Staphylococcal Penicillinase Plasmids: Studies on the Reversion of a Temperature-sensitive Replication Mutant to Temperature Stability

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SUMMARY

A culture of *Staphylococcus aureus* harbouring a temperature-sensitive plasmid carrying genes for resistance to penicillin and cadmium ions, loses this plasmid and the associated phenotype when grown at the restrictive temperature. However, incubation at the restrictive temperature in the presence of cadmium ions results in a small number of cadmium-resistant cells. The properties of these surviving cells have been investigated and evidence is presented in support of the hypothesis that at least some of these cadmium-resistant cells are the result of integration of the plasmid into some other replicon, possibly the chromosome.

INTRODUCTION

In most penicillin-resistant strains of *Staphylococcus aureus*, the penicillinase structural gene responsible for this resistance is carried on a plasmid (Novick, 1963). Present on this same plasmid may be genes conferring resistance to various inorganic ions (Novick & Roth, 1968) and erythromycin (Mitsuhashi, Hashimoto, Kono & Morimura, 1963). These plasmids can be divided into at least two groups on the basis of compatibility: a type I and a type II plasmid can be maintained within the same cell but two type I or two type II plasmids cannot be stably maintained (Novick, 1967*a, b*). One of the possible explanations for these results is that there is a specific site within the cell for the maintenance of each type and that, if this site is occupied, then a second plasmid of the same compatibility group cannot be stably maintained (cf. Jacob, Brenner & Cuzin, 1963). It is possible that the postulated site is part of the membrane and that this membrane site is essential for replication and segregation of the plasmid.

In the course of investigations on plasmid replication, we isolated a temperature-sensitive mutant of a penicillinase plasmid (Johnston & Dyke, 1971). Evidence was presented to show that, at the restrictive temperature, the mutant plasmid does not replicate and that, since the bacteria continue to grow and divide, plasmid-negative variants are formed. This mutant plasmid, like that from which it was derived, confers resistance to cadmium ions, so that if the bacterium is grown at the restrictive temperature in the presence of cadmium ions, only revertants that can replicate and express a cadmium-resistance gene will survive. This paper is an account of studies on the properties of such revertants. Our evidence supports the hypothesis that the most common means of reversion to temperature-stable replication of the cadmium-resistance gene results from integration of the entire plasmid into some other replicon – most probably the chromosome.

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METHODS

Chemicals. Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium) was a gift of Boots Pure Drug Co. Lysostaphin was from Cambrian Chemicals Ltd, Croydon, Surrey. 2-[¹⁴C]thymidine (250 μ Ci/mg) and 6-[³H]thymidine (107.5 mCi/mg) were from the Radiochemical Centre, Amersham, Bucks.

Media. These were as described by Johnston & Dyke (1971) except that for the radio-active labelling experiments cells were grown in modified Kloos & Pattee medium (KP medium) (Kloos & Pattee, 1965). KP medium contains in 1 l of distilled water: K_2HPO_4 , 7 g; KH_2PO_4 , 2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; tri-sodium citrate, 0.5 g; glycine, 50 mg; L-glutamic acid, 100 mg; L-aspartic acid, 90 mg; L-serine, 30 mg; L-methionine, 20 mg; L-cysteine, 20 mg; L-alanine, 60 mg; L-lysine HCl, 50 mg; L-arginine HCl, 50 mg; L-threonine, 30 mg; L-phenylalanine, 40 mg; L-tryptophan, 10 mg; L-leucine, 90 mg; L-isoleucine, 30 mg; L-proline, 80 mg; L-valine, 80 mg; L-histidine, 40 mg; nicotinic acid, 1 mg; thiamine-HCl, 1 mg; sodium thioglycollate, 200 mg; glucose, 5 g; and 0.04 ml of a trace element solution.

The trace element solution contained (per litre): $CuSO_4 \cdot 5H_2O$, 2.5 g; $ZnSO_4 \cdot 7H_2O$, 2.5 g; $FeSO_4 \cdot 7H_2O$, 2.5 g; $MnCl_2 \cdot 4H_2O$, 1.0 g; and conc. HCl, 50 ml.

