Constitutive Penicillinase Formation in \textit{Staphylococcus aureus} Owing to a Mutation Unlinked to the Penicillinase Plasmid

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Previously described penicillinase-constitutive mutations in \textit{Staphylococcus aureus} are caused by genetic lesions in a regulator gene (or genes) on the penicillinase plasmid in close linkage to the structural gene. This report describes a new class (R2-) of penicillinase-constitutive mutants of \textit{S. aureus} unlinked to the plasmid. By transductional analysis, the penicillinase plasmids in these mutants were wild type. Wild-type plasmids transduced into penicillinase-negative (plasmid loss) derivatives of R2- mutants produced penicillinase constitutively in amounts comparable to a fully induced culture of the wild-type strain. Penicillinase production in R2- mutants was maximal at 30 to 32°C and was much reduced at 40°C.

The structural gene for penicillinase in \textit{Staphylococcus aureus} is usually borne on a plasmid (8, 9, 14), although there is evidence for a chromosomal location in a few strains (1, 10, 20). The enzyme is inducible (6), and its rate of formation is controlled by a regulator \((i)\) gene closely linked to the structural gene (14, 21). The product of the \(i\) gene is a diffusible cytoplasmic repressor. Richmond (22) presented evidence for another regulatory locus, also on the plasmid, which restores inducibility to a class of noninducible mutants. He suggested that the product of this locus and that of the \(i\) gene may cooperate in the regulation of penicillinase, perhaps by forming a dimeric repressor. Mutant strains of \textit{S. aureus} that form increased amounts of penicillinase constitutively have been isolated. Those that have been analyzed by transduction bear their genetic lesion on the penicillinase plasmid, usually in the \(i\) gene, and, in any case, very close to the structural gene (15, 16). Thus, when the penicillinase plasmid is transduced from such mutants to penicillinase-negative recipients, constitutivity is cotransduced in all cases.

During the course of studies of the regulation of staphylococcal penicillinase formation, we obtained plasmid constitutive mutants like the \(i\) mutants described above; in addition, we found another class of constitutive mutants, in which the site of mutation is unlinked to the penicillinase plasmid. In this paper, we report the isolation and some properties of these mutants.


\textbf{Materials and Methods}

\textit{Organisms.} Table 1 lists the strains of \textit{S. aureus}. Penicillinase production was associated in each case with resistance to HgCl\(_2\), tested according to Green (7). Strains producing no enzyme were sensitive to HgCl\(_2\).

Strain 8325xw was R. P. Novick's transducing of staphylococcal plasmid-propagating strain 47 (PS47) from the Public Health Laboratory Service, Colindale, London (14). In this paper, we refer to this strain only as 8325xw and reserve the designation PS47 for the strain received with a set of typing phages from the Communicable Disease Center, Atlanta, Ga.

Mutants constitutive for penicillinase are indicated by the letter \(K\) following the strain designation; the number after \(K\) identifies each separately isolated mutant, e.g., A5K11. Transductants prepared in our laboratory are indicated by the designation of the recipient strain followed by a solidus and then the source of the penicillinase plasmid. Penicillinase-negative strains obtained by plasmid loss are designated by (N) as a suffix to the designation of the parental strains (16).

Strains were phage-typed either in the Bacteriology Laboratory, Illinois Public Health Department, Chicago, through the courtesy of Richard Morrissey, or in this laboratory, according to Blair and Williams (2). Our set of 22 phages and their propagating strains were furnished by the Communicable Disease Center, Atlanta, Ga.

\textit{Media.} LY medium contained 0.3% enzymatic lactalbumin hydrolysate (Nutritional Biochemicals
**Table 1. Staphylococcal strains—sources and properties**

