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STABILITY OF PENICILLINASE PLASMIDS IN STAPHYLOCOCCUS AUREUS.

A dissertation submitted for the
degree of Doctor of Philosophy
at the University of Oxford.

by

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CONTENTS

	Page
CHAPTER I - INTRODUCTION.	1
Factors that affect the stability of the plasmid	3
(a) The effect of temperature	3
(b) Effect of acridines	4
(c) Effect of ethidium bromide	6
Mutations that affect the stability of penicillinase plasmids	8
The interaction of the penicillinase plasmid with the chromosome	12
The lysogenic response of a temperate phage	16
CHAPTER 2 - MATERIALS AND METHODS.	19
Chemicals and enzymes	19
Media	20
Bacteriophages	20
Propagation of phage	21
Transduction procedure	21
Nomenclature	22

Bacteria and plasmids 23

Plate assays for production of penicillinase 26

Optical density readings 27

Growth rate of bacteria in the presence of ethidium bromide and acridine orange 28

The elimination of penicillinase plasmids by growth in ethidium bromide or acridine orange 28

Controlled experiment on the elimination, by ethidium bromide, of the penicillinase plasmid from strain 1071(Pl_{1071}) 29

Uptake of ethidium bromide by staphylococci 30

Mutagenesis by ethylmethane sulphonate 30

Isolation of uustable and temperature-sensitive mutants 31

Segregation kinetics for mutants affected in stability of the plasmid 31

Uv light irradiation of cells and phage 33

Mutagenesis by uv irradiation for [penI] and [penZ] mutants 33

Mapping of plasmids 34

Estimation of the stability of the temperature-sensitive plasmid and a temperature-stable revertant at 42 C 35

Transposition of *ero* and *penZ* from the plasmid to the chromosome 36

Tests for the chromosomal location of plasmid-derived genes 36

Excision of integrated plasmids by a superinfecting plasmid 37

Radioactive labelling of plasmid DNA 37

Extraction of plasmid DNA 38

Chromatography 39

Ultracentrifugation 40

CHAPTER 3 - THE EFFECT OF ACRIDINE DYES AND ETHIDIUM BROMIDE ON THE STABILITY OF PENICILLINASE PLASMIDS IN STAPHYLOCOCCI. 42

Introduction 42

Elimination of penicillinase plasmids by ethidium bromide 42

Elimination of penicillinase plasmids by acridine orange 43

The resistance of strain 1044(PI_{1044}) to ethidium bromide 44

Uptake of ethidium bromide by strains of staphylococci 45

Uptake of ethidium bromide at 0 C and in the presence of sodium azide 46

The location of the genetic determinants for resistance to ethidium bromide 47

Discussion 49

CHAPTER 4 - THE ISOLATION AND CHARACTERISATION OF MUTANTS IN WHICH A PENICILLINASE PLASMID IS UNSTABLE. 52

Introduction 52

The isolation of unstable and temperature-sensitive mutants 53

Tests for plasmid or host linkage of mutations 54

Segregation kinetics for the SI-plasmid 55

Stability of $\underline{mcr}_1$ and $\underline{mcr}_{11}$ plasmids in S2 56

Complementation of SI 57

Discussion 58

CHAPTER 5 - INTEGRATION OF THE PLASMID INTO THE CHROMOSOME. 60

Introduction 60

A. Integration of the Plasmid into the chromosome 61

Reversion to temperature stability of SI 61

B. A Specific Mechanism for Integration of the Plasmid into the Chromosome 64

Isolation of an integration-deficient mutant 65

The relationship between homology and frequency of integration 66

The reversion of the SI-*ero* linked plasmids 67

The transduction of the chromosomal *ero* 68

Isolation of further [*int*] mutants 69

Discussion 70

CHAPTER 6 - EXCISION OF AN INTEGRATED PLASMID FROM THE CHROMOSOME. 77

Introduction 77

Excision of an integrated plasmid by a superinfecting plasmid 78

Excision of an integrated plasmid by the plasmid-derived chromosomal *ero* 80

Excision of the chromosomal *ero* by a superinfecting plasmid 81

Excision of chromosomal penicillinase genes by a superinfecting plasmid 82

Discussion 82

CHAPTER 7 - MAPPING THE INTEGRATION AND EXCISION FUNCTIONS AND THEIR SITE OF ACTION ON THE PLASMID 85

Location of *int* on the plasmid 85

Location of *xis* on the plasmid 87

The plasmid site of action of *int* and *xis* 87

CHAPTER 8 - A POSSIBLE PENICILLINASE PLASMID 'TERMINATION' FUNCTION 91

Introduction 91

S1[*int*]-plasmid map 92

Discussion 92

CHAPTER 9 - PURIFICATION OF PLASMID DNA 96

Introduction 96

Labelling and extraction of plasmid DNA 97

Isolation of plasmid DNA 98

Ultracentrifugation 100

Discussion 101

CHAPTER 10 - DISCUSSION. 103

REFERENCES 113

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ABSTRACT.

The stability of the penicillinase plasmids of Staphylococcus aureus has been investigated both by the effects of various agents on the elimination of the plasmids and by the characterisation of mutations influencing the stability.

Growth of plasmid-carrying strains in the presence of ethidium bromide enhanced the spontaneous rate of loss of plasmids in all seven strains of S.aureus tested. In one of these strains, growth in media containing acridine orange had a similar effect on plasmid loss. One of the strains grew in the presence of high concentrations of ethidium bromide. This resistance to ethidium bromide is determined by a gene located on the penicillinase plasmid and appears to be due, at least in part, to a permeability effect.

Two types of mutant affected in the stability of the penicillinase plasmids have been isolated from S.aureus PS80 after treatment with ethyl methane sulphonate. In one, the mutation is present on the penicillinase plasmid and results in an inability to replicate at 42 C; replication at 30 C being unaffected. The second type of mutation is not located on the plasmid and causes instability of penicillinase plasmids of both compatibility groups but does not

affect a plasmid conferring resistance to tetracycline.

Reversion of the temperature-sensitive plasmid to temperature stability has been studied. Evidence is presented to show that this reversion is the result of integration of the plasmid into another replicon, probably the bacterial chromosome. Deletions encompassing a particular region of the plasmid reduce the frequency of this reversion. It is suggested that this region includes a gene specifying a protein involved in the integration process.

A penicillinase plasmid that has a gene conferring resistance to erythromycin (ero) has been used to integrate a fragment of this plasmid into the chromosome of S.aureus PS80. The fragment includes only the ero gene of the known plasmid genes. After transduction into this constructed strain, the temperature-sensitive plasmid integrated at a considerably increased rate. Such an integration can be effected at sites distant from the ero, since linkage between the integrated plasmid and the chromosomal ero is not obligatory. It is suggested that the effect is due to the fragment including,in addition to the ero gene, a gene int that produces a protein involved in recombination between a specific site on the plasmid and sites on the chromosome.

An integrated plasmid can be excised by a superinfecting plasmid. This excision is apparently independent of any recombination between the integrated and superinfecting plasmid. A gene xis that specifies a protein responsible for

this excision is proposed. *xis* activity functions independently of *int* product.

Deletion mapping involving irradiation of transducing phage normally gives the same linear sequence of plasmid genes. This implies that the circular DNA in a host is ruptured at a specific site. Deletion mapping of a plasmid without this putative site results in an altered linear sequence. It is proposed that this site be called *end*.

The location on the plasmid of the genes specifying these functions is discussed and a tentative plasmid map presented.

In view of the similarities between the integration and excision functions specified by the penicillinase pladmid and those specified by temperate phage, such as lambda, it is proposed that temperate phages are the precursors of staphylococcal penicillinase plasmids.

It has proved impossible to remove all plasmid-like DNA from a penicillinase plasmid negative *S.aureus* PS80. Nevertheless there is some indication that the temperature-sensitive plasmid DNA can be separated from other plasmid-like DNA by centrifugation in neutral sucrose gradients.

CHAPTER I

INTRODUCTION

Strains of Staphylococcus aureus are usually resistant to penicillin because they produce the enzyme penicillinase, which can destroy the antibacterial activity of the penicillin. It was observed many years ago that a culture of penicillin-resistant staphylococci gave rise to penicillin-sensitive variants (Voureka, 1948) and, because of it's potential medical importance has been studied extensively since that time.

Barber (1949) examined 32 strains of penicillin - resistant staphylococci that had been stored for 5-12 months. Two strains gave only penicillin-sensitive colonies and fifteen strains gave mixtures of penicillin-sensitive and penicillin-resistant colonies. Of another six strains all gave some penicillin-sensitive colonies after repeated subculture over 9 months. Thus penicillin-resistance was unstable in some two-thirds of the strains she examined.

Loss of penicillin-resistance from cultures of staphylococci has been reported by Fairbrother, Parker and Eaton (1954), Novick (1963), May, Houghton and Perret (1964)

and Asheshov (1966.a.). The facts that the rate of loss of penicillin-resistance from a culture is much higher than can be explained by spontaneous mutation and that derived penicillin-sensitive variants did not revert to penicillin-resistance were two of the pieces of evidence that led Novick (1963) to suggest that the genes conferring penicillin-resistance are located on a piece of extrachromosomal DNA, the penicillinase plasmid. The penicillin-sensitive organisms arise as a result of the loss of the entire plasmid DNA from the cell and consequently the loss of the phenotypic characters coded for by genes on the plasmid, such as penicillinase production.

Richmond (1966) studied the stability of the penicillinase plasmid in 67 independently isolated penicillin-resistant strains of S.aureus. Twenty-five thousand colonies of each strain were screened for the presence of any colonies that did not produce penicillinase. In 34 of the strains, one or more colonies were found that did not produce the enzyme, and had presumably lost the plasmid. Dyke, Parker and Richmond (1970) derived plasmid-negative variants from 95 of 96 independently isolated strains of 'hospital' staphylococci.

Factors that affect the stability of the plasmid.

One method of investigating the frequent appearance of penicillin-sensitive colonies in a resistant culture was to find factors which increased this instability of the penicillinase gene. Voureka (1948) found that some growth conditions may increase the proportion of penicillin-sensitive staphylococci in a culture. Thus growth of a penicillin-resistant strain of S.aureus in mixed culture with some other organism, for example Streptococcus haemolyticus, gave penicillin-sensitive staphylococci. She suggested that this was due to the activity of some substance produced by the associated organism. Then Chandler, Davidson, Long and Monnier (1951) and Chandler and Goltz (1952) reported a marked decrease in penicillinase production and the appearance of penicillin-sensitive organisms after growth of penicillin-resistant staphylococci in the presence of aureomycin. Fairbrother et.al. (1954) were unable to confirm that growth in either mixed culture or the presence of other antibiotics significantly affected the proportion of penicillin-sensitive variants.

(a) The effect of temperature.

Bondi, Kornblum and de Saint Phalle (1953) tested the effect of a wide variety of cultural conditions on the frequency of production of penicillin-sensitive variants in a

strain of penicillin-resistant S.aureus and found little effect except during growth at high temperature (45 C), when there was a 20-fold increase in occurrence of penicillin-sensitive colonies. Fairbrother et.al.(1954) confirmed this effect of temperature for some, but not all, strains of S.aureus. They concluded that some inherited factor controls the production of penicillinase and an uneven distribution of the factor between the daughters of a division resulted in penicillin-sensitive organisms.

May et.al.(1964) and Asheshov (1966a) have also reported an accelerated rate of loss of the penicillinase plasmid from some strains by growth of the organisms at a higher temperature. May et.al. suggest that the loss may result because the replication rate of the plasmids at elevated temperatures is less than that of the host cell, so that the plasmid is diluted out of the culture.

(b) Effect of acridines.

In 1952 Lederberg and his co-workers discovered the episome, F, and also reported the occasional loss of the factor from a cell; a similar phenomenon was found by Hayes (1953). This loss is increased by growth in the presence of cobalt or nickel ions (Hirota, 1956), however acriflavine, an acridine dye, was found to be more effective than either cobalt or nickel ions in converting F^+ cells to F^-

(Hirota and Iijima, I957). The eliminating action of acridine dyes on F was clearly shown by Hirota (I960) who found acridine orange to be more effective than acriflavine, proflavine or acridine yellow.

The acridine dyes are all planar molecules that can intercalate between the adjacent base pairs of a double stranded DNA molecule with consequent extension of the DNA backbone (Lerman, I96I). Why this should affect extrachromosomal elements specifically and result in their elimination from a cell is not known. Intercalation may not even be the prime cause of the elimination.

The experiments of Hirota on the elimination of F resulted in a number of investigations on the elimination of other episomes by acridine dyes. Resistance transfer factors were eliminated by acridine dyes from Shigella flexneri and Escherichia coli (Watanabe and Fukasawa, I96I). Resistance to erythromycin, lincomycin and oleandomycin in S.aureus was eliminated by overnight growth in the presence of acriflavine (Mitsuhashi, Morimura, Kono and Oshima, I963). Penicillin resistance was lost from I7 of I8 S. aureus strains by treatment with acriflavine (Hashimoto, Kono and Mitsuhashi, I964) and Harmon and Baldwin (I964) eliminated penicillinase plasmids using acridine orange.

However Novick (I963) using acridine orange and

following the procedure of Hirota (1960) was unable to demonstrate increased incidence of penicillinase-negative organisms. Similarly Sneath, Asheshov and Richmond (reported in Richmond, 1968) in independent experiments were unable to show any elimination of penicillinase plasmids by acridine dyes. Although subsequently Asheshov (1969) reported the elimination of penicillinase plasmids from strain PS80 after growth in the presence of an acridine dye.

Whether acridine dyes do, in fact, have any effect on the stability of penicillinase plasmids in S.aureus is not clear. Richmond (1968) concludes that they probably do not eliminate these plasmids. He believes the conflicting reports could be due to the fact that neither Hashimoto nor Baldwin studied a statistically significant number of colonies from an untreated culture to act as a control rate of loss.

(c) Effect of Ethidium bromide.

Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium) is, like the acridines, a planar molecule that intercalates between adjacent base pairs in the DNA molecule (Waring, 1966; Lepecq and Paoletti, 1967). In 1969 Bouanchoud, Scavizzi and Chabbert tested ethidium bromide as an agent in the elimination of penicillinase plasmids. Penicillinase producing strains were grown overnight in the

presence of ethidium bromide, and the plasmid was eliminated with high frequency (8 to 100%) from five strains. These strains were all resistant to mercuric ions, a resistance whose genetic determinants are usually located on the penicillinase plasmid (Richmond and John, 1964), and mercury-resistance and penicillinase production were co-eliminated in all five cases. With four mercury-sensitive strains, there was no elimination of penicillin resistance. It appeared that for mercury-resistant-organisms at least, ethidium bromide is more efficient in the elimination of penicillin-resistance than acridines; plasmid negative variants were more often found and the concentration of ethidium bromide necessary for this elimination was lower than the acriflavine concentrations previously reported.

Thus growth in the presence of ethidium bromide, or at high temperature or possibly in the presence of acridine dyes increases the probability of a penicillinase-plasmid negative variant appearing from some strains of S.aureus. Attempts at correlation of plasmid stability with other strain characteristics have met with very limited success. Among the 67 strains studied by Richmond (1968) there was no such correlation apparent. However, Asheshov (1966a) found that the twelve strains (out of 50 examined) that had lost their resistance to penicillin all belonged to the 52/52A/80/81 phage-typing complex.

Any such correlation is probably only quantitative since Dyke, Parker and Richmond (1970) obtained plasmid-negative variants from 95 out of 96 strains tested.

Mutations that affect the stability of penicillinase plasmids in S.aureus.

A different way by which information about the phenomenon of plasmid stability has been obtained is through the study of mutations that affect this stability. First, let us consider the related problem of compatibility.

Staphylococcal penicillinase plasmids found in strains of phage-typing patterns of Group I or Group III can be divided into two categories: $\underline{mcr}_I$ and $\underline{mcr}_{II}$. A strain harbouring an $\underline{mcr}_I$ plasmid can accept and form a stable plasmid diploid with a $\underline{mcr}_{II}$ plasmid but not another $\underline{mcr}_I$ plasmid while, conversely, a strain harbouring a $\underline{mcr}_{II}$ plasmid can form a stable plasmid-diploid with a $\underline{mcr}_I$ plasmid but not an $\underline{mcr}_{II}$ plasmid. Thus an $\underline{mcr}_I$ plasmid is compatible with a $\underline{mcr}_{II}$ plasmid but not with a $\underline{mcr}_I$ plasmid.

The replicon model (Jacob, Brenner and Cuzin, 1963) provides a possible molecular basis for compatibility of this type. For each replicon in the cell (penicillinase plasmid in this case) it is suggested that there is a membrane attachment site with distributive and possibly replicative functions. The compatibility groups may reflect the existence

of a discrete membrane attachment site for each group; a corollary to this is that there might be a discrete plasmid locus responsible for the interaction of the plasmid with the membrane attachment site of Jacob et.al. Such a region may well express itself both in terms of the known compatibility characteristics of penicillinase plasmids, i.e. such a locus may be a member of a pair of alleles, and also in terms of stability of the plasmids.

Evidence for a plasmid region implicated in compatibility and stability was first presented by Novick (1967a), who studied deletions of the plasmid PI_{258} (see section on nomenclature). A map of this plasmid is given in Figure I.I. The genes for resistance to arsenate and cadmium ions are included as these have been shown to be located on the penicillinase plasmid (Novick, 1967a). A region has been labelled mcr in anticipation of this discussion.

Among the plasmids with deletions obtained by Novick were a large class with ero as the only known plasmid gene. Strains harbouring these deleted fragments accepted superinfection by either mcr_I or mcr_{II} plasmids, and the erythromycin resistance gene remained physically separate from the superinfecting plasmid. Further, the transduction frequency of ero was stimulated by ultraviolet light irradiation of transducing phage, a characteristic of a

Figure I.I. The order of genes on the penicillinase plasmid, PI_{258} (after Novick, I967a).

penI is a control gene for penicillinase production.

penZ the structural gene for penicillinase.

asa the gene conferring resistance to arsenate-ions

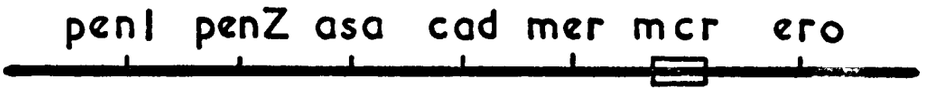
cad the gene conferring resistance to cadmium-ions.

mer the gene conferring resistance to mercury-ions.

mcr represents a region involved in plasmid maintenance, compatibility and replication.

ero the gene conferring resistance to the antibiotic erythromycin.

