>
<td>+ +</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>PS80[penI penZ]</td>
<td>PS80.d. (P1₉₇₈₉)</td>
<td>- -</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>PS80[penI penZ] (P1₉₇₈₉)</td>
<td>PS80.d. (P1₉₇₈₉ penI penZ)</td>
<td>- -</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>PS80[penI penZ](P1₉₇₈₉ penI penZ)</td>
<td></td>
<td>+ +</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>147(P1₉₇₈₉)</td>
<td></td>
<td>+ +</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>258(P1₉₇₈₉)</td>
<td></td>
<td>+ +</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>PS80(1₉₇₈₉)</td>
<td></td>
<td>+ +</td>
<td>+ + + + + +</td>
</tr>
</tbody>
</table>

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screened.

After screening approximately 10,000 colonies by this method, a colony temperature-sensitive for production of penicillinase was found. This is the temperature-sensitive mutant that was used in these studies.

The mutants unstable for both plasmid compatibility groups were also isolated from PS80.d(Pl*788 penI penZ). EMS-treated cells were plated on 0.3CY, incubated at 37°C, stained with PNCB, and inspected with a plate microscope. Highly sectored colonies were selected for further study.

Transduction experiments. These were carried out as described by Asheshov (1) with the difference that after incubation for 3 hr to allow phenotypic expression, cells were plated directly onto CY-agar containing 13.3 μg of cadmium acetate/ml. The plates were incubated at 30°C until cadmium-resistant transductants had grown, usually after about 40 hr.

Segregation experiments. For the temperature-sensitive mutant. Cells were grown in TSB at 30°C for 16 hr, inoculated into fresh TSB, and incubated at 30°C until in exponential phase (usually 2 hr). These cells were used to inoculate a flask of fresh, warm TSB at 42°C, to give a concentration of cells of 5 × 10⁶/ml. Samples were withdrawn from this flask at 20-min intervals; after appropriate dilution, they were spread on CY agar and incubated at 30°C for 24 hr. The extinction of all samples was measured. Plates were developed by the starch-iodine method, and the per cent of penicillinase-negative colonies was determined for each time point.

When growing in broth, staphylococci tend to form clumps. This tendency is least apparent during the exponential phase of growth, when inspection under a light microscope indicates that, in strain PS80 at least, cells are predominantly in ones, twos, and threes. The segregation kinetics of the temperature-sensitive mutant were determined during exponential growth; to minimize effects owing to clumping, cells were shaken vigorously before plating out. In addition, when scoring colonies after plating, those that were half (or more) penicillinase-negative were scored as negative.

For the unstable mutant. The inoculum was taken from cells grown in TSB at 30°C for 16 hr. Cells (5 × 10⁶) were inoculated into fresh TSB and incubated at 42°C for 8 hr. A sample of cells from the 16-hr inoculum was plated on CY agar and incubated for 16 hr at 37°C. After the cells had grown for 8 hr at 42°C, a further sample was plated on CY agar and incubated for 16 hr at 37°C. The colonies were stained by the starch-iodine method, and the proportion of penicillinase-negative colonies was determined.

No allowance was made for the clumping of the cells during growth, since the results for loss of plasmids due to this mutation are not absolute, but are a comparison of the number of penicillinase-negative colonies before the defined incubation, with the number of penicillinase-negatives after incubation.

For tetracycline plasmid. The same procedure was followed as for the segregation of penicillinase plasmids from the unstable mutant. After the plates had been incubated for 16 hr at 37°C, they were replicated to CY agar containing 5 μg of tetracycline/ml, and the tetracycline-sensitive colonies were counted.

Optical density readings. A Unicam SP600 spectrophotometer was used. Readings were taken at 675 nm in cells of 1-cm light path.

Irradiation of phage for penicillinase-negative mutants. Phage propagated on the appropriate donor was irradiated for 15 sec with an Hanovia Chromatolite ultraviolet lamp. The phage, suspended in one part TSB to two parts phage buffer (9), was held 12 cm from the source. The irradiated phage was used to transduce cadmium-resistance to PS80.d. Transductants were stained with starch-iodine, and a penicillinase-negative colony was selected.

RESULTS

Plasmid or host linkage of mutations. Of the functions necessary for replication of a plasmid, some are provided by the plasmid and some by the cell. To determine whether the temperature-sensitive and unstable mutations were host- or plasmid-linked, a plasmid from the mutated cells was transduced into PS80.d., and the stability of the plasmid was then determined. In addition, mutant host cells which lost their plasmid during growth at 42°C were selected and used as recipients for a stable, wild-type, mcr plasmid in a transduction. The stability of the mcr plasmid in these mutant hosts was then determined (Table 2).

The temperature-sensitive mutation was found to be plasmid-determined, and the mutation has been named seg-l. The full genotype is PS80.d(Pl*788 seg-l penI penZ). For simplification in this paper, we referred to this plasmid as the S1 plasmid.

The mutation leading to instability of the plasmid was host-determined, and the mutation
has been named seg-2. The full genotype is PS80.d.seg-2. For simplification, it is referred to as the S2 mutation.

Segregation kinetics for S1. Jacob et al. (7) found that a temperature-sensitive F-lac plasmid in *Escherichia coli* gives a dilution curve with a slope of −0.3 at the nonpermissive temperature. This particular negative slope is indicative of a situation in which plasmids do not replicate at the nonpermissive temperature, but survive, so that they are progressively diluted out of the culture. Slopes less steep than −0.3 indicate some replication at the temperature of the experiment, and steeper slopes indicate destruction of existing plasmids. A defect involving plasmid distribution but not replication would give a convex dilution curve.

The rate of loss of the S1 plasmid from PS80.d.(PI<sub>1050</sub> seg-2 pen<sub>1</sub> penZ) was determined (Fig. 2). The slope obtained indicates a temperature-sensitive replication function. However, it is less steep than −0.3 so there is some replication at 42 C. Hirota, Ryter, and Jacob (6) have shown that, at temperatures slightly below the nonpermissive temperature, the slope of the dilution curve for a temperature-sensitive F-lac in *E. coli* may be increased by small changes in temperature. We think it is probable that, at some temperature a little above 42 C, the S1 plasmid will cease to replicate.

Stability of mcr<sub>1</sub> and mcr<sub>11</sub> plasmids in S2. Inspection of colonies of the S2 mutant stained with PNCB indicated that mcr<sub>1</sub> and mcr<sub>11</sub> plasmids were lost from the cell at high frequency. The stability of mcr<sub>1</sub> and mcr<sub>11</sub> plasmids was then determined quantitatively (Table 3). Both mcr<sub>1</sub> and mcr<sub>11</sub> are unstable in S2, but there are minor quantitative differences in that mcr<sub>11</sub> plasmids are less affected by the S2-mutation than mcr<sub>1</sub> plasmids. This was also true of two subsequent S2-type mutants isolated by the selection for stability of mcr<sub>1</sub> plasmids.

Stability of the tetracycline plasmid is not de-

### Table 2. Plasmid or host location of mutations

<table>
<thead>
<tr>
<th>Transduction</th>
<th>Recipient strain</th>
<th>No. of transductants examined</th>
<th>Stability of plasmid in transductants</th>
<th>Location of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS80(PI&lt;sub&gt;1079&lt;/sub&gt; pen&lt;sub&gt;1&lt;/sub&gt; penZ)</td>
<td>Plasmid negative variant of the temperature-sensitive mutant PS80.d.</td>
<td>160</td>
<td>Stable</td>
<td>Plasmid</td>
</tr>
<tr>
<td>Temperature-sensitive mutant</td>
<td>1054</td>
<td>65</td>
<td>Temperature-sensitive</td>
<td>Plasmid</td>
</tr>
<tr>
<td>Temperature-sensitive mutant</td>
<td>9</td>
<td>Temperature-sensitive</td>
<td>Plasmid</td>
<td></td>
</tr>
<tr>
<td>PS80(PI&lt;sub&gt;1079&lt;/sub&gt; pen&lt;sub&gt;1&lt;/sub&gt; penZ)</td>
<td>Plasmid negative variant of the unstable mutant PS80.d.</td>
<td>135</td>
<td>Unstable</td>
<td>Host</td>
</tr>
<tr>
<td>Unstable mutant</td>
<td>62</td>
<td>Stable</td>
<td>Host</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2.** Segregation kinetics and growth curve for PS80.d.(PI<sub>1050</sub> seg-2 pen<sub>1</sub> penZ) at 42 C. Symbols: Δ, segregation kinetics of the control strain PS80.d.(PI<sub>1050</sub> penZ); •, segregation kinetics for PS80.d.(PI<sub>1050</sub> seg-2 pen<sub>1</sub> penZ); O, growth curve of the control strain PS80.d.(PI<sub>1050</sub> pen<sub>1</sub> penZ); @, growth curve for PS80.d.(PI<sub>1050</sub> seg-1 pen<sub>1</sub> penZ).
not been possible to attempt complementation between PS80.d.(P11768 seg-l penI penZ) and PS80(P11768 penI penZ) but we have constructed diploids in two other ways.

First, the fact that in wild-type PS80 the penicillinase genes are chromosomal and do not interfere with the replication and stability of a resident plasmid allows the construction of a stable partial diploid. Phage propagated on strain PS80.d.(P11768 seg-l penI penZ) was used to transduce cadmium resistance to PS80. All 68 transductants obtained were found to be temperature-sensitive. We conclude that there is no complementation of the S1 plasmid by the penI penZ region of the plasmid, at least when the latter is integrated into the chromosome.

Second, we have constructed plasmid diploids of the S1 plasmid with a plasmid of mcr1 type. Phage propagated on PS80(P11147) was irradiated and used to transduce cadmium resistance to strain PS80.d., and a penicillinase-negative transductant was isolated. A preparation of phage propagated on PS80.d.(P11768 seg-l penI penZ) was used to transduce penicillinase into this penicillinase-negative mutant. Transductants were selected on CY agar containing 0.2 μg of benzyl-penicillin/ml. All five transductants tested were temperature-sensitive. We conclude that there is no complementation of the temperature-sensitive defect in the S1 plasmid (mcr1 type) by proteins produced by a mcr1 plasmid.

**DISCUSSION**

The S1 plasmid has a mutation that affects its stability. Our results (Fig. 2) suggest that the mutation may affect the replication of the plasmid and, since this result in temperature-sensitivity, it is probable that a defective protein is produced at the restrictive temperature. An alternative is that the structure of the plasmid DNA itself is affected by the high temperature so that it is not attached correctly to the hypothetical membrane attachment site.

If a temperature-sensitive protein is produced, it should be possible to demonstrate complementation by a suitable non-mutant plasmid. Although neither we, nor Novick (10) in a more extensive study, were able to demonstrate complementation, the tests we used have drawbacks. In one case, we used strain PS80 with a chromosomal penI penZ region to provide the complementing proteins; however, it is not certain that this region includes DNA coding for the defective proteins in S1. Even if this DNA is present on the chromosome, it may very well not be expressed. The second complementation test employed a plasmid of different compatibility group, and therefore it was presumably different in at least one function of replication and maintenance for a mcr1 plasmid. It could be that the S1 plasmid is defective in this different function. An explanation for the lack of complementation apart from the deficiencies in our tests is that the S1 defect is in a protein that is not freely diffusible. We have no evidence on this possibility.

The S2 mutation is in a host-directed function that is necessary for replication or maintenance of mcr1 and mcr11 plasmids but not necessary for the replication and maintenance of a tetracycline plasmid. This mutation does not affect the growth rate of the host. These results suggest that the maintenance of penicillinase plasmids requires a component which is common to the two compatibility groups. But this component is not involved in the maintenance of all plasmids. One interpretation is that a particular species of membrane protein has been adopted as attachment site for penicillinase plasmids.

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**LITERATURE CITED**