<table>
<thead>
<tr>
<th>Source</th>
<th>Phage type</th>
<th>Antibiotic sensitivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pen</th>
<th>Ery</th>
<th>Kan</th>
<th>Chl</th>
<th>Te</th>
<th>Str</th>
<th>Penicillase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;3&lt;/sup&gt;</td>
<td>52/52A/80/81</td>
<td>R S S S MR R</td>
<td>2.5</td>
<td>380</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sup&gt;5&lt;/sup&gt;</td>
<td>52/52A/80/81</td>
<td>R MR* S S S MR R</td>
<td>2</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52/52A/80</td>
<td>R S S S R R</td>
<td>3</td>
<td>390</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;4&lt;/sup&gt;</td>
<td>(29)/79</td>
<td>R S S S S S</td>
<td>2</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8&lt;sup&gt;8&lt;/sup&gt;</td>
<td>80/81</td>
<td>R S S S S R S</td>
<td>2.2</td>
<td>240</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>258&lt;sup&gt;d&lt;/sup&gt;</td>
<td>80/81</td>
<td>R R* S S R S</td>
<td>3</td>
<td>250</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8325&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
<td>47/53/55/77</td>
<td>R S S S S S</td>
<td>2.5</td>
<td>202</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U9W&lt;sup&gt;f&lt;/sup&gt;</td>
<td>80/81</td>
<td>R MR* S S S R R</td>
<td>2.6</td>
<td>265</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS47&lt;sup&gt;e&lt;/sup&gt;</td>
<td>47/53/55/77</td>
<td>S S S S S R</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52/52A/80</td>
<td>S S S S S MR</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29</td>
<td>S S S S S MR</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52/52A/80</td>
<td>S S S S S S</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The abbreviations stand for penicillin, erythromycin, kanamycin, chloramphenicol, tetracycline, and streptomycin, in that order; S, sensitive; R, resistant; MR, moderately resistant.

<sup>b</sup> Isolated from patients at Michael Reese Hospital.

<sup>e</sup> Erythromycin resistance was unlinked to the penicillinase plasmid.

<sup>d</sup> Kindly given by R. P. Novick.

<sup>e</sup> Kindly given by J. N. Baldwin.

<sup>f</sup> Communicable Disease Center, Atlanta, Ga.

Corp.), 0.3% yeast extract, (Difco), 0.05 M sodium phosphate, 2.7 mM KCl, and 0.42 mM MgSO<sub>4</sub>. The final pH was 7.2. The broth was sterilized in an autoclave, and 0.1% glucose was added aseptically. CY medium was prepared according to Novick (14).

**Brain Heart Infusion (BHI), Heart Infusion (HI), and Nutrient Broth (NB) were Difco products. Media containing 0.15 units of benzylpenicillin per ml are designated 0.15 u. pen.**

*Assays.* From an overnight culture on 0.15 u. pen. BHI-agar, three or four well-isolated colonies were transferred to 10 ml of L broth and grown with shaking for 2 hr at 30°C to a density of 0.025 mg/ml, measured by optical density at 540 m<sub>u</sub> in a Coleman Junior spectrophotometer. The culture was diluted with 15 ml of L broth and divided among three flasks. One flask was grown at 30°C, another at 40°C. Each was sampled at 0.2 mg per ml, diluted again with an equal volume of broth, grown one more generation, and sampled again. The mean of these two samples was taken as the penicillinase activity at each temperature. The culture in the third flask was induced by the addition of cephalosporin C (40 μg/ml) or, in a few cases, meccillin (0.5 μg/ml). It was grown for 3.5 hr with shaking at 30°C and then sampled. All samples were collected in cold gelatin-chloramphenicol solution (0.1 ml each of 0.5% gelatin and 0.1% chloramphenicol). Penicillinase was assayed according to Perret (18) as modified by Novick (13). Results are expressed as units of enzyme per milligram (dry weight). Penicillinase phenotypes were scored on colonies grown overnight at 30 to 32°C on unbuffered 0.3 CY plates by the N-phenyl-1-naphthylamine-azo-o-carboxybenzene (PNCB) method of Novick and Richmond (16). This procedure was also used to check the constitutivity of samples of cultures taken at the time of assay. This precaution was necessary since some mutants lost the penicillinase plasmid so rapidly as to invalidate penicillinase determinations. In the assays reported herein, the samples contained not more than 10% penicillinase-negative clones.

**Transduction.** For transduction of 80/81 strains, we used typing phage 80; for transduction of strain 010, phase 29. These phages were grown on the appropriate hosts by the soft-agar overlay method (2). For transduction to PS7 or PS47 and its derivatives, we induced the lysogenic host, PS47, or 8325<sup>a</sup>, with ultraviolet (14). We obtained a few transductants of PS47 with the A5 plasmid by the use of phage 80.