The distribution of genes does not represent actual physical distance


penI
penZ
asa
cad
mer
mcr
ero

chromosomal gene (Arber, I960), or more accurately of a gene without linked functions that are necessary for existence as an autonomous replicon. It is probable that the ero fragments have become integrated into the host chromosome and replicate as part of the chromosome. Such fragments are thought to be unable to exist as independent plasmids because a region essential for the maintenance of the plasmid state is deleted. Comparison of this class of deleted plasmids, possessing only ero, with other deletions indicated that this maintenance region is located between mer and ero (Figure I.I.).

Since recombination between ero and a superinfecting mcr_{II} were all of mcr_{II} compatibility type (ero was derived from a mcr_{I} plasmid), the ero fragment does not appear to possess a compatibility region either. The regions for maintenance and compatibility and replication have not been separated from each other (see below) so that the whole region is termed mcr.

It is now possible to consider mutations affecting the stability of penicillinase plasmids. Richmond (I966) reported the isolation of a strain with a plasmid-borne mutation causing instability of that plasmid, independent of the host cell. During the transduction of PI_{8325} from it's naturally occurring host to strain I47, certain transductants were obtained wherein PI_{8325} was highly

unstable and were lost from the cell at a frequency of about I per 50 divisions. Richmond (I968) investigated the genetic region governing stability and compatibility, using this mutant plasmid, and was unable to separate these functions by recom bination. The unstable PI_{8325} was transduced into I47(PII_{I47}), and as a rare event the resident plasmid, (PII_{I47}), became unstable. In all cases in which this happened the compatibility characteristics of the (PII_{I47}) were found to have changed, in that it was now compatible with another, stable, PII_{I47}. This suggests that the stability and compatibility regions are close to one another and may even be the same.

Novick (I967a) isolated a mutant host strain (258-94) in which $\underline{mcr}_I$ plasmids are highly unstable, while the stability of $\underline{mcr}_{II}$ plasmids is unaffected. Thus at least one component of the maintenance system in the host is different for each of the two groups. Novick, by similar experiments to those of Richmond, was also unable to separate the functions for stability and compatibility.

Then in I967 Novick (I967b) isolated a different sort of mutant affected in plasmid stability, these are mutants temperature sensitive for penicillinase production as a result of failure of plasmid replication at 42 C. At 30 C replication of the plasmids, and hence penicillinase production, is normal. A total of 24 such mutants were

isolated, thirteen of these proved to be host-determined mutations and eleven were plasmid-determined. Using the host-determined mutants, Novick further investigated the behaviour of wild type plasmids having the compatibility type opposite to that present in the original strains. Some of these mutants could not maintain plasmids of either compatibility type at 42 C, while in others plasmids of opposite mer type were maintained at 42 C, but were less stable than in a wild type host strain.

Novick was unable to demonstrate complementation of any of these temperature-sensitive defects by wild-type plasmids of opposite compatibility type.

The interaction of the penicillinase plasmid with the chromosome.

Although the ability to produce penicillinase can be lost from many strains of S.aureus, there are strains in which loss of this ability has not been demonstrated, (Poston, I966; Asheshov, I966b; Harmon, Baldwin, Tien and Critz, I966; Miller and Harmon, I967; Sweeney and Cohen, I968; Dyke, Parker and Richmond, I970). Asheshov (I966b) found that in strain PS80 (NCTC. No. 9789) penicillinase production is a highly stable character - in one experiment no penicillinase-negative colonies were found among I00,000 examined. The mercury-resistance gene is linked to

the plasmid penicillinase gene in most strains of S.aureus (Richmond and John, 1964), and Asheshov found mercury-sensitive colonies. So in strain PS80 the gene for mercury resistance seems to be a plasmid gene but not linked to the penicillinase gene. Furthermore the transduction frquency of the penicillinase genes in PS80 is stimulated by uv irradiation of the transducing phage - a characteristic of a gene without linked replicon functions (Arber, 1960). Novick (1963) had shown that irradiation of transducing phage gave an exponential decrease in frequency of transduction of penicillinase genes for which there was other evidence that these genes were plasmid borne. So it is probable that the penicillinase genes in PS80 are chromosomal in location.

Genes that are on the chromosome are more stable than those on a plasmid. Thus a mechanism whereby the plasmid genes could become integrated into the chromosome of a cell and so increase their stability may be an advantage to the organism. There is some evidence for an interaction between the plasmid and the chromosome in S.aureus PS80 (Asheshov, 1969). In the wild type the penicillinase genes are chromosomal in location and the resident plasmid carries genes conferring resistance to arsenate - , cadmium- , and mercury-ions but does not carry the penicillinase genes. However when the strain is maintained on nutrient agar, a change occurs in it's genetic constitution such that

the penicillinase genes become linked to the plasmid. This is believed to be due to duplication of the penicillinase genes from the chromosome to the plasmid.

Similarly, the gene conferring resistance to cadmium-ions is most usually plasmid borne but in a few strains is chromosomal (Novick and Roth, 1968; Dyke, Parker and Richmond, 1970). It is possible that the resistance genes found at present on the plasmid may have been aquired from the chromosome by one or more recombinational events. Novick (1967a) demonstrated such an event when he forced the ero gene from (PI_{258}) into a host chromosome and subsequently obtained evidence of transfer of this fragment to a superinfecting plasmid. Novick explains this transfer by a recombinational exchange between regions of homology on the fragment and on the superinfecting plasmid.

Richmond and Johnston (1968) constructed a chromosomal ero by the method of Novick and then demonstrated the segregation of this gene in the presence of a superinfecting plasmid. With no penicillinase plasmid in the cell the gene was not lost, but when a plasmid was introduced into the cell by transduction two genetic conformations were found among the transductants. In the first a merodiploid is formed, the plasmid remains in an extrachromosomal state, and the ero of the recipient remains integrated. In the second the plasmid markers have become

stable, presumably by integration into the chromosome. This second type subsequently behaved in one of two ways: either the plasmid genes are integrated irreversibly so that they become stable chromosomal markers or they are unstable there and return freely to the extrachromosomal state, when the chromosomal ero may also be excised from the chromosome.

Novick (I968) showed by a different series of experiments probable integration of a plasmid into the chromosome. Heat-stable revertants of a mcr_I temperature-sensitive plasmid were obtained. These were at least I000 times more stable than the temperature-sensitive mutant, and each can accept a superinfecting plasmid of the mcr_I compatibility type, which is normally incompatible. The increased stability did not appear to be due to a host mutation overcoming the temperature-sensitive mutation, since a superinfecting temperature-sensitive plasmid remains temperature sensitive. Neither was it due to a back mutation, since the temperature-sensitive mutation was recovered in outcross by transduction. Novick concludes that the stability is the result of integration into some other replicon, most probably the chromosome.

The lysogenic response of a temperate phage.

Temperate phages, such as λ , have evolved a highly sophisticated mechanism of integration into, and excision from the host chromosome. There is some evidence for a similar interaction of the penicillinase plasmid with the chromosome and, since some of the genes normally present on the plasmid may be found on the chromosome, it can be argued that when chromosomal these genes are analogous to prophage. For this reason I would like to consider the lysogenic response.

During lysogeny the viral genome becomes integrated into the bacterial chromosome and replicates as part of the chromosome. The integrated phage genome is said to be in the prophage state. In phage lambda lysogeny is established only after the completion of two separate phage-directed events: repression of those viral functions necessary for lytic development, and integration of the phage genome into the host DNA. Induction of the prophage to enter the lytic cycle requires release of repression, followed by phage-directed excision of the prophage DNA. Only the integration and excision steps will be discussed here.

Campbell (I962) suggested that both integration and excision take place by recombination events. According to this model a prior condition for integration is the circularisation of the phage DNA by joining of the two

ends. Next a recombination event takes place between a special region of homology on the phage DNA ($\underline{att}_P$) and a region of homology on the bacterial chromosome ($\underline{att}_B$), resulting in the linear insertion of the phage DNA into the bacterial chromosome. Excision of the prophage occurs by another recombination event between the, now, hybrid att regions to reform a circular phage molecule and the bacterial chromosome. It seems likely that the Campbell model is, in broad outline, an accurate description of integration (Signer, 1968).

At a number of stages in the Campbell model, enzymes could play a part; two of these are the recombination steps of integration and excision. Zissler (1967) was the first to isolate mutants of λ affected in the mechanism involved in prophage integration. Experiments involving measurement of recombination in conditions where the normally available recombination systems were blocked by mutation (a $\underline{rec}^-$ bacterial host, and a $\underline{red}^-$ phage), showed the presence of a third recombination system, catalysed at least in part by the protein product of the int gene (Weil and Signer, 1968; Echols, Gingery and Moore, 1968). The int product catalysed recombination between specific sites (att) on the phage genome and on the bacterial

chromosome.

An active *int* gene is also necessary for excision of λ from the chromosome (Gottesman and Yarmolinsky, I968). Whether the *int* gene determines entirely the site-specificity of both integration and excision was not clear until separate excision defective mutants were found - *xis*$^-$ (Guarneros and Echols, I970; Kaiser and Masuda, I970). *xis* is functionally distinct from *int*, although the product of *int* is also essential for prophage excision. *xis* appears to be essential for excision but not for integration. The interrelationship between the *int* and *xis* products is not known, but it is possible that *xis* might specify a protein that modifies a bifunctional *int* protein so that excision is favoured.

CHAPTER 2

MATERIALS AND METHODS.

Chemicals and enzymes.

Ethidium bromide - (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium) was a gift of Boots Pure Drug Company.

Acridine orange - (2:8 bis-dimethylamino acridine) was obtained from British Drug Houses, Poole, England.

N-phenyl-1-naphylamine-azo-O-carboxybenzene (PNCB) - was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, England.

Lysostaphin - from Dr. Walter A. Zygmunt of Mead Johnson Research Center, Evansville, Indiana.

Polyethylene glycol - from British Drug Houses, Poole, England.

Sephadex - from Pharmacia, Uppsala, Sweden.

Nitrocellulose - from Serva Entwicklungslabor, Heidelberg, Germany.

Media.

For liquid culture of organisms, 3% Oxoid tryptone soya broth (TSB) was used in all cases.

Solid media were 0.5 CY-agar and 0.3 CY-agar(Novick, 1963), see Table 2.1.

Phage was assayed on 0.5 CY-agar with 0.004M $CaCl_2$ added.

Phage buffer was used in the propagation of phage (Novick, 1963) (Table 2.1).

For radioactive labelling of DNA, cells were grown in CY-medium. This is the same as 0.5 CY-agar, but containing 1% yeast extract, 1% hydrolysed casein and without the agar.

CY-agar, TSB, and CY-medium were sterilised by autoclaving at 15 lb pressure/sq.in. for 15 minutes, and phage buffer by autoclaving at 10 lb pressure/sq.in. for 10 minutes.

Bacteriophages.

Phages 53 and 80 of the International Phage Typing Series were used as transducing phages.

Table 2.I. The composition of CY-agar and phage buffer.

	Phage Buffer	0.3 CY	0.5 CY
β-glycerophosphate	0.06 M	-	0.06 M
$MgSO_4$	0.001 M	0.001 M	0.001 M
$CaCl_2$ [I.]	0.004 M	-	-
NaCl	0.085 M	-	-
Glucose[I.]	-	-	0.028 M
Gelatin	0.1% (w/v)	-	-
Trace metals[2.]	-	0.02 ml/l	0.02ml/l
Yeast extract (Difco)	-	0.3% (w/v)	0.5% (w/v)
Acid hydrolysed casein(Difco)	-	0.3% (w/v)	0.3% (w/v)
Agar (Difco)	-	1.5% (w/v)	1.5% (w/v)

I. Added after autoclaving.

2. 0.5% (w/v) $CuSO_4$; 0.5% (w/v) $ZnSO_4$; 0.5% (w/v) $FeSO_4$; 0.2% (w/v) $MnCl_2$; 10% (v/v) HCl.

Propagation of phage.

10 ml of TSB was mixed with 20 ml phage buffer, about 2×10^8 colony forming units (cfu) of bacteria were added, and phage to give a multiplicity of infection (moi) between 0.3 and 0.7. A number of different moi were usually tried; the culture that had been inoculated with the lowest moi and that gave the least turbidity after approximately 6 hr incubation at 30 C was harvested. Phage lysates were clarified by centrifugation at 2000g for 10 min and any remaining bacteria were removed by filtration through a sintered glass 5/2 funnel.

Transduction procedure.

A modification of the method of Asheshov (1966b) was used. An exponentially growing culture of the recipient strain in TSB at about 2×10^8 cfu/ml was diluted 3 fold with phage buffer. This was incubated at 30 C with gentle shaking for 30 min to allow phage adsorption to occur. Sodium citrate to a final concentration of 0.02 M was then added to prevent further phage adsorption, and the mixture was centrifuged. The cells were resuspended in TSB containing 0.02 M sodium citrate, incubated a further 2-3 hr to allow phenotypic expression, and then plated out.

Selection was on CY-agar, with additions as follows: for erythromycin resistance - 20µg erythromycin/ml (CY-ero agar); for cadmium resistance 13.3µg cadmium acetate/ml (CY-cad agar); for penicillin resistance - 0.2µg benzyl penicillin/ml (CY-pen agar); for resistance to ethidium bromide - 80µg ethidium bromide/ml (CY-eb agar).

Nomenclature.

The convention of Peyru, Wexler and Novick (1969) has been used. Each plasmid is uniquely identified by an indication of the naturally occurring strain in which it was found. The only plasmid marker always mentioned is the determinant of incompatibility set, and this is designated as either PI or PII.

The following symbols are used:

penZ - refers to the structural gene for penicillinase.

penI - is the gene controlling inducible synthesis of penicillinase.

asa - is the gene determining resistance to arsenate-ions.

cad - is the gene determining resistance to cadmium-ions.

mer - is the gene determining resistance to mercury-ions.

mcr - refers to the region of the plasmid responsible for maintenance, compatibility and replication, and is essential for survival of the plasmid as a replicon.

The Roman numeral following mcr (either $_{I}$ or $_{II}$) refers to the incompatibility set.

ero - refers to the gene determining resistance to the antibiotic erythromycin.

tet - refers to the gene determining resistance to the antibiotic tetracycline.

Letters without parentheses following the strain designation refer to the chromosomal genes; plasmid genes are enclosed in round parentheses.

Square parentheses are used to indicate that the genes enclosed within these parentheses have been deleted from the genome.

The strain number alone indicates a plasmid negative variant of that strain.

Bacteria and plasmids.

The genotypes of the naturally occurring plasmids found in the strains used in these studies are given in Table 2.2.

Strains 1051(PI_{1051}), 1071(PI_{1071}), 13313(PI_{13313}), 3804(PI_{3804}), 1044(PI_{1044}) and 1054(PI_{1054}) are strains of 'hospital staphylococci' referred to in a survey by Dyke and Richmond (1967).

Table 2.2. The strains used and their nomenclature

Strain	Abbreviated nomenclature	Chromosomal genes			Plasmid genes						Replication
		penI	*penZ*	*ero*	*penI*	*penZ*	*ase*	*cad*	*mer*	*ero*	
524(PI_{524})	-	-	-	-	+	+	+	+	+	-	normal
LA75(PI_{LA75})	-	-	-	-	+	+	-	-	-	-	normal
IO44(PI_{IO44})	-	-	-	-	+	+	-	-	-	-	normal
IO5I(PI_{IO5I})	-	-	-	-	+	+	+	+	+	-	normal
IO54(PI_{IO54})	-	-	-	-	+	+	-	-	-	-	normal
IO7I(PI_{IO7I})	-	-	-	-	+	+	-	-	-	-	normal
3804(PI_{3804})	-	-	-	-	+	+	-	-	+	-	normal
I33I3(PI_{I33I3})	-	-	-	-	+	+	-	-	-	-	normal
I47(PII_{I47})	-	-	-	-	+	+	+	+	+	-	normal
258(PI_{258})	-	-	-	-	+	+	+	+	+	+	normal
8325(PI_{258}·*penZ*-40I.*cad*-52)	-	-	-	-	+	40I	+	52	+	+	normal
PS80	-	+	+	-	-	-	-	-	-	-	-
PS80.*penZ*-50I.	-	+	50I	-	-	-	-	-	-	-	-
PS80(PI_{9789})	-	+	+	-	-	-	+	+	+	-	normal
PS80(PI_{9789}·*penI*.*penZ*)	-	+	+	-	+	+	+	+	+	-	normal
PS80(PI_{9789}·*seg*-I)	-	+	+	-	-	-	+	+	+	-	temperature sensitive
PS80[*penI*.*penZ*]	PS80.d.	-	-	-	-	-	-	-	-	-	-
PS80[*penI*.*penZ*](PI_{9789})	PS80.d.(PI_{9789})	-	-	-	-	-	+	+	+	-	normal
PS80[*penI*.*penZ*](PI_{9789}·*penI*.*penZ*)	PS80.d.(PI_{9789}·*penI*.*penZ*)	-	-	-	+	+	+	+	+	-	normal
PS80[*penI*.*penZ*](PI_{9789}·*seg*-I.*penI*.*penZ*)	PS80.d.(SI)	-	-	-	+	+	+	+	+	-	temperature sensitive
PS80[*penI*.*penZ*](PI_{9789}·*seg*-I.*penI*.*penZ*.*ero*)	PS80.d.(PI_{9789}·SI-*ero*)	-	-	-	+	+	+	+	+	+	temperature sensitive
PS80[*penI*.*penZ*].*ero*	PS80.d.*ero*	-	-	+	-	-	-	-	-	-	-
PS80[*penI*.*penZ*](PII_{I47})	PS80.d.(PII_{I47})	-	-	-	+	+	+	+	+	-	normal
PS80[*penI*.*penZ*](PI_{258})	PS80.d.(PI_{258})	-	-	-	+	+	+	+	+	+	normal
PS80[*penI*.*penZ*](PI_{258}·*penZ*-40I.*cad*-52)	PS80.d.(PI_{258}·*penZ*-40I.*cad*-52)	-	-	-	+	40I	+	52	+	+	normal

Strain 524 was isolated by Dr. C.H. Lack from a case of osteomyelitis (Rogers, 1953).

