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Nature and Interactions of the Genetic Elements Governing Penicillinase Synthesis in *Staphylococcus aureus*

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ABSTRACT

NOVICK, RICHARD P. (The Rockefeller Institute, New York, N.Y.), AND MARK H. RICHMOND. Nature and interactions of the genetic elements governing penicillinase synthesis in *Staphylococcus aureus*. J. Bacteriol. **90**:467–480. 1965.—It has been found previously that penicillinase-producing strains of *Staphylococcus aureus* each harbor an extrachromosomal element, or plasmid, which apparently carries all the genetic information necessary for penicillinase synthesis. These plasmids behave in a manner consistent with their being small chromosomelike structures in that they comprise linkage groups containing several markers and in that they undergo such genetic events as mutation, segregation, and recombination. There is currently no evidence for conjugal cell-cell transfer of the plasmids or for a state of stable integration into the staphylococcal chromosome. A certain amount of variability has been encountered among the penicillinase plasmids harbored by different staphylococcal strains. It has been found that: (i) there are at least three molecular variants of the enzyme itself; (ii) most, but not all, of the penicillinase plasmids carry a genetic determinant of resistance to mercuric ion; (iii) plasmids carried by a very small number of the strains bear a determinant of resistance to erythromycin; (iv) the plasmids determine the fraction of penicillinase excreted into the medium during growth, and this also varies from strain to strain. The penicillinase plasmids appear to behave as integral genetic structures. The entire known linkage group is transduced intact, and is occasionally lost completely as a spontaneous occurrence during the growth of the organisms. Rarely, the plasmid markers dissociate during transduction, resulting in transduced clones which have inherited only a part of the plasmid linkage group. Similarly, dissociation occurs as a spontaneous event during normal growth, also resulting in rare clones which appear to have lost one or more but not all of the plasmid markers. When crosses are performed between two plasmid-harboring strains, a plasmid heterozygote is formed. In most cases, this persists for only one or a few cell divisions before segregating, with or without the formation of recombinant plasmids. In two instances thus far observed, the heterozygote persists as a stable plasmid heterodiploid, in which the continued presence of both plasmids can be readily demonstrated. The fate of the heterozygote, i.e., early segregation or persistence as a heterodiploid, depends upon which particular pair of plasmids is involved in the cross. This observation has led to the hypothesis of a plasmid-linked determinant of plasmid compatibility. A pair of plasmids is considered compatible if it can form a stable heterodiploid, incompatible if it cannot.

Evidence has previously been presented that the genetic determinant of penicillinase formation in *Staphylococcus aureus* is incorporated in an extrachromosomal element or "plasmid" (Novick, 1963); this conclusion has recently been supported by other workers (Harmon and Baldwin, 1964; Hashimoto, Kono, and Mitsuhashi, 1964).

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Briefly, the evidence is as follows. (i) The penicillinase determinant is lost freuqently ($\backsim 10^{-3}$ per cell per generation in many strains) and irreversibly (Barber, 1949; Bondi, Kornblum, and de Saint Phalle, 1953; Fairbrother, Parker, and Eaton, 1954; Novick, 1963). Certain other markers which, by the criterion of cotransduction, are linked to the penicillinase locus are lost coordinately with it. Among these are the locus or loci concerned with the inducibility of penicil-

linase synthesis (Novick, 1963) and a locus responsible for resistance to mercuric chloride (Richmond and John, 1964). Hashimoto, Kono, and Mitsuhashi (1964) reported that resistance to macrolide antibiotics (notably erythromycin) appears to be inherited extrachromosomally, linked to penicillinase, in two strains of *S. aureus*. In strain 258 (kindly supplied by S. Mitsuhashi) we found, in confirmation of the results of Hashimoto et al. (1964), that the penicillinase, macrolide resistance, and mercuric chloride resistance properties are all lost coordinately and are all cotransduced. (ii) Irradiation of transducing lysates with ultraviolet light produces exponential inactivation of the ability to transduce the penicillinase genes (*see* Garen and Zinder, 1955; Arber, 1960; Luria, Adams, and Ting, 1960). (iii) The appearance of segregants which have lost these properties is promoted in a population by growth in the presence of a variety of ordinarily nonmutagenic agents. Among these agents are high temperature (Bondi et al., 1953; May, Houghton, and Perrett, 1964), novobiocin (Novick, *unpublished data*), quinine, detergents, glucose, and tetracycline (Borowski, 1964), and acridine dyes (Harmon and Baldwin, 1964; Hashimoto et al., 1964), although in our hands acridine orange has been ineffective (Novick, 1964; P. H. A. Sneath, *personal communication*). It is possible that these agents inhibit selectively the replication of the penicillinase elements in a manner akin to the process of elimination of F episomes by acridine dyes (Hirota, 1960). It is also possible, however, that, in the presence of these agents, spontaneous penicillinase-negatives have a growth advantage and are thereby selected. In none of the examples cited above is there evidence in favor of one or the other of these possibilities. As yet, there is no evidence for a specific relationship between the penicillinase element and the staphylococcal chromosome, nor has infective cell-to-cell transfer of the capacity to produce penicillinase been observed. Consequently, we refer to the element carrying the penicillinase genes as a plasmid (*see* Lederberg, 1952), despite certain clear similarities between it and the resistance-transfer factors of the Enterobacteriaceae (Watanabe, 1963) which are considered to be episomes.

The pattern of plasmid-borne markers is variable among naturally occurring penicillinase-producing strains of *S. aureus*; the available evidence suggests, in fact, that there exists a family of related extrachromosomal elements, each harbored initially by a different wild-type host strain and each able to undergo recombination with the others. These elements can be transferred by transduction among various staphylococcal strains; the host organisms appear to play a relatively neutral role with regard to expression of most markers.

The introduction of a penicillinase plasmid into a host organism already harboring one (differently marked) produces what may be regarded as a heterozygote. This heterozygote may either segregate early, with or without recombinant formation, or it may persist as a stable plasmid heterodiploid. The heterodiploid survives successive subcultures, but segregants harboring one or the other of the original parental plasmids or harboring a recombinant plasmid appear continually at a relatively low frequency. The fate of the plasmid heterozygote depends upon which particular pair of plasmids is involved. From this, it is inferred that there is a plasmid-borne determinant of plasmid compatibility, i.e., the ability of a pair of plasmids to persist in the same host as a heterodiploid.

In this paper is presented a description of the properties of several penicillinase plasmids and their markers and some observations on their behavior in transductional crosses. The nature and occurrence of the plasmid heterodiploids are also considered.

In the following paper is presented a study of the control of penicillinase formation as analyzed through the plasmid heterodiploids (Richmond, 1965*a*).

MATERIALS AND METHODS

Strains and nomenclature. The strains of *S. aureus* used in these experiments are listed in Table 1. They are derived from naturally occurring strains—524SC (Rogers, 1953), NCTC 8325, 147 (Segalove, 1947; Richmond, 1965*b*), and 258 (Mitsuhashi et al., 1963). Strain Q4 was derived from 8325 by ultraviolet (UV) "curing" of 8325 of at least two prophages. We have modified the nomenclature of Novick (1963) for penicillinase-producing staphylococci to take into account the several different penicillinase plasmids which have been found and to provide flexibility for the inclusion of various plasmid-linked markers in the strain designations. We have also changed the designation of constitutive mutants from *c* to i^- since inducibility is now known to be the dominant state in penicillinase synthesis (Richmond, 1964, 1965*a*). We distinguish between the host organisms and their plasmids by retaining the original strain numbers of the hosts and inserting in parentheses a representation of the genotype of the plasmid involved. The generic Greek letter refers to the plasmid as a whole. It is followed by letters and subscript figures indicating the markers which it carries and which are pertinent to the cross under consideration. The complete genotypes of naturally occurring and mutant strains are given in Tables 1 and 2. The symbol *p* refers

TABLE 1. *Characterization and origins of the strains used*

Strain and genotype of penicillinase region	Derivation	Parental strain or source[a]	Donor	Recipient	Penicillinase type	Enzyme levels (units/mg, dry wt)		*Hg*[b]	*em*[c]
						Induced	Uninduced		
1. 8325 (=PS47)	Naturally occurring	Colindale	—	—	—	—	—	S	S
2. 8325(αi^+p^+) (=8325(α_w)	Transductant	—	524SC	No. 1[d]	A	10	300	R	S
3. 8325($\alpha i_1^-p^+$)	Spontaneous	No. 2	—	—	A	400	400	R	S
4. 8325($\alpha i^+p_2^-$)	EMS[e]-induced	No. 2	—	—	A_2[f]	0.3	15	R	S
5. 8325($\alpha i_{35}^-p_2^-$)	EMS-induced	No. 4	—	—	A_2	14	16	R	S
6. 8325($\beta i^-_{220}p^+$)	Transductant	—	No. 9	No. 1	C	150	250	R	S
7. 147(βi^+p^+) (=147(β_w)	Naturally occurring	Segalove (1947)	—	—	C	10	250	R	S
8. 147(N)	Spontaneous loss of plasmid	No. 7	—	—	—	—	—	S	S
9. 147($\beta i_{220}^-p^+$)	EMS-induced	No. 7	—	—	C	150	250	R	S
10. 147($\alpha i_1^-p^+$)	Transductant	—	No. 3	No. 8	A	350	350	R	S
11. 258(γi^+p^+) (=258(γ_w)	Naturally occurring	Mitsuhashi et al. (1963)	—	—	A	15	250	R	R
12. 258(N)	Spontaneous loss of plasmid	No. 11	—	—	—	—	—	S	S
13. 258($\gamma i_{443}^-p^+$)	EMS-induced	No. 11	—	—	A	250	250	R	R
14. 258($\gamma i^+p_{401}^-$)	EMS-induced	No. 11	—	—	A	1	18	R	R
15. 12865(δi^+p^+) (=12865(δ_w)	Naturally occurring	Colindale	—	—	B	1	50	R	S
16. V119(ϵi^+p^+) (=V119(ϵ_w)	Naturally occurring	Vanderbilt	—	—	A	—	—	S	S
17. V137(ζi^+p^+) (=V137(ζ_w)	Naturally occurring	Vanderbilt	—	—	C	—	—	S	S
18. 346(ηi^+p^+) (=346(η_w)	Naturally occurring	Colindale	—	—	C	—	—	R	S
19. 8835/62(θi^+p^+) (=8835/62(θ_w)	Naturally curring	Colindale	—	—	A	—	—	R	S
20. Q4	UV "cured" of two prophages	8325	—	—	—	—	—	S	S
21. Q4(γi^+p^+) (=Q4(γ_w)	Transductant	—	No. 11	No. 20	A	18	250	R	R
22. Q4($\gamma i_{443}^-p^+$)	Transductant	—	No. 13	No. 20	A	250	250	R	R
23. Q4($\beta i_{220}^-p^+$)	Transductant	—	No. 9	No. 20	C	150	250	R	S

[a] Colindale = Cross Infection Reference Laboratory, Central Public Health Laboratory, London, England; Vanderbilt = isolated from clinical material at Vanderbilt University Hospital, Nashville, Tenn.

[b] *Hg* = mercuric ion. R indicates that single colony-forming units can grow on plates with 2×10^{-5} M Hg^{++}. S indicates that single colonies cannot grow on 2×10^{-6} M Hg^{++}.

[c] *em* = erythromycin. R indicates that normal growth occurs in the presence of erythromycin, 400 μg/ml. S indicates that no growth occurs in the presence of erythromycin, 1 μg/ml.

[d] Numbers refer to tabulation in first column.

[e] EMS = ethyl methane sulfonate.

[f] A_2 enzyme is a mutated penicillinase having approximately 5% of the specific enzyme activity of the normal type A enzyme.

to the structural gene for penicillinase and i, to a penicillinase inducibility locus; mutations in the p and i loci are numbered in series. We use a separate set of numbers for the family of structural mutants and another for the family of constitutive mutants fathered by each plasmid, the appropriate number of the mutant being placed as a subscript after i or p. The wild-type or naturally occurring state is designated w where necessary.

Strains that carry no penicillinase plasmid fall into two classes: those that are naturally occurring plasmid-negatives and those that are plasmid-loss variants of penicillinase-positive cultures. The former are designated simply by their strain numbers, e.g., 8325, and the latter have (N) suffixed, e.g., 147(N), for emphasis. In cases where recombination has occurred between different plasmids, the designation indicates which of the markers in the recombinant comes from each of the parent plasmids, as far as can be ascertained. Thus, Q4($\alpha i^+p^+ - - - \gamma em^r$) designates strain Q4 carrying a recombinant element, the penicillinase region being that of α and the erythromycin-resistance marker contributed by γ. In plasmid heterodiploids, the respective plasmid genotype designations are separated by an oblique stroke, e.g., 147($\alpha i^+p_2^-/\beta i_{220}^-p^+$).

The second column of Table 1 indicates the route by which the strain was obtained, and the third column indicates the source in the case of naturally occurring strains and the parentage in the case of mutants. The next two columns indicate the donor and recipient of the cross by which those strains which are transductants were constructed. The next two columns indicate the basal and methicillin-induced penicillinase activities [units per milligram (dry weight) of organisms] of those strains for which these measurements have been carried out, and the final two columns list the sensitivity or resistance of the strains to mercuric ion and to erythromycin.

The bacteriophage strains used for transduction were 80 (of the International Typing Series), 80α, 47′, and P147. Phage 80α appears to be a host-induced modification of phage 80 (Novick, 1963). Phage 47′ is a temperate phage carried by strain 8325, and P147 is a temperate phage carried by strain 147.

Media. The media used, CY and 0.3CY, were described previously (Novick, 1963) with the exception that 0.05 M tris(hydroxymethyl)aminomethane (Tris) was substituted for sodium β-glycerophosphate in certain cases. BY medium was used to prevent the lysis of transductants when the recipients were phage-sensitive. Its components were, per liter: Brain Heart Infusion (Difco), 18 g; yeast extract (Difco), 10 g; and agar (Difco), 15 g; the pH was adjusted to 7.6.

Growth conditions, UV induction of phages, production of mutations with ethyl methane sulfonate (EMS), penicillinase assay methods, and transduction procedures have been described previously (Novick, 1963). Penicillin G was a gift of Chas. Pfizer & Co., Inc., New York, N.Y.; methicillin was a gift of Bristol Laboratories, Syracuse, N.Y.; erythromycin was a gift of Eli Lilly & Co., Indianapolis, Indiana.

Exopenicillinase measurements. Because the extracellular fraction of total penicillinase activity varies during the growth of the cultures (Novick, 1962), this determination was always carried out with cultures that had been grown under standard conditions. Exponentially growing cells were inoculated into prewarmed CY medium to give a density of 0.02 mg (dry weight) per ml and shaken at 37 C for 3.5 hr. The cultures were then iced, samples were centrifuged at 4 C for 10 min at 8,000 $\times$ g, and the penicillinase activities of the whole cultures and supernatant fluids were determined. In the case of inducible strains, methicillin was included in the medium as inducer at a concentration of 1.5×10^{-6} M. This inclusion had no detectable effect on the liberation of penicillinase.

Detection of penicillinase in colonies. Penicillinase activity was detected by two methods. The starch-iodine technique of Perret (1954) was used only where great sensitivity was needed, as it gives a positive result for colonies with as little penicillinase activity as 0.05 units per mg (dry weight) of organisms. A modification of the *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene (PNCB) test of Novick (1963) was developed which gave very good resolution of penicillinase-positive and -negative subclones within single colonies.

For this test, the organisms were grown at 37 C on unbuffered 0.3CY agar with an initial pH of 7.6. After 48 hr of incubation, the plates were dried at 37 C for 2 hr with lids off. The agar surface was then flooded with 1.5 ml of an 0.25% (w/v) solution of the acid-base indicator, PNCB, in *N*,*N*-dimethyl formamide with 6% (v/v) 1 N NaOH. The plates were allowed to dry in the hood, and the stain was developed by flooding with 1.5 ml of an aqueous penicillin G solution. The acid released by the hydrolysis of penicillin changed the indicator from yellow to purple. The penicillin concentration used depended on the expected penicillinase activity of the colonies. Thus, for organisms with low activity (from 2 to 10 units per mg, dry weight), 10% (w/v) penicillin was used; for intermediate activity (from 10 to 50 units per mg, dry weight), 3% penicillin was used; and for high activity (>50 units per mg, dry weight), 1% penicillin was used. The lower limit of detectability of penicillinase activity in colonies was about 2 units per mg (dry weight). Viable organisms could readily be recovered from stained colonies for up to 6 hr; after 18 hr, there was usually little or no residual viability. In Fig. 1 is shown a photograph of colonies of a mixed culture of penicillinase-positive and -negative organisms. The dark areas are penicillinase-positive. The sharp resolution of adjacent positive and negative areas should be noted. This is accounted

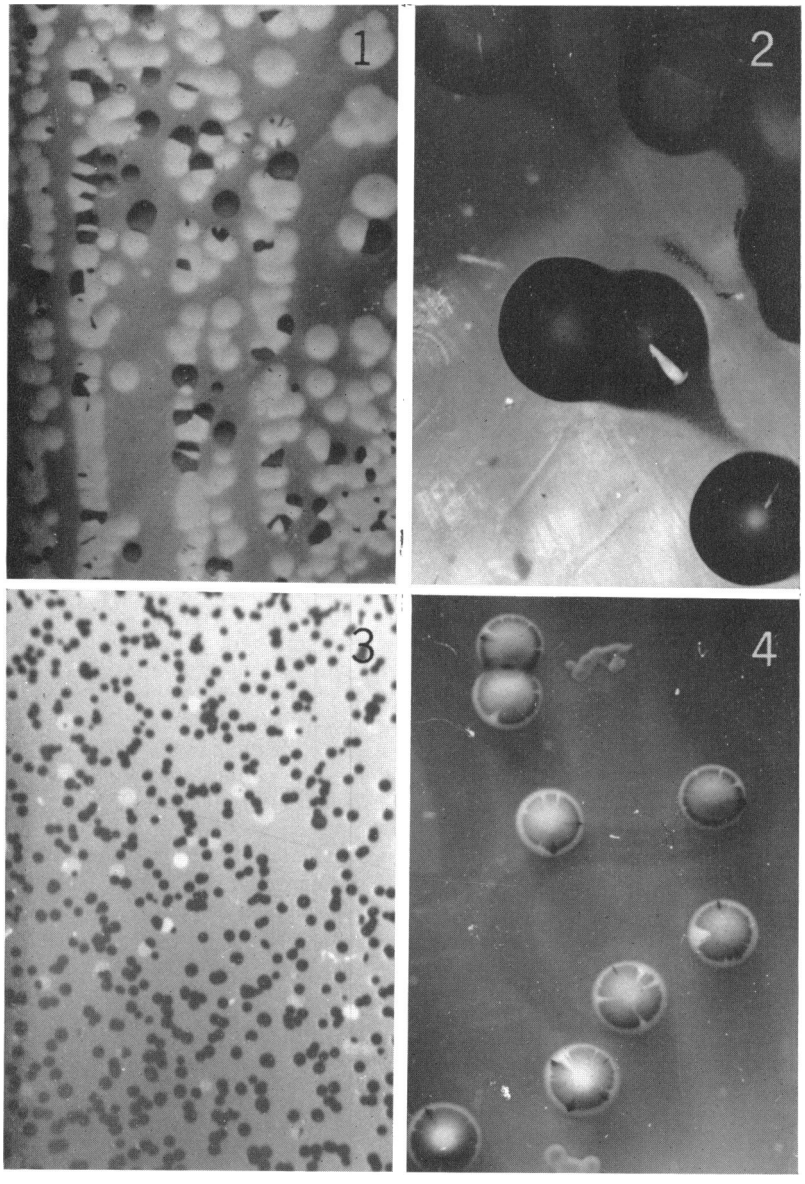

FIG. 1. *Colonies of a mixed culture of penicillinase-positive and -negative staphylococci stained for penicillinase. The dark colonies and areas represent penicillinase-positive clones. The light represent penicillinase-negatives.*

FIG. 2. *Colonies of a penicillinase-positive culture stained for penicillinase. The sharply defined white sectors seen in three of the colonies represent spontaneous penicillinase-negative segregant subclones.*

FIG. 3. *Colonies of an EMS-treated, penicillinase-positive culture plated as a soft-agar overlay and subsequently stained for penicillinase. The white colonies represent plasmid-loss variants or penicillinase-negative mutants.*

FIG. 4. *Colonies of a plasmid merodiploid, 8325($\alpha i^+ p_2^-/\beta i_{220}^- p^+$), stained for penicillinase. The dark sectors represent segregant clones harboring the parental β plasmid (constitutive for penicillinase C production); the light sectors represent segregant clones harboring the parental α plasmid (inducible for pencinillinase A_2 production).*

TABLE 2. *Markers carried by some of the penicillinase plasmids in Staphylococcus aureus*

Plasmid	Penicillinase type	*Hg*	*em*	*exo**	Extracellular fraction of penicillinase (per cent of total)	Original host strain
α	A	R	S	+	45	524SC
β	C	R	S	−	5–10	147
γ	A†	R	R	+	25	258
δ	B	R	S	−	5–10	12865
ϵ	A†	S	S	+	45	V119
ζ	C†	S	S	+	40	V137
η	C†	R	S	+	50	346
θ	A†	R	S	−	10	8835/62

* *exo* = extracellularity of penicillinase. Plus indicates liberation of 25% or more. Minus indicates liberation of 10% or less.

† Classified by serological criteria.

for by the insolubility of the purple, acidic form of the indicator.

This test was used mainly for detecting spontaneous penicillinase-negative segregants (Fig. 2), for scoring transductional crosses, and for observing segregation patterns in certain transductant clones (see Fig. 4). In addition, it was used for detecting penicillinase-negative and -constitutive mutants after mutagenic treatments of wild-type populations. For this purpose, the treated organisms were plated as overlays in 1.5 ml of soft (1%) agar and overlaid with an additional 1.5 ml of sterile 1% agar to prevent spreading surface growth. The test was carried out as described; by this method, 10^5 colonies could easily be screened on a single plate. Figure 3 shows a photograph of a small area of a plate with 10^4 colonies of an EMS-treated culture; the white colonies are penicillinase-negative mutants or plasmid-loss segregants.

A further application of the test was a rapid screening test for penicillinase inducibility. For this purpose the cultures to be tested were spotted on plain 0.3CY plates and on 0.3CY plates containing 10^{-6} M methicillin as an inducer of penicillinase. After 24 hr of incubation at 37 C, the inducible cultures gave a much more rapid color reaction on the methicillin plates than on the plates lacking inducer.

Transduction procedures. The production of transducing lysates and the infection of recipient cultures were described previously (Novick, 1963). When the donor carried erythromycin resistance linked to the penicillinase locus, selection of transductants was usually for erythromycin resistance, penicillinase production being scored as an unselected marker by the PNCB test. For optimal recovery of erythromycin-resistant transductants, a 3-hr period was required for phenotypic expression. The transduction mixtures were plated in 2-ml overlays of molten 1% agar, incubated at 37 C for 3 hr, and then overlaid with an additional 1.5 ml of molten 1% agar containing 400 μg of erythromycin per ml. The final erythromycin concentration was thus about 20 μg/ml. Plates were scored after a further 36 hr at 37 C. Selection of penicillinase-producing transductants on the basis of penicillin resistance was also carried out as described (Novick, 1963); where the recipient strain was a mutant with low residual penicillinase activity, careful adjustment of the selective penicillin concentration and of the number of recipient organisms plated was essential. A detailed description of the procedure for penicillin selection against strain 8325($\alpha i^+ p_2^-$), a mutant with 5% of wild-type penicillinase activity, is given in the following paper (Richmond, 1965*a*). The conditions for penicillin selection against other mutants with different residual penicillinase activities would be different and would have to be worked out in each case.

RESULTS

Genetic Characteristics of Plasmids

A description of eight different plasmids encountered to date is presented in Table 2. The assignment of Greek letter status to a newly observed element depends solely upon its having a novel combination of markers. We have studied particles α, β, and γ almost exclusively; the others are included to illustrate the additional diversity which has been encountered and to demonstrate certain specific markers. A brief description of the various markers follows.

Penicillinase. The penicillinase locus is carried by all of the plasmids listed, as it has so far been instrumental in the identification of the elements. However, as will be described in a later section, certain segregants may occur which appear to have deletions for one or another part of the plasmid. We therefore anticipate a series of naturally occurring plasmids homologous in part to the members of the present series but not carrying the penicillinase marker.

The penicillinase locus itself is complex and consists of a structural locus, a classical inducibility locus analogous to the *i* locus for the β-galactosidase operon in *Escherichia coli*, and possibly a second control locus which appears to fill some of the criteria for an operator. The behavior of the *i* locus will be described in the following paper (Richmond, 1965*a*). The region as a whole has not yet been mapped in any detail, but it is at least likely that the structural (*p*) and inducibility (*i*) loci are closely linked (Novick, 1963).

By serological and enzymological criteria, the penicillinases of different, naturally occurring staphylococcal strains have been differentiated into three variants or classes, indicated as A, B, and C in Table 1 (Richmond, 1965*b*). Two of these, types A and C produced by strains harboring the

plasmids α and β, respectively, have been purified and their amino acid compositions have been determined (Richmond, 1965*b*). Chromatography of tryptic digests shows that enzyme A has a few differences in primary sequence from enzyme C. Enzymes A and C are found in staphylococcal strains belonging to both phage groups I and III, but penicillinase B has been found only in strains that fall into phage group II.

A variety of structural and control mutations involving the penicillinase loci of the α-plasmid have been produced with EMS, and have been described previously (Novick, 1963). The types and frequencies of mutations induced in the other plasmids have conformed thus far to the pattern observed with α. It is perhaps consistent with its extrachromosomal state that the penicillinase region is highly susceptible to mutagenic attack by EMS; structural and control mutations have each been obtained at a frequency approaching 1% with this agent.

Resistance to mercuric ion (Hg). It has been known for some time that there is an association between penicillinase production in staphylococci and resistance to mercuric ion (Moore, 1960; Richmond et al., 1964). However, the actual genetic linkage of these two characters has been established only recently (Richmond and John, 1964). Resistance to $HgCl_2$ is inconvenient as a selective marker because there is only a 10-fold difference in level of resistance between resistant and sensitive organisms. Further, the resistance level is enhanced by increasing the size of the test inoculum, an effect which complicates the scoring of the marker. Nevertheless, it has been possible to show that resistance to mercuric ion is virtually always eliminated and transduced coordinately with penicillinase (Richmond and John, 1964).

Resistance to macrolide antibiotics (abbreviated em, for erythromycin). The only strains that have thus far been described in which macrolide resistance is linked to penicillinase production are those reported by Hashimoto et al. (1964). Twenty-five additional strains that were erythromycin-resistant and penicillinase-positive were screened by selecting penicillinase-loss variants and testing for concomitant loss of erythromycin resistance. No further instance of association of the two was found by this test. Thus, it appears that this association is infrequent. There is at present no evidence bearing on the question of whether or not the erythromycin-resistance marker was acquired from the chromosome by the plasmid in a recombinational event akin to that involved in the acquisition of chromosomal markers by the F factor of *E. coli* (Jacob and Adelberg, 1959).

Like the mercuric-resistance marker, resistance to erythromycin is coeliminated and cotransduced with penicillinase at a frequency greater than 99%.

Extracellularity (exo). Staphylococcal penicillinase is partially extracellular in most strains studied. Measurement of the proportion of free and cell-bound penicillinase has been carried out with several strains, with the use of constitutive mutants to avoid possible variability due to interstrain differences in the induction process. The results (Table 2) indicate that, in strains carrying α, γ, or η plasmids, 25 to 45% of the enzyme is liberated into the medium. In strains carrying plasmids β or θ, only 5 to 10% of the enzyme is liberated.

The proportion of enzyme present in the extracellular form is a genetic characteristic of the plasmid and not of the host strain harboring it. When strain 147($\alpha i_1^- p^+$) was constructed by transducing the α plasmid from 8325($\alpha i_1^- p^+$) to 147 (*N*), it was found to release 41% of its penicillinase into the medium under standard conditions; this liberation pattern is the same as that originally observed for the donor strain 8325($\alpha i_1^- p^+$). Further, when 8325($\beta i_{220}^- p^+$) was constructed by transduction of the β plasmid from 147($\beta i_{220}^- p^+$) to 8325, it was found to release 5%

TABLE 3. *Cotransduction of the genetic determinants responsible for the degree of extracellularity of penicillinase together with the penicillinase control and structural loci*

Strain	Genotype	Total penicillinase [units/mg (dry wt) of bacteria]	Extracellular penicillinase [units/mg (dry wt) of bacteria]	Per cent extracellular
Donor strain ...	8325 ($\alpha i_1^- p^+$)	358	116	45
Acceptor strain ...	147 (N)	Nil	Nil	—
Transductants.	147($\alpha i_1^- p^+$)	358*	146*	41
Donor. strain ...	147($\beta i_{220}^- p^+$)	245	20 (max)	8
Acceptor strain ...	8325	Nil	Nil	—
Transductants.	8325($\beta i^-_{220} p^+$)	245	12	5

* Indicates results that are the mean of 12 measurements on separately isolated penicillinase-producing transductants. In all the cultures, the degree of extracellularity was between 37 and 44%.

of its enzyme and thus likewise to have the liberation pattern characteristic of the donor strain, $147(\beta i_{220}^{-} p^{+})$. These results (Table 3) suggest that the degree of extracellularity of penicillinase A is under the control of plasmid α, and that of penicillinase C is under the control of plasmid β. In neither case was the host strain involved found to have any specific influence on the release of the enzyme.

It is possible that the degree of extracellularity is a property of the enzyme molecule itself. Against this possibility is a series of results with certain other naturally occurring staphylococcal strains. Thus, a strain carrying plasmid θ produces a penicillinase that behaves serologically like penicillinase A but has a very different liberation pattern from strains carrying plasmid α (which all produce penicillinase A). Similarly, a strain carrying plasmid η makes a penicillinase that is serologically indistinguishable from penicillinase C but releases 50% of its enzyme, in contrast to strains carrying plasmid β. The latter all make penicillinase C but release only 5 to 10%. These results are shown in Table 2.

In support of the possibility that the degree of extracellularity is a property of the enzyme molecule are some results described in the accompanying paper (Richmond, 1965*a*). In this case, it appears that the two distinct varieties of penicillinase made by a plasmid heterodiploid are released independently of one another and in the same proportions that are observed in their liberation from cells carrying only single plasmid types.

Further investigation will, therefore, be necessary before it can be decided to what degree the liberation process is determined by a property of the enzyme molecule and to what degree by the plasmid governing its production.

Genetic Behavior of Plasmids

Integrity of plasmids. The three plasmids, α, β, and γ, that have thus far been studied in crosses behave ordinarily as discrete genetic units. That is, the markers which they bear seem usually to be all transduced together in crosses and all to disappear from the host strains when spontaneous loss occurs. Linked transduction and spontaneous loss could be most conveniently studied with strains harboring the γ plasmid which carries erythromycin resistance as well as mercuric ion resistance and the penicillinase loci. In an experiment to isolate and test spontaneous-loss variants, three 0.3CY plates were each seeded with 3×10^4 colony-forming units of strain $258(\gamma i_{443}^{-} p^{+} Hg^{r} em^{r})$ in soft-agar overlays, incubated for 18 hr at 37 C, and then stained for penicillinase. A total of 110 penicillinase-negative colonies were detected and isolated, and all were found to have lost erythromycin resistance and mercuric ion resistance as well as penicillinase. The same type of experiment with strains harboring plasmid α has shown that mercuric ion resistance is virtually always missing from spontaneous penicillinase-loss variants. The results with plasmid γ are shown in Table 4.

Similarly, the plasmid linkage group is generally transferred intact by transducing phage from penicillinase-positive donor strains to plasmid-negative recipients. This linked transfer was demonstrated by a transductional cross between $258(\gamma_w)$, donor, and 8325, recipient, mediated by phage 80. The phage-infected recipient culture was plated for selection with penicillin and separately with erythromycin. All of 400 transductants selected for penicillin resistance were found also to have inherited resistance to erythromycin. On the other hand, on staining 4,880 erythromycin-resistant colonies for penicillinase, 4,875 were found to be positive. Comparable results have been obtained for cotransduction of penicillinase production and resistance to mercuric ion (Richmond and John, 1964).

Although the plasmid linkage group is for the most part quite stable, several instances of dissociation between the markers have been observed. Typical results are shown in Table 5 and include the experiment just cited, in which 5 of 4,880 transductants failed to receive the penicillinase loci. In subsequent experiments, this type of clone varied from 0.1 to 1% of the total erythromycin-resistant transductants. It appears on the following grounds that the penicillinase locus has been deleted in all of 80 of the $pen^{s} em^{r}$ transductants that have been examined: (i) none had detectable penicillinase activity [in over 100 apparent point mutants for penicillinase, we have been unable to find a single one that did *not* have measurable residual penicillinase activity (*see* Novick, 1963)]; (ii) 5 of the 80 had failed to acquire mercuric ion resistance from the donor; (iii) of 17 that were examined in greater detail, all failed to give penicillinase-positive revertants on plating 3×10^9 colony-forming units of each on plates containing 0.1 μg of penicillinase per ml; (iv) these same 17 were used as recipients in a cross with $Q4(\beta i_{220}^{-} p^{+})$ as donor, and erythromycin-resistant, penicillinase-positive transductants were selected. In each cross, over 100 transductants were examined, and all were found to be constitutive for penicillinase, suggesting that the recipient strains were all lacking the *i* locus. This apparently means that the recipient strains had received neither the *i* locus nor the *p* locus

TABLE 4. *Spontaneous loss and dissociation of plasmid markers*

Strain	Medium	Total colonies scored	Clones harboring dissociant plasmids		
			Genotype	Total	Frequency
258($\gamma i_{443}^{-}p^{+}Hg^{r}em^{r}$).....	0.3CY	9×10^{4}	plasmid^{-}*	110	1.2×10^{-3}
258(γ_w).....	0.3CY + erythromycin, 10 μg/ml	3×10^{5}	pen^{s}†$Hg^{r}em^{r}$	7	2×10^{-5}
258(γ_w).....	0.3CY + penicillin, 0.6 μg/ml	1.8×10^{4}‡	$i^{+}p^{+}Hg^{s}em^{s}$	2	1×10^{-4}

* Plasmid^{-} signifies that the entire plasmid linkage group is absent.
† pen^{s} signifies that the strain is as sensitive to penicillin as plasmid^{-} variants.
‡ Scored by replication.

TABLE 5. *Dissociation of plasmid markers during transduction*

Donor	Recipient	Selective agent	Total transductants scored	Clones harboring dissociant plasmids		
				Genotype	Total	Frequency
147($\beta i^{+}p^{+}Hg^{r}$)	147(N)	Penicillin	304*	$i^{+}p^{+}Hg^{s}$	2	7×10^{-3}
147($\beta i^{+}p^{+}Hg^{r}$)	147(N)	$Hg(Cl)_2$	148*	$pen^{s}Hg^{r}$	2	1×10^{-2}
258($\gamma i^{+}p^{+}Hg^{r}em^{r}$)	8325	Penicillin	400†	$i^{+}p^{+}em^{s}$	0	$<2.5 \times 10^{-3}$
258($\gamma i^{+}p^{+}Hg^{r}em^{r}$)	8325	Erythromycin	4,880	$pen^{s}Hg^{r}em^{r}$	3	6×10^{-4}
				$pen^{s}Hg^{s}em^{r}$	2	4×10^{-4}
Q4($\gamma i^{+}p^{+}Hg^{r}em^{r}$)	Q4	Erythromycin	6,632	$pen^{s}Hg^{r}em^{r}$	77	1.2×10^{-2}
				$pen^{s}Hg^{s}em^{r}$	3	5×10^{-4}

* Data taken from (Richmond and John1964).
† Scored by ratioeplicn.

from the original donor, 258(γ_w), in the transductional cross during which they did receive the erythromycin-resistance locus.

For these reasons, those few clones which failed to inherit one or more of the plasmid markers can be regarded as harboring dissociated plasmids. In most cases, the transductant clones harboring dissociated plasmids were pure; occasionally they occurred in sectored colonies which also contained clones harboring complete donor type plasmids. This observation suggests that the dissociation of plasmid markers can occur either before the formation of the transducing phage particle or after the introduction of the plasmid into the recipient. No dissociated plasmids lacking erythromycin resistance but retaining penicillinase were detected in any of the 400 transductant clones selected for penicillinase and scored for erythromycin resistance.

Dissociated plasmids have also been observed in crosses using 147(β_w) as donor and 147(N) as recipient (Richmond and John, 1964). In this case, with selection for penicillin, about 1% of transductants failed to receive resistance to mercuric ion; with selection for mercuric resistance, about 1% failed to receive penicillinase (Table 5).

Plasmid dissociation has also been observed as an occasional spontaneous event apart from the process of transduction. This was observed with strain 258 (γ_w). To eliminate from consideration those clones which had lost the entire plasmid, erythromycin (10 μg/ml) was included in the agar. When 3×10^{4} colony-forming units were plated on each of 10 plates and incubated at 37 C for 18 hr, on staining for penicillinase, a total of 7 penicillinase-negative colonies was observed and isolated. These clones were all resistant to mercuric ion, but in other respects the plasmids were similar to the dissociated plasmids observed in the transduction experiments (see Table 4).

In a similar experiment, about 600 colony-forming units were spread on each of thirty 0.3CY plates containing 0.6 μg of benzyl penicillin per ml (to eliminate complete loss variants) and incubated for 24 hr at 37 C. The plates were then replicated onto erythromycin (10 μg/ml) plates and, after further incubation, two colonies were detected that failed to grow in the presence of erythromycin. Both were found to be penicillinase-

positive but to have lost erythromycin resistance. They were also sensitive to mercuric ion. Thus, spontaneous dissociation appears to be similar to dissociation during transduction but less frequent. Dissociated plasmids carrying all possible marker combinations have not yet been sought, but among the dissociation patterns studied, no restriction has been found. It appears probable that dissociation involves physical loss of part of the plasmid, and it is expected that further study of dissociation patterns and recombination between strains harboring dissociated plasmids will provide information about the physical organization of these elements.

Interactions of Plasmids

By using appropriately marked strains, it has been possible to introduce penicillinase plasmids into plasmid-harboring hosts. It is proposed that the resultant cells, harboring two distinctly marked elements, be called plasmid heterozygotes. It has been found that when two plasmids are in a single cell they interact in different ways with one another and with their host, depending upon which particular plasmids are present. In the following presentation of observations on the fate of such plasmid heterozygotes, the terms "isogenic" and "heterogenic" are used in reference to pairs of plasmids. By "isogenic" is meant derived from the same plasmid line, differing at one or a few point mutational sites, but otherwise isologous. By "heterogenic" is meant derived from different naturally occurring plasmid lines, differing in an unknown number of regions of unknown extent, but presumably homologous, at least for the penicillinase region.

Fate of isogenic plasmid heterozygotes. In this category, only crosses between strains harboring α plasmids have been analyzed. For one such cross, 8325($\alpha i_1^- p^+$) (donor) × 8325 ($\alpha i^+ p_2^-$) (recipient), phage 47′ was obtained by UV induction of the donor, and 2.5×10^7 plaque-forming units (PFU) were used to infect the acceptor culture at a calculated multiplicity of 1.1. After infection, washing of the infected cells, plating, and incubation, 250 penicillin-resistant transductants were obtained. This gave a yield of about 10^{-5} transductants per PFU, a typical result for this phage.

The transductants were isolated and tested for penicillinase inducibility and activity in comparison with stock cultures of the donor strain, 8325($\alpha i_1^- p^+$), and with $i^+ p^+$ and $i^- p^-$ strains corresponding to the possible recombinant types in the cross, i.e., 8325($\alpha i^+ p^+$) and 8325($\alpha i_1^- p_2^-$). The results are listed in Table 6. Of the 250 transductants, 231 were indistinguishable from the donor strain, 12 had the $i^- p^-$ phenotype, and 7 had the $i^+ p^+$ phenotype. Thus, both reciprocal recombinant types were observed, occurring at comparable frequencies corresponding to roughly 4% of the overall transduction rate.

From the results of this experiment, it is suggested that the heterozygote usually segregates early, giving, with respect to the penicillinase locus, a donor-type and a recipient-type daughter cell. Of these, only the former can grow into a colony; the latter would be indistinguishable from the rest of the unchanged acceptor cells. Occasionally, recombination occurs prior to segregation, giving $i^+ p^+$ or $i^- p^-$ types, but it has not yet been ascertained whether the reciprocally recombinant plasmids are both produced in the same recombination event. The results of other crosses between strains harboring α plasmids have been

TABLE 6. *Transductional cross involving two strains harboring isogenic plasmids*

Designation	Genotype	No. of colonies	Per cent of total transductants	Penicillinase levels (units/mg, dry wt)	
				Uninduced	Induced
Parental types					
Acceptor	8325($\alpha i^+ p_2^-$)	—	—	0.3*	15
Donor	8325($\alpha i_1^- p^+$)	—	—	360	370
Transductant types					
Donor	8325($\alpha i_1^- p^+$)	231	92	360	350
Recombinants	8325($\alpha i^+ p^+$)	7	3	4.7	300
	8325($\alpha i_1^- p_2^-$)	12	5	14	16
Stock cultures corresponding to recombinant phenotypes	8325($\alpha i^+ p^+$) [= 8325(α_w)]	—	—	5.2	310
	8325($\alpha i_{35}^- p_2^-$)	—	—	14	16

* All enzyme activities represent the means of six determinations.

presented previously (Novick, 1963). In these crosses, recombination between different structural mutants as well as between structural and control mutants was observed.

Fate of heterogenic plasmid heterozygotes. In this category, three different crosses have been analyzed, involving α/γ, α/β, and β/γ heterozygotes. The α/γ heterozygotes segregated early, similarly to the α/α type, whereas the α/β and β/γ heterozygotes persisted as stable plasmid heterodiploids.

Fate of α/γ heterozygotes. For a cross between Q4($\gamma i_{443}^{-}p^{+}em^{r}$), donor, and 8325($\alpha i^{+}p_{2}^{-}$), recipient, phage 47′, propagated on the donor strain, was used to infect the recipient at a calculated multiplicity of 2. Selection was carried out for erythromycin resistance, and a total of 2,712 colonies was obtained on 10 plates. On staining for penicillinase by the PNCB test, the great majority appeared pure and was of the donor type, that is, $i^{-}p^{+}em^{r}$; about one-sixth appeard pure and was of the recombinant type, $i^{+}p^{-}em^{r}$; 2% were sectored, each containing one or more stable subclones of genotypes $i^{-}p^{+}em^{r}$ (donor) and $i^{+}p^{-}em^{r}$ (recombinant); a very small number of colonies was of the recombinant phenotype, $i^{+}p^{+}em^{r}$, and none was detected of the recombinant type, $i^{-}p^{-}em^{r}$. These results are shown in Table 7.

Again, in this cross, it is clear that the heterozygote most often underwent early segregation without the formation of recombinants for the test markers. In about 18% of the cases, recombination between the penicillinase and erythromycin regions occurred, giving pure or mixed $i^{+}p^{-}em^{r}$ clones (the reciprocal in this case, $i^{-}p^{+}em^{s}$, would have been lost because of the selection for erythromycin resistance). The occurrence of sectored colonies indicates that the heterozygote can survive at least one division cycle. If the assumptions are made that the gross appearance of a colony is a reflection of events which occurred very early in its growth, and that the heterozygote has a constant probability of segregating at each cell division, then it can be estimated that about 5% of α/γ plasmid heterozygotes survive a cell division.

Fate of α/β and β/γ heterozygotes. When a cross was performed between 147($\beta i_{220}^{-}p^{+}$), donor, and 8325($\alpha i^{+}p_{2}^{-}$), recipient, in which phage P147 obtained by UV induction of the donor strain was used and selection was for penicillin resistance, 120 colonies were obtained on two plates. Twelve were isolated, and all were found to have the wild, $i^{+}p^{+}$, phenotype. These, however, were not harboring recombinant plasmids, as they were observed to segregate pure-breeding clones carrying plasmids corresponding to the original parental types, $i^{-}p^{+}$ and $i^{+}p^{-}$. These transductant clones, therefore, represent persistent plasmid heterodiploids, 8325($\alpha i^{+}p_{2}^{-}/\beta i_{220}^{-}p^{+}$); a photograph of heterodiploid colonies, stained for penicillinase, is shown in Fig. 4. The dark sectors represent segregants harboring the $i^{-}p^{+}$ parental plasmid; the light sectors represent segregants harboring the $i^{+}p^{-}$ parent; the basal penicillinase activity of the heterodiploid is responsible for the intermediate depth of stain over the bulk of the colonies.

Stable $i^{+}p^{+}$ and $i^{-}p^{-}$ recombinant plasmids have been found among the segregants of the α/β heterodiploid but only after UV irradiation (Richmond, 1965*a*). A detailed analysis of the

TABLE 7. *Transductional cross involving two strains harboring heterogenic plasmids*

Designation	Genotype	No. of colonies	Per cent of total transductants	Penicillinase levels (units/mg, dry wt)	
				Uninduced	Induced
Parental types					
Acceptor	8325($\alpha i^{+}p_{2}^{-}$)	—	—	0.3	15
Donor	Q4($\gamma i_{443}^{-}p^{+}em^{r}$)	—	—	250	250
Transductant types					
Donor	8325($\gamma i_{443}^{-}p^{+}em^{r}$)	222	82	250	250
Recombinants	8325($\alpha i^{+}p_{2}^{-}$---γem^{r})	427	16	0.3	15
	8325(αi^{+}---$\gamma p^{+}em^{r}$)*	11	0.4	10	150
	8325(αp_{2}^{-}---$\gamma i_{443}^{-}em^{r}$)	0	<0.04	—	—
Mixed clones†	8325($\gamma i_{443}^{-}p^{+}em^{r}$) + 8325($\alpha i^{+}p_{2}^{-}$---$\gamma em^{r}$)	52	2	—	—

* The genotype assigned this class is tentative.
† See toxt.

properties of this heterodiploid is presented in the accompanying paper (Richmond, 1965*a*).

The zygotes formed in one other cross, 258($\gamma i^+ p_{401}{}^- em^r$), donor, × 147($\beta i_{220}{}^- p^+$), acceptor, also persisted as stable heterodiploids, 147($\beta i_{220}{}^- p^+ / \gamma i^+ p_{401}{}^- em^r$), which likewise continually produced donor-type and recipient-type segregants. No stable i^+p^+ or i^-p^- recombinants have been observed, but segregant clones harboring the reciprocally recombinant plasmids $i^+p^-em^s$ and $i^-p^+em^r$ commonly appeared.

DISCUSSION

The results presented here and elsewhere (Harmon and Baldwin, 1964; Hashimoto et al., 1964) support the conclusion that the synthesis of penicillinase in *S. aureus* is determined by an extrachromosomal element (Novick, 1963). This element, on the basis of evidence currently available, does not qualify as an episome, because there is as yet no definite evidence that it has a specific relationship to the host chromosome (Hayes, 1964), and it has, therefore, been termed a plasmid, since this is felt to be the more general term (Lederberg, 1952).

It has been found that there are several molecular variants of the enzyme (Richmond, 1965*b*), and that in naturally occurring strains of *S. aureus* there is considerable diversity in the markers linked to the penicillinase locus. These observations show that there are a number of related but genetically distinct plasmids which control penicillinase formation. These different plasmids appear to be at least partially homologous in that all carry a penicillinase locus and in that recombination between them has been found in the cases thus far studied.

Present evidence suggests that the pattern of plasmid-borne markers is not random but that there are certain characteristics which are plasmid-specific. Furthermore, in the case of penicillinase production and resistance to mercuric ion, these markers appear always to be plasmid-carried. Thus, no naturally occurring strain of *S. aureus* has been described in which the penicillinase determinant is not lost spontaneously; if initially present, resistance to mercuric ion is nearly always eliminated with penicillinase (Richmond and John, 1964). The genetic status of the determinant(s) of erythromycin resistance in this organism is more complex, there being at least two phenotypically distinct types of resistance to this drug (Garrod, 1957). It may be that one is typically chromosomal, and the other extrachromosomal, but there is not yet any evidence bearing upon this point.

So far as concerns the known markers, the entire linkage group appears to be lost, occasionally, as a spontaneous event during the normal growth of the cell. Whether such spontaneous loss is the result of a distributive or of a replicative error has not been determined. If the plasmid is indeed lost in its entirety, then it is clear that it controls no indispensable cellular function. Again, with the proviso that only known markers are being considered, the entire element appears to be transduced as a unit, and no difference has been observed in the properties of transductants obtained with any of the three transducing phages that have been used.

If the element is indeed transduced intact, an upper limit to its size would be the amount of genetic material which can be carried by a transducing phage particle. By analogy with transductions carried out with coliphage P1, this amounts to about 1% of the total bacterial genome (Lennox, 1955).

The evidence presented here suggests that the penicillinase plasmids are a series of small, discrete genetic structures which are harbored by various hosts and which can be transferred from one host to another with relative impunity. Evidence has been presented that, when two genetically distinguishable penicillinase plasmids are present in the same cell, they may behave in either of two specific ways: by early segregation with or without recombination, or by the formation of a persistent plasmid heterodiploid. In both cases, segregants have been observed that harbor recombinant as well as parental-type plasmids. The observation of these two alternative modes of behavior among otherwise very similar extrachromosomal elements leads us to postulate a genetic determinant of compatibility. That is, two different elements that can form a stable plasmid heterodiploid are regarded as compatible; two that cannot, as incompatible. That the determinant of compatibility is plasmid-borne is suggested by the observation that the same relationship *vis à vis* compatibility holds for the various pairs of plasmids in each of three different host strains thus far examined.

Each of the plasmids examined in this study can be assigned unequivocally to one of two compatibility groups. These groups, designated I and II, are delimited as follows: each plasmid is incompatible with all the members of its own group but compatible with all the members of the other group. Since any two isogenic plasmids (e.g., two differently marked plasmids) are incompatible, it follows that any two compatible plasmids are necessarily heterogenic, at least with respect to that part of the plasmid genome which is responsible for the compatibility type.

Of the plasmids examined in most detail, α and γ characterize compatibility group I, and β

defines compatibility group II. Several other naturally occurring plasmids have recently been screened, and all have been found to fall into compatibility group I (Richmond, Brit. Med. Bull., *in press*). Thus, β is the sole member of compatibility group II. It will, therefore, be important to survey other naturally occurring staphylococcal strains to see whether the compatibility types are consistent, whether other types of interaction can occur, and whether additional compatibility groups exist.

A close analogy to the behavior of the penicillinase elements is seen in the λ-434 bacteriophage system described by Kaiser and Jacob (1957). In this case, two λ genomes or two 434 genomes cannot lysogenize the same cell (except in rare cases). However, a λ and a 434 can co-lysogenize without mutual interference. Furthermore, any two of these elements can undergo recombination; apparently any of the known loci can participate, with the exception of the loci which determine the site of attachment of the phage genome to the bacterial chromosome. Thus, the isogenic prophages are incompatible, whereas the compatible ones are heterogenic at least for the determinant of attachment sites.

The concept of compatibility also seems to apply to the behavior of some other extrachromosomal particles. The naturally occurring "resistance transfer factors" in the coliforms have recently been separated into two distinct classes (fi^+ and fi^-; Watanabe et al., 1964) which are roughly comparable to the compatibility types described for the penicillinase plasmids. Compatibility between the colicin V determinant and the episome, F, both of which determine fertility in *E. coli*, was noted by Kahn and Helinski (1964), but in this case establishment of the heteroploid class was rare and segregation of the parental types occurred at high frequency. Other colicin factors which do not have a fertility determinant are fully compatible with F. Pairs of F and F′ factors in various combinations do not ordinarily appear to be compatible (Cuzin, 1962; Scaife and Gross, 1962; Echols, 1963; Maas, 1963). Rare clones harboring two such elements have, however, been reported (Cuzin, 1962; Echols, 1963; Maas, 1963), but the nature of the association between the two elements in these cases has not been established and it is, therefore, not clear how they fit in with the idea of compatibility expressed above.

A possible model for the compatibility phenomenon has been provided by a suggestion of Jacob, Brenner, and Cuzin (1963). According to this model, there is an intracellular binding site (or sites) for the F particle in *E. coli*; attachment to this site has the dual function of regulating the number of F particles in each cell and of ensuring the proper distribution of F particles during cell division. If there is a similar mechanism for the regulation and distribution of penicillinase plasmids in *S. aureus*, then it might be supposed that incompatible plasmids must compete for the same site while different sites would exist for compatible ones. Similar models have been suggested by Watanabe et al., (1964) and by Kahn and Helinski (1964) to explain the behavior of resistance-transfer episomes and colicin-determining episomes, respectively. For the penicillinase plasmids, a necessary consequence of an attachment site model is that it would account for but a single plasmid in each cell except in the case of the heterodiploids.

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