The transduction procedure was that of Pattee and Baldwin (17) with minor modifications. Cells were grown overnight on HI-agar, washed off with NB, and adjusted to a concentration of 10<sup>9</sup> colony forming units/ml. Phase 80 or 29 was added at a multiplicity of about 1.0, induced phase from 8325<sup>a</sup> or PS47 at a multiplicity of about 0.1. Phage and cocci (10<sup>8</sup> colony forming units) in 2 ml NB containing CaCl<sub>2</sub> (400 μg/ml) were incubated for 45 min at 37°C with gentle shaking. We added 2.5 ml of BHI, centrifuged the culture, washed the cells with 2.5 ml of BHI, and resuspended them in 0.5 to 1.0 ml of BHI. Samples (0.1 ml) were plated on 0.15 u. pen. BHI-agar and incubated overnight at 37°C. Transductant colonies were subcultured twice on 0.15 u. pen. agar for purification. Phage sterility controls were run with each transduction. Recipient organisms without phage were plated on 0.15 u. pen. agar in each experiment, but never yielded spontaneous, penicillinase-positive mutants. The frequency of transduction...
mediated by phage 80 ranged widely from $10^{-4}$ to $10^{-8}$, the common range being $10^{-4}$ to $10^{-5}$. The frequency of transduction with induced phage from PS47 or R325sa was about $10^{-4}$. In some transductions of the 258 plasmid, we selected for erythromycin resistance and examined penicillinase production as the unselected marker (16).

**Derivation of mutant strains.** Cultures grown overnight in L broth were treated with ethyl methane sulfonate according to Novick (14). The cells were plated on unbuffered 0.3 CY medium, covered with a thin layer of 0.5% agar, and incubated overnight at 30 to 32 C. Constitutive colonies were detected by the PNCB method and were purified by two or more successive subcultures of single colonies at 30 to 32 C.

Penicillinase-negative derivatives were obtained either spontaneously or after growth of the parental strain at 42 C (11). They too were selected on 0.3% CY plates by the PNCB method. Presumably negative colonies were streaked again on HI-agar for purification. Loss of the penicillinase plasmid was confirmed as follows. (i) Penicillinase activity was not detected by quantitative assay of dense suspensions nor by qualitative tests on starch-iodine plates (18). (ii) Standard antibiotic sensitivity paper discs impregnated with 10 µg of benzylpenicillin produced zones of inhibition on heavily inoculated plates as wide as those produced with naturally occurring penicillin-susceptible strains. (iii) HgCl₂ resistance, which is borne on the penicillinase plasmid, was lost in all cases, and erythromycin resistance was lost from strains bearing the 258 plasmid (16, 23).

**RESULTS**

**Penicillinase-constitutive mutants.** In our initial studies with strain A5, the genetic lesions in all but 1 of 24 constitutive mutant strains, selected from five mutagenic treatments, were found to be unlinked to the penicillinase plasmids. With the same single exception, all strains produced large amounts of penicillinase, ranging, for the most part, from 300 to 400 units/mg. Phage 80 grown on each of seven mutant strains selected from two mutagenic treatments transduced wild-type inducible penicillinase production into naturally occurring penicillinase-negative or cured, wild-type inducible strains. A representative experiment is summarized in Table 2, which also shows that the same transduction into strain A5K11(N), a cured derivative of the donor strain, yielded only constitutive progeny. Experiments with other A5 mutants gave similar results. Phage grown on the exceptional, weakly constitutive strain (A5K22) transduced similarly weakly constitutive penicillinase production to negative strains. This strain was considered to be plasmid-constitutive.

To assess the properties of all our A5 mutants, we transduced one of several wild-type plasmids (A3, A5, 258, B8) into negative strains derived from each of 23 A5 mutants. Each of 319 penicillinase-positive transductants isolated in pure culture was constitutive, producing the enzyme in amounts comparable to the original mutant (Table 3). Wild-type penicillinase plasmids were recovered from these transductants by outcrossing to naturally occurring negative strains or to negative strains derived either from wild-type penicillinase-positive or plasmid-constitutive strains.

**Mutant A5K22 could not be tested in this way since we were unable to obtain a penicillinase-negative derivative from it.**

Evidently, strain A5 is susceptible to a mutation that imposes constitutive penicillinase production upon normal penicillinase plasmids. The mutation is obviously unlinked to the plasmid. We refer to this phenotype as penicillinase R² by analogy with a class of mutations affecting regulation of alkaline phosphatase in *Escherichia coli* described by Garen (see Discussion). Although we apply this name to all mutant strains of this class, we recognize that there is, as yet, no evidence that they bear lesions at a single locus.