I am indebted to Dr. D.H. Bouanchaud for sending me strain LA75(PI_{LA75}).

Strain PS80 (NCTC 9789) is the propagating strain for phage 80 of the International Phage Typing Series. The wild type has a chromosomal penI.penZ region (Asheshov, 1966b), and also harbours a mcr_I plasmid, which does not carry penicillinase genes (Table 2.2). When the strain is maintained on nutrient agar a change occurs in it's genetic constitution, in that these distinct genetic elements - the chromosomal penI.penZ genes and the plasmid - become linked and cotransducible. The change is believed to be due to duplication of the penicillinase genes, one copy remaining on the chromosome and one becoming incorporated into the plasmid (Asheshov, 1969). The plasmid now carries the penI.penZ genes in addition to the ion-resistance genes and it's genotype is: PS80(PI_{9789}·penI.penZ).

The strain of PS80 harbouring the plasmid that confers resistance to tetracycline, PS80(T_{10501}), was obtained from Dr. E.H. Asheshov.

The mcr_1 plasmid that confers resistance to erythromycin originated in strain 258(PI_{258}) (Mitsuhashi, Hashimoto, Kono and Morimura, 1965). Phage 80 was propagated on this, in broth, and used to transduce

erythromycin resistance to PS80.

PS80.d. was derived by Dr. K.G.H. Dyke (Johnston and Dyke, 1971a). A (PI_{258}) plasmid was transduced into PS80(PI_{9789}.penI.penZ) by selecting on CY-ero agar. 20% of the transductants were resistant to cadmium and mercury ions, and to erythromycin, but produced no detectable penicillinase, either in the presence or absence of inducer. He believes the penicillinase genes to have been lost from the chromosome by a process analogous to superinfection curing. A cadmium-sensitive colony was isolated after growth in broth at 43 C and replica plating to CY-cad agar, this was also found to be sensitive to arsenate, mercury and erythromycin, and this variant became PS80.d.

PS80.d.(PI_{9789}.penI.penZ.[asa.cad.mer]) was constructed by Dr. K.G.H. Dyke (Johnston and Dyke, 1971b). Phage 80 was propagated on PS80(PI_{9789}.penI.penZ) that had previously been irradiated with ultraviolet (uv) light; and this phage preparation used to transduce penicillin resistance to PS80.d. A cadmium-sensitive transductant was picked off after replica plating to CY-cad agar and was also found to be sensitive to arsenate and mercury ions.

The mcr_{11} plasmid used in these experiments was derived from strain 147 (Segalove, 1947). Phage 53 was propagated on this strain and the plasmid transduced to

PS80.d., by selection for cadmium resistance. One transductant was obtained and was checked for compatibility with known $\underline{mcr}_1$ plasmids to ensure that it was $\underline{mcr}_{11}$.

I am indebted to Dr. R.P. Novick for supplying strain 8325(PI_{258}.penI.penZ-401.cad-52). Phage 53 was propagated on this and used to transduce erythromycin resistance to strain 1054; then phage 80 was propagated on one of these transductants and used to transduce erythromycin resistance to PS80.d.

Where necessary plasmid-negative variants of the strains were obtained by growth in TSB at 42 C for 6 hr. After plating out, in the case of penicillinase-producing plasmids, colonies were developed with starch iodine and a penicillinase-negative colony selected. For penicillinase-negative plasmids, colonies were replica plated onto CY-cad agar, and a cadmium-sensitive colony selected.

Plate assays for production of penicillinase.

Two methods were used:

(a). A modification (Dyke, Jevons and Parker, 1966) of the method of Novick (1963). Colonies were grown on 0.5 CY-agar containing 0.3 (w/v) starch (Connaught Medical Research Laboratories, Toronto, Canada). The plates were

flooded with a solution of phosphate buffer at pH 7.0 containing 24 mg bezylpenicillin/ml, and 0.032 M iodine in 0.12 M potassium iodide.

(b). The N-phenyl-1-naphthylamine-azo-o-carboxybenzene (PNCB) stain of Novick and Richmond (1965) was also used. Cells were spread on 0.3 CY-agar with an initial pH of 7.6, and when grown the plates were dried at 37 C for 2 hr with their lids off. The agar was then covered with 1.5 ml of a 0.25% (w/v) solution of the acid-base indicator PNCB, in N,N-dimethyl formamide with 6% (v/v) 1N NaOH. The plates were dried in a fume cupboard, and the stain was developed by flooding with 1.5 ml of 1% (w/v) aqueous benzylpenicillin solution. The acid released by the hydrolysis of penicillin by penicillinase changed the indicator from the orange, soluble form, to the insoluble purple form. This stain gave a sharp resolution between adjacent penicillinase positive and negative regions of a colony. Viable organisms could be recovered from stained colonies for up to 6 hr.

Optical Density readings.

The optical density (OD) of bacterial cells in TSB and of ethidium bromide in 0.1 M phosphate buffer (pH 7.0) was read using a Unicam SP600 spectrophotometer, with

cells of 1 cm light path, and at wavelengths of 675 nanometres (nm; 10^{-9}m) and 480 nm respectively.

The OD of DNA solutions was read at 260 nm, using a SP500 spectrophotometer, also with cells of 1cm light path.

Growth rate of bacteria in the presence of ethidium bromide and acridine orange.

5×10^7 cfu from a 16 hr culture in TSB were inoculated into flasks containing 10 ml of fresh TSB and either ethidium bromide, or acridine orange, at various concentrations. These flasks were incubated at 37 C and the OD measured at regular intervals. The growth of cells in a control flask, not containing any drug, was also followed.

The elimination of penicillinase plasmids by growth in the presence of ethidium bromide or acridine orange.

5×10^7 cfu from a 16 hr culture in TSB were inoculated into a flask containing 10 ml of fresh TSB and either ethidium bromide or acridine orange. As a control the same number of cells were inoculated into a flask containing TSB. Flasks were incubated at 37 C for 16 hr, after which appropriate dilutions of the culture were plated out on

CY-agar. Plates were incubated at 37 C for 16 hr, and the colonies were then assayed for the production of penicillinase by the starch-iodine technique, and the percent penicillinase negative colonies calculated.

Controlled experiment on the elimination, by ethidium bromide, of the penicillinase plasmid from strain 1071(PI_{1071}).

The following flasks were prepared: two control flasks each containing 10 ml TSB; two test flasks, containing 10 ml TSB and ethidium bromide at concentrations of 8×10^{-6} M and 10^{-5} M respectively. 4×10^{7} cfu from a 16 hr culture in TSB were inoculated into each of the flasks; a sample from this cuture was also plated on CY-agar to establish the initial percent of penicillinase-negative colonies.

Flasks were shaken at 37 C and growth in each flask was followed. When the first control flask reached a total of 8×10^{8} cfu it was diluted and plated, and as the flasks containing ethidium bromide grew to that number of cells they too were plated out. The second control flask was incubated for the same length of time as was taken by the flask containing 10^{-5} M ethidium bromide to reach a total of 8×10^{8} cfu - by which time it had grown to a total of

9×10^9 cfu.

All plates were incubated at 37 C for 16 hr, assayed for penicillinase production by the starch-iodine technique, and the percent penicillinase-negative colonies calculated.

Uptake of ethidium bromide by strains of S. aureus.

10^{10} cfu of bacteria from an overnight culture in TSB were resuspended in 10 ml of 0.1 M phosphate buffer (pH 7.0), containing the concentration of ethidium bromide to be tested. Flasks were shaken at 37 C or 0 C, and samples were taken at intervals, centrifuged at 2000g for 15 min, and the OD at 480 nm of the ethidium bromide remaining in the supernatant measured.

When uptake in the presence of 10^{-2} M sodium azide was measured, the cells were preincubated with azide in the phosphate buffer for 1 hr.

Mutagenesis by ethylmethane sulphonate (EMS)

The method is that of Novick (1963). 0.08 ml of EMS was added to 2 ml of an overnight culture in TSB. The cells were incubated at 30 C for 25 min, diluted with 10 ml of sterile distilled water and centrifuged. They were resuspended in 10 ml TSB, incubated a further 3 hr before plating out for isolation of the required mutant.

Isolation of unstable and temperature-sensitive mutants.

Both kinds of mutant were obtained by EMS treatment of PS80.d.(PI_{9789}.penI.penZ).

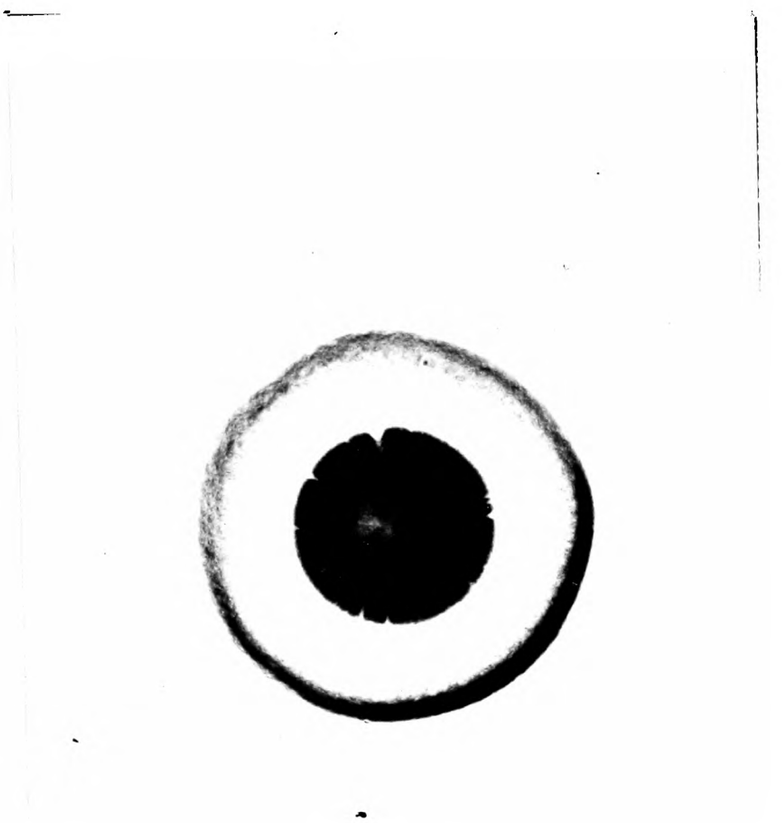
Temperature-sensitive mutant: EMS-treated cells were plated on 0.3 CY-agar to give about 250 colonies per plate. The plates were incubated at 30 C for 24 hr, by which time the colonies were about 1 mm in diameter, and then transferred to 42 C for 15 hr. Colonies were stained with PNCB and inspected under a plate microscope. By following this procedure, colony centres will have grown at 30 C and the peripheral parts at 42 C; colonies that are temperature sensitive for penicillinase production are those with dark (purple) centres, surrounded by light (orange) areas (Figure 2.1). In this way large numbers of colonies could be very rapidly screened.

Unstable mutants: EMS-treated cells were plated on 0.3 CY-agar, incubated at 37 C, stained with PNCB and inspected under a plate microscope. Highly sectored colonies were selected for further study.

Segregation kinetics for mutants affected in stability of the plasmid.

For the temperature-sensitive mutant: Cells were grown in TSB at 30 C for 16 hr, inoculated into fresh TSB and

Figure 2.I. A colony temperature sensitive for penicillinase production, after growth at 30 C and then 42 C.



incubated at 30 C until in exponential phase (usually 2 hr). 5 x 10^7 cfu were inoculated into 10 ml of fresh, warm TSB at 42 C. Samples were withdrawn from this flask at 20 min intervals and after appropriate dilution were spread on CY-agar. The OD of all samples was measured. Plates were incubated at 30 C for 24 hr and then developed by the starch-iodine method to determine the percent of penicillinase-negative colonies.

Staphylococci growing in broth tend to form clumps. This tendency is least apparent during the exponential phase of growth, when inspection under a light microscope indicates that, in strain PS80 at least, cells are predominantly in ones, twos and threes. The segregation kinetics for the temperature-sensitive mutant were determined during exponential growth, and to minimise clumping, before plating out cells were shaken vigorously. In addition, when screening colonies after plating, those that were half, or more, penicillinase negative were scored as negative.

For the unstable mutant: 5 x 10^6 cfu from a 16 hr culture in TSB, incubated at 30 C, were inoculated into 10 ml of fresh TSB and incubated at 42 C for 8 hr. A sample of cells from the 16 hr inoculum was plated on CY-agar, as was a sample after growth in broth at 42 C. Plates were incubated at 37 C for 16 hr. The colonies

were developed with starch-iodine and the percent penicillinase-negative colonies determined. No allowance was made for the clumping of the cells during growth, since the results for loss of plasmids from this mutant are not absolute, but are a comparison of the proportion of penicillinase-negative colonies before and after a defined incubation.

For the tetracycline plasmid: The same procedure was followed as for the unstable mutant, immediately above. After the plates had been incubated for 16 hr at 37 C, they were replicated to CY-agar containing 5µg tetracycline/ml and the percent of tetracycline-sensitive colonies determined.

Uv light irradiation of cells and phage.

An Hanovia Chromatolite uv lamp was used. The material to be irradiated, either exponentially growing cells in TSB, or phage suspended in TSB diluted with two parts phage buffer, was 12 cm from the lamp.

Mutagenesis by uv irradiation for [penI] and[penZ] mutants.

Phage propagated on the appropriate donor was irradiated for 10 sec and used to transduce cadmium

resistance to PS80.d. Exponentially growing cells were irradiated for 15 sec, diluted and plated immediately on CY-cad agar.

Colonies were tested for penicillinase production with starch-iodine, and the desired mutants isolated.

Such mutants are believed to be deletions because,
(a) no revertants were found of any of the penicillinase-negative mutants in more than 10^{10} cells plated on CY-pen agar.
(b) none produced any detectable penicillinase and all known point mutations in the penZ gene produce some penicillinase (Dr. V. Dyke, personal communication).
(c) two have lost both penZ and asa together.

By analogy it is assumed that the constitutive penicillinase mutants have part or all the penI gene deleted.

Mapping of plasmids.

Phage was propagated on cells harbouring the plasmids to be mapped. Approximately 5×10^9 phage were irradiated for 15 sec and then used to transduce penicillin -, or cadmium -, or erythromycin-resistance to PS80.d. All transductants were tested for: production of penicillinase

by the use of starch-iodine; resistance to erythromycin, by replication to CY-ero agar and resistance to arsenate, cadmium and mercury by the use of paper discs. Each disc was 5 mm in diameter and was impregnated with 250 µg of sodium arsenate, or 26.6 µg of cadmium acetate, or 0.25 µg of phenyl mercuric nitrate. The transductants to be assayed for ion resistance were grown in TSB for 6 hr, a single streak of this was made on CY-agar and the discs were placed on this streak.

Estimation of the stability of the temperature-sensitive plasmid and a temperature-stable revertant at 42 C.

Cells were grown in TSB at 30 C for 18 hr, a sample was diluted and plated on CY-agar to determine the initial percent of penicillinase negatives. The 18 hr culture was diluted in fresh TSB to 5×10^5 cfu per ml, incubated at 42 C for 4 hr and plated on CY-agar. All the plates were incubated at 30 C and the percent of penicillinase-negative colonies was dtermined by staining with starch-iodine.

Transposition of ero and penZ from the plasmid to the chromosome.

Phage propagated on PS80(PI_{258}) or PS80.d. containing the temperature-sensitive plasmid was irradiated with uv light for 45 sec and used to transduce erythromycin resistance or penicillin resistance to PS80.d., selection was on CY-ero agar or CY-pen agar. Erythromycin plates were incubated at 37 C, and the penicillin plates at 42 C.

For an integrated erythromycin-resistance gene, a transductant producing no penicillinase and sensitive to arsenate -, cadmium - and mercury-ions was selected for further study. In the case of the transposed penicillinase gene, a transductant sensitive to the three metal ions was selected.

Tests for the chromosomal location of plasmid-derived genes.

(a). Compatibility with both mcr_1 and mcr_{11} plasmids.

(b). In creased stability e.g. ero: after 8 hr growth in TSB at 43 C, no erythromycin-sensitive colonies were found in 9484 examined, a control PS80(PI_{258}) gave 1.9% erythromycin-sensitive colonies; one of the strains with transposed penI.penZ genes: no penicillinase-negative colonies in 13248 colonies examined, a control penicillinase plasmid gave 1.3% penicillinase-negative colonies.

Excision of integrated plasmids by a superinfecting plasmid.

The genotype of the appropriate transductants was checked and then a single colony was grown in TSB at 30 C for 18 hr. This 'initial' culture was diluted in TSB , in duplicate, to 5 x 10^5 cfu per ml and incubated for 7 hr, one at 30 C and the other at 42 C. The 'initial' and the two 'final' cultures were diluted and plated on CY-agar. Half the plates from the'initial' and each of the two 'final' flasks were incubated at 30 C and the other half were incubated at 42 C. Plates were either developed with starch-iodine (for an integrated temperature-sensitive plasmid) or replicated to CY-cad agar (for an integrated penicillinase-negative, temperature-sensitive plasmid) and the percent of penicillinase-negative or cadmium-sensitive colonies determined. Each figure quoted in Tables 6.1 and 6.2 represents the result of screening about 2000 colonies.

Radioactive labelling of plasmid DNA.

Cells to be labelled were grown for 16 hr in 70 ml CY-medium, supplemented with 250 µg deoxyadenosine/ml and 250 µg uridine/ml (Boyce and Setlow, 1962); and 25 µCi of ^{3}H-thymidine (25 Ci/mmole) per ml. Cells without penicillinase plasmids were shaken at 37 C and those containing temperature-sensitive plasmids were grown at 30 C.

