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# Naturally Occurring Penicillinase Plasmids in *Staphylococcus aureus*

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A series of plasmids harbored by naturally occurring penicillin-resistant strains of *Staphylococcus aureus* were surveyed with a view toward exploring the variability in plasmid-linked marker patterns. Plasmids were transduced from their natural hosts to either of two plasmid-negative laboratory strains by selection for cadmium resistance, and the transductants were tested for all other markers previously found to be plasmid-linked. All of the strains that were able to serve as genetic donors to one of the two stock strains could donate cadmium and lead resistance as linked, plasmid-borne markers. Among the other plasmid markers, a wide variety of patterns was found, including four plasmids that did not carry the penicillinase determinant. Each of the 26 plasmids studied, including the latter 4, was found to belong to one of the two incompatibility sets of penicillinase plasmids previously identified. With the exception of the penicillinase-negative plasmids, which were found in both sets, all the plasmids of incompatibility set I directed the production of penicillinase type A; those belonging to set II directed either type A or type C. Those of set II without exception increased the sensitivity of their host strains to bismuth ion; those of set I carried determinants of bismuth resistance or did not affect the sensitivity of their host to this ion. No other perfect correlations between markers were encountered; in particular, there was no correlation between penicillinase serotype and the excretion of the enzyme. This finding allows the prediction that there is, in addition to all of the markers thus far identified, a plasmid-linked determinant of penicillinase excretion.

In previous investigations of extrachromosomal resistance factors carried out in this laboratory (10-12) and in Richmond's laboratory (16), three different naturally occurring staphylococcal penicillinase plasmids were studied in some depth. Other investigators (2, 3) have utilized strains harboring different plasmids that have not been fully characterized.

To broaden and systematize the study of these extrachromosomal resistance factors and to gain information on the variety of naturally occurring marker patterns within the overall plasmid group, we have undertaken a study of plasmids harbored by a series of naturally occurring penicillin-resistant staphylococcal strains. These strains have been phage-typed and tested for antibiotic resistance, penicillinase type, and penicillinase excretion pattern by Dyke and Richmond (5), who isolated from some of them penicillinase-negative variants which they screened for concomitant loss of resistance to arsenate, cadmium, and mercury (K. Dyke and M. Richmond, *personal communication*). To verify the linkage of markers that we have come to associate with the plasmid genome (namely, resistance to cadmium, lead, bismuth, arsenate, arsenite, and mercuric ions, and to erythromycin) and to evaluate possible host effects on the expression of the various genes, we transduced the plasmids from their natural hosts into one of two standard test strains. The analysis of these transductants revealed a considerable variety of marker patterns within an apparently homologous series of plasmids. In many cases, our results paralleled the preliminary observations of Dyke and Richmond. In others, we found that the wild-type hosts altered the expression of certain plasmid markers; in still others, we found that the usual plasmid genes were not all on one linkage group as revealed by transduction.

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## MATERIALS AND METHODS

**Organisms.** The staphylococcal strains studied are listed in Table 2. Most of the tests were carried out on strains (isolated in various British hospitals) kindly supplied to us by Keith Dyke, and on two standard strains, namely, 8325, a naturally occurring penicillinase-negative strain used for propagation of phage 47 of the International Typing Series, and 147(N), a plasmid-negative variant of a penicillinase-producing strain (19). Naturally occurring donor strains are not listed individually except when their properties differed significantly from transductants.

Phage 80 of the International Typing Set, P147 (15), and P11, a temperate phage carried by 8325, were used as transducing phages. Phages were propagated in lysing broth and were sterilized by membrane filtration.

Test plasmids for incompatibility tests were $PI_{258}$ (*penI443 cad-52*) and $PII_{147}$ (*penI220 cad-11* ··· *ero*$^+$); the latter is a recombinant with the *ero* marker from $PI_{258}$ and the other markers from $PII_{147}$. These plasmids were carried in P11 lysates of 8325 derivatives harboring them.