**TABLE 2. Transduction of penicillinase plasmids from an R² mutant**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transductants scored</th>
<th>Penicillinase phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS47</td>
<td>23</td>
<td>Wild type</td>
</tr>
<tr>
<td>05</td>
<td>98</td>
<td>Wild type</td>
</tr>
<tr>
<td>A5(N)</td>
<td>10</td>
<td>Wild type</td>
</tr>
<tr>
<td>A5K11R²-(N)</td>
<td>47</td>
<td>Constitutive</td>
</tr>
<tr>
<td><strong>Donor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5K11R²</td>
<td></td>
<td>Constitutive</td>
</tr>
</tbody>
</table>

**TABLE 3. Transduction of a wild-type penicillinase plasmid into negative derivatives from R² and plasmid-constitutive mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transductants scored</th>
<th>Penicillinase phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>4</td>
<td>Wild type</td>
</tr>
<tr>
<td>A10K1(N)</td>
<td>8</td>
<td>Wild type</td>
</tr>
<tr>
<td>B8K8(N)</td>
<td>7</td>
<td>Wild type</td>
</tr>
<tr>
<td>A5K6R²-(N)</td>
<td>12</td>
<td>Constitutive</td>
</tr>
<tr>
<td>A5K11R²-(N)</td>
<td>7</td>
<td>Constitutive</td>
</tr>
<tr>
<td><strong>Donor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Strain A5 readily lost its penicillinase plasmid spontaneously, and its R\(^2\) mutants did so at rates that varied with different mutants but were usually much greater than that of the parental strain. Revertants of A5R\(^2\) mutants to wild-type inducible phenotypes were looked for on PNCB plates, either with or without preliminary ultraviolet radiation. None was found among many thousands of colonies of mutants A5K11 or A5K15.

We looked for R\(^2\) constitutive mutants in other penicillinase-producing strains by transducing a wild-type inducible penicillinase plasmid into one or two negative strains of each constitutive mutant. Recovery of constitutive transductants was taken to signify an R\(^2\) mutant and recovery of wild-type transductants, a plasmid mutant. Again, in each transduction, the progeny were uniformly of the same class. To check further the constancy of the R\(^2\) phenotype in any individual mutant, we isolated 12 negative strains from 1 R\(^2\) mutant (258K2) and 12 from a plasmid mutant (258K1). The 258 plasmid was transduced into each penicillinase-negative strain and transductants were selected for erythromycin resistance. When the recipient strains were derived from the R\(^2\) mutant, all of 665 transductants were penicillinase-constitutive by the PNCB test. When the recipients were derived from the plasmid mutant, all of 631 transductants gave PNCB tests comparable to wild-type strains.

We have produced R\(^2\) mutations in the naturally occurring, penicillinase-producing strains 258 and U9W, in five naturally occurring negative strains bearing a transduced A5 penicillinase plasmid (05, 010, 012, PS7, and PS47), and in the transductant strain 8325\(\alpha\),. These include all strains of \textit{S. aureus} in which we have systematically looked for R\(^2\) mutants. The ratio of plasmid mutants to R\(^2\) mutants varied from strain to strain: 1:24 for strain A5, 2:12 for 8325\(\alpha\),, 9:12 for A5(N)/258, and 9:20 for 258.

Effect of temperature on penicillinase activity of R\(^2\) mutants. The penicillinase activity of R\(^2\) mutants was maximal in cultures grown at 30 to 32 C, but was progressively reduced in cultures grown at higher temperatures. Strain 258 R\(^2\) mutants grown at 40 C had a mean penicillinase activity about one-fifth as great as at 30 C (Table 4). For individual 258 R\(^2\) mutants, this ratio ranged from 0.08 to 0.3, and for plasmid mutants, from 0.5 to 1.2. Mutants of strains 8325\(\alpha\), and A5 gave generally similar results. In addition to temperature, the physiological age of the culture and the composition of the broth modified the expression of penicillinase activity in some mutants. We shall report in another publication details of these properties (Cohen and Sweeney, \textit{in preparation}). In this paper, we wish only to point out the magnitude of the temperature effects in R\(^2\) mutants. Some R\(^2\) mutants grown in 0.3 CY medium at 37 C and then stained by the PNCB method with 1% penicillin solution were not identifiable as being penicillinase-constitutive and could therefore be overlooked in the primary screening for mutants. For example, when strain 258 was treated with ethyl methyl sulfonate in the usual way and then plated at 37 C, only 1 of 8

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Strain & \multicolumn{2}{|c|}{Plasmid-constitutive mutants} & \multicolumn{2}{|c|}{R\(^2\) constitutive mutants} & \multicolumn{2}{|c|}{R\(^2\) transductants} \\
& Uninduced & Induced (30 C) & Uninduced & Induced (30 C) & Uninduced (30 C) & Induced (30 C) \\
\hline
8325\(\alpha\), & (2) 71-105 & 89 & 61-115 & 89 & 204-430 & 138-280 & 177 & 10-50 & 27 & 171-330 & 242 & (12) 122-175\(^b\) & 144 & 183-322 & 243 \\
A5 & (1) 9 & 8 & 28 & & (23) 246-510 & 352 & 17-187 & 104 & 278-680 & 408 & (6) 274-473\(^c\) & 356 & 286-396 & 341 \\
\hline
\end{tabular}
\caption{Penicillinase activity of plasmid and R\(^2\) mutants\(^*\)}
\end{table}

\(^*\) Number of mutants tested is indicated in parentheses. The results are expressed as units of penicillinase per milligram. The first row of numbers for each strain gives the range; the second row gives the mean.

\(^a\) Transduced by plasmid from strain 258.
\(^b\) Transduced by plasmid from strain 8325\(\alpha\),.
\(^c\) Transduced by plasmid from strain A5.
mutants was R²−, whereas 20 of 29 mutants detected after culture at 30 C were R²−.

The correlation between temperature sensitivity and R²− mutants was not perfect. In some strains, a few plasmid mutants were temperature-sensitive. The sensitivity usually moderate, the cocci grown at 40 C containing about one-half as much enzyme as at 30 C. In two plasmid mutants of strain A5(N)/258, the ratio of the penicillinase activities at the two temperatures was 0.4. Conversely, a few R²− mutants were only slightly temperature-sensitive and contained, like some plasmid mutants, about one-half as much penicillinase at 40 as at 30 C. In all strains, however, on the average, the ratio of the penicillinase activities at 40 C to those at 30 C was much lower for R²− than for plasmid mutants.

Expression of penicillinase production in R²− mutants. In general, the amount of penicillinase produced in R²− mutants appeared to be determined by the mutation. That is, strains obtained by transduction of wild-type plasmids into the cured mutants produced penicillinase in amounts comparable to the original mutants, provided that the transduced plasmid could be induced to similarly high levels in its native host. Thus, all of our constitutive R²− mutants in strains A5 and 258 produced upwards of 200 units/mg, about as much enzyme as fully induced cultures of the parental strains; this, in turn, was the level attained in the same R²− mutants harboring plasmids transduced from wild-type 80/81 strains (Table 4). Induction of the R²− mutant evoked either slight further increase in penicillinase or none at all. R²− mutants in strain 8325αwere qualitatively similar but produced lower amounts of enzyme. Several R²− mutants in PS47/A5 produced still less enzyme, 50 to 100 units of penicillinase/mg, and could be induced to slightly higher levels of enzyme, 100 to 200 units/mg. Comparable amounts of penicillinase were produced by the 8325α plasmid transduced into the penicillinase-negative derivatives of these mutants.

Some strains of S. aureus, resistant to penicillin only, are poorly inducible. They produce about one-half to one-sixth as much enzyme on full induction as do multiresistant strains such as A5 (24). Strain B4 is one of these poorly inducible strains, producing 60 to 90 units/mg when fully induced. Six constitutive mutants of this strain produced similarly restricted amounts of penicillinase, from 33 to 78 units/mg.

R²− mutants of strain 010 carrying a B4 plasmid transduced by phage 29 produced 59 units of enzyme/mg (Table 5). The same mutant carrying the A5 plasmid produced 395 units/mg and, carrying the 258 plasmid, produced 236 units/mg. Therefore, in this 010 R²− mutant, the concentration of enzyme corresponded to the fully induced, or nearly fully induced, level characteristic of each plasmid examined.

With another relatively poorly inducible plasmid, we obtained a somewhat different result. Strain B8 produced 150 to 200 units of penicillinase when fully induced. This plasmid produced 400 units/mg in negative derivatives of either of two R²− mutants of strain A5, about twice as much as that formed on induction of the same plasmid in its native host or in a negative derivative obtained from the unmutated A5. This result suggests that some R²− mutants may be able to disclose the potential penicillinase-forming capacity of certain plasmids more efficiently than do conventional methods of induction.

**DISCUSSION**

Our results establish the presence of a locus or loci unlinked to the penicillinase plasmid which, in the mutant state at least, influences the regulation of staphylococcal penicillinase synthesis. These mutants have been found, among others, in S. aureus 8325α and 258, strains in which

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**Table 5. Penicillinase production in an R²− mutant by a plasmid transduced from a poorly inducible strain**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Derivation</th>
<th>Penicillinase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uninduced</td>
</tr>
<tr>
<td>B4</td>
<td>Wild type</td>
<td>2</td>
</tr>
<tr>
<td>010/B4</td>
<td>Transductant</td>
<td>2</td>
</tr>
<tr>
<td>010/A5</td>
<td>Transductant</td>
<td>3</td>
</tr>
<tr>
<td>010/A5/K1</td>
<td>R²− constitutive mutant of 010/A5</td>
<td>395</td>
</tr>
<tr>
<td>010/A5/K1(N)/258</td>
<td>Transductant of B4 plasmid into negative derivative of 010/A5/K11</td>
<td>59*</td>
</tr>
<tr>
<td>010/A5/K1(N)/258</td>
<td>Transductant of 258 plasmid into negative derivative of 010/A5/K11</td>
<td>236</td>
</tr>
</tbody>
</table>

* Mean of three assays on each of four transductants.
excellent evidence has been adduced for the presence on the penicillinase plasmid of a regulator gene closely linked to the structural gene for the enzyme. We assume that the same is true for the other strains we have studied, and this is supported by our isolation from them of typical plasmid-constitutive mutants.

A number of possible mechanisms for the R2− mutants may be suggested. The mutation may cause the synthesis of an internal inducer of penicillinase. Two cyclic peptides have been reported to be inducers or substrates of staphylococcal penicillinase (25). More recently, extracts of Bacillus cereus or S. aureus containing chemical fragments of the cell wall were found to induce penicillinase in B. cereus [J. H. Ozer, D. L. Lowery, and A. K. Saz, Bacteriol. Proc., p. 124, 1967; A. K. Saz, unpublished observations cited by Pollock (19)]. Possibly, a mutation affecting some aspect of cell wall synthesis may lead to accumulation of such peptides and, in consequence, to internal induction of penicillinase.

Another possibility is that normal regulation requires the cooperation of another substance produced by an R2 gene, assumed to be present in penicillinase-positive or -negative staphylococci. Mutation at either the i or the R2 gene would yield penicillinase-constitutive organisms forming increased amounts of enzyme. Taking into consideration Richmond's second plasmid regulatory locus, this hypothesis would require the cooperation of three elements in the normal uninduced state, a perhaps embarrassingly large number.

Still a third possibility is that the R2− mutants contain an informational suppressor that interferes with the translation of the messenger produced by the normal i gene. Although a suppressor is recognized ordinarily by its effect on another mutation, some suppressors may affect the translation of some normal genes (5, 26). In this connection, it may be significant that many R2− mutants form smaller colonies on agar media than do their parental strains or plasmid mutants.

The current state of knowledge of the regulation of protein synthesis in bacteria has been reviewed recently (27). Our observations appear to suggest a pattern of regulation of penicillinase synthesis in S. aureus analogous to the alkaline phosphatase system in E. coli (3) and B. subtilis (12). This enzyme also has two regulatory loci, one (R1) closely linked to the structural gene, the other remote from it (R2). Mutation at either site is sufficient cause for constitutive production of enzyme. There is evidence that the R2 locus in E. coli is responsible for the production of at least one specific protein. The concentration of this protein responds to genetic or environmental factors in parallel with that of alkaline phosphatase. On this evidence, Garen and Otsuji (4) reasoned that the R2 protein was not the repressor itself but that it might be a component of the repressor or be required for the function of repressor.

The R2− staphylococcal mutants may lend variety to the study of regulation. Other inducible systems studied to date are for the most part subject to catabolite repression. Repressible systems are open to influence by variations in concentration and kind of corepressor. Staphylococcal penicillinase offers the advantages for further investigation of a simple, apparently purely inducible system uninfluenced by catabolite repression.

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LITERATURE CITED