Extraction of plasmid DNA

The method used, with minor modifications, was that of Rush, Gordon, Novick and Warner (1969). The labelled cells were centrifuged at 0 C and washed four times with separate 20 ml amounts of cold 0.15 M EDTA, pH 7.0. They were centrifuged a fifth time and resuspended in 25 ml 0.15 M EDTA containing 30% (w/v) NaCl and 50 µg lysostaphin per ml. This was shaken for 30 min at 37 C, centrifuged, resuspended in 10 ml 0.15 M EDTA and frozen. Lysis was usually complete after thawing and shaking for a short while at 37 C.

At room temperature, the pH of the extremely viscous lysate was raised to 12.3 with 1N NaOH, and incubated for 3 min, with vigorous stirring. Then it was neutralised with 6N HCl. The resulting heavy precipitate was removed after centrifugation at 4 C. Ribonuclease (Seravac Laboratories, Maidenhead, England), heated to 80 C for 10 min to destroy deoxyribonuclease activity, was added to the supernatant (10 µg/ml), which was then incubated at 80 C for 3 min and centrifuged at 2000g at 4 C. This supernatant comprised the crude plasmid DNA extract.

Chromatography.

(a). Sephadex chromatography.

Sephadex G100 was allowed to swell in distilled water, at room temperature, for 5 days. To destroy any nuclease activity present, 1N NaOH was added to raise the pH to 12, and the Sephadex was soaked at this pH for 2 hr (Boedtker, 1968). After de-aerating, a 500 ml column (4 cm diameter) was prepared on which the crude DNA extract was chromatographed at 4 C. 2 x SSC was used as the elution buffer (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate in distilled water). 5 ml fractions were collected.

The fractions comprising the first peak obtained (see Chapter 9) were combined, concentrated using polyethylene glycol, and passed through a nitrocellulose column.

(b). Nitrocellulose chromatography.

Nitrocellulose was thoroughly ground in 2 x SSC with a mortar and pestle. The ground nitrocellulose was put into a 1.5cm diameter column, and packed down with a glass rod so that the final bed volume, 20 ml, was about one-third that of the unpacked volume (Boezi and Armstrong, 1967). Before use the column was washed with about 500 ml of 2 x SSC.

After being used once, the column was unpacked, the nitrocellulose reground in distilled water with a mortar

and pestle, and re-packed. It was washed with four separate 200 ml amounts of distilled water and then with 500 ml 2 x SSC before being used again (Boezi and Armstrong,1967).

All the DNA passing directly through the nitrocellulose was collected and concentrated in polyethylene glycol. This is the plasmid DNA preparation.

Ultracentrifugation.

Linear sucrose gradients (5 ml) were prepared from 5 and 20% sucrose in 0.5 M sodium chloride, 0.01 M potassium phosphate (pH 6.8). A 0.2 ml shelf of 60% sucrose was placed at the bottom of each cellulose nitrate tube. 0.1 ml of the material to be centrifuged was layered onto the top of the gradient. A Spinco SW 39 rotor was used and sedimentation was at 35,000 rpm for 3 hr. The centrifuge was allowed to coast, unbraked, to a stop.

Gradients were fractionated from the bottom by puncturing the tube with a stainless steel needle. Two drop fractions were collected on Whatman Paper Chromedia GF 82, dried, and the radioactivity counted in a 2-(4-t-butylphenyl) -5-(4-biphenyl)-1,3,4,-oxydiazole scintillator (5 mg/ml in toluene) in a Wallac Liquid Scintillation Counter, model Decem NTL^{314}. All samples were counted for at least 1000 counts.

CHAPTER 3

THE EFFECT OF ACRIDINE DYES AND ETHIDIUM BROMIDE ON THE STABILITY OF PENICILLINASE PLASMIDS IN STAPHYLOCOCCI.

Introduction.

Studies on the effect of various agents on the elimination of penicillinase plasmids are important for several reasons. The increased rate of loss of characters from a cell due to particular cultural conditions is one of the characteristics of an extrachromosomal element. Further, easy elimination of plasmids from cells facilitates genetic studies, hence the requirement for agents which can accelerate the loss of these elements from a cell. Finally, such studies may be an aid in investigating further the molecular basis of the stability of plasmids and episomes. For example, why should ethidium bromide eliminate plasmids from five mercury-resistant strains but not from four mercury-sensitive strains of staphylococci (Bouanchoud et.al., 1969)?

The acridines are primarily of interest because of their mutagenic effects and the carcinogenicity of the related benzacridines. They are planar molecules and Lerman (1961) showed that the drug can intercalate between adjacent base pairs of DNA and cause extension and unwinding of the DNA backbone. Whether they do in fact increase the rate of loss of plasmids from staphylococci is not certain (cf. Richmond, 1968).

Ethidium bromide, a trypanocidal drug, is, like the acridines a planar molecule that can intercalate between base pairs of DNA (Waring, 1966; Lepecq and Paoletti, 1967). Lepecq and Paoletti have further shown that at low salt concentrations there is a second mode of binding, which seems to be electrostatic, between the positively charged dye and the negatively charged phosphate groups on the DNA molecule. Is ethidium bromide more effective than acridines in eliminating plasmids?

Elimination of penicillinase plasmids by ethidium bromide.

Strains were grown in the presence of various concentrations of ethidium bromide and a concentration of the drug which increased the mean generation time (MGT) approximately six-fold was used to test for

elimination of the plasmid. Results for the strains of staphylococci that were tested are given in Table 3.I.

Although there was evidence of elimination from all strains tested, a more carefully controlled experiment was performed, using strain I07I. This strain was chosen because the penicillinase plasmid was eliminated with high frequency, and because a comparison of the growth of I07I(PI_{I07I}) with I07I, in the presence of ethidium bromide showed that both grew at approximately the same rate. This rules out the possible explanation that the results were due to selection by ethidium bromide of I07I against I07I(PI_{I07I}).

There was no loss of plasmids from either control (Table 3.2.), but there was loss in both flasks containing ethidium bromide, less in that containing 8×10^{-6}M than in the one containing 10^{-5}M.

Elimination of penicillinase plasmids by acridine orange.

The growth rates of the strains referred to in Table 3.I. were determined in the presence of various concentrations of acridine orange. Concentrations that increased the MGT of each strain some six-fold were used in experiments to test elimination of the plasmid. There was no increase in frequency of loss of the plasmid in any of the strains tested, except strain

Table 3.I. The production of penicillinase negative variants from strains of staphylococci by the action of ethidium bromide.

Strain	Concentration ethidium bromide used (M)	Total penicillinase negatives per number of colonies screened		Percent penicillinase negative colonies	
		Control	Test	Control	Test
524	8×10^{-6}	0/3001	21/4103	0.00	0.53
1051	10^{-5}	5/7360	587/5850	0.07	9.78
1071	10^{-5}	0/5001	529/3150	0.00	17.06
13313	10^{-5}	4/4101	25/4240	0.10	0.60
3804	10^{-5}	2/3015	5/3001	0.07	0.17
1044	5×10^{-4}	2/2200	416/9500	0.10	4.37
LA75	10^{-5}	3/5060	102/2750	0.05	3.71

Table 3.2. Production of penicillinase negative variants from strain 107I (PI_{107I}) after growth during exponential phase in the presence of ethidium bromide.

	Initial inoculum	Control flasks		Test flasks	
		1[a]	2[b]	8×10^{-6} M ethidium bromide	10^{-5} ethidium bromide
Total colonies counted	7200	4100	4000	1900	1100
Total penicillinase negatives[c]	1	0	0	3	8
Percent penicillinase negatives	0.014	0	0	0.16	0.72

a. This control was grown to the same OD as the two test flasks, but in absence of ethidium bromide.

b. This control was grown, without ethidium bromide, for the length of time taken by the test flask containing 10^{-5} M ethidium bromide to reach a total of 8×10^{8} cfu.

c. Colonies that were one half, or more, penicillinase negative were scored as negatives.

$IO44(PI_{IO44})$. Elimination by acridine orange was attempted under different conditions (pH and temperature) and using different concentrations of acridine orange, but without success. The frequency of loss was only increased in strain $IO44(PI_{IO44})$. A comparison of the frequency of loss of penicillinase plasmids from this strain using ethidium bromide and using acridine orange is given in Table 3.3. Though the concentration of ethidium bromide used was higher, this is, to a large extent, artificial as $IO44(PI_{IO44})$ was found to be unusually resistant to the drug and in fact has a plasmid-borne gene conferring this resistance (Johnston and Dyke, 1969).

An attempt was made to repeat the controlled experiment done with ethidium bromide on strain $IO7I(PI_{IO7I})$, using acridine orange and strain $IO44(PI_{IO44})$, but without success. Under the conditions of that experiment there was no loss of the plasmid from $IO44(PI_{IO44})$.

<u>The resistance of strain $IO44(PI_{IO44})$ to ethidium bromide.</u>

It is apparent from Table 3.I. that a much higher concentration of ethidium bromide is required to inhibit growth of $IO44(PI_{IO44})$ than is required for the other strains tested. Strain $IO44(PI_{IO44})$ can grow at a

Table 3.3. The elimination of the penicillinase plasmid from strain IO44 (PI_{IO44}.) by the action of ethidium bromide and acridine orange.

Drug	Concentration used (M).	Percent penicillinase negative colonies.	
		Control	Test
Ethidium bromide	$5 \times IO^{-4}$	0.IO	4.37
Acridine orange	$4 \times IO^{-5}$	0.I3	3.59

a concentration of the drug (2×10^{-5}M) that was inhibitory to all other strains tested, (Figure 3.I.). Even at an ethidium bromide concentration of 2×10^{-4}M, there was substantial growth of I044(PI_{I044}).

It seemed possible that an investigation of this resistance might help toward an understanding of the mechanism of action of ethidium bromide in causing instability and, if the resistance were due to an alteration in the function(s) necessary for stability, then something might also be learnt of this system. The mechanism of resistance of I044(PI_{I044}) to ethidium bromide was therefore investigated.

Uptake of ethidium bromide by strains of staphylococci.

After some strains had grown in TSB containing ethidium bromide, the cells assumed a pinkish colour, as if they had taken up the drug. This was not the case with strain I044(PI_{I044}). A simple explanation for the resistance of I044(PI_{I044}) to ethidium bromide may be that this strain does not take up ethidium bromide, while sensitive strains do. Experiments were done to test this.

First, the absorption spectrum of ethidium bromide in 0.I M phosphate buffer at pH 7.0 was measured; 480 nm was found to be the optimum wavelength for measuring the concentration of the drug.

Figure 3.I. The effect of ethidium bromide on the growth of strains of S.aureus.

Cultures grown in TSB at 37 C for I6 hr were diluted with fresh TSB containing ethidium bromide. The diluted cultures were shaken at 37 C and growth was followed by measurement of OD at 675 nm.

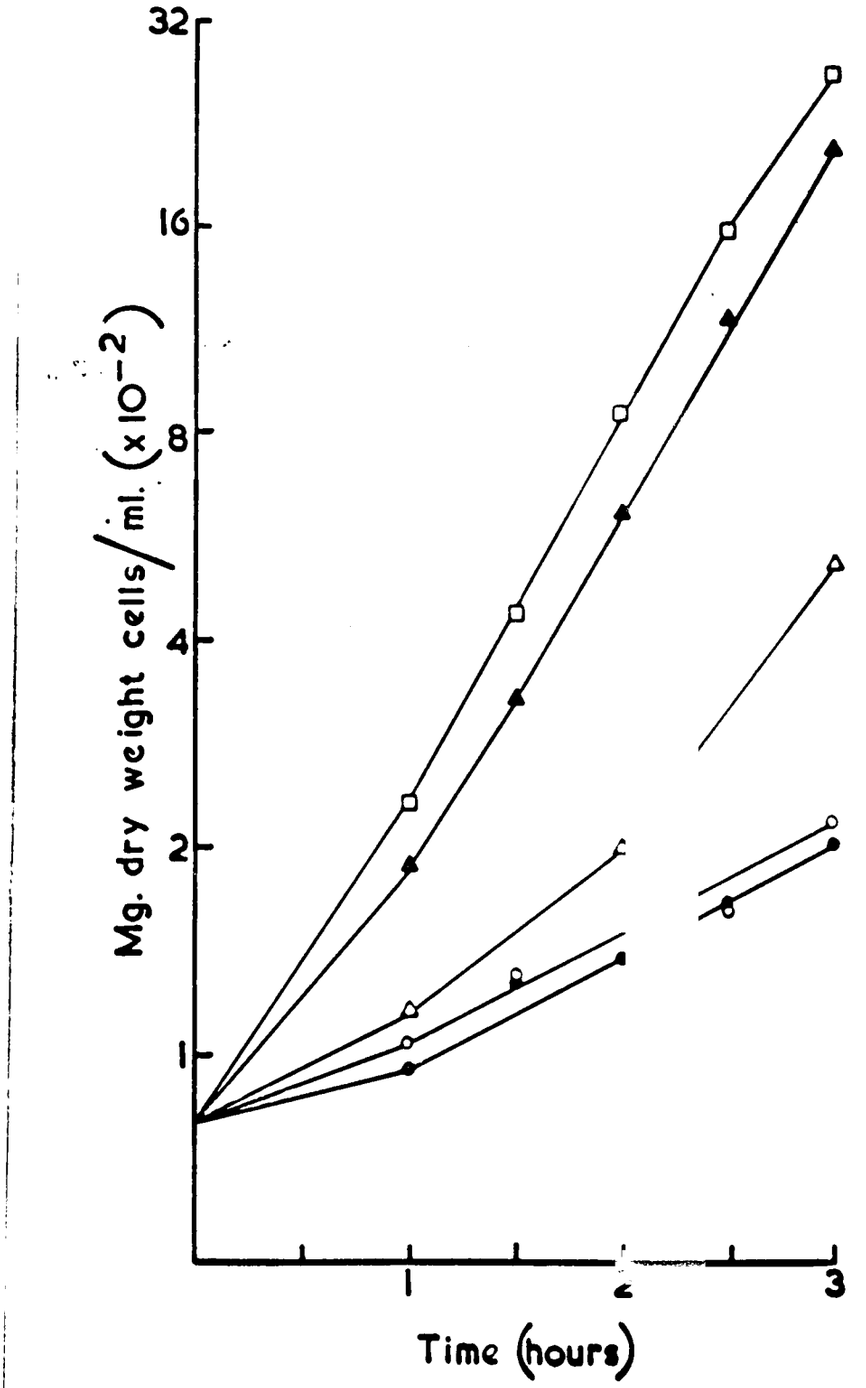
□ Control: I044(PI_{I044}), I044, 524(PI_{524}) in the absence of ethidium bromide.

▲ I044(PI_{I044}) in 2 x $I0^{-5}$M ethidium bromide.

● 524(PI_{524}) -do-

○ I044 -do-

△ I044(PI_{I044}) in 2 x $I0^{-4}$ M ethidium bromide.


32
16
8
4
2
1
Mg. dry weight cells/ml. (x10⁻²)
1
2
3
Time (hours)

The relationship between concentration of ethidium bromide and absorption was found to be linear (Figure 3.2.).

The uptake of ethidium bromide by strains 524(PI_{524}), 107I(PI_{107I}) and 1044(PI_{1044}) is given in Table 3.4. There is no uptake by the resistant strain 1044(PI_{1044}), but substantial uptake by 524(PI_{524}), the strain most sensitive to ethidium bromide, and slightly less uptake by 107I(PI_{107I}), which is marginally more resistant than 524(PI_{524}) (Table 3.I.).

At concentrations of ethidium bromide that inhibit (4×10^{-4}M) and completely prevent (2×10^{-3}M) growth of 1044(PI_{1044}), there is uptake of the drug (Table 3.5). In this table the uptake of a sensitive strain is also given for comparison.

<u>The uptake of ethidium bromide at 0 C and in the presence of sodium azide</u>.

Since the resistance of 1044(PI_{1044}) to ethidium bromide may involve exclusion of the drug from the cell, it is possible that energy is involved in this process. The uptake of ethidium bromide by 1044(PI_{1044}), 1044 and 524(PI_{524}) at 0 C and in the presence of 10^{-2}M sodium azide was measured (Table 3.6.). For controls, uptake at 37 C and in the absence of azide, see Table 3.4.

Figure 3.2. Relationship between concentration of ethidium bromide and OD at 480 nm.

The ethidium bromide was dissolved in 0.01 M phosphate buffer (pH 7.0) and OD was read at a wavelength of 480 nm.