Nomenclature. This was described by Johnston & Dyke (1971). Chromosomal genes are represented without parentheses, while genes carried on a plasmid are within parentheses, (). Deletion of gene(s) is represented by their inclusion within brackets, [].

Transduction procedure. Transductions were carried out as previously described (Johnston & Dyke, 1971). Selection was on casein hydrolysate-yeast agar (CY agar) with additions as follows: for erythromycin resistance, 20 μ g erythromycin/ml (CY-ero agar); for cadmium resistance, 13.3 μ g cadmium acetate/ml (CY-cad agar); for penicillin resistance, 0.2 μ g benzyl penicillin/ml (CY-pen agar).

Plate assay for penicillinase. A modification of the starch-iodine method of Perret was used (Dyke, Jevons & Parker, 1966).

Irradiation of cells and phage. An Hanovia 'Chromatolite' ultraviolet lamp was used. The material to be irradiated, either exponentially growing cells in tryptone soya broth (TSB) or phage suspended in TSB diluted with two parts phage buffer (Novick, 1963), was 12 cm from the lamp.

Bacteriophage. The bacteriophage was phage 80 of the International Staphylococcal Phage Typing Set. Phage was propagated as described by Novick (1963).

Bacteria and plasmids. Table 1 lists some of the characteristics of the bacterial strains used.

Strain ps80(NCTC9789) is the propagating strain for phage 80 of the International Phage Typing Set. This strain has a chromosomal *penZ* gene and plasmid *asa cad mer* genes (Asheshov, 1966). Phage propagated on ps80 can sometimes cotransduce the *asa cad mer* and *penZ* genes and this linkage group behaves as a plasmid in the recipient (Asheshov, 1969). Strain ps80.d was derived by elimination of plasmid (PI₉₇₈₉) and by deletion of the *penI penZ* genes from the chromosome (Johnston & Dyke, 1971).

The temperature-sensitive plasmid was that described by Johnston & Dyke (1971) and designated (PI₉₇₈₉ *seg-1 penI penZ*). A brief summary of the properties of this mutant is as follows: it was derived by mutagenesis of strain ps80[*penI penZ*](PI₉₇₈₉ *penI penZ*); it is phenotypically temperature sensitive for production of penicillinase; this defect is believed to be in replication of the plasmid, with reduction of replication at 42 °C. In a different set of experiments it has been shown that the peak of radioactivity that corresponds to the

