Associated Diploids Involving Penicillinase Plasmids in *Staphylococcus aureus*

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SUMMARY

Staphylococcus aureus penicillinase plasmid diploids of the type ($\alpha.i^+p^+_A/\beta.i^-p^+C$), when constructed in strain 147, normally segregated one of the two parental genotypes at a frequency of about 1/5000 divisions, and recombinants were rare. In about 2% of the diploid clones, however, segregation occurred at a much higher frequency and in these clones both plasmids might be lost together. Examination of these 'unstable' diploids suggests that their two plasmids are associated to form a single structure, so that they are subsequently lost or transduced together. The ease of formation of associated diploids suggests that the penicillinase plasmids may be circular.

INTRODUCTION

The genes responsible for penicillinase synthesis in *Staphylococcus aureus* are usually carried on a plasmid, an extrachromosomal piece of DNA (Novick, 1963). Apart from the penicillinase-controlling and structural genes, a number of other genetic markers have been located on the penicillinase plasmids, and a number of distinct plasmid types can be identified on the basis of the markers they carry (Novick & Richmond, 1965; Richmond, 1965*a*; Dyke & Richmond, 1967).

Transduction of a penicillinase plasmid to a strain already containing another, leads to one of two situations, depending on the pair of plasmids involved. With some pairs, recombination occurs and is followed by elimination of one of the parental plasmid types. With other pairs, however, diploids are formed (Richmond, 1965*b*; Novick & Richmond, 1965). Normally, these diploids—such as the diploid 147($\alpha.i^+p^+/\beta.i^-p^+$) which is formed by transducing strain 147($\beta.i^-p^+$) with phage propagated on strain 8325($\alpha.i^+p^+$)—segregate to the two possible parental haploid states, 147($\alpha.i^+p^+$) and 147($\beta.i^-p^+$), at a frequency close to the rate at which the relevant plasmids are lost from a haploid cell, and recombination is rare.

The use of a special dye reagent (see later) allows this process to be seen by observing stained colonies growing on the surface of agar, constitutive segregants appear as dark sectors in a pale purple colony (see fig. 4 in Novick & Richmond, 1965). When the dye is used in this way, however, a few colonies are found in which segregation appears to occur at a much higher rate. Further examination of these 'unstable' diploids shows that, in addition to a high rate of segregation of the α -plasmid, a very high rate of loss of both the α - and β -plasmids occurs, to give rise to penicillin-sensitive organisms. This high rate of loss to the α -plasmid in a small proportion of the clones was found to be characteristic of the α -plasmid when present in *Staphylococcus aureus*

strain 147, whether alone or accompanying another plasmid (Richmond, 1966*a*). However, the β -plasmid (the one carried by strain 147 in nature) shows an abnormally high rate of loss only in the presence of an 'unstable' α -plasmid. The instability of the β -plasmid in the presence of α is almost certainly due to association of α and β so that they act as a single unit whose stability is controlled by the α -component. Furthermore, examination of the behaviour of such α/β 'associated' diploids shows that recombinants are common among the segregants obtained from this diploid, whereas segregants from 'stable' diploids are almost invariably parental in type. This suggests that a genetic interaction between plasmids is possible in the 'unstable' diploids which is not possible when the diploids are in the stable state. The simplest mechanism whereby two plasmids can interact to form an associated diploid, from which recombinants can arise easily, is one in which the plasmids are circular.

METHODS

Organisms. The strains of *Staphylococcus aureus* used in these experiments were derived from three naturally occurring strains—NCTC 8325, 147 (Segalove, 1947; Richmond, 1965*c*) and 258 (Mitsuhashi, Morimura, Kono & Oshima, 1963). The following nomenclature has been used throughout. The host strains and the plasmids are distinguished by keeping the original strain numbers of the hosts and inserting the genotype of the plasmid in parenthesis. The Greek letter refers to the plasmid as a whole. It is followed by letters and subscript figures indicating the markers carried by the plasmid and which are relevant to the cross under consideration. The symbol p refers to the structural gene for penicillinase, p_A being the gene for the A-type and p_C for the C-type penicillinase (Richmond, 1965*c*). The symbol i refers to an inducibility gene and i^+ is the induced state since this is dominant to the constitutive (i^-) in diploids (Richmond, 1965*b*). The symbols Hg^R and em^R refer to markers conferring mercury and erythromycin resistance, respectively.

Strains carrying no penicillinase plasmid fall into two categories: those that are naturally occurring penicillinase-less strains and those that have lost their penicillinase plasmid to become penicillin sensitive. The former are designated by their strain number alone: the latter have the suffix (N), 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 8325($\alpha.i^+p^+Hg^R \dots \gamma em^R$) designates strain 8325 carrying a recombinant plasmid, the penicillinase and mercury regions being those from α and the erythromycin being from plasmid γ . In plasmid diploids the plasmid genotypes are separated by an oblique stroke, e.g. 147($\alpha.i^+p^+/\beta.i^-p^+$).

Diploid 147($\alpha.i^+p^+ \dots \gamma.em^R/\beta.i^-_{223}/p^+.em^S$) was constructed by transducing strain 147($\beta.i^-_{223}/p^+.em^S$) with phage obtained by u.v.-irradiating strain 8325 ($\alpha.i^+p^+ \dots \gamma.em^R$). Similarly, strain 147 ($\alpha.i^-p^+ \dots \gamma.em^R/\beta.i^+p^+.em^S$) was made by transducing strain 147($\beta.i^+p^+.em^S$) with phage raised on strain 8325($\alpha.i^-p^+ \dots \gamma.em^R$); and diploid 147($\alpha.i^+p^+.Hg^R \dots \gamma.em^R/\beta.i^-_{223} p^+.Hg^R.em^S$) was made with phage from strain 8325($\alpha.i^+p^+.Hg^R \dots \gamma.em^R$) using strain 147($\beta.i^-_{223} p^+.Hg^R.em^S$) as recipient. In all cases the transductants were selected on plates containing 10 μ g. erythromycin/ml. and the diploid character of the transductants detected by examining their segregation patterns. The method whereby strains 8325($\alpha.i^+p^+ \dots \gamma.em^R$),

8325($\alpha.i^{-}p^{+} \dots \gamma.em^B$) and 8325($\alpha.i^{+}p^{+}.Hg^S \dots \gamma.em^B$) are obtained from strains 8325($\alpha.i^{+}p^{+}.em^S$), 8325($\alpha.i^{-}p^{+}.em^S$) and 8325($\alpha.^{+}p^{+}.Hg^S.em^S$), respectively, is described by Richmond (1966*b*). Strain 147(N) is the penicillinase-less variant obtained spontaneously from strain 147($\beta.i^{+}p^{+}$) (Novick & Richmond, 1965).

Media. The composition of the CY medium used was described by Novick (1963). When colonies were to be stained with the *N*-phenyl-naphthylamine-azo-*o*-carboxybenzene (PNCB) dye reagent, they were grown on A¹ agar. This medium has the composition of Andrade agar (Kogut, Pollock & Tridgell, 1965) but omits the indicator. The test for mercury resistance or sensitivity was done on the peptone agar described by Moore (1960).

Staining of colonies. The production of penicillinase by colonies was normally detected by the penicillin + iodine method (Novick & Richmond, 1965) and the PNCB dye reagent was reserved for detecting sectorized colonies. Staining with the dye was done as described by Novick & Richmond (1965). The test is qualitative but, in general, colonies producing much penicillinase stain dark purple, those producing less are pale purple and those producing no enzyme are orange. The presence of constitutive segregant sectors in diploid colonies thus appear as dark purple wedges round the edge of pale purple colonies when the diploids are plated out on agar lacking inducer. Any penicillinase-less sectors appear as orange wedges under similar conditions.

Resistance markers. Erythromycin-resistant strains were detected by using discs containing 10 μ g. erythromycin base/disc; resistance to $HgCl_2$ was tested by the method of Green (1962).

Materials. *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene was obtained from British Drug Houses (Poole, Dorset, England). Benzylpenicillin was obtained from Glaxo Ltd., and the methicillin used for induction experiments was part of a generous gift from the Beecham Research Laboratories.

RESULTS

'Stable' and 'unstable' diploids

The diploid 147($\alpha.i^{+}p^{+} \dots \gamma.em^B/\beta.i^{-}p^{+}.em^S$) was grown exponentially in CY medium and about 500 colony-forming units plated on A₁ agar. The plates were incubated overnight, stained with the PNCB dye reagent, and developed with penicillin as described in Methods. Two types of sectorized colony were observed. (1) Colonies with one or two fine dark (constitutive) sectors; these will be called 'stable' clones. (2) Colonies with many dark (constitutive) flecks, mostly towards the periphery of the colony. In these colonies the rim was often pale purple or even orange (penicillin-less) and substantial orange sectors were often seen. These colonies will be called 'unstable' clones.

The appearance of these two types of colony suggested that segregation was occurring at different rates and, since the α -plasmid was known to enter an unstable state in strain 147 (Richmond, 1966*a*), the proportion of the segregants in each type of clone was measured.

For this purpose, six entire colonies of each type were picked from the surface of agar after treatment with PNCB + benzylpenicillin and subcultured in 2 ml. CY medium containing 5000 units sterile staphylococcal penicillinase to destroy excess penicillin.

The tubes were shaken for 4 hr at 35° and about 15,000 colonies from each tube plated on CY+ starch agar. After incubation overnight, the plates were stained with the penicillin/iodine reagent and a differential count made to determine the proportion of constitutives and negatives on each plate. Although this method allowed ready scoring of these two segregant classes, it did not allow inducible segregants, such as 147(α .i⁺p⁺... γ .em^R), to be distinguished from the parent diploid and, for the purposes of this experiment, the constitutives and negatives are quoted as a proportion of all colonies (Table 1). As expected from previous work, the constitutive segregant—147(β .i⁻p⁺.em^S)—was present at a frequency of about 1/5000 colonies in the relatively stable diploid clones and all those picked (24/24) were erythromycin-sensitive. Penicillinase-less colonies were extremely rare; in fact, only one was found among the 40,000 or so colonies screened from the stable diploids.

Examination of the unstable diploids showed a very different picture. Constitutive segregants occurred at a very variable frequency but the lowest was about 1/600 (Table 1). Among these, erythromycin-resistant recombinants were relatively common, amounting to between 10 and 20% of all the constitutives isolated from the six parent clones. However, it was the incidence of penicillinase-less cocci in these clones

Table 1. *Staphylococcus aureus*: proportion of constitutive and negative segregants obtained from stable and unstable clones of the diploid 147(α .i⁺p⁺... γ .em^R/ β .i⁻p⁺em^S)

	Total number of clones examined (approx.)	Number of constitutive segregants	Constitutives as % of total colonies	Number of i ⁻ .em ^R segregants	Number of penicillinase-less segregants	Penicillinase-less as % of total colonies
Stable diploids						
1	24,000	6	0.025	0	1	0.004
2	14,000	6	0.043	0	0	—
3	9,000	2	0.022	0	0	—
4	11,000	4	0.036	0	0	—
5	10,000	2	0.020	0	0	—
6	15,000	4	0.026	0	0	—
Unstable diploids						
1	11,000	18	0.16	2	107/11,000*	0.97
2	16,000	41	0.26	4	47/2,000*	2.35
3	8,000	23	0.29	3	407/2,000*	20.3
4	10,000	107	1.07	19	681/2,000*	34.0
5	10,000	61	0.61	8	127/2,000*	6.4
6	10,000	44	0.44	5	69/2,000*	3.5

* Smaller total samples screened because of the high incidence of negatives.

that was most surprising. In all cases the proportion of these cocci was far greater than the proportion of constitutives, and in one case they appeared as frequently as 1:3 (Table 1). All were erythromycin-sensitive.

The higher incidence of penicillinase-less than constitutive segregants in the last experiment could be due to a number of causes. For example, the unstable α -plasmid (carrying the i⁺ gene) might destabilize the β -plasmid by competing for an attachment site in the cell. Alternatively, the α -plasmid might join the β to form a double plasmid whose stability was determined by the α component. If the second possibility were correct, a plasmid of this type might be transduced *en bloc* and this possibility is supported by the finding that co-transduction of two plasmids occurs, as a rare

event, in certain other diploid strains (Novick, 1965).

If the coordinate loss of both the α - and β -plasmid from the unstable version of the diploid $147(\alpha.i^+p^+ \dots \gamma.em^R/\beta.i^-p^+.em^R)$ was due to the association of the two plasmids, then the frequency with which the diploid culture formed penicillinase-less segregants at a given point in its growth would be related to the frequency with which transducing phage raised on these unstable diploids at that time could co-transduce both plasmids to a suitable recipient. There are certain technical difficulties, however, against determining the frequency with which a clone is undergoing segregation to the negative state, at a given moment in its history. In any segregating culture the number of segregants appearing *de novo* during a single generation is a small proportion of the total number of segregants, since most of the negatives present will have arisen by division of those formed earlier in the life of the culture. As a result, to obtain an accurate measure of the co-ordinate rate of loss of both plasmids during a single generation, a very large number of colonies would have to be examined to obtain an estimate of worth-while precision. As an alternative, it was decided to compare the rate at which both components of the diploid were co-transduced with the proportion of penicillinase-less organisms present at the time the transducing phage was grown on the strain. Under these circumstances, there should be an approximately linear relationship between these two quantities, particularly since the unstable state of the α -plasmid in strain 147 changes to the stable relatively infrequently (Richmond, 1965*b*).

To test this possibility, the diploid $147(\alpha.i^-p^+ \dots \gamma.em^R/\beta.i^+p^+.em^R)$ was grown on the surface of agar, and 4 stable and 10 unstable colonies picked after staining with PNCB + penicillin, as described above. This diploid, rather than the one used in the previous experiment, was chosen to simplify the identification of haploid and associated diploid transductants (see later). After transfer to 3.0 ml. fresh CY medium, the colonies were grown to a concentration of about 10^9 organisms/ml. and the culture then divided into two parts. One part (1.0 ml.; culture 1) was used to provide serial dilutions for plating about 10,000 colony-forming units on CY + starch plates to determine the proportion of cocci in the clones which were 'negatives' at this stage: the other part (2.0 ml.; culture 2) was centrifuged, the cocci resuspended in 2.0 ml. physiological saline and u.v.-irradiated to induce the carried phages. Transducing phage, prepared in this way, was then used to infect strain 147(N) at a multiplicity of 1.1 p.f.u./c.f.u. and the transductants selected with erythromycin. Selection by this means allowed isolation of any transduced diploids, together with the $\alpha \dots \gamma$ haploid—i.e. $147(\alpha.i^-p^+ \dots \gamma.em^R)$ —and any recombinants of the type $147(\beta.i^+p^+ \dots \gamma.em^R)$.

The strain $147(\alpha.i^-p^+ \dots \gamma.em^R)$ was constitutive and could be readily distinguished therefore from the transduced diploid which was inducible. To distinguish the inducible diploid transductants from the inducible recombinant $147(\beta.i^+p^+ \dots \gamma.em^R)$, colonies were streaked on CY + starch agar lacking inducer and the presence of constitutive segregants sought by staining with penicillin + iodine. The frequency of segregation of the diploids, and the characteristically large amounts of penicillinase synthesized by the constitutive segregants, ensured that the diploids were distinguished easily from haploid transductants such as $147(\beta.i^+p^+ \dots \gamma.em^R)$.

The proportion of negatives present in each of the 10 unstable diploid clones and the proportion of em^R transductants which were diploids is shown in Table 2. By and large there was an approximate correspondence between the proportion of negatives found in an unstable diploid clone and the ability of that clone to act as a source of

transducing phage capable of transferring both components of the diploid. As far as the 4 stable clones are concerned, a single penicillinase-less segregant was obtained in one of the clones tested. Furthermore, no examples of transduced diploids were found among the 50 or so *em^R* transductants examined from each clone (Table 2). In all clones the *em^R* transductants were all identical with the stock strain 147($\alpha.i^{-}p^{+} \dots \gamma.em^{R}$).

These results suggested that the two plasmid components of an unstable diploid associated to form a structure capable of being transduced by a single phage and liable to be lost as a single event. The absence of the phenomenon of co-transduction from stable clones and their property of segregating the two component plasmids separately suggested that association of the two plasmids is rare in stable diploids.

Table 2. *Staphylococcus aureus*: comparison of the behaviour of stable and unstable versions of the diploid 147($\alpha.i^{-}p^{+} \dots \gamma.em^{R}/\beta.i^{+}p^{+}.em^{R}$), (a) as a source of penicillinase-less variants, and (b) as a source of phage capable of transducing both plasmids from the diploid to strain 147(N)

	Total number of colonies examined	Number of penicillinase- less variants	Penicillinase-less variants as % of total colonies	Total number of <i>em^R</i> transductants	Total number of constitutive <i>em^R</i> transductants	Number of diploid transductants
Stable diploids						
1	11,000	1	0.009	946	922	0/50
2	12,000	0	—	654	636	0/50
3	10,000	0	—	962	944	0/50
4	8,500	0	—	922	914	0/50
Unstable diploids						
1	628	45	7.1	841	607	7/25
2	714	192	27	888	240	20/25
3	396	87	22	619	301	16/25
4	841	302	35	712	81	20/25
5	627	81	13	397	233	7/25
6	388	70	18	581	239	9/25
7	912	218	24	577	209	14/25
8	902	180	20	828	322	12/25
9	688	337	49	618	37	21/25
10	703	232	33	714	238	18/25

Recombination in stable and unstable diploids

The results shown in Table 1 suggest, on the basis of very small samples, that recombination between the β - and γ -plasmids (i.e. the appearance of ($\beta.i^{-} \dots \gamma.em^{R}$) recombinants) was more common in unstable than in stable plasmid diploids. To test this, constitutive segregants from stable and unstable clones of the diploid 147($\alpha.i^{+}p^{+} \dots \gamma.em^{R}/\beta.i^{-}p^{+}.em^{R}$) were picked and screened to see whether they were erythromycin resistant or sensitive. In all, 67 constitutives were picked from the stable diploid and only one was erythromycin resistant, whereas of the 81 constitutives picked from unstable diploids, 13 were resistant to erythromycin. The twelve-fold greater abundance of erythromycin-resistant recombinants among the unstable diploids shows that recombination, at least between the β and γ plasmids, was more common in the unstable than in the stable colonies.

Similarly, in a separate experiment, examination of the constitutive segregants from the diploid $147(\alpha.i^+p^+Hg^s \dots \gamma.em^s/\gamma.i^-p^+Hg^s.em^s)$ showed that recombinants involving the Hg marker also occurred more frequently in unstable than in stable diploid clones.

DISCUSSION

The behaviour of the two plasmids which comprise the diploids studied here was very different, depending whether they were present in a stable or in an unstable clone. In the stable state, each component of the diploid segregated to leave a haploid coccus containing the other plasmid, and penicillinase-less clones only arose by the step-wise loss of both plasmids. Propagation of phage on a stable diploid followed by transduction to a suitable penicillinase-less recipient allowed transfer of either plasmid (but not both) and recombinants were only formed rarely between the plasmid components of a stable diploid. Among the unstable diploids, however, three characters appeared to be correlated with the unstable state: (1) a high frequency of co-ordinate loss of both plasmids from the diploid; (2) a high frequency of co-transduction of both plasmids to a suitable penicillinase-less recipient when the diploid is used as a source of transducing phage; (3) the appearance of recombinants at a relatively high frequency among the segregants from an unstable diploid. The first two of these characters suggest that in unstable diploids the two plasmids behave as a single unit. These properties, taken in conjunction with the relatively high frequency of recombination found among the segregants from such diploids, suggests that the connexion formed between the two plasmids may be similar to that formed on integration of an F-factor into the *Escherichia coli* chromosome. Campbell (1962) has suggested that F-factors may be circular and experiments on the mechanism of integration of the F-factor into the *E. coli* chromosome (Broda, Beckwith & Scaife, 1964) are consistent with the idea that a circular F-particle forms an 'open-eight' structure with the circular *E. coli* chromosome. Similarly, if it be postulated that penicillinase plasmids are circular, an associated α/β plasmid could arise by a single cross-over between α and β to form an 'open-eight' structure (Fig. 1). Such a structure could then act as a single unit both with respect to the loss of α and β from the cell or co-transduction of α and β by transducing phage. Furthermore, segregation of a single plasmid from such a diploid would require a return to the unassociated state as a first step and this would involve a cross-over between the two components of the 'open-eight' structure in the opposite sense to the one that established the structure. This second cross-over would re-establish single plasmids wherever it occurred on the opposed circles of the open-eight structure, and plasmids arising by this process would therefore be expected to contain recombinants in proportion to the distances which separate the markers on the two parental plasmids. The relatively high proportion of recombinants among the segregants from associated diploids in these experiments, when compared with segregants from stable clones, is entirely consistent with associated diploids being structures of the 'open-eight' type.

The appearance of associated plasmids in the unstable but not the stable diploid suggests that it is only in the unstable clones that the plasmids come into close enough contact within the cell for direct genetic interaction to occur. Jacob, Brenner & Cuzin (1963) postulated that there is a membrane attachment site for each distinct genetic element within the bacterial cell, and Novick (1966) produced evidence that sites of this type may exist for the penicillinase plasmids of *Staphylococcus aureus*. The

facility with which genetic interaction occurs in the unstable diploids, as compared with the behaviour of the stable diploids in the experiments reported above, suggests that the stable and unstable states might be a reflexion of two different membrane sites of attachment for the α -plasmid in strain 147: one which allows genetic interaction with β and the other which prevents it.

Fig. 1. A possible mechanism for the formation and separation of associated plasmid diploids in *Staphylococcus aureus*. (a) Normal diploids. (b) First stage of formation of associated diploids: apposition of the two circles. (c) Formation of the associated diploid by a cross-over. (d) Re-formation of two circles by a cross-over in the opposite sense from the first. (e) Final form: note new position of em^R marker in relation to pC^+ .

If genetic interaction between plasmids can only occur via the associated state in an unstable diploid, it is necessary to account for the presence of any recombinants among the segregants from the stable diploids (Table 1). Previous work (Richmond, 1966) has shown that the α -plasmid can move from the stable to the unstable state (and vice versa) in *Staphylococcus aureus* strain 147, and every colony of a stable diploid will therefore contain some cocci carrying the α -plasmid in the unstable state. The incidence of unstable plasmids in a predominantly stable clone (Richmond, 1966a) is sufficient to account for the number of recombinants among the segregants from stable plasmid diploids.

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