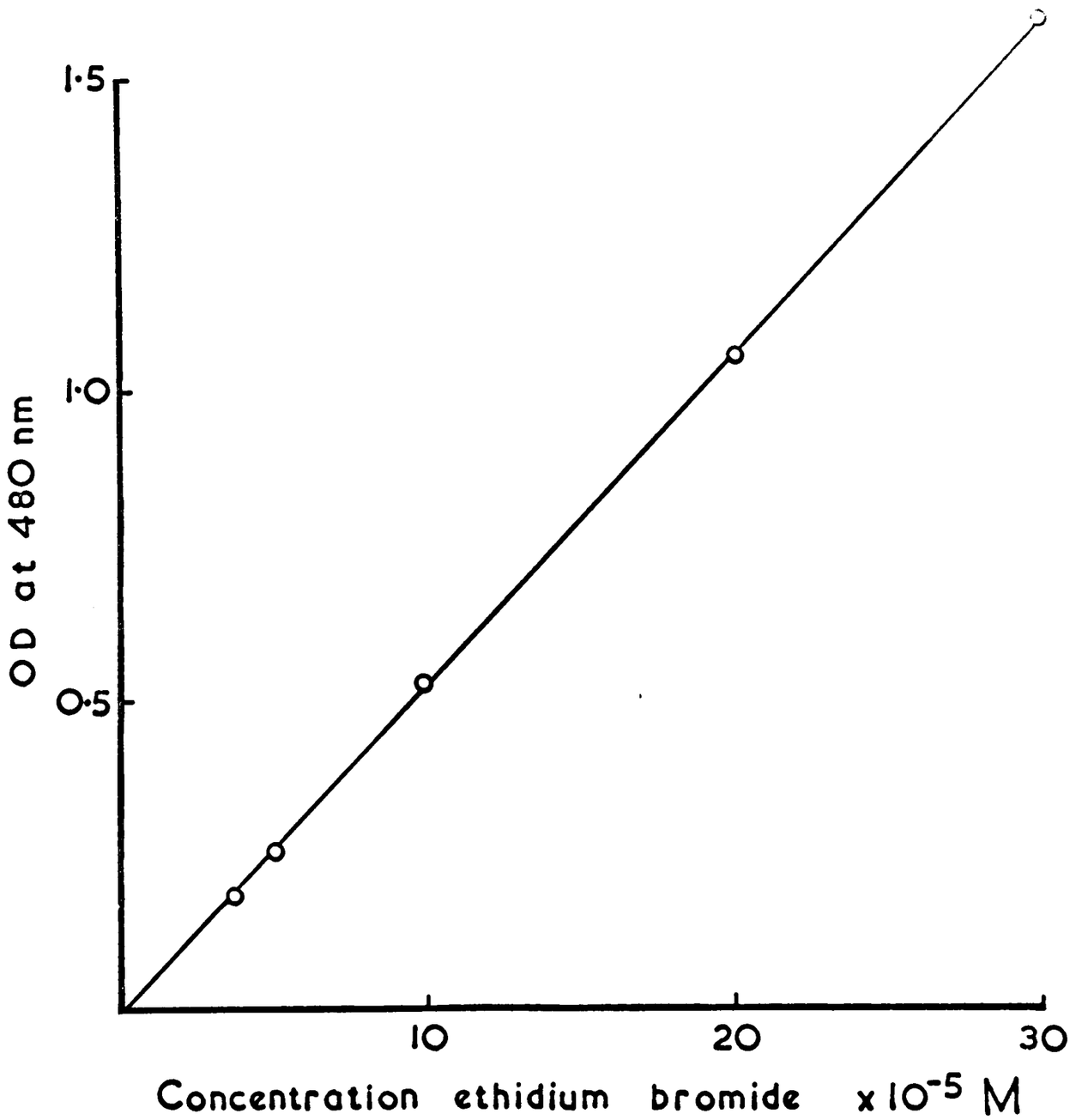

1·5
1·0
0·5
OD at 480 nm
10
20
30
Concentration ethidium bromide x 10⁻⁵ M

Table 3.4. The uptake of ethidium bromide from a 2 x 10^{-4}M solution by strains of staphylococci.

Time (minutes)	Absorbancy of supernatant fluid after incubation with strain:			
	524(PI_{524})	1071(PI_{1071})	1044(PI_{1044})	1044
0	1.18	1.20	1.20	1.20
12	0.70	0.96	1.20	-
24	0.50	0.84	1.20	-
30	0.39	0.80	1.20	0.78
60	0.24	0.62	1.20	0.70

Table 3.5. The uptake of ethidium bromide by strain I044(PI_{I044}) at concentrations inhibiting growth of the strain.

Time (minutes)	Absorbancy of supernatant fluid after incubation with:			
	4×10^{-4}M ethidium bromide		2×10^{-3}M ethidium bromide	
	I044(PI_{I044})	I044	I044(PI_{I044})	524(PI_{524})
0	2.10	2.10	10.60	10.60
12	-	-	10.05	9.80
25	-	-	9.90	9.80
30	1.93	1.66	-	-
36	-	-	9.90	9.50
60	1.90	1.60	9.80	9.30

Table 3.6. The uptake of ethidium bromide from a 2 x 10^{-4}M solution by strains 524(PI_{524}), 1044(PI_{1044}) and 1044, at 0 C and in the presence of sodium azide.

Time (minutes)	Absorbancy of supernatant fluid after incubation:					
	At 0 C			In the presence of sodium azide		
	1044(PI_{1044})	524(PI_{524})	1044	1044(PI_{1044})	524(PI_{524})	1044
0	I.I8	I.I8	I.I6	I.20	I.20	I.20
20	I.I8	I.I0	-	-	I.06	-
30	I.I8	I.08	I.I6	I.20	-	0.94
40	I.I8	I.07	-	-	I.00	-
60	I.I8	I.04	I.I4	I.20	0.99	0.88

From these results it seems that in sensitive cells there is a system that actively takes up ethidium bromide and this system is absent or masked in IO44(PI_{IO44}).

The location of the genetic determinants for resistance to ethidium bromide.

The penicillinase plasmid was eliminated from IO44(PI_{IO44}) after growth in the presence of 4 x IO^{-5}M acridine orange. This penicillinase-negative variant, strain IO44, was tested and found to be as sensitive to ethidium bromide as the other strains tested (the minimum inhibitory concentration of ethidium bromide necessary to prevent growth for 24 hr was determined in TSB at 37 C with an inoculum of 4 x IO^{6} cfu/ml; IO44(PI_{IO44}) = 5.75 x IO^{-4}M and IO44 = 3.5 x IO^{-5}M). Three different isolates of IO44 had also lost their resistance to ethidium bromide along with the ability to produce penicillinase. Strain IO44 behaved just as did the other sensitive strains examined (Tables 3.4., 3.5., 3.6.).

Phage 53 propagated on IO44(PI_{IO44}) could be used to transduce resistance to ethidium bromide back to strain IO44, by selecting transductants on CY-eb agar. All such transductants produced penicillinase and behaved exactly

as did I044(PI_{I044}). When transductants were selected on CY-pen agar, all were also resistant to ethidium bromide. The gene for resistance to ethidium bromide must therefore be located on the penicillinase plasmid (Johnston and Dyke, I969).

Attempts to transduce (PI_{I044}) to other strains of staphylococci susceptible to phage 53, by selection on CY-eb agar were unsuccessful. This transduction was attempted many times with strains I07I, I33I3 and 524 as recipients but was never successful. When the transduction was done with selection on CY-pen agar, penicillin-resistant transductants were obtained, but none of these were resistant to ethidium bromide.

It seemed possible that absorption was only part of the mechanism of resistance to ethidium bromide, so further experiments were done. With I044(PI_{I044}) there is an increased lag period when growing in the presence of ethidium bromide, before exponential growth begins. This could be consistent with the synthesis of an enzyme for degradation of ethidium bromide. So I044(PI_{I044}) was grown in TSB containing ethidium bromide for I6 hr, the cells were centrifuged down and various dilutions of the ethidium bromide remaining in the supernatant were added to a culture of 524(PI_{524}) . There was no detectable

difference in inhibition of growth compared to the same concentration of ethidium bromide with no prior incubation with I044(PI_{I044}), so that biological activity of the ethidium bromide was unaltered. Furthermore there was no change in the absorption spectrum of the drug after growth of I044(PI_{I044}).

Discussion.

The results given in Tables 3.I. and 3.2. suggest that ethidium bromide can cause the elimination of penicillinase plasmids from some strains of staphylococci. Since the cells were in contact with ethidium bromide for I6 hr (Table 3.I.) any eliminating effect may have occurred only when the cells were in stationery phase. To ensure that the eliminating effect was due to the activity of ethidium bromide, the controlled experiment on I07I(PI_{I07I}) was done (Table 3.2.). Cells were only in contact with the drug during lag and early exponential phases of growth, yet cells in both flasks containing ethidium bromide showed increased loss of the plasmid. There was no loss from either control.

In the strains tested ethidium bromide is more efficient than acridine orange in increasing the loss of penicillinase plasmids. The plasmid was eliminated from only one strain

by acridine orange - strain I044(PI_{I044}). However the rate of loss was not sufficient to allow a repeat of the controlled experiment done with I07I(PI_{I07I}).

The molecular basis for the apparently selective effect of ethidium bromide on plasmids is not known. However Bauer and Vinograd (1968 and 1970) have shown that ethidium bromide has an increased affinity for right-handed supercoiled circles of DNA in vitro. It is possible that the specific effect of ethidium bromide may be accounted for by this fact; that in the cell plasmids are in right-handed supercoils and so plasmid DNA is more susceptible to ethidium bromide than the DNA of the chromosome. The concentration that will eliminate plasmids may be that concentration which will exploit this differential; too high a concentration causing intercalation of the drug into the chromosome, killing the cell and a lower concentration not having sufficient effect to eliminate the plasmid.

It was not possible to establish any correlation between frequency of elimination by ethidium bromide and any other phenotypic characters of the strains used, such as antibiotic resistances, ion resistances or phage typing pattern. I was unable to confirm the finding of Bouanchoud et.al. that ethidium bromide eliminates penicillinase plasmids only from mercury-resistant organisms. Strain I07I(PI_{I07I}) a strain showing frequent loss of the plasmid, is

mercury-sensitive. The plasmid was also eliminated from strain LA75(PI_{LA75}) (Table 3.I), one used by Bouanchoud et.al. that was apparently resistant to elimination. The discrepancy probably arose as Bouanchoud et.al. did not study a sufficient number of colonies.

The resistance of IO44(PI_{IO44}) to ethidium bromide seems to be due to permeability alone. No effect of the organism on the drug was detected. A genetic determinant for the resistance is located on the penicillinase plasmid, yet it was not possible to transduce this resistance to any other strain, though transfer of the plasmid was demonstrated. This could be explained by the existence of at least two determinants for resistance to ethidium bromide, one on the plasmid (PI_{IO44}), and one on the chromosome of strain IO44.

CHAPTER 4

THE ISOLATION AND CHARACTERISATION OF MUTANTS IN WHICH A PENICILLINASE PLASMID IS UNSTABLE.

Introduction.

In their replicon hypothesis Jacob et.al.(1963) postulated that for each replicon in the bacterial cell there is a membrane attachment site with the functions of distribution and possibly replication. It might be possible to separate the different functions necessary for the stability of a plasmid by analysis of mutations causing instability; such mutations might, for example, be separated into complementation groups. On the basis of the replicon hypothesis one might expect two types of instability mutant: one, a host mutation in the plasmid attachment site and the other a mutation in the locus on the plasmid responsible for the attachment. Plasmid - and host-determined mutations causing instability in penicillinase plasmids have been isolated (Novick, 1967b).

For the isolation of unstable mutants in this study, strain PS80 was chosen, primarily because there is evidence of an interaction between the plasmid and the chromosome. In PS80 the penI.penZ genes are stable and are located on the chromosome, while the naturally occurring plasmid does not possess penicillinase genes and has the genotype: (PI_{9789}.asa.cad.mer). Occasionally however, the penicillinase genes are found linked to the plasmid (Asheshov, 1969); this probably occurs by gene duplication, since the penicillinase genes are not lost from the chromosome.

Thus PS80 seemed to provide a diverse genetic situation in which to study stability. Furthermore it was possible, in view of the interaction between plasmid and chromosome, that the chromosomal penicillinase genes might have associated with them plasmid DNA which might contain genes that can complement unstable plasmid mutants.

The isolation of unstable and temperature-sensitive mutants.

The temperature-sensitive mutant was obtained by EMS treatment of PS80.d.(PI_{9789}.penI.penZ). Approximately 10,000 colonies were screened in the manner described and a colony temperature sensitive for production of

production of penicillinase was found. This is the temperature-sensitive mutant that has been used in these studies and the mutation has been named seg-I; for simplification it is referred to as the SI-mutation.

The unstable mutants were also isolated after EMS treatment of PS80.d.(PI_{9789}.penI.penZ). Three separate mutants were isolated after screening approximately 10,000 colonies. Mutants of this sort were selected on the basis of the highly sectored nature of the colonies, which implied frequent segregation of the plasmid, and hence instability. The first of these mutants was named seg-2; for simplification it is referred to as the S2-mutation. Two further unstable mutants subsequently isolated were found to be phenotypically identical to seg-2, however all the experiments with an unstable mutant described here were done with seg-2.

Tests for plasmid or host linkage of mutations.

To determine whether the SI - and S2-mutations were host - or plasmid-linked, a plasmid from the mutated cells was transduced into PS80.d. and the stability of the plasmid was then determined (by plate assay for production of penicillinase by colonies of SI, after overnight incubation at 42 C; and for S2 by inspecting colonies, stained with PNCB, for sectoring after overnight incubation at

37 C). In addition mutant host cells which had lost their plasmid during growth at 42 C were selected and used as recipients for a stable, wild-type, $\underline{mcr}_I$ plasmid in a transduction experiment. The stability of the $\underline{mcr}_I$ plasmid in these mutant host cells was determined (Table 4.I).

The SI-mutation is solely plasmid-linked, it is also temperature-sensitive in strain I054 (Table 4.I), and the full genotype of the SI-plasmid is: (PI_{9789}.<u>seg</u>-I.<u>penI</u>.<u>penZ</u>).

The S2-mutation is host-linked. The genotype of the S2-mutant cells is PS80.d.<u>seg</u>-2 . Two subsequent mutants isolated by the same criteria as <u>seg</u>-2 were also host-linked.

<u>Segregation kinetics for the SI-plasmid</u>.

Jacob <u>et.al</u>.(I963) found that a temperature-sensitive F-<u>lac</u> plasmid in <u>E.coli</u> gives a dilution curve with a slope of -0.3 at the non-permissive temperature. This particular negative slope is indicative of a situation in which plasmids do not replicate at the non-permissive temperature, but survive, so that they are progressively diluted out of the culture. Slopes less steep than -0.3 indicate some replication at the temperature of the experiment, and steeper slopes indicate destruction of existing plasmids. A defect involving plasmid distribution but not replication would give a convex dilution curve.

Table 4.I. Plasmid or host linkage of mutations.

Transduction		Number of transductants examined	Stability of plasmid in transductants	Location of mutation
Donor of plasmid	Recipient strain			
PS80(PI$_{9789}$•penI.penZ)	Plasmid negative variant of temperature sensitive strain	I60	Stable	Plasmid
Temperature sensitive mutant	PS80.d.	65	Temperature sensitive	Plasmid
Temperature sensitive mutant	I054	9	Temperature sensitive	Plasmid
PS80(PI$_{9789}$•penI.penZ)	Plasmid negative variant of unstable mutant	I35	Unstable	Host
Unstable mutant	PS80.d.	62	Stable	Host

The rate of loss of the SI-plasmid from PS80.d. was determined (Figure 4.I.). The slope obtained indicates some replication at 42 C, as it is less steep than -0.3.

Stability of mcr_I and mcr_{II} plasmids in S2.

Inspection of colonies of the S2-mutant stained with PNCB indicated that both mcr_I and mcr_{II} plasmids were lost from the cell at high frequency. The stability of mcr_I and mcr_{II} plasmids was therefore determined quantitatively (Table 4.2). mcr_I and mcr_{II} plasmids are unstable in S2, but there are minor quantitative differences in that mcr_{II} plasmids are slightly less affected by the S2-mutation than mcr_I plasmids.

Both mcr_I and mcr_{II} plasmids were also affected in the two further unstable mutants isolated in the same way as had been S2.

Stability of the tetracycline plasmid is not affected in any one of these three mutants. In one experiment involving PS80.d.*seg*-2(T_{I050I}), 4 out of I298 colonies were tetracycline sensitive, compared to 3 out of I203 in a control PS80.d.(T_{I050I}).

Figure 4.I. Segregation kinetics and growth curve for SI in PS80.d. at 42 C.

△ Segregation kinetics of the control strain PS80.d.(PI_{9789}.penI.penZ) .

▲ Segregation kinetics for PS80.d.(SI).

○ Growth curve for the control strain, PS80.d.(PI_{9789}.penI.penZ)

● Growth curve for PS80.d.(SI).

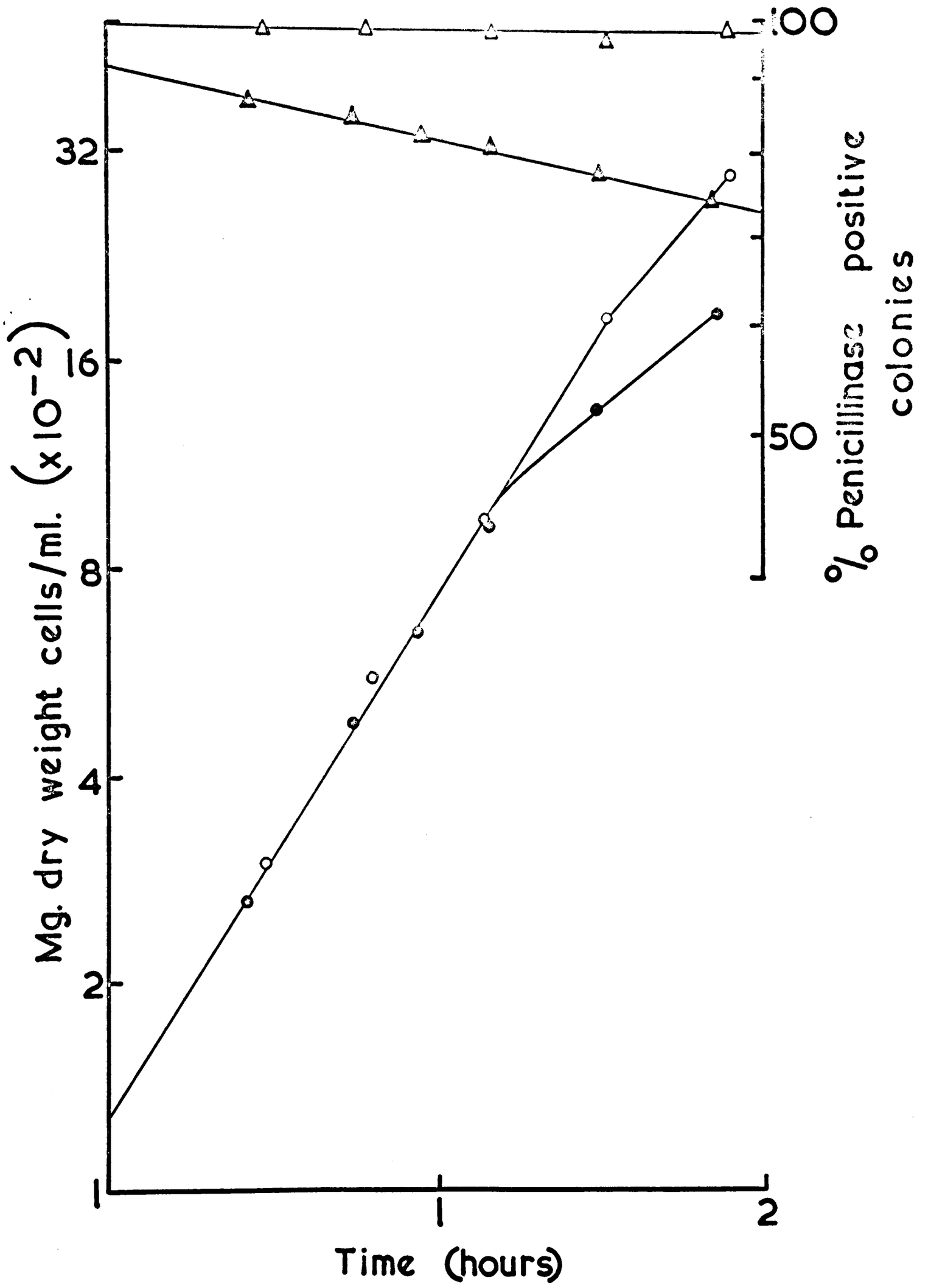

100
32
16
8
4
2
1
50
Mg. dry weight cells/ml. (x10⁻²)
% Penicillinase positive colonies
1
2
Time (hours)

Table 4.2. Stability of $\underline{mcr}_I$ and $\underline{mcr}_{II}$ plasmids in S2.

Strain	Total colonies counted		Number of Penicillinase-negative colonies		Percent penicillinase-negative colonies	
	Initial	Final	Initial	Final	Initial	Final
Control, PS80(PI_{9789}·penI.penZ)	4909	3763	43	8I	0.88	2.I5
S2(PI_{9789}·penI.penZ)	I0050	8680	62I	5I03	6.I8	58.75
Control, PS80(PII_{I47}·)	5003	60I0	40	I09	0.80	I.8I
S2(PII_{I47}·)	769I	5224	3I3	2I3I	4.07	40.79

Complementation of SI.

It is difficult to test for complementation between penicillinase plasmids because of the problem of incompatibility. For this reason it has not been possible to investigate complementation between PS80.d. (PI_{9789}·seg-I.penI.penZ) and PS80.d.(PI_{9789}·penI.penZ), but diploids were constructed in two other ways.

First, the fact that in wild-type PS80 the penicillinase genes are chromosomal and do not prevent the replication of a superinfecting plasmid allows the construction of a partial diploid. SI was transduced into PS80, by selecting transductants on CY-cad agar. Sixty-eight were obtained and were tested for stability of the plasmid by assaying for cadmium resistance. Colonies derived from the transductants were grown overnight on CY-agar at 42 C and replicated to CY-cad agar. All were cadmium sensitive and hence temperature sensitive.There is therefore no complementation of the SI-plasmid by the penI.penZ region on the chromosome.

Secondly, a diploid was constructed of the SI-plasmid with a plasmid of mcr_{II} type. Phage propagated on PS80.d.(PII_{I47}) was irradiated and used to transduce cadmium-resistance to PS80.d., and a penicillinase-negative transductant isolated. SI was transduced into this strain by selection on CY-pen agar. Five transductants were

obtained, all were temperature sensitive. There is no complementation of the temperature-sensitive defect in the SI-plasmid by proteins produced by a $\underline{mcr}_{II}$ plasmid.

Discussion.

The SI-plasmid has a temperature-sensitive mutation that causes the plasmid to be diluted out of the culture at high temperature; this may be due to non-replication of the plasmid at the high temperature. The mutation is solely plasmid-linked. The results given in Figure 4.I. indicate that there is some replication at 42 C - Jacob et.al. found that complete cessation of replication gives a dilution curve of slope -0.3 and the slope obtained for SI is less steep than -0.3. Hirota, Ryter and Jacob (1968) have shown that at temperatures slightly below the non-permissive temperature, the slope of the dilution curve for a temperature-sensitive F-lac in E.coli may be increased by small rises in temperature. It is probable therefore that at some temperature a little above 42 C, the slope of the dilution curve for SI will be -0.3. Since the mutation is temperature dependent it is probable that a defective protein is produced at the restrictive temperature.

If a temperature-sensitive protein is produced, it

should be possible to demonstrate complementation by a suitable wild-type plasmid. However there was no complementation of the SI-plasmid, but both tests used have limitations. It is not certain that the chromosomal penI.penZ region in PS80 includes DNA coding for the defective SI-plasmid protein. Even if this DNA is present on the chromosome it may very well not be expressed. The second complementation test employed a plasmid of different compatibility group, and therefore different in at least one function of maintenance from a mcr_I plasmid - and possibly SI is defective in this different function. Another possibility is that the SI-plasmid has a defect in a protein that is not freely diffusible.

The S2-mutation is in a host-directed function that is necessary for maintenance of mcr_I and mcr_{II} plasmids but not necessary for the maintenance of a tetracycline plasmid. This mutation does not affect the growth rate of the host. These results suggest that the maintenance of penicillinase plasmids requires a component that is common to the two compatibility groups. But this component is not involved in the maintenance of all plasmids, or of the chromosome. If the 'stability' of the chromosome were affected by the S2-mutation, this would presumably be reflected in an altered growth rate.

CHAPTER 5

INTEGRATION OF THE PLASMID INTO THE CHROMOSOME.

Introduction.

Genes located on the plasmid are lost from the cell at rates greater than mutation rates. Penicillinase genes located on the chromosome are more stable than those on the plasmid (Asheshov, 1966b). Thus a mechanism whereby plasmid markers could become integrated into the chromosome of a cell, and so have increased stability, would probably be an advantage to the cell. In view of this I have studied integration of the penicillinase plasmid into the chromosome of strain PS80.

A. Integration of the Plasmid into the Chromosome.

This was studied by investigating the reversion of the temperature-sensitive plasmid, SI, to temperature stability. It seemed likely, in view of the work of Novick (1968), that at least some revertants would become temperature stable by integration into another replicon, probably the chromosome.

Reversion to temperature stability of SI.

When 10^6 cfu of PS80.d.(SI) were spread on CY-cad agar and incubated at 42 C for 18 hr, on average (5 plates, each of 10^6 cfu) some 33 cadmium-resistant colonies grew. Since the gene for resistance to cadmium-ions is located on the plasmid, and the plasmid is temperature sensitive, no colonies would be expected after incubation of CY-cad agar plates at 42 C. However the experiment has been repeated more than 15 times and cadmium resistant colonies are always found; the average (of 5 plates per experiment) for each experiment has always been between 31 and 35 cadmium-resistant colonies per 10^6 cfu plated, with exactly 33 by far the most common. The plasmid is now more stable at 42 C than the wild-type mcr_I plasmid from which it was derived, and very much

more stable than the SI-plasmid (Table 5.I). Colonies obtained in this way will be referred to as revertants.

Phage propagated on these stable revertants and used to transduce cadmium resistance to PS80.d. gave transductants that were all (more than a thousand were tested) temperature sensitive for penicillinase production. So the original mutation causing the plasmid to be temperature sensitive is still present, but masked, in the revertants.

A second $\underline{mcr}_I$ plasmid was transduced into the revertants to give a stable, plasmid diploid. This is an unusual situation because a superinfecting incompatible plasmid cannot usually establish itself in the recipient, except by recombination with the resident plasmid or by displacing the resident plasmid (Richmond, 1968). A possible interpretation of the stable plasmid diploid is that the putative membrane attachment site for the plasmid was no longer occupied, so that the superinfecting plasmid was able to be maintained. In experiments in which this second $\underline{mcr}_I$ plasmid was temperature sensitive it was found that the second plasmid was lost after growth at 42 C.

Of the various possibilities for stabilisation of the temperature-sensitive mutation, integration into the chromosome seems most likely. Linkage between the stabilised plasmid and a gene known to be on the chromosome

Table 5.I. The stability at 42 C of the penicillinase genes of (PI_{9789}.*penI*.*penZ*), the SI-plasmi and a SI revertant, in host PS80.d.

Grown at 42 C for:	Percent penicillinase-negative colonies		
	(PI_{9789}. *penI*.*penZ*)	SI-plasmid	SI-revertant
0 hr	0.8	I.8	0.I2
4 hr	I.0	63.0	0.34

would be strong evidence for an integration event. In staphylococci however, very few chromosomal genes have been identified. One such in strain PS80 is the structural gene for penicillinase (Asheshov, 1966b). I therefore looked for linkage between a reverted temperature-sensitive, cadmium-resistant, but penicillinase-negative plasmid and the chromosomal penicillinase gene. A SI-plasmid from which the penicillinase gene had been deleted (see 'Material and Methods') was transduced into PS80, giving transductants with the genotype: PS80(PI_{9789}·seg-I). Cells from one of the transductants were spread on CY-cad agar and incubated at 42 C; cadmium-resistant, temperature-stable revertants were isolated. Phage was propagated on two of these; one of the preparations gave no cotransduction of the plasmid cad gene and the chromosomal penZ but the other gave 100% cotransduction of these genes, and all these transductants contained plasmids that were temperature sensitive for both cadmium resistance and penicillinase production.

In further experiments a plasmid gene, erythromycin resistance from a (PI_{258}) plasmid, was forced into the chromosome by a modification of the method of Novick (1967). Subsequently, linkage between this and a reverted SI-plasmid was investigated. A SI-plasmid was trans- into strain PS80.d.ero, selecting for cadmium resistance,

and temperature-stable revertants obtained. Phage was propagated on a number of these revertants and used to transduce erythromycin resistance to PS80.d.; all transductants were tested for cotransduction of the penZ gene. Phage propagated on four of 32 revertants showed cotransduction of the two markers, but at different frequencies: 0.6%, 3.7%, 8.0%, 14.6% of the erythromycin-resistant transductants possessed the penZ gene as well. These four all have the genotype, PS80.d.(PI_{9789} seg-I.penI.penZ.ero); the plasmid, including ero, is temperature sensitive. Phage propagated on the remaining 28 revertants did not cotransduce ero and penZ.

These results suggest that temperature-stable reversion is due to integration of the plasmid into the chromosome.

B. A Specific Mechanism for Integration of the Plasmid into the Chromosome.

In view of the probable integration of the plasmid into the chromosome, an obvious question is whether there is any specific mechanism responsible for the integration event. One approach to answering this is to look for

mutations on the SI-plasmid that decrease the frequency of integration.

Isolation of an integration-deficient mutant.

Regions of the SI-plasmid were deleted by irradiation with uv light; all such deletions were then checked for frequency of integration (reversion to temperature stability) in PS80.d. A gene producing a protein involved in mediation of the integration event might be linked to one of the known genes of the plasmid and by investigating integration frequency of deletions of each of the genes in turn, it was thought that it might be possible to isolate an integration-defective mutant.

One of the first deletions examined was for the penicillinase region. In an earlier series of experiments the SI-plasmid was transduced into PS80.penZ-50I.; 63 cadmium-resistant transductants were obtained, one of which produced no penicillinase. This was found to give fewer temperature-stable revertants than SI (Table 5.2), and is thought to be defective in some mechanism for integration into the chromosome. It arose as the result of a spontaneous deletion during transduction of the plasmid, and it has been named SI[int]; the plasmid is

Table 5.2. Frequency of temperature-stable reversion of SI and SI[*int*]

Plasmid	Number of revertants in 10^6 cells						
	Host cell genotype: PS80						
	PS80.d. (1)	Wild-type (2)	Plasmid derived chromosomal genes				
			ero (3)	*penI*.*penZ* (4)	*penI*.*penZ* (5)	*penI*.*penZ* (6)	*penI*.*penZ* (7)
SI	33	36	c.1000	37	73	42	75
SI[*int*]	3	3	c.1000	3	3	3	3

resistant to arsenate - , cadmium - and mercury-ions and only penI.penZ of the known plasmid genes are absent.

The relationship between homology and frequency of integration.

The frequency of integration of the SI-plasmid in the presence of chromosomal penI.penZ genes was investigated. SI was transduced into PS80 and the number of temperature-stable revertants determined; there was only a slight increase, SI[int] was not affected at all and reverted with the usual low frequency (Table 5.2, column 2).

Next,the frequency of reversion of SI and SI[int] in PS80.d.ero (the strain with the plasmid-derived chromosomal ero) was determined. There was a very large increase in the number of revertants for both SI and SI[int]. The plates for each contained some 60 normal-sized colonies and a large number of smaller colonies of different sizes, which made precise counting difficult. It was not possible to decrease the number of cfu plated when estimating the frequency of reversion as there is an inoculum effect. One-tenth the number of cells plated did not result in one-tenth the usual number of revertants, but

considerably fewer than expected. An approximation of the total number of visible colonies when 10^6 cfu are plated is given in Table 5.2(column 3). Plates for SI and SI[int] were indistinguishable.

In view of this unexpected result further experiments on the effect of homology on the frequency of integration were carried out. It was found possible to force the penI.penZ genes from the plasmid to the chromosome, just as had been done with the ero gene (see 'Material and Methods'). Four separate plasmid-derived, chromosomal, penicillinase strains were constructed; the SI-plasmid was transduced into these, and the frequency of integration determined (Table 5.2, columns 4,5,6,7). There was no increase comparable with that found in PS80.d.ero.

An attempt was made to force a cad gene into the chromosome from (PI_{9789}.seg-I.[penI.penZ.asa]) plasmid, but without success, so it was not possible to do similar experiments with a cad chromosomal gene.

The reversion of the SI-ero linked plasmids.

Four of the preparations of phage propagated on SI-revertants in PS80.d.ero cotransduced ero and the plasmid markers. Since there may now be more homology

between the SI-ero plasmids and the chromosome, the frequency of reversion of these four plasmids was determined.

All four reverted with a very much greater frequency than SI in PS80.d.ero. Plates were covered with small colonies, too numerous to count, to give almost confluent growth. There was an observable difference in integration frequency between the reverted plasmid which showed 0.6% cotransduction with the chromosomal ero and that which showed 14.6% cotransduction, the former reverting with greater frequency than the latter.

The transduction of the chromosomal ero.

A possible explanation of the increased frequency of integration in the presence of the plasmid-derived chromosomal ero is that ero has the integration function linked to it. If this is correct then a preparation of phage transducing the chromosomal ero should cotransduce the integration function. Phage was therefore propagated on PS80.d.ero on seven separate occasions; these preparations were found to transduce erythromycin resistance with frequencies varying from I per 5×10^5 phage to I per 2×10^6 phages. In this system, this is a figure typical for transduction of a plasmid gene

rather than a chromosomal gene (Arber, 1960; Asheshov, 1966b, 1969); yet transductants from each transduction were tested for stability of ero by growth at 42 C, and for absence of mcr, by testing for compatibility with both mcr_I and mcr_{II} plasmids, and in every case ero behaves as a chromosomal gene.

There was a very large range in size of transductant colonies on the selective plates but they did not appear to be abortive transductants since on continued incubation the smaller colonies continued to grow until eventually all the colonies were approximately the same size. Furthermore the transductants, both large and small colonies, remain resistant to erythromycin when tested subsequent to subculture in absence of the antibiotic.

Isolation of further [int] mutants.

The original integration-deficient mutant also lacked penZ, so that in looking for further [int]-mutants I looked first at deletions of the plasmid penZ region. Ten [penI], sixty-two [penZ] and two [penZ.asa] deletion mutants of SI were obtained by irradiating phage grown on PS80.d.(SI) and these mutants were tested for reversion to temperature stability. All gave the

normal or a slightly reduced frequency of reversion. The integration function, therefore, did not seem to be linked to either penI, penZ or asa when the plasmid is being carried by phage (i.e. is probably in the linear form).

The original [int] was a spontaneous deletion that occurred during transduction of the SI-plasmid. It is possible that the deletion occurred when the plasmid was either in the donor cell or in the recipient cell, in either of which it would have been circular (Rush et.al., 1969). I therefore irradiated cells containing the SI-plasmid and subsequently picked colonies that either produced no penicillinase or produced the enzyme constitutively. These were then tested for integration frequency (Table 5.3). One of the presumed [penI.penZ] mutants was indistinguishable from the original [int] mutant (Table 5.3, columns I and 2) and three [penI] mutants integrated at a much reduced frequency (Table 5.3, columns 3,4,5).

Discussion.

The possible mechanisms that might have accounted for the suppression of the temperature-sensitive defect in the stable revertants are:

Table 5.3. Frequency of temperature-stable reversion of SI[*int*] and other integration-deficient mutants.

Number of revertants in PS80.d. per 10^6 cells					
SI[*int*] (I)	[*penI*.*penZ*] (2)	[*penI*] (3)	[*penI*] (4)	[*penI*] (5)	[*ero*] (6)
3	3	9	9	7	6

(i). Back mutation.

(ii). A second mutation in the host cell overcoming the temperature-sensitive defect.

(iii). Integration into another replicon.

The first possibility can be ruled out since phage propagated on a revertant and subsequently used to transduce cadmium resistance, gave transductants that were temperature sensitive for production of penicillinase. A change in the host cell can also be discounted since a second, identical temperature-sensitive plasmid could be maintained in the revertant cell at the permissive temperature but was lost on transfer to the higher, non-permissive temperature. Integration into another replicon and replication as part of that replicon seems the most likely means of stabilisation of the plasmid. That this second replicon is the chromosome is not certain. It seems probable on the basis of the linkage found between a reverted temperature-sensitive plasmid and either the chromosomal penZ gene in PS80 or the chromosomal erythromycin-resistance gene. It would have been more satisfactory to have shown that the revertant plasmid genes become linked to genes that specified enzymes involved in biosynthetic pathways. However experiments of this sort present difficulties because the staphylococcal chromosome (and in particular the chromosome of strain

PS80) is unmapped.

Integration seems to be the result of a recombination event mediated by a protein specific for integration and produced by a gene, *int*, on the plasmid. An explanation based on homology and a host-directed recombination event seems inadequate but cannot be ruled out, particularly as all these experiments were performed using a *rec*$^+$ host (a *rec*$^-$ mutant of strain PS80 has not been isolated). The *rec* system of PS80.d. is probably responsible for the residual integrations of SI[*int*] into the chromosome (3 per 10^6 cfu), hence there is only an eleven-fold reduction in integration frequency compared to SI.

If the general recombination system of the cell were alone responsible for the integration of the SI-plasmid then it would be expected to catalyse recombination between any region on the plasmid and any region of homology on the chromosome, as outlined by Campbell (1962). The difference in reversion frequency between SI and SI[*int*] would then be accounted for by assuming that the original deletion of the *penI.penZ* from the chromosome of PS80 to give PS80.d. was not complete and that some of the *penI.penZ* genetic material remains on the chromosome. This would provide some homology with the corresponding region on the SI-plasmid but not with SI[*int*], which has the penicillinase region deleted, so

that different frequencies of integration would be obtained. This is unlikely however, for two reasons,

(i). If it were true there should be a substantial increase of integration frequency with SI in PS80, since the penI.penZ region on the plasmid is isogenic with the chromosomal penI.penZ. However, there was only a slight (insignificant) increase in the number of revertants (Table 5.2, column 2). SI[int] is not affected and reverts at it's usual rate.

(ii). The sixty-two [penZ] and two [penZ.asa.] deletions of SI integrated at the normal or only a slightly reduced rate in PS80.d., while SI[int] is substantially affected.

Furthermore the frequency of integration of SI in the presence of the plasmid-derived chromosomal penI.penZ region is at most doubled (Table 5.2, columns 4, 5, 6, 7). The increase in integration frequency in the presence of the plasmid-derived chromosomal, penicillinase genes over the integration in the presence of the normal chromosomal penicillinase genes, is probably due to transposition of cryptic plasmid DNA along with the penicillinase region. Different amounts of this cryptic DNA would account for the differences in integration frequency between the four strains possessing transposed penicillinase genes. However there was no increase comparable with that in

PS80.d.*ero*. Neither was the integration frequency of SI[*int*] influenced at all by the presence of transposed penicillinase genes on the chromosome, while it integrated at the same rate as SI in PS80.d.*ero*.

It is possible that this increase in integration in the presence of the plasmid-derived *ero* gene is due to linkage, on the plasmid, between the *int* locus and *ero*. When *ero* is transposed from the plasmid to the chromosome the putative *int* gene is carried with it, and it is the protein produced by this *int* gene that is responsible for the increase in integration frequency in PS80.d.*ero*. Were the increase in integration in this strain due only to increased homology with the plasmid, there should be frequent association of the *ero* with the reverted plasmid. In fact only four in 32 tested showed linkage. The *int* product therefore appears to act *trans*. The isolation of intermediate [*int*] mutants (Table 5.3, columns 3, 4, 5, 6), however, is an indication that this explanation may be an over-simplification and that there may be other functions involved in addition to a *trans*-acting protein produced by *int*, e.g. the host cell *rec* system.

The efficient transduction of the chromosomal *ero* may be a result of it's linkage with *int*. Possibly this linkage is so close that all *ero* fragments transferred by the phage possess the *int* gene as well, and therefore

integrate into the recipient cell chromosome and survive. The transductant colonies obtained in this transduction are of heterogeneous size, yet there appear to be no abortive transductants (cf. Novick, 1967a). The difference in size can be explained if small colonies represent bacteria in which the integration of ero was later than in the larger colonies.

It is possible that when int was transposed with ero to the chromosome, the system controlling production of the int protein was disrupted with the result that the int protein is produced constitutively. This would account for the large increase in frequency of integration in the presence of the chromosomal int. If this int protein is being produced constitutively it is probable that control of int expression acts only cis.

The superinfecting plasmid presumably possesses the necessary control system, but seems unable to repress synthesis of the chromosomal int product.

Whether the presence of int alone makes possible the transposition of ero from the plasmid to the chromosome is not certain, though it is probable. The transposition of the penI.penZ genes from the plasmid to the chromosome could be explained by homology with remnants of the penI.penZ genes on the chromosome of PS80.d. as explained above, which would allow a normal, host-

directed recombination event to occur. By contrast it was not possible to transpose cadmium resistance from plasmid (PI_{9789}·seg-I.[penI.penZ.asa].cad.mer) to the chromosome, though many attempts were made. There is no int gene associated with cad, nor any known homology with the chromosome.

In this discussion it has been assumed that the integration function on (PI_{258}), the parent plasmid of the chromosomal ero.int, is equivalent to that on (PI_{9789}·penI.penZ), from which SI[int] is derived. Though this is likely it has not been proved. However, supposing that they were not equivalent, the existence of an integration function still is probable, for the facts remain that deletion of a specific region of the plasmid results in a reduced integration frequency and the plasmid-derived chromosomal ero mediates a trans integration event, since association between an integrated plasmid and ero is not obligatory.

CHAPTER 6.

EXCISION OF AN INTEGRATED PLASMID FROM THE CHROMOSOME.

Introduction.

It is unlikely that the integration of the penicillinase plasmid into the chromosome is irreversible, a mechanism whereby the plasmid can be excised from the chromosome again is probable. An SI-revertant was superinfected with plasmid (PI_{258}.[penZ]), by selecting transductants on CY-ero agar. When the transductants were plated for single colonies, they appeared to be segregating penicillinase-negative colonies with high frequency. To examine this further, experiments on the segregation of integrated plasmids were done.

Excision of an integrated plasmid by a superinfecting plasmid.

Revertants of SI and SI[int] were superinfected with plasmid (PI_{258}.penZ-40I.cad-52) by selecting transductants on CY-ero agar. The segregation of SI and SI[int] was then determined (Table 6.I and 6.2, compare columns 2 and 4 - in each case column 2 is a control without any superinfecting plasmid, and segregation of the integrated plasmid in these cases is probably due to self-excision or spontaneous excision due to the host rec system). The results indicate excision directed by the superinfecting plasmid. Flasks were incubated at 30 C and 42 C, and half the plates from each flask were incubated at 30 C and half at 42 C. There is thus an internal control since incubation of the cells on plates at 42 C will not permit an excised temperature-sensitive plasmid to persist in the cytoplasm, whereas when incubated at 30 C an excised temperature-sensitive plasmid may be able to establish itself by displacing the superinfecting plasmid (see Richmond, I968). There should therefore be less excision when cells have been incubated at 30 C, than when they have been incubated at 42 C. This was found to be the case, both after growth in broth (Table 6.I, column 3; Table 6.2, column 3 - compare the number of

Table 6.I. Excision of an integrated SI[*int*] - plasmid from the chromosome of PS80.d.

	Percent cadmium-sensitive colonies					
Superinfecting plasmid	None		(PI_{258}·*penZ*-40I.*cad*-52)		(PI_{9789}·*penI*.*penZ*.[*asa*.*cad*.*mer*])	
Temperature of incubation of plates	30 (I)	42 (2)	30 (3)	42 (4)	30 (5)	42 (6)
Cells(a) from initial inoculum	0.07	I.05	2.I	39.2	55.5	I00
Cells(b) after growth in broth at 30 C	- (0/760)	I.42	2.4	78.I	54.5	90.6
Cells(c) after growth in broth at 42 C	0.I9	0.93	I7.6	I9.8	98.2	I00

Table 6.2. Excision of an integrated SI plasmid from the chromosome of PS80.d. and PS80.d.*ere*.

	Percent penicillinase-negative colonies					
Superinfecting plasmid	None		(PI_{258}-*pen*Z-40I.*cad*-52)		None	
Host	PS80.d.		PS80.d.		PS80.d.*ere*	
Temperature of incubation of plates	30 (I)	42 (2)	30 (3)	42 (4)	30 (5)	42 (6)
Cells(a) from initial inoculum	0.I2	0.39	4.6	25.7	0.59	I.0I
(b) after growth in broth at 30 C	0.65	I.I4	I0.5	42.0	0.86	I.9I
(c) after growth in broth at 42 C	0.I2	0.30	I9.5	2I.0	0.3I	0.37

negatives after growth in broth at 30 C, with those after growth at 42 C, in both cases) and after incubation on plates (e.g. Table 6.I, columns 3 and 4, after growth in broth at 30 C).

In certain instances there is apparently less excision in flasks incubated at 42 C, when compared with those incubated at 30 C. An explanation for this could be that the protein responsible for excision is naturally temperature sensitive (see 'Discussion', this chapter).

To reduce the likelihood of a recombinational event between the integrated plasmid and the superinfecting plasmid leading to excision, two plasmids with a minimum of apparent homology were chosen for a segregation experiment - (PI_{9789}·penI.penZ.[asa.cad.mer.]) as the superinfecting plasmid and as the integrated plasmid the SI[int]-revertant of genotype: (PI_{9789}·seg-I.[penI.penZ].asa.cad.mer). If there were any segregation of the integrated plasmid due only to recombination with the superinfecting plasmid, some of the superinfecting plasmids would be expected to have become temperature sensitive. To test this, after the experiment all the plates - those from the 'initial inoculum' and those from the broth cultures grown at 30 C and at 42 C - that had been incubated at 42 C were stained with starch-iodine to

test for presence of the penicillinase plasmid. Any that were temperature sensitive by recombination with SI[int] would have been lost from the cells during growth at 42 C to give penicillinase-negative colonies. However there was no increase in the number of penicillinase-negative colonies, above those to be expected from growth of (PI_{9789}) at 42 C (see Table 5.I). There was no evidence of any recombination, yet excision of the integrated SI[int] was highly efficient (Table 6.I, columns 2 and 6).

The efficiency of the excision function is shown by column 5 in Table 6.I, where 55% of the initial culture is already cadmium sensitive. This is almost certainly not due merely to an early recombinational event, since (a) the result is reproducible and (b) incubation of the plates at 42 C gives 100% cadmium-sensitive colonies.

Excision of an integrated plasmid by the plasmid-derived chromosomal ero.

A SI-revertant in PS80.d.ero was constructed and the rate of segregation of the SI-plasmid determined (Table 6.2, column 6). The SI-plasmid was lost from the cell at only a slightly higher rate than in the SI-revertant control (Table 6.2, column 2). The integrated ero does not have the ability to excise linked to it, although int, the gene

responsible for integration is associated with it. Alternatively the excision function may be linked to ero, but is repressed when ero is chromosomal.

Excision of the chromosomal ero by a superinfecting plasmid.

(PI_{9789}.penI.penZ) was transduced into PS80.d.ero. This is the parent plasmid of (PI_{9789}.penI.penZ[asa.cad.mer]), a plasmid shown to have the excision function (Table 6.I, columns 5 and 6). However there was no significant excision of the integrated ero (Table 6.3, columns I and 2).

In addition, to check for loss of ero from the chromosome by recombination, as opposed to the action of an excision protein, segregation of ero in the presence of a superinfecting temperature-sensitive plasmid, SI, was also determined. An ero gene segregated from the chromosome by recombination with a superinfecting, temperature-sensitive plasmid, would remain in the cell but would be temperature-sensitive and there would then be increased loss of ero when plates were incubated at 42 C. However again there was no significant loss of ero from the chromosome (Table 6.3, columns 3 and 4).

Table 6.3. Excision of the chromosomal *ero* by a superinfecting plasmid.

	Erythromycin-sensitive colonies			
Superinfecting plasmid	(PI_{9789}·*penI*.*penZ*.)		SI	
Temperature of incubation of plates	30 (1)	42 (2)	30 (3)	42 (4)
Cells(a) from initial inoculum	0/1190	1/1497	0/1400	1/1304
(b) after growth in broth at 30 C[I]	2/1750	0/2160	0/2017	0/1978

I. Cells were not grown in broth at 42 C as in one experiment the superinfecting plasmid was temperature-sensitive.

4/13000

Excision of chromosomal penicillinase genes by a superinfecting plasmid.

(PI_{258}·penZ-40I.cad-52), a plasmid shown to have the excision function (Tables 6.I and 6.2, columns 3 and 4), was transduced into PS80. There was no significant excision of the chromosomal penicillinase genes (Table 6.4, columns I and 2).

Segregation of the penicillinase genes was also examined in the presence of the temperature-sensitive plasmid (PI_{9789}·seg-I), for the same reason as above. There was no significant loss of the penicillinase genes (Table 6.4, columns 3 and 4).

Discussion.

An excision-specific function seems indicated by the results given in Tables 6.I and 6.2. That it is distinct from, and independent of, the integration function is probable because:

(i). In the presence of the DNA associated with the transposed ero gene, integration frequency is greatly increased (Table 5.2, column 3), but excision is only slightly, if at all, affected (Table 6.2, compare columns I and 2 with columns 5 and 6).

Table 6.4. Excision of the chromosomal penZ by a superinfecting plasmid.

	Penicillinase-negative colonies			
Superinfecting plasmid	(PI_{258}·penZ-40I. sad-52)		(PI_{9789}·seg-I)	
Temperature of incubation of plates	30 (I)	42 (2)	30 (3)	42 (4)
Cells(a) from initial inoculum	0/1870	I/1776	3/2500	I/2897
(b) after growth in broth at 30 C[I]	2/3530	0/3280	2/3098	4/4680

I. Cells were not grown in broth at 42 C as in one experiment the superinfecting plasmid was temperature-sensitive.

3/5000 10/13000

(ii). The integration-defective mutant, SI[*int*], excises itself from the chromosome with approximately the same frequency as SI (Tables 6.I and 6.2, columns I and 2).

Since an integrated plasmid can be excised by a superinfecting plasmid the excision function is presumably a *trans* acting protein and it is produced by a gene which has been named *xis*. From the results in Tables 6.I and 6.2 the *xis* protein appears to be naturally temperature sensitive. There is less excision in cultures incubated at 42 C than in those incubated at 30 C, particularly when the plates were also incubated at 42 C (e.g. Table 6.I, column 4; Table 6.2, columns 4 and 6). However there is some indication that re-integration of excised plasmids can occur (Table 6.I, columns 5 and 6, compare cells from (a), with cells from (b)).This does not seem to be sufficient to explain the differences between cells incubated in broth at 30 C with those incubated at 42 C (Table 6.I, columns 2, 4; Table 6.2, columns 2, 4, and 6). If reintegration alone accounted for this difference, there would be a similar difference between the plates incubated at 30 C, but in every case, except Table 6.2, column I, there is an increase between cells from (b) and cells from (c). The fact that in every case the number of penicillinase-negative cells or cadmium-sensitive cells after growth in broth at 42 C is only slightly increased by incubation on plates at 42 C,

as compared with plates incubated at 30 C, is an indication that little further excision occurs at 42 C and that the excision protein is probably temperature sensitive.

The chromosomal penI.penZ genes of strain PS80 are not excised by a superinfecting plasmid. This, together with the fact that they do not complement a temperature-sensitive plasmid (Chapter 4), suggests that there is little or no cryptic plasmid DNA associated with these genes. It is possible, though, that any such DNA may be repressed when in this situation.

If the penI.penZ genes are not excised by a superinfecting plasmid, what is the origin of PS80.d.? When (PI_{258}) was transduced into PS80(PI_{9789}.penI.penZ) the putative membrane attachment site was occupied so that (PI_{258}) could not establish itself but may have integrated into the chromosome. Once integrated it could be excised by the superinfecting plasmid (PI_{9789}.penI.penZ). If (PI_{258}) integrated near the chromosomal penI.penZ genes (as did PI_{9789}.seg-I. - see Chapter 5) then the excised plasmid could be (PI_{258}.penI.penZ) and such a plasmid could recombine with the (PI_{9789}.penI.penZ) plasmid to give plasmids of genotype (PI_{258}.[penI.penZ]asa.cad.mer.ero) and (PI_{9789}.penI.penZ.penI.penZ.asa.cad.mer) and the latter could be lost from the cell to give PS80.d.(PI_{258}.[penI.penZ].asa.cad.mer.ero).

CHAPTER 7

MAPPING THE INTEGRATION AND EXCISION FUNCTIONS AND THEIR SITE OF ACTION ON THE PLASMID.

A. Location of int on the plasmid.

The original integration-defective mutant also had the penI.penZ genes deleted, yet further penI.penZ deletions, obtained by uv irradiation of transducing phage, integrated at a normal rate (see Chapter 5, 'Isolation of further [int] mutants'). However the deletion giving rise to SI[int] was a spontaneous deletion that probably occurred while the plasmid was in a cell i.e. while the plasmid DNA is a covalently closed circle (Rush et.al.,1969). These results suggest that penI.penZ are closely linked to int in the circular form but not in the linear form.

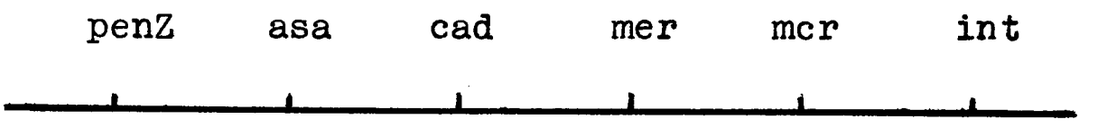
The SI-plasmid was mapped by analysing deletions caused by uv irradiation of transducing phage (Table 7.I).

Table 7.I. Deletions of the SI-plasmid.
(Abbreviations as in Fig.I.I.).

Selective resistance	Number of transductants	Transductant phenotype			
		penZ	asa	cad	mer
Cadmium	651				
	31				
	5				
Penicillin	402				

——————→ gene present.

The gene order is (abbreviations as used in Figure I.I; *int* - gene specifying the proposed integration protein):



int is placed to the right of *mcr* because it is not linked to *penI.penZ* in the linear form and SI[*int*] possesses all the other genes shown.

The results presented in Chapter 5 provide strong evidence for a close linkage between *int* and *ero*. Consideration of the (PI_{258}) map given in Figure I.I indicates that this linkage is consistent with the location of *int* given above, as the *ero* gene is also situated on the DNA to the right of *mcr* on the linear map, as given in Figure I.I. The relative order of *ero* and *int* has not

been established, and their positions, given in Figure 7.1, are interchangeable. To separate them, further mapping with temperature-sensitve (PI_{258}) is required.

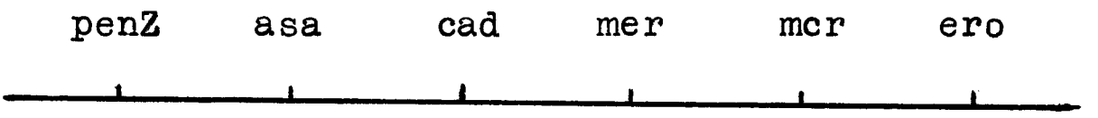
B. Location of xis on the plasmid.

The location of the excision gene(s) onthe plasmid is not known, though the SI[int]-plasmid appears to possess the function since it can excise itself from the chromosome (Table 6.1, column 2), and it is [penI.penZ]. Also the (PI_{9789}.penI.penZ[asa.cad.mer]) excises with great efficiency (Table 6.1., column 6), so the most likely site of xis is near to mcr.

C. The plasmid site of action of int and xis.

Is the int product specific for a small piece of plasmid DNA or is it a general recombinase system? If the int protein catalysed general recombination, homology would be expected to be very important and integration of the plasmid would most often occur at sites of such homology, e.g. the plasmid-derived chromosomal ero. In fact only 4 out of 32 SI-revertants in PS80.d.ero showed linkage with the ero. The genes on two of these plasmids have been

mapped by deletion during transduction (Table 7.2). Both give the following map (abbreviations as in Figure 1.1):



In both plasmids ero has become linked to the plasmid in the same place. This result can be explained if it is assumed that the integration protein has a specific site of action on the plasmid and that there are many sites on the chromosome (or at least 8 - if there is an equal chance of integration at a suitable site, for only 4 out of 32 plasmids integrated close to ero), one of which is close to the chromosomal ero.

The site of action (att_p) of the integration protein is likely to be the site of action of the excision function in order that exchanges can occur - such exact exchanges have

Table 7.2. Deletions of two SI-*ero* recombinant plasmids. (Abbreviations as in Fig.I.I.).

Selective resistance	Number of transductants	Transductant phenotype				
		penZ	*asa*	*cad*	*mer*	*ero*
Recombinant A						
Erythromycin	465					
	4					
	I					
	7					
Cadmium	218					
	5					
	I					
	2					
	I					
Penicillin	203					
	I					

P.T.O.

Table 7.2. (contd.).

Selective resistance	Number of transductants	Transductant phenotype				
		penZ	asa	cad	mer	ero
Recombinant B						
Erythromycin	329					
	3					
	5					
	2					
	10					
Cadmium	586					
	4					
	8					
	1					
Penicillin	102					

———— gene present.

not been demonstrated but seem probable.

$\underline{att}_p$ is probably genetically distinct from *int* because:

(i). A SI[*int*]-plasmid that has integrated into the chromosome can be efficiently excised by a superinfecting plasmid (Table 6.1, columns 4 and 6).

(ii). The plasmid-derived *ero* fragment is not excised by a superinfecting plasmid (Table 6.3, columns 5 and 6), yet it possesses *int*.

(iii). SI[*int*] integrates at high frequency in PS80.d.*ero* (Table 5.2, column 3).

The latter two reasons depend on the equivalence of the *int* and *xis* functions on the SI-plasmid with those on (PI_{258}) - from which is derived the chromosomal *ero*. This equivalence has not been demonstrated.

A possible plasmid map incorporating *int* and $\underline{att}_p$ is given in Figure 7.1, this is in the circular form to facilitate later discussion. The location of *int* on this map has already been discussed; $\underline{att}_p$ is between *int* and *mcr* because the SI[*int*] plasmid seems to possess $\underline{att}_p$ - due to the high efficiency of integration in the presence of *int* protein i.e. in PS80.d.*ero*, and it is efficiently excised by a superinfecting plasmid. It has also been placed between *ero* and *mcr* because the integrated *ero* fragment has *int* but not $\underline{att}_p$ (it is not excised by a

Figure 7.I. A possible location of the *int* gene and it's site of action (att_p).

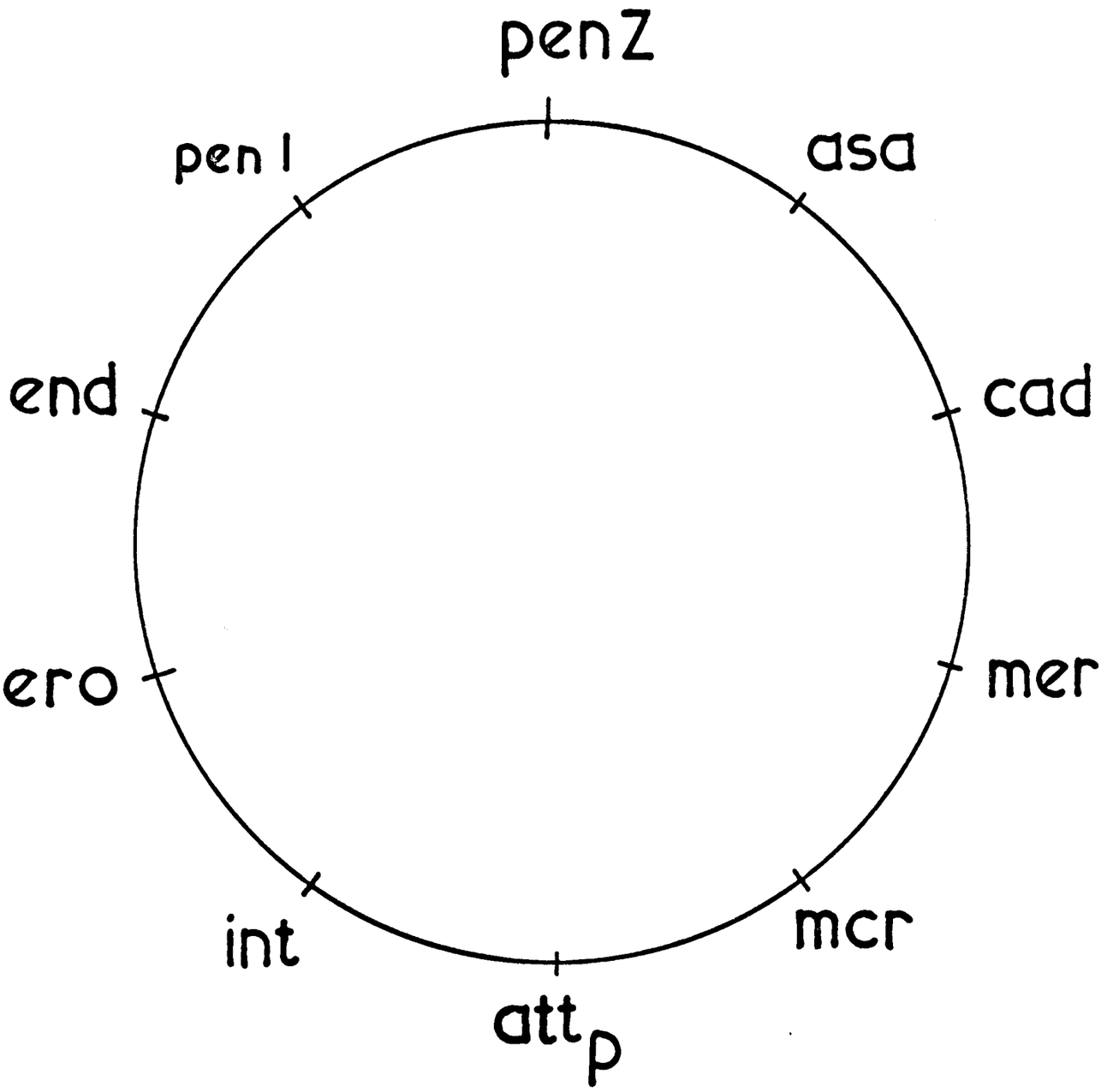
Abbreviations as in Figure I.I.

int the gene for the integration function.

att_p the site of action of the *int* protein.

end the site at which the circular form of the plasmid breaks to give the linear sequence of genes found in transducing phage.

The distribution of genes does not represent physical distance.


penZ
asa
cad
mer
mcr
attp
int
ero
end
penI

superinfecting plasmid - Table 6.3). Richmond and Johnston (1969) reported the segregation of a chromosomal ero in the presence of a superinfecting plasmid. It is possible that when this ero gene was transposed from (PI_{258}) to the chromosome, att_p was carried with it.

The location of att_p in Figure 7.1 explains the map obtained for the two SI-ero recombinant plasmids. att_p determines that ero, or any other chromosomal gene, will, in the presence of an active int gene, be picked up onto the plasmid in the same place. A model outlining the events leading to this association between the SI-plasmid and the chromosomal ero is given in Figure 7.2. The integration event is likely to be similar to that described by Campbell (1962), but I have no direct evidence for this.

Figure 7.2. A possible sequence of events leading to linkage of the chromosomal *ero* with the SI-plasmid.

I. The integration of the plasmid into the bacterial chromosome - probably by a Campbell (1962) integration event. Integration is by the *int* product acting on the plasmid site of action, att_p, and a chromosome site/region of homology.

P.T.O.

1.

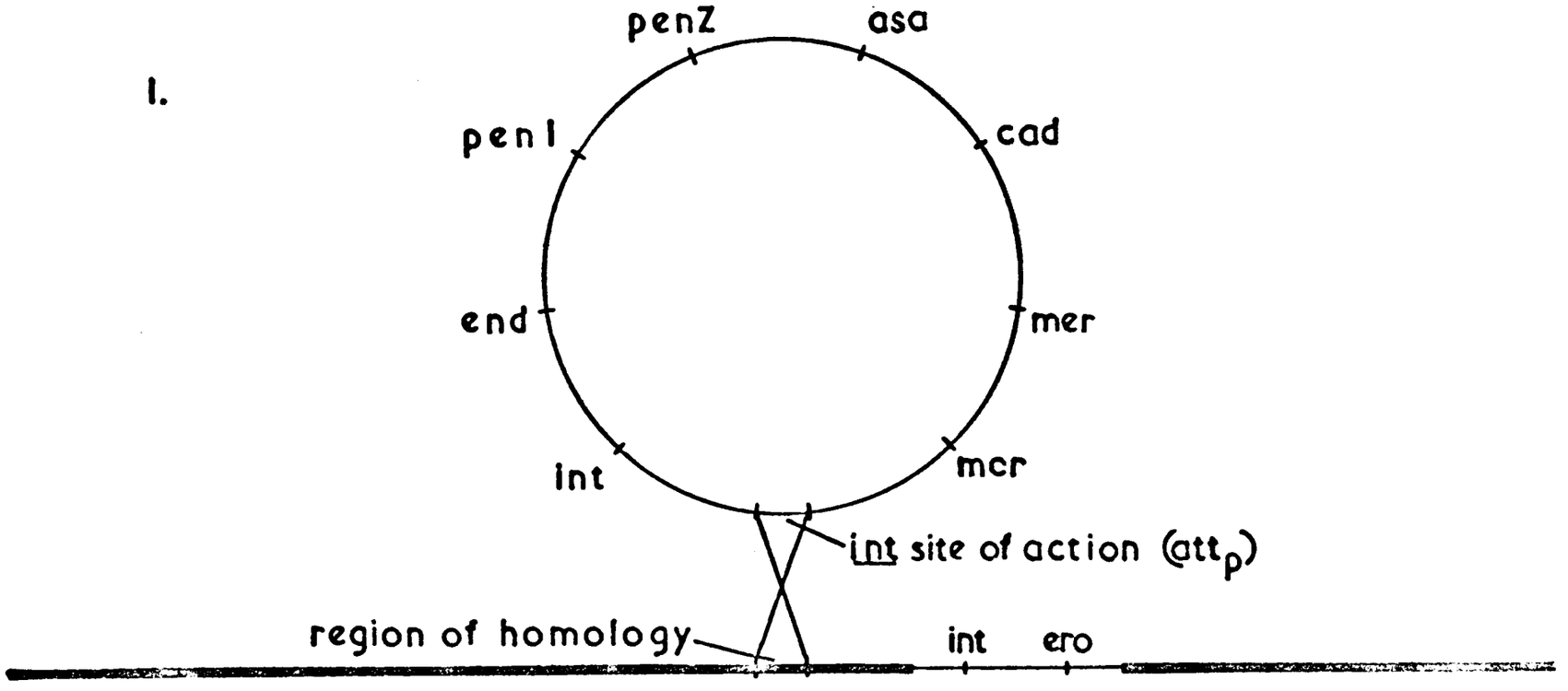

penZ
asa
penI
cad
end
mer
int
mcr
int site of action (att$_p$)
region of homology
int
ero

Figure 7.2. (contd.)

2. The integrated plasmid. In this example the plasmid is integrated near to a chromosomal ero and int previously derived from a plasmid. Transducing phage picks up as one piece of DNA both the revertant plasmid and the ero-int region linked to some bacterial chromosomal DNA.

P.T.O.

2.

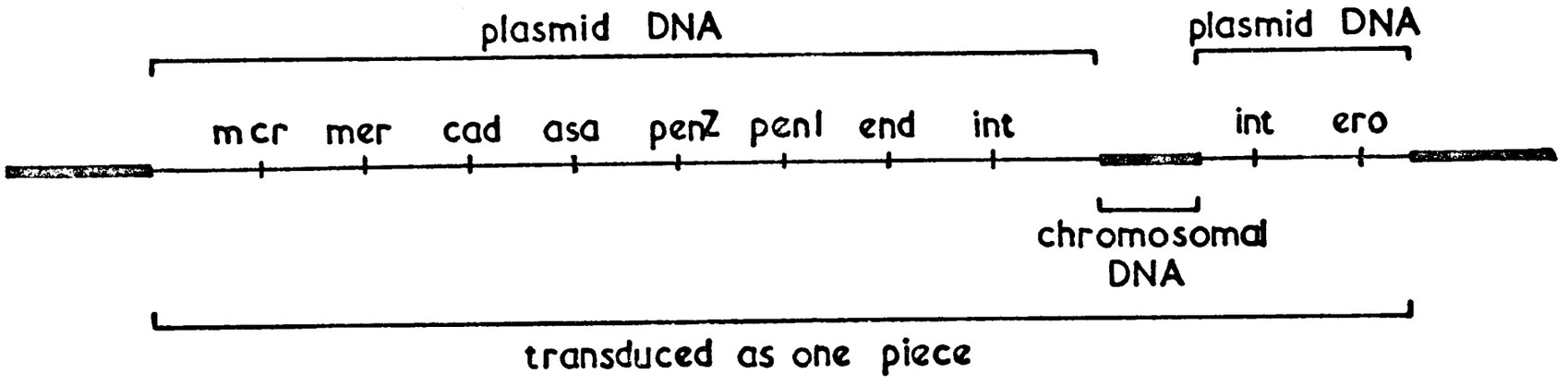
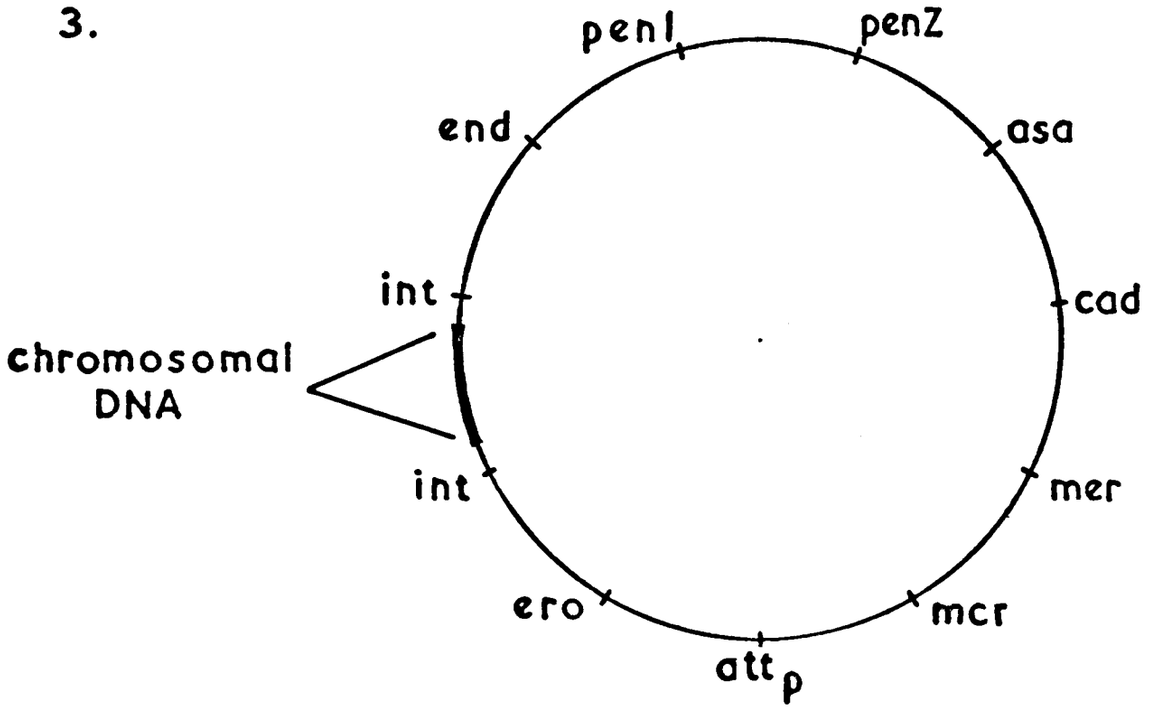

plasmid DNA
plasmid DNA
mcr
mer
cad
asa
penZ
penI
end
int
int
ero
chromosomal DNA
transduced as one piece

Figure 7.2. (contd.)

3. The enlarged plasmid in the recipient cell.

3.


penI
penZ
end
asa
int
cad
chromosomal
DNA
int
mer
ero
mcr
attp

CHAPTER 8

A POSSIBLE PENICILLINASE PLASMID 'TERMINATION' FUNCTION.

Introduction.

The results of Rush et.al (1969) indicate that the penicillinase plasmid is circular when in the bacterium. Deletion mapping of the plasmid performed by irradiation of transducing phage gives a linear, unique, sequence of markers (Figure 1.1 and Tables 7.1 and 7.2). These facts imply that a plasmid breaks between pen and ero (see Figure 1.1 and Figure 7.1). I have assumed that this break occurs at a specific site which has been called end (Figure 7.1).

The integration defective mutant of SI has a deletion encompassing both penZ and int, so that the proposed end is deleted in SI[int]. In the absence of end the plasmid must break at a different site and as a result may give a new linear

arrangement of genes inside the transducing phage. The SI[*int*] plasmid was therefore mapped.

SI[int]-plasmid map.

The SI[*int*]-plasmid was mapped by analysis of deletions caused by uv irradiation of transducing phage. Transductants were selected for resistance to cadmium ions.

Of 525 transductants, nine were (*asa*.*cad* [*mer*].*mcