Regulation of Penicillinase Synthesis: a Mutation in *Staphylococcus aureus* Unlinked to the Penicillinase Plasmid That Reduces Penicillinase Inducibility

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A mutant of *Staphylococcus aureus* strain 655 was isolated that is restricted in penicillinase induction. Wild-type plasmids that bear penicillinase determinants could not be fully induced in this mutant, 655par-1; hence, the responsible mutation is not located on the plasmid. Mutant plasmid P1147, penI443, which produces penicillinase constitutively in wild-type cells, was fully constitutive for penicillinase production when it was harbored by mutant 655par-1. Therefore, the bacterial mutation does not interfere directly with the transcription of the penZ gene or translation of the penicillinase messenger ribonucleic acid. Mutant plasmid PII147, penI220 was fully inducible in the mutant bacterium, even though the wild-type plasmid PII147 was only partially inducible in the par-1 mutant. Thus, in the presence of inducer, complementation appears to occur between the product of the par-1 gene and the product of the penI220 gene. These results suggest that the par-1 gene codes for a penicillinase antirepressor.

In *Staphylococcus aureus*, the penicillinase structural gene (penZ) and the regulatory gene that specifies the cytoplasmic penicillinase repressor (penI) are usually borne on a plasmid along with a number of other genetic determinants. The penZ gene and the penI gene are closely linked (12, 17). Genetic analysis of the regulation of penicillinase synthesis has demonstrated two classes of mutants that are magnoconstitutive for penicillinase production. One group possesses a mutation in the penI gene (16); the second group, designated R2* by Cohen and Sweeney (3), contains a mutation unlinked to the penicillinase plasmid, and presumably is located on the bacterial chromosome. It has been postulated that the product of the unlinked regulatory gene assists in the inactivation of the cytoplasmic penicillinase repressor specified by the penI gene (6, 7). Several other classes of mutants in which the rate of penicillinase synthesis is altered have been isolated from *S. aureus*, and they appear to fall into three classes (designated according to the nomenclature proposed by Collins et al. (4a)). The first class are micro-inducible mutants which synthesize a normal amount of penicillinase protein with a low specific activity and are probably structural gene mutations (11). The second class consists of micro-inducible and micro-constitutive mutants which synthesize penicillinase indistinguishable from that of the wild type and carry an intact penI gene. Mapping experiments suggest that these mutations lie between the penI gene and the penZ gene, and it has been suggested that these are operator mutations (17). Mutants of the third class possess a mutation located on the plasmid which restores inducibility to a class of uninducible mutants. Richmond suggested that the product of this locus and that of the penI gene may cooperate in the regulation of penicillinase by forming a dimeric repressor (18).

During the course of our studies concerning the regulation of penicillinase formation, we sought to investigate the function of the unlinked regulatory gene and the role, if any, of the "bound" penicillin (6, 7). To this end, we isolated a series of mutants that were micro-inducible for penicillinase synthesis. Most mutants were of the two micro-inducible classes described above; in addition, we have
found a meso-inducible mutant in which the site of mutation is unlinked to the penicillinase plasmid. In this paper, we report the isolation and some of the properties of this mutant.

**MATERIALS AND METHODS**

**Strains.** The meso-inducible mutant 655par-1 was isolated from a novobiocin-resistant mutant of strain 655 (13). The abbreviated symbol par (7) designates the locus governing formation of the proposed penicillinase antirepressor. Strain 8325-C_{sdc}, which contains the chromosomal penicillinase linkage group found in strain 55C1, was kindly provided by Sidney Cohen. Strain 152 (13) was frequently used as a recipient in transduction experiments with strain 655.

**Plasmids.** Table 1 lists the derivation of plasmids, expressed in the nomenclature of Peyret et al. (15), used in this investigation. The plasmid present in wild-type strain 655 has been established as type I by a test-cross (15) with plasmid Pl_{1443} (penI443 cad-78). This type I plasmid displaced the resident plasmid in 655, indicating that both plasmids belong to the same maintenance-compatibility set, mercuric sulfide. The plasmid in strain 655 confers resistance to arsenate and cadmium inhibition of growth, as do the other plasmids employed in this study.

**Media.** P and D medium was prepared according to Pattee and Baldwin (13), and 0.3 Cy medium, according to Novick (11). Brain Heart Infusion (BHI) and Nutrient Broth are Difco products. Cell dilutions were performed in a buffered salts solution of the following composition (grams/liter): K_{2}HPO_{4}, 7.0; KH_{2}PO_{4}, 2.0; sodium citrate 5H_{2}O, 0.5; MgSO_{4}·7H_{2}O, 0.1; and (NH_{4})_{2}SO_{4}, 1.0, pH 7.0.

**Transduction.** Virulent phage were grown on the appropriate hosts by the soft-agar overlay method (2). The temperate phage were induced with ultraviolet light (11).

The transduction procedure was essentially that of Pattee and Baldwin (13) except for the addition of 10^{-3} M CaCl_{2} to the transduction mixture (1). Phage 80, 80α, or 83 was added at a multiplicity of about 1.0; induced plasmid from 8325-C_{sdc} was added at a multiplicity of about 0.1. Samples were propagated on BHI Agar plates which contained 0.15 units of benzylpenicillin per ml, 0.125 to 0.25 mM Cd(NO_{3})_{2}, or 10 mM Na_{2}HAsO_{4} with a BHI Agar overlay; the plates were incubated overnight at 37 C. Transductants were subcultured three times on selective media for purification.

**Assay.** Strains which were to be assayed for penicillinase activity were grown overnight on a BHI Agar slant [0.25 mM Cd(NO_{3})_{2} added if the strain contained a plasmid]. Cells obtained from the slant were used to inoculate P and D medium, and were allowed to grow to exponential phase at 37 C. Cell growth was measured with a Zeiss spectrophotometer at a wavelength of 535 nm. The culture was diluted with P and D medium to an absorbancy of 0.06 and divided into two flasks, one of which contained the gratuitous inducer 2(2-carboxyphenyl) benzoyl-6-aminopenicillanic acid (CBAP; kindly donated by Imperial Industries, Ltd.) at 7.25 μM (8).

After the cultures had grown at 37 C for four generations, samples for penicillinase activity were transferred to polyethylene tubes containing chloramphenicol (200 μg/ml final concentration) and maintained in ice until assayed. Penicillinase activity was assayed by the standard Perret procedure (14). A unit of penicillinase is defined as the amount of enzyme activity required to hydrolyze 1 μmole of benzylpenicillin per hr at pH 6.4 and 30 C. Specific activity is defined as units of penicillinase per milligram (dry weight) of cells (1 ml of a culture with an absorbancy of 1.0 at 535 nm contains 0.28 mg (dry weight) of cells).

Penicillinase phenotypes of colonies grown on unbuffered 0.3 Cy agar plates with or without 7.25 μM CBAP were tested by the N-phenyl-1-naphthylamine-α,α,α-trihydroxybenzene (PNCB) method of Novick and Richmond (12).

**Nitrosoguanidine mutagenesis.** Cells from an 18-hr BHI Agar slant were washed in citrate buffer (0.03 M sodium citrate, pH 5.0) and resuspended in citrate buffer containing 200 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml (Aldrich Chemical Co., Milwaukee, Wis.). The mixture was incubated at 37 C for 1 hr with gentle shaking; then the cells were collected, washed, and resuspended in cold P and D medium.

Penicillinase-negative or plasmid-negative derivatives. Plasmid-negative derivatives were obtained by growing the parental strain at 42 C (10). Loss of the penicillinase plasmid was confirmed by assaying dense cell suspensions for penicillinase activity by the Perret procedure and by the simultaneous loss of resistance to Na_{2}HAsO_{4} and Cd(NO_{3})_{2}.

**RESULTS**

**Mutant isolation.** After treatment with nitrosoguanidine as described in Materials and Methods, the cultures were diluted and plated on unbuffered 0.3 Cy medium containing 7.25 μM CBAP. Those colonies which did not show an induced level of penicillinase with the PNCB color-indicator method were purified by

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Derivation</th>
<th>Source</th>
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<tbody>
<tr>
<td>Pl_{443}</td>
<td>Naturally occurring</td>
<td>P. A. Pattee</td>
</tr>
<tr>
<td>Pl_{1443}</td>
<td>Naturally occurring</td>
<td>R. P. Novick</td>
</tr>
<tr>
<td>PenI443</td>
<td>Penicillinase constitutive repressor mutation</td>
<td>R. P. Novick</td>
</tr>
<tr>
<td>P_{54s}</td>
<td>Naturally occurring</td>
<td>S. Cohen</td>
</tr>
<tr>
<td>P_{147}</td>
<td>Naturally occurring</td>
<td>R. P. Novick</td>
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<tr>
<td>PenI220</td>
<td>Penicillinase repressor mutation</td>
<td>R. P. Novick</td>
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*Pl_{1443}, Pl_{54s}, and Pl_{147}, were previously designated γ, α, and β, respectively (15).*
three successive subcultures of single colonies at 37 C. In one experiment, 16 micro-inducible or micro-constitutive mutants and 15 plasmid-negative isolates were found among 32,000 colonies examined. In the second experiment, 47 micro-inducible or micro-constitutive mutants and 48 plasmid-negative isolates were found among 60,000 colonies examined. Induced and uninduced levels of penicillinase activity produced by each mutant were assayed quantitatively (14). Lysates of these mutants were transduced into strain 152. Transductants were selected by their resistance to arsenate and then replicated onto BHI Agar plates which contained 1 lµg of penicillin per ml. Transductant colonies were subcultured three times for purification, and were then assayed quantitatively (14) for uninduced and induced levels of penicillinase activity. The lysate from one mutant, designated 655par-1, transduced a wild-type plasmid into strain 152. In all other cases, micro-constitutive or micro-inducible levels of penicillinase activity were detected in the transductants, indicating that the mutation was located on the plasmid. The mutation in 655par-1 had a pleiotropic effect, since the doubling time of the mutant in P and D medium was 60 min whereas the wild-type strain, 655, had a doubling time of 36 min. Also, mutant 655par-1 was slightly more sensitive to Cd(NO₃)₂ than the wild-type strain, 655; hence, the concentration of Cd(NO₃)₂ in the selective medium was reduced to 0.125 mM when selecting for crosses with strain 655par-1.

Expression of PI plasmids in mutant 655par-1. PI plasmids were transduced into plasmid-negative strains of 655 and 655par-1. The basal level of penicillinase produced by wild-type PI plasmids borne by strain 655par-1 was approximately 30% of that produced by plasmids of the same genotype harbored by the parent strain 655 (Table 2). Similarly, the induced level of penicillinase produced by wild-type PI plasmids borne by strain 655par-1 was approximately 15 to 20% of that produced by the wild-type PI plasmids borne by the parental strain (Table 2). However, when the penicillinase-constitutive mutant plasmid PI₃₅₈ penI443 was borne by strain 655par-1, the uninduced and induced levels of penicillinase activities differed little from 655(PI₃₅₈ penI443) in that penicillinase synthesis was fully constitutive (Table 2). These results show that the mutation in strain 655par-1 does not directly impede the transcription of the penZ gene nor the translation of penZ gene messenger ribonucleic acid (RNA).

Expression of PII plasmids in mutant 655par-1. The expression of the penicillinase group on the wild-type plasmid PII₁₄₇ was also altered by the mutation in strain 655par-1 (Table 3). As in the case of type I plasmids, both the basal and induced levels of penicillinase synthesis directed by plasmid PII₁₄₇ were lower in mutant 655par-1 than in the parental strain.

An unexpected result was obtained when the mutant plasmid PII₁₄₇ penI220 was transduced into strains 655 and 655par-1 (Table 3). This plasmid is reported to contain a mutation in the pen₁ gene, which codes for the cytoplasmic penicillinase repressor, and has been described as a penicillinase-constitutive mutant plasmid (16). However, the mutation must be a missense mutation because, even though the uninduced enzyme activity for this plasmid was four times higher than for the wild-type plasmid, it could still be induced. This observation strongly suggests that the penI220 mutation results in a partially active repressor. Not only was the mutant plasmid inducible in strain 655, it was also inducible in the mutant strain 655par-1. It should be noted that this was the only penicillinase operon which was essentially fully inducible in strain 655par-1.

All wild-type penicillinase operons examined, whether on a PI or PII plasmid or on the chromosome, were meso-inducible in strain 655par-1. The fact that the mutant plasmid, which produces an altered penicillinase repressor, could be induced to a higher level, and that the strain 655par-1 than could the wild-type plasmid, suggests that the products of the two mutant genes, par₁ and penI220, may interact. This, in turn, suggests that the product of the gene which bears the par₁ mutation interacts directly with the penicillinase repressor.

Chromosomal penicillinase linkage group in par₁. A penicillinase linkage group (C₁₅₁₀) that becomes integrated into the bacterial chromosome was transduced into strains 655 and 655par-1. It has been reported that this linkage group when integrated is not derepressed spontaneously by a certain class of R⁻ mutants (4). However, as in the case of wild-type plasmids (Tables 2 and 3), both the basal level and induced level of penicillinase produced by the chromosomal linkage group were reduced by the par₁ mutation (Table 3). Thus, the position effect observed for the R⁻ mutants (4) could not be demonstrated for the par₁ mutation.
Qualitative properties of penicillinase repressor. The inducibility of plasmid PII147 pen1220 in mutant 655par-1 (see Table 3) suggests the possibility that the repressor produced by plasmid PII147 may differ qualitatively or quantitatively from the repressor produced by type I plasmids. Therefore, the kinetics of penicillinase induction and de-induction in strains 655(PI258) and 655(PII147) were compared. As shown in Fig. 1, the time lapse between the addition of inducer and the achievement of the maximal rate of penicillinase synthesis was approximately 17 min for strain 655(PI258) and approximately 24 min for strain 655(PII147). Conversely, the kinetics of deinduction produced upon the removal of exogenous inducer from the two strains (Fig. 2) show that strain 655(PII147) de-induces at a faster rate than does strain 655(PI258). These results suggest that more molecules of penicillinase repressor are synthesized by plasmid PII147 than by plasmid PI258. This, in turn, suggests that the promoters or operators that control the expression of these genes must differ.

**DISCUSSION**

Cohen and collaborators have described a locus or loci (R2) unlinked to the penicillinase plasmid which, in the mutant state at least, participates in the regulation of staphylococcal penicillinase synthesis. The precise function of this locus, or loci, is unknown; however, a mutation in this locus renders many wild-type PI plasmids phenotypically constitutive for penicillinase synthesis. It has been proposed that the locus codes for a penicillinase antirepressor substance (6), but this notion was questioned (4). Our results with mutant 655par-1 confirm the existence of such a locus...
or loci and provide further evidence that the product of this locus is a substance that functions as a penicillinase antirepressor. However, it has not been established, as yet, whether the par-I mutation and the R2- mutations are in the same gene or in different genes.

If the par-I mutation and the R2- mutations are in the locus which codes for a penicillinase antirepressor (6, 7), there are two central questions that must be answered: (i) why are penZ genes that are integrated into the host chromosome less susceptible to derepression by the R2- mutations than penZ genes that are carried by a plasmid (4), and (ii) why are PI plasmids but not PII plasmids phenotypically constitutive in some of the R2- hosts (4). A plausible explanation for the difference in the expression of the chromosomal and plasmid penicillinase genes in the R2- mutants is that the penI gene when located on the chromosome is under the control of a different promoter than when it is located on the plasmid. As a result of this position effect, transcription of the penI gene proceeds at different frequencies, depending upon the relative efficiency of the promoters that govern the frequency of its transcription. In turn, the expression of the penZ gene is regulated by the availability of the penicillinase repressor. Likewise, the difference in the genetic response between PI and PII plasmids in an R2- host can be explained in these terms. That is to say, data presented in Fig. 1 and 2 suggest that more penicillinase repressor is synthesized by a PII147 plasmid than by a PII148 plasmid. This observation indicates that the promoter regions that control the transcription of the penI genes in the two types of plasmids are indeed different. The additional repressor that apparently accumulates in a cell which contains a PII147 plasmid may act to prevent derepression of the penZ gene by the R2- mutation. Since the penicillinases specified by the penZ genes of PI plasmids and PII plasmids are known to differ (15, 16), it is likely that the penicillinase repressors (i.e., the products of the penI genes) coded by the two types of plasmids also differ. Hence, there may be both qualitative and quantitative differences in the penicillinase repressors produced by the two types of plasmids. Thus, the two questions stated above appear to be readily resolved.

As shown in Tables 2 and 3, all of the wild-type plasmids that we have examined are meso-inducible for penicillinase when carried by mutant 655par-1. However, when the mutant bacterium 655par-1 harbors the mutant plasmid PII148, penI443, penicillinase synthesis is fully constitutive. These results show that the mutation in strain 655par-1 neither interferes directly with the transcription of the penZ gene nor with the translation of the penZ gene messenger RNA. It can be concluded, therefore, that the mutation in strain 655par-1 hinders the normal inactivation of wild-type penicillinase repressor, the synthesis of which is coded by the penI gene. In addition, mutant plasmid PII147, penI220 is fully inducible in the bacterial mutant 655par-1 even though the wild-type plasmid PII147 is only partially inducible in the par-1 mutant (see Table 3). These latter observations suggest that in the presence of inducer complementation occurs between the products of the par-1 and penI220 mutant genes. Such complementation suggests that the par gene codes for a penicillinase antirepressor that can interact with the product of the penI gene, thus permitting expression of the penZ gene. A working model for the regu-

![FIG. 2. Penicillinase deinduction kinetics. Cells were grown as described in Fig. 1. When exponential growth had established, 5 μM Fe++ (9) and 7.25 μM CBAP (final concentrations) were added. At 60 min after the addition of the inducer, the cells were collected quickly on a membrane filter, washed, and suspended in fresh warm P and D medium supplemented with 5 μM Fe+++. Samples (3 ml) were taken from each flask at zero time and at 2-min intervals thereafter. The differential rates of penicillinase synthesis after the removal of exogenous CBAP from strains 655(PII148), O, and 655(PII148), X, are presented.](http://jb.asm.org/Downloaded from http://jb.asm.org/ on October 25, 2017 by guest)
lution of penicillinase that is consistent with these and other data has been constructed (7).

ACKNOWLEDGMENTS

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LITERATURE CITED