**Media.** CY broth, 0.3 CY medium, phage agar, phage buffer (9), and GL-agar (0.3 CY + 0.1% glycerol) were used exclusively in these studies. Lysing broth was a 1:1 mixture of CY broth and phage buffer. GL-cad-agar was GL-agar + $5 \times 10^{-5}$ M cadmium nitrate. GL-cad-ero-agar was GL-cad-agar + erythromycin (100 $\mu$g/ml).

**Cultures.** Culture conditions were similar to those described previously (12). Bacterial dry weights were estimated turbidimetrically with a Coleman Junior spectrophotometer read at 540 nm in 19-mm cuvettes. In exponential CY cultures at 37 C, an optical density of 0.1 = 0.045 mg (dry weight/ml).

**Tests.** Disc tests for resistance were carried out as previously described (12). Resistance to K(BiO)·$C_4H_5O_6$ was tested by twofold serial tube dilution tests, and by inoculating CY plates supplemented with different concentrations of the salt. The results of disc and tube sensitivity tests were evaluated when possible by comparison of plasmid-positive strains with isogenic plasmid-negative strains.

Penicillinase-production was tested on plates by the iodine-starch (13) and acid-base indicator (9) techniques. For quantitative measurement of penicillinase activities, the iodometric method of Perret (13) as modified by Novick (9) was employed. One penicillinase unit = 1.0 $\mu$mole of benzylpenicillin destroyed per hr at 30 C and *p*H 5.9. Penicillinase was induced with methicillin as previously described (8).

**Transduction procedures.** The production of transducing lysates and the infection of recipient cultures were described previously (9). When necessary to prevent killing of transductants by phage, 0.03 ml of antiphage serum ($K \cong 200$) was added to each overlay at the time transduction mixtures were plated. Cadmium-resistant transductants were selected on GL-cad-agar. Erythromycin-resistant transductants were selected on GL-agar with 1 $\mu$g of erythromycin/ml and were purified on GL-agar with 100 $\mu$g of erythromycin/ml.

**Incompatibility tests.** Unknown plasmids were tested for incompatibility with known ones by a pair of crosses. The rationale for this test is that, on selection for a donor plasmid marker, a resident plasmid will be "displaced" if it is incompatible with the donor plasmid but not if it is compatible. Known test plasmids of genotypes *penI*$^-$ *penZ*$^+$ *cad*$^-$ $mcr_{I}$ *ero*$^+$ and *penI*$^-$ *penZ*$^+$ *cad*$^-$ $mcr_{II}$ *ero*$^+$, respectively, were transduced in turn into a recipient strain harboring an unknown, of genotype *penI*$^+$ *penZ*$^+$ *cad*$^+$ $mcr_2$ *ero*$^-$, with selection for erythromycin resistance.

The transductions were carried out in duplicate. One plate of each pair was stained for penicillinase activity; the other was replicated to a GL-ero-cad plate. In these tests, excluding physical recombination which occurs generally in no more than about 10% of primary transductants, four outcomes are theoretically possible, as diagrammed in Fig. 1.

(i) The $mcr_{I}$ test plasmid displaces the resident plasmid, giving constitutive, CadS transductants, while the $mcr_{II}$ forms a heterodiploid with it, giving inducible (*penI*$^+$ is *trans*-dominant) CadR transductants that produce occasional constitutive, CadS segregants. In this case, the conclusion is that the unknown is $mcr_{I}$.

(ii) The $mcr_{I}$ gives heterodiploids; the $mcr_{II}$ displaces. In this case, the unknown is classed as $mcr_{II}$.

(iii) Both $mcr_{I}$ and $mcr_{II}$ give heterodiploids. In this case, the unknown belongs to a third incompatibility set, $mcr_{III}$. In the event that several plasmids were to test as $mcr_{III}$, each would be tested against all the others to identify additional incompatibility sets.

(iv) Both $mcr_{I}$ and $mcr_{II}$ displace the resident plasmid, giving donor type, EroR transductants. In this case, we discard the Jacob-Brenner-Cuzin theory (6) and the concept of incompatibility sets, and begin all over again.

In the case of penicillinase-negative plasmids, that part of the test involving penicillinase testing was dispensed with and an attempt was made to demonstrate CadR-CadS and EroR-EroS heterozygosity.

## RESULTS

Our initial approach consisted in attempting to propagate a series of transducing phages on each of the potential donor strains. Where lysates were obtained, attempts were made to transduce cadmium resistance into either 8325 or 147(N). In each cross, at least 5 and usually 10 CadR transductants were purified and analyzed for cotransduction of other markers.

Starting with 52 different penicillin-resistant strains of *Staphylococcus aureus*, we were able to propagate at least one of the transducing phages on 39. With 23 of the resulting lysates we were able to obtain cadmium-resistant transductants of at least one of the two standard strains. In 14 of the 16 cases in which we were unable to get CadR transductants, the donor strain had tested as resistant to cadmium. In 19 of 23 cases, the CadR transductants inherited a penicillinase locus from the donor along with a cluster of ion resistance markers. In the other four cases, the donor

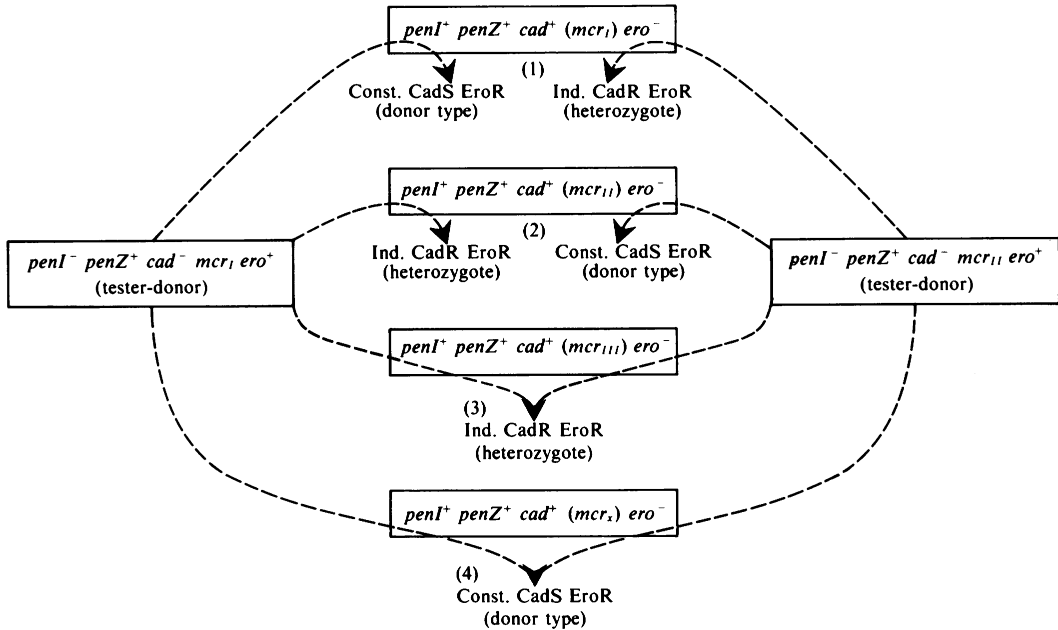


FIG. 1. *Incompatibility tests. Genotypes of donor test plasmids are given in the boxes at left and right. Genotypes of unknown recipient plasmids are given at center. Lines with arrows represent crosses and point to phenotypes of major transductant class. Numbers refer to different possibilities as explained in text. The mcr region of each unknown plasmid is hypothetical and is therefore placed in parenthesis. Const. and Ind. = constitutive and inducible for penicillinase production. For other abbreviations, see Table 3.*

penicillinase marker failed to cotransduce with cadmium resistance. In another, 55C1, Sweeney and Cohen (20) identified both a plasmid-linked and a nonplasmid-linked determinant of penicillinase production. Experiments are currently in progress to decide whether in these strains the penicillinase region is chromosomal (1, 14) or is borne by a separate plasmid. Asheshov and Dyke (2) found in strain 9789 that a nonplasmid-linked penicillinase determinant could under special conditions be incorporated into the plasmid linkage group.

We feel justified in concluding that the linkage groups transduced are extrachromosomal and are related to the known penicillinase plasmids on the basis of two criteria. First, mutual cotransduction frequencies of linked markers were always close to 100% as has been found previously with plasmid markers. Second, each of the newly analyzed linkage groups was found to be incompatible with one or another of the known plasmids and therefore to belong to one of the two known incompatibility sets.

**Nomenclature.** In a previous publication (11), a nomenclature scheme for the plasmids was suggested in which any newly studied plasmid with a different combination of the (then) known plasmid markers was to be designated by a different Greek letter. As additional plasmid markers were discovered (10), this scheme became more and more unwieldy (16, 18), and it finally became apparent that it was theoretically as well as operationally unworkable. We have come to the conclusion, finally, that the only reasonable scheme is one in which each plasmid is uniquely identified by an indication of the naturally occurring strain in which it was found. We feel also that the determinant of incompatibility set is the only plasmid marker of sufficient taxonomic significance to be included in the designation. Therefore, we now suggest that plasmid-harboring strains be designated as follows: 8325($PI_{524}$) is 8325 carrying a plasmid of *mcr* type I originally found in strain 524; 147($PI_{258}$ *cad-52*) is 147 carrying an $mcr_I$ plasmid originally found in strain 258, now carrying a mutation to cadmium sensitivity. All plasmid-linked markers not listed as such are assumed to be wild type.

As noted (12), some plasmids render their hosts more sensitive to bismuth. We have previously referred to this effect as "dominant" sensitivity, for lack of a better term (12). Perhaps "expressible" sensitivity would be better, since the marker is recessive to *plasmid-linked* bismuth resistance.

Designations of recombinant plasmids and

plasmid heterozygotes would be essentially as before. It seems appropriate in this context, however, to introduce changes in marker designation sufficient to bring the plasmid nomenclature into conformity with that proposed by Demerec et al. (4). These designations are listed in Table 1.

**Patterns of plasmid-linked markers.** The characteristics of strains harboring 26 naturally occurring plasmids, including the three that had been studied previously (11, 12), are listed in Table 2. In most cases, the marker pattern of the transductant strain did not differ significantly from that of the donor. In these cases, results for the transductant clone are listed. When differences were found, results for both donor and transductant are listed.

Several patterns of plasmid-linked markers were found with different frequency among the strains analyzed. These patterns are arranged in Table 1 according to the number of markers identified—ranging from $PI_{258}$, which carries all of the markers listed in Table 1, to $PI_{1071}$, which carries only *pen*, *lea*, and *cad*. We have no information bearing on whether the regions corresponding to "missing" markers are present and carry different information or are physically absent.

The absolute level of resistance determined by a particular ion resistance locus was relatively constant among the different plasmids examined. However, striking host-specific differences in expression of particular genes were observed. For example, the *asa* locus of $PI_{1036}$ was expressed nearly 20 times as well in 147 as in its original host, as indicated by relative resistance levels. Examples of host-specific variations in expression are shown in Table 3.

TABLE 1. *Penicillinase plasmid markers*

| New designation[a] | Previous designation | Function |
|---|---|---|
| *penB* | ($i_2$) | Maximum level of penicillinase production |
| *penI* | ($i_1$) | Penicillinase inducibility |
| *penZ* | (p) | Penicillinase structure |
| *asa* | ($AsO_4^-$) | Arsenate resistance |
| *asi* | ($AsO_2^-$) | Arsenite resistance |
| *bis* | ($Bi^{+++}$) | Bismuth ion resistance or sensitivity |
| *lea* | ($Pb^{+++}$) | Lead ion resistance |
| *cad* | ($Cd^{++}$) | Cadmium ion resistance |
| *mer* | ($Hg^{++}$) | Mercuric ion resistance |
| *mcr* | (mc) | Plasmid maintenance, compatibility, and replication |
| *ero* | (em) | Erythromycin resistance |
| *exo* | | Excretion of penicillinase |

[a] Nomenclature of plasmid markers as changed to conform with the proposals of Demerec et al. (4) for a standard nomenclature in microbial genetics.

Plasmids carrying determinants of resistance to cadmium and to lead were harbored by all of the penicillin-resistant strains examined, whether or not the penicillinase gene belonged to the plasmid linkage group. In the four strains in which the penicillinase determinant was not cotransduced with cadmium, the cadmium linkage groups were evidently closely related to the penicillinase plasmids in that they carried many of the other plasmid markers and showed appropriate compatibility behavior.

The strains listed in Table 2 were also tested for resistance to a large number of other salts (*see* reference 12 for the list of compounds). Two of the plasmids were found to confer a slight but significant increase in sensitivity to sodium silicate. There were no other significant findings.

**Penicillinase production.** The donor strains were tested by Dyke and Richmond (*personal communication*) for penicillinase serotype (15). Limited past experience has been that this serotype is host-independent; thus, penicillinase serotypes are listed for the newly analyzed plasmids according to the findings of Dyke and Richmond for the original strains.

The amount of penicillinase synthesized by standard hosts harboring the naturally occurring plasmids was determined in cells grown in CY broth with and without methicillin. All of the strains were inducible; the penicillinase activity found ranged in uninduced cells from 3.3 to 14 units/mg (dry weight), and in induced cells from 98 to 310 units/mg. Staphylococcal penicillinase has been found to be an extracellular enzyme (8); there are, however, two distinct patterns of its liberation by staphylococcal cultures (5): those in which 20 to 60% is in the supernatant fluid (high liberators), and those in which $< 10\%$ is in the supernatant fluid (low liberators).

The transductants harboring naturally occurring plasmids all fell into the same two classes: "high" and "low" liberation. Of the strains tested, 16 belong to the first group, liberating more than 20% of their tested penicillinase activity. Five strains showed extracellularity of penicillinase between 0 and 12%. No significant differences were found between the degree of extracellularity of penicillinase in original donor strains, as determined by Dyke and Richmond (*personal communication*), and in transductants.

## DISCUSSION

One of the hopes of this study was to analyze a representative sample of naturally occurring

TABLE 2. *Patterns of plasmid-linked markers*[a]

| Strain | *pen* | *asa* | *asi* | *bis* | *lea* | *cad* | *mer* | *mcr* | *ero* | *exo* |
|---|---|---|---|---|---|---|---|---|---|---|
| 8325($PI_{258}$)[b] | A | + | + | + | + | + | + | I | + | + |
| 8325($PI_{524}$)[b] | A | + | + | + | + | + | + | I | − | + |
| 147($PI_{13333}$) | A | + | + | + | + | + | + | I | − | + |
| 9033 (wild) | A | − | − |  | + | + | + |  | − | + |
| 147($PI_{9033}$) | A | + | + | + | + | + | + | I | − | + |
| 147($PI_{13371}$) | A | + | + | + | + | + | + | I | − | + |
| 8325($PI_{6193}$) | A | + | + | + | + | + | + | I | − | + |
| 147($PI_{3743}$) | A | + | + | − | + | + | + | I | − | + |
| 8325($PI_{3719}$) | A | + | − | + | + | + | + | I | − | + |
| 147($PI_{1042}$) | A | + | + | + | + | + | + | I | − | − |
| 1036 (wild) | A | − | − |  | + | + | − |  | − | − |
| 147($PI_{1036}$) | A | + | + | + | + | + | + | I | − | − |
| 147($PI_{6187}$) | A | + | + | + | + | + | − | I | − | + |
| 3721 (wild) | A | + | + |  | + | + | + |  | − | + |
| 8325($PI_{3721}$) | A | + | − | − | + | + | − | I | − | + |
| 3761 | A | − | − |  | + | − | − |  | − | − |
| 8325($PI_{3761}$) | A | + | − | − | + | + | − | I | − | − |
| 8325($PI_{13313}$) | A | − | + | − | + | + | − | I | − | − |
| 8325($PI_{3773}$) | A | − | − | − | + | + | − | I | − | + |
| 8325($PI_{55C1}$) | A | − | − |  | + | + | + | I | − | +[c] |
| 8325($PI_{1071}$) | A | − | − | − | + | + | − | I | − | − |
| 9789 (wild) | + | + | + |  | + | + | + |  | − |  |
| 147($PI_{9789}$) | − | + | + |  | + | + | + | I | − |  |
| 147($PII_{10496}$) | A | + | + | ES | + | + | + | II | − | + |
| 147($PII_{6907}$) | C | + | + | ES | + | + | − | II | − | + |
| 8325($PII_{3772}$) | A | + | + | ES | + | + | − | II | − | + |
| 147($PII_{147}$)[b] | C | + | − | ES | + | + | + | II | − | − |
| 8325($PII_{3804}$) | C | − | − | ES | + | + | − | II | − | − |
| 3755 (wild) | A | + | + |  | + | + | − |  | − |  |
| 147($PII_{3755}$) | − | + | + | ES | + | + | − | II | − | − |
| 1008 (wild) | C | + | + |  | + | + | − |  | − | + |
| 147($PII_{1008}$) | − | + | + | ES | + | + | + | II | − |  |
| 17810 (wild) | A | + | + |  | + | + | − |  | − | + |
| 147($PII_{17810}$) | − | + | − | ES | + | + | − | II | − |  |

[a] A and C refer to naturally occurring variations of penicillinase serotype (16). See Table 3 for explanation of marker abbreviations. A plus or a minus sign signifies a marker is phenotypically identifiable or is not. A blank space indicates that the test was not done or is inapplicable. ES = expressible sensitivity.

[b] $PI_{258}$, $PI_{524}$, and $PII_{147}$ were previously designated $\gamma$, $\alpha$, and $\beta$, respectively (10–12, 15, 16, 18).

[c] S. Cohen (*personal communication*).

strains. In practice, we have fallen somewhere short of this objective for a number of reasons. First, a significant omission was of plasmids carried by strains belonging to phage group II, which no one has succeeded in transducing into a group I or III strain for comparative purposes, and for compatibility studies. Second, although the group I and group III strains are probably a reasonable sample of what is prevalent in British hospitals, there were two possible causes of bias in selection: there was a limitation imposed by the inability of our transducing phages to propagate on some strains, and another imposed by the failure of some of the lysates of CadR strains to transduce cadmium resistance to the standard recipients. Insofar as these failures were due to modification-restriction systems, they introduced a bias that is probably due at least in part to genetic distance, which forced us to end up examining a relatively homogeneous group of strains.

Among the wild-type strains examined, none of the successful donors carried erythromycin resistance, a marker that has been found to be associated with penicillinase production in a series of Japanese strains (7). In a series of 25 American PenR EroR strains examined previously, in no case was erythromycin resistance coeliminated from penicillinase-negative segregants (R. Novick, *unpublished data*). Evidently, the plasmid linkage of erythromycin resistance found in Japan is a somewhat unusual occurrence. It could

TABLE 3. *Expression of resistance in different host strains*

| Strain | Salt | MIC[a] |
|---|---|---|
| | | M |
| 3761 | $Na_2AsO_4$ | $6.2 \times 10^{-3}$ |
| 8325($PI_{3761}$) | | $2.5 \times 10^{-2}$ |
| 1036 | | $7.5 \times 10^{-4}$ |
| 147($PI_{1036}$) | | $1.25 \times 10^{-2}$ |
| 8325 | | $6.2 \times 10^{-4}$ |
| 147(N) | | $6.2 \times 10^{-4}$ |
| 3761 | $Cd(NO_3)_2$ | $1.2 \times 10^{-4}$ |
| 8325($PI_{3761}$) | | $6.2 \times 10^{-3}$ |
| 8325 | | $1.6 \times 10^{-5}$ |

[a] Minimal inhibitory concentrations as obtained by twofold serial dilution test.

be the result of a local recombination event occurring in Japan or perhaps, since there is clearly more than one generic type of erythromycin resistance, it might be that a particular erythromycin determinant has evolved in Japan and happens to be or to become penicillinase-plasmid linked, whereas western EroR determinants, evidently genetically different, either are chromosomal or are borne by some other plasmid.

The assignation of plasmids to the same incompatibility set amounts to a prediction that they are themselves incompatible, a prediction that has not yet been tested for any of the new plasmids described here. Accepting for the moment the assumption that this prediction will prove true, we should like to suggest that elements of the same incompatibility set are much more closely related to one another than they are to elements of other incompatibility sets. We say this for the following reasons: evidence has been presented previously that a specific region of the plasmid, the *mcr* region, which is responsible for compatibility, is in some way attuned to a host maintenance system specific for elements of the same incompatibility set, and is required for autonomous replication (10). Two plasmids in the same incompatibility set are evidently attuned to the same host maintenance system and are therefore closely homologous or identical with respect to the *mcr* region. Two plasmids in different incompatibility sets are attuned to different host maintenance systems and are therefore less closely related. All of 24 plasmids examined thus far could be assigned unequivocally to either of the two known incompatibility sets defined by $PI_{524}$ and $PII_{147}$, the first two plasmids examined (11). There are no examples of plasmids showing behavior that would place them between the two or, incidentally, in a third set. This lack of intermediate types is probably indicative of fairly stringent structural requirements, *vis à vis* attunement to host maintenance systems, for autonomous survival; if elements belonging to the two sets have common ancestry, then intermediate types must have been selected against, in favor of their differentiated and fully adapted descendants.

In the general patterns of plasmid-linked markers, there is only one consistent difference between the $mcr_{I}$ and $mcr_{II}$ elements, namely, their reaction toward bismuth; it is presumably of taxonomic significance that all of the $mcr_{II}$ elements have the expressible bismuth sensitivity allele, whereas the $mcr_{I}$ elements are either bismuth-resistant or indifferent. Among other markers, possibly significant correlations include the finding that all $mcr_{I}$ $pen^{-}$ plasmids coded for penicillinase of serotype A; among the $mcr_{II}$ set, some coded for penicillinase A, some for C. Also, three of the four $pen^{+}$ plasmids were $mcr_{II}$. There was no correlation between penicillinase serotype and excretion pattern. If differences in serotype reflect differences in primary structure, then it would seem reasonable to conclude that primary structure per se does not determine excretion pattern and that a separate plasmid-linked locus is therefore responsible. Richmond (15) found that whatever does determine excretion pattern is *cis*-dominant in plasmid heterodiploids, so that the mechanism of action of the excretion (*exo*) determinant must be very interesting indeed.

We have not attempted to study determinants of penicillinase carried by staphylococcal strains belonging to phage group II; this study is planned and is expected to be informative, since the variety of penicillinase [type B of Richmond (17)] produced by these strains is rather different than those produced by strains of groups I and III. If the penicillinase determinants in group II strains are extrachromosomal, we shall not be surprised if they belong to one or more new incompatibility sets.

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