Table 1. *Strains used and their nomenclature*

Strain	Abbreviated nomenclature	Chromosomal genes			Plasmid genes						Replication
		<i>penI</i>	<i>penZ</i>	<i>ero</i>	<i>penI</i>	<i>penZ</i>	<i>asa</i>	<i>cad</i>	<i>mer</i>	<i>ero</i>	
PS80(PI ₉₇₈₉)	—	+	+	—	—	—	+	+	+	—	Normal
PS80(PI ₉₇₈₉ <i>penI penZ</i>)	—	+	+	—	+	+	+	+	+	—	Normal
PS80[<i>penI penZ</i>]	PS80.d	—	—	—	—	—	—	—	—	—	—
PS80[<i>penI penZ</i>]	PS80.d.	—	—	—	+	+	+	+	+	—	Normal
(PI ₉₇₈₉ <i>penI penZ</i>)	(PI ₉₇₈₉ <i>penI penZ</i>)	—	—	—	+	+	+	+	+	—	TS*
PS80[<i>penI penZ</i>]	PS80.d.(S1)	—	—	—	+	+	+	+	+	—	TS*
(PI ₉₇₈₉ <i>seg-1 penI penZ</i>)	—	—	—	—	+	+	+	+	+	+	Normal
8325	—	—	—	—	+	401	+	52	+	+	Normal
(PI ₂₅₈ <i>penZ-401 cad-52</i>)	—	—	—	—	+	+	—	—	—	—	Normal
1054(PI ₁₀₅₄)	—	—	—	—	+	401	+	52	+	+	Normal
PS80[<i>penI penZ</i>]	PS80.d.	—	—	—	+	401	+	52	+	+	Normal
(PI ₂₅₈ <i>penZ-401 cad-52</i>)	(PI ₂₅₈ <i>penZ-401 cad-52</i>)	—	—	—	+	+	—	—	—	—	Normal
PS80[<i>penI penZ</i>]	PS80.d.—	—	—	—	+	+	—	—	—	—	Normal
(PI ₉₇₈₉ <i>penI penZ</i> [asa cad mer])	(PI ₉₇₈₉ <i>penI penZ</i> [asa cad mer])	—	—	—	—	—	+	+	+	—	TS*
PS80[<i>penI penZ</i>]	PS80.d.(PI ₉₇₈₉ <i>seg-1</i>)	—	—	—	—	—	+	+	+	—	TS*
(PI ₉₇₈₉ <i>seg-1</i>)	—	—	—	—	—	—	—	—	—	—	—
PS80[<i>penI penZ</i>] <i>ero</i>	PS80.d. <i>ero</i>	—	—	—	+	+	+	+	+	+	Normal
PS80[<i>penI penZ</i>](PI ₂₅₈)	PS80.d.(PI ₂₅₈)	—	—	—	+	+	+	+	+	+	Normal

* TS = temperature sensitive.

penicillinase plasmid DNA is absent in this strain when the culture is grown at 42 °C in the presence of radioactive precursors of DNA, but is present when the incubation is at 30 °C (J. C. Measures, unpublished results). The observed lack of penicillinase production at high temperature is a result of loss of the plasmid from the bacteria after growth at 42 °C. The mutation is solely plasmid linked.

We are indebted to Dr R. Novick for sending us strain 8325(PI₂₅₈ *penZ-401 cad-52*). Phage 53 was propagated on this and used to transduce erythromycin resistance to strain 1054 (Johnston & Dyke, 1971); then phage 80 was propagated on one of these transductants and used to transduce erythromycin resistance to PS80.d, to give PS80.d.(PI₂₅₈ *penZ-401 cad-52*). PS80.d.(PI₉₇₈₉ *penI penZ*[asa cad mer]) was constructed as follows: phage 80 was propagated on PS80(PI₉₇₈₉ *penI penZ*) that had previously been irradiated with u.v. light and this phage preparation was used to transduce penicillin resistance to PS80.d. A cadmium-sensitive transductant was picked after replica plating to CY-cad agar and was also found to be sensitive to arsenate and mercury ions. To be sure that this penicillinase gene was plasmid borne, the stability of the gene during growth at 43 °C was determined. It was lost from the cell with a frequency characteristic of a plasmid gene. (After 4 h growth in TSB at 43 °C, 1.2 % of 3480 colonies tested did not produce penicillinase.)

Strain PS80.d.(PI₉₇₈₉ *seg-1*) was constructed as follows: phage propagated on PS80.d.(S1) was irradiated with u.v. light for 10 s and then used to transduce cadmium resistance to PS80.d. Transductants were tested for penicillinase production, and one that produced no penicillinase but was resistant to arsenate, cadmium and mercury ions and was temperature-sensitive for replication was selected.

Strain PS80.d.*ero* was constructed as follows: phage 80 propagated on PS80.d.(PI₂₅₈) was irradiated with u.v. light for 45 s and then used to transduce erythromycin resistance to

ps80.d. A transductant producing no penicillinase and sensitive to arsenate, cadmium and mercury ions was selected for further study. The erythromycin resistance of this transductant is stable (after 8 h growth in TSB at 42 °C, no erythromycin-sensitive colonies were found among 9484 examined; ps80.d.(PI₂₅₈) gave 1.9 % erythromycin-sensitive colonies in a control experiment). Further, the transductant will accept a PI₉₇₈₉ plasmid at a tenfold higher frequency than will ps80.d.(PI₂₅₈ *penZ-401 cad-52*). Since both PI₂₅₈ and PI₉₇₈₉ belong to the same compatibility group, this result suggests that the compatibility has been lost. These results are consistent with a transposition of the *ero* gene from the plasmid to the chromosome (Novick, 1967*b*) or possibly some other replicon.

Estimation of the stability of the S1 plasmid in ps80.d and a S1 revertant at 42 °C. Cells were grown in TSB at 30 °C for 18 h. The 18 h culture was diluted in fresh TSB to 5×10^5 colony forming units (c.f.u.)/ml, incubated at 42 °C for 4 h and then diluted and plated on CY agar. All plates were incubated for approx. 24 h at 30 °C and then the proportion of penicillinase-negative colonies was determined with starch-iodine.

When the effect of ethidium bromide on loss was studied this procedure was modified as follows: the 18 h culture was diluted to 5×10^5 c.f.u./ml with fresh TSB, with and without 9×10^{-6} M-ethidium bromide. The cultures were then incubated for 5 h at 42 °C and afterwards diluted and plated for estimation of the proportion of penicillinase-negative colonies as above.

DNA-labelling. Cultures were grown for at least two generations in KP medium supplemented with 250 µg 2-deoxyadenosine/ml (Boyce & Setlow, 1962) plus either 6-[³H]thymidine at 5 µCi/ml or 2-[¹⁴C]thymidine at 1 µCi/ml. Cells were harvested at an $E_{675 \text{ nm}}$ of 0.8, equivalent to 2×10^8 c.f.u./ml.

Sucrose gradient velocity centrifugation. A suspension of 2.5×10^9 cells/ml PNE buffer (0.06 M-KH₂PO₄; 0.04 M-K₂HPO₄; 0.1 M-NaCl; 0.01 M-ethylene diamine tetra-acetate; pH 7.4) was treated with 15 µg lysostaphin/ml for 10 min at 37 °C. The suspension was rapidly frozen and thawed and incubated for 30 min at 37 °C to complete the lysis.

Linear gradients (4.9 ml) were prepared by means of a two-chamber mixing device from 10 and 40 % (w/v) sucrose in PNE buffer over a shelf of 0.5 ml of a solution of CsCl (3.4 g CsCl in 3 ml 50 % (w/v) sucrose in PNE buffer) in cellulose nitrate tubes. The gradients were stored at 2 °C for 2 h.

A sample (0.1 ml) of the cell lysate was carefully layered on to the top of the gradient and the tubes were centrifuged in a SW50.1 rotor in a Spinco Model L2-65B centrifuge at 40000 rev./min for 3 h at 5 °C. The centrifuge was braked to 5000 rev./min and allowed to coast to a stop.

Gradients were fractionated by puncturing the bottom of the tube with a stainless steel needle. Two-drop fractions were collected on Whatman Paper Chromedia GF82. This paper was soaked in 5 % (w/v) trichloroacetic acid (TCA) at 2 °C for 20 min, washed with 5 % (w/v) TCA and then with ethanol by suction on a porous polythene strip. The washed papers were dried at 80 °C *in vacuo* for 30 min and then inserted into glass vials with 2.5 ml 2-(4-t-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (butyl PBD; 5 g/l in toluene) for determination of radioactivity.

RESULTS

Reversion to temperature stability of the temperature-sensitive plasmid S1

When 10^8 c.f.u. of a strain carrying the temperature-sensitive plasmid are plated on CY-cad agar and then incubated at 42 °C for 18 h, on average some 33 cadmium-resistant colonies appear. The experiment has been repeated many times and cadmium-resistant

Table 2. The stability at 42 °C of the penicillinase genes of *PS80.d* harbouring plasmid *PI*₉₇₈₉ *penI penZ*, the *S1* plasmid, and a *S1* revertant

Grown at 42 °C for (h)	Penicillinase-negative colonies (%)		
	<i>PI</i> ₉₇₈₉ <i>penI penZ</i>	<i>S1</i> plasmid	<i>S1</i> revertant
0	0.8	1.8	0.12
4	1.0	63.0	0.34

At least 1000 colonies were tested for each of the figures given in the table.

Table 3. The stability at 42 °C of penicillinase genes of *PS80.d* harbouring plasmid *PI*₉₇₈₉ *penI penZ*, *S1* plasmid, and *S1* revertant, in the presence and absence of 9×10^{-6} M ethidium bromide

Grown at 42 °C for (h)	Penicillinase-negative colonies (%)					
	<i>PI</i> ₉₇₈₉ <i>penI penZ</i>		<i>S1</i> plasmid		<i>S1</i> mutant	
	- EtBr	+ EtBr	- EtBr	+ EtBr	- EtBr	+ EtBr
0	0.8	0.8	1.7	1.7	0.0	0.0
5	1.11	16.0	56.7	68.5	0.105	0.17

EtBr = ethidium bromide.

At least 3000 colonies were tested for each of the figures given in the table.

colonies were always found. Cadmium-resistant colonies obtained in this way will be called revertants. Under the same conditions, 10^6 c.f.u. of *PS80.d* (*PI*₉₇₈₉ *penI penZ*) gave confluent growth on the plate.

The stability of the penicillinase genes in these cadmium-resistant colonies has been measured at 42 °C and compared with the stability of both wild-type and the derived temperature-sensitive plasmid. The results (Table 2) indicate that the penicillinase genes are very much more stable than the penicillinase gene in the *S1* plasmid and of rather greater stability than the *PI*₉₇₈₉ *penI penZ* plasmid. Loss of penicillinase is assumed to be equated to loss of the plasmid since in all cases tested (> 100 for each plasmid) resistance to cadmium ions was also lost.

Growth of plasmid-containing strains of *Staphylococcus aureus* in the presence of ethidium bromide results in loss of the plasmid (Bouanchaud, Scavizzi & Chabbert, 1968). The experiment on stability was repeated in the presence of 9×10^{-6} M-ethidium bromide. The results (Table 3) confirm those of Table 2 in that the cadmium-resistant temperature-stable revertant loses its cadmium resistance at a low rate compared with the temperature-sensitive plasmid. The revertant is more stable than the parent plasmid-containing strain.

Transduction of cadmium resistance from revertants

Phage 80 propagated on ten independently isolated revertants and used to transduce cadmium resistance to *PS80.d* gave transductants that were all (more than 1000 tested for each transduction) temperature sensitive for penicillinase production. Thus the temperature-sensitive mutation is present but masked in the revertants.

Interaction of revertants with a second plasmid

When a second plasmid is transduced into a *Staphylococcus aureus* that already harbours a plasmid of the same compatibility type, then the second plasmid may establish itself in the recipient either by recombination with the resident plasmid or by displacing it (Richmond, 1968).

Phage 80 propagated on $\text{ps80.d.}(PI_{258} \text{ penZ-401 cad-52})$ was used to transduce erythromycin resistance into a revertant of $\text{ps80.d.}(SI)$. Transductant colonies were isolated, grown in TSB medium at 30 °C, then subcultured to fresh TSB and growth continued at 30 °C. After a total of 14 generations, the culture was plated for single colonies, the plates incubated at 30 °C, and the colonies tested for penicillinase production and for resistance to erythromycin. All colonies were resistant to erythromycin and 2.4 % had lost the ability to produce penicillinase. The transductants are thus moderately stable and the small loss of the revertant phenotype is not associated with loss of the superinfecting plasmid.

In a second transduction, phage 80 was grown on $\text{ps80.d.}(PI_{9789} \text{ seg-1 penI penZ})$ and used to transduce penicillin-resistance into a revertant of $\text{ps80.d.}(PI_{9789} \text{ seg-1})$ [$\text{ps80.d.}(PI_{9789} \text{ seg-1})$ has the penicillinase gene deleted]. The transductants were tested for stability of penicillinase production as described for Table 2. After 4 h growth in TSB medium at 42 °C followed by plating and incubation of plates at 30 °C, some 57 % of the colonies were penicillinase negative. All 20 penicillinase-negative colonies tested were resistant to cadmium ions. The cells had thus lost the temperature-sensitive plasmid but had retained the revertant cadmium resistance.

Association between revertant cadmium resistance and a chromosomal gene

If the reversion to temperature stability is due to integration into a different replicon, such as the chromosome, then it should be possible to obtain co-transduction of a plasmid gene and a chromosomal gene. There is good evidence that the *penZ* gene is chromosomal in strain ps80 (Asheshov, 1966) so we looked for linkage between the penicillinase gene and cadmium resistance in a revertant derived from $\text{ps80.}(PI_{9789} \text{ seg-1})$. Phage 80 was propagated on two of the revertants; one of the phage preparations gave no co-transduction of *cad* and *penZ* (173 transductants tested) but the other gave 100 % co-transduction of these genes (153 transductants tested) and all the transductants were temperature sensitive for both penicillinase and cadmium resistance. In a control when phage is propagated directly on $\text{ps80.}(PI_{9789} \text{ seg-1})$ there is no co-transduction of cadmium resistance and penicillinase.

The erythromycin-resistance gene can be transposed from the plasmid to another replicon – probably the chromosome (Novick, 1967*b*). We have constructed such a strain (see Methods). It is sensitive to cadmium, arsenate and mercury and does not produce penicillinase A. $(PI_{9789} \text{ seg-1 penI penZ})$ plasmid was transduced into this strain ps80.d.ero selecting for cadmium resistance, and then temperature-stable revertants were obtained in the usual way. Phage 80 was propagated on a number of these revertants and used to transduce erythromycin resistance to ps80.d. ; all transductants were tested for co-transduction of the *penZ* gene. Phage propagated on 4 of 32 revertants show co-transduction of the two markers, but at different frequencies: 0.6, 3.7, 8.0 and 14.6 % of the erythromycin-resistant transductants possessed the *penZ* gene as well, and these were all temperature sensitive. All the transductants that co-transduced penicillin resistance and erythromycin resistance had plasmids that were temperature sensitive in that both resistances were lost together at very

Fig. 1. Velocity sedimentation of DNA from *S. aureus* ps80.d.S1 and ps80.d.S1 revertant. Cells of ps80.d.S1 were grown in the presence of [³H]thymidine at 30 °C and cells of a cadmium-resistant revertant derived from ps80.d.S1 were grown at 30 °C in the presence of [¹⁴C]thymidine. The cells were mixed, lysed with lysostaphin, and 0.1 ml of the lysate placed on top of a 10 to 40 % (w/v) linear sucrose gradient (4.9 ml). The gradient was centrifuged at 5 °C and 40000 rev./min in a Spinco model L2-65B with a SW50.1 rotor for 3 h. About 50 fractions were obtained from each gradient and the amount of radioactivity precipitated by cold 5 % (w/v) trichloroacetic acid was determined for each fraction. The c.p.m. have been adjusted so that the amount of radioactivity sedimenting to the bottom of the gradient is the same for both ¹⁴C and ³H. ○—○, ³H-radioactivity (ps80.d.S1); ●.....●, ¹⁴C-radioactivity (ps80.d.S1 revertant).

high frequency during growth at 42 °C. Phage propagated on the remaining 28 revertants did not co-transduce *ero* and *penZ*.

Physical studies on a revertant

A culture of ps80.d.(PI₉₇₈₉ *seg-1 penI penZ*) that had been labelled with [³H]thymidine at 30 °C was mixed with a culture of a revertant of the same strain that had been grown in [¹⁴C]thymidine at 30 °C. The mixture was lysed as described in Methods and a sample placed on top of a 5 to 40 % sucrose gradient and centrifuged.

Fractions from the gradients were collected, precipitated with 5 % cold trichloroacetic acid and radioactivity determined as described in Methods. The results (Fig. 1) show that there is very little radioactivity present in the peaks corresponding to the penicillinase plasmid for the revertant. The position of the penicillinase plasmid in this system has been extensively studied (J. C. Measures, unpublished).

DISCUSSION

The possible mechanisms that might account for the loss of temperature sensitivity of the plasmid are either reversion or suppression. Reversion is ruled out because it has been shown that the temperature-sensitive mutation is still present in the revertants so that the mutation is suppressed and not reverted.

There are several ways in which suppression might operate: (i) a second mutation such as a deletion or frame-shift on the plasmid; (ii) a second mutation on some other DNA element such as the chromosome; (iii) an integration of the plasmid into some other replicon – such an integration might be specific in that the integration disrupts a gene necessary for the expression of the temperature sensitivity.

The first explanation is ruled out since transduction of cadmium resistance from a revertant to a ps80.d gave rise to temperature-sensitive transductants. The plasmid thus retains the temperature-sensitive mutation and does not carry a suppressor mutation.

The second explanation seems improbable since it has been shown that a second plasmid, isogenic for the temperature-sensitive defect, retains its temperature sensitivity in the revertant. There is thus no cytoplasmic factor, specified by some other piece of DNA, capable of suppressing the defect in the plasmid.

We are thus left with the third possibility, that of integration into some other replicon. There is no rigorous evidence for this mechanism but there are several pieces of evidence that support the possibility of integration.

First, we have evidence of linkage between the cadmium-resistance gene of the plasmid and a chromosomal penicillinase gene in the revertant. This linkage is unlikely to be a fortuitous association between the two genes during replication since in one case there is 100 % co-transduction. The fact that there was no co-transduction in the other revertant tested suggests that there is no one specific region of the chromosome into which the plasmid must integrate. This latter conclusion is supported by the evidence for linkage between the plasmid penicillinase gene and the chromosomal erythromycin gene since these two genes are co-transducible in four revertants but not in a further twenty-eight. It would be more convincing to show that there is linkage between a plasmid gene and an auxotrophic marker. Experiments are in progress to investigate this possibility.

Secondly, we have shown from the sucrose gradient results that there is very little, if any, DNA that behaves as a penicillinase plasmid in a revertant. This is evidence that the sedimentation properties of the plasmid DNA are changed but it does not prove that the plasmid DNA is integrated into the chromosome or any other replicon.

Thirdly, we have been able to construct a plasmid diploid in which two normally incompatible plasmids co-exist. The experiments reported in this paper suggest that the two plasmids are physically separate and are not associated diploids (see Richmond, 1968). Thus either plasmid can be lost without affecting the other. A possible interpretation is that there is a site essential for replication of the plasmid and that in the revertant this site has become vacant and so can be used by the normally incompatible plasmid. There is no good evidence for such an interpretation.

One of our main pieces of evidence depends on the supposition that the *ero* gene is chromosomal in ps80.d. *ero*. All our evidence is that the *ero* gene is not on a plasmid in this strain, but it is possible that it is on a plasmid for a small part of the cell cycle or in a small proportion of the population and that our methods do not detect it. Thus the observed linkage between the *ero* gene and the S1 plasmid in some revertants could have occurred while the *ero* gene was extrachromosomal rather than while the *ero* was in the chromosome. It could be argued that the small amount of plasmid DNA found in the gradients prepared from revertants is due to this small fraction of plasmid in the cultures, rather than our explanation which is that some excision of the plasmid has occurred.

We suggest that all this evidence leads to the hypothesis that the most likely cause of suppression of the temperature-sensitive mutation in some of the revertants is integration into some other replicon, very probably the chromosome. Further evidence is required before this can be more than a very tentative hypothesis. It is unlikely that the hypothesis applies to every revertant, since true reversion and other forms of suppression will occur, but we suggest that integration is one form of reversion in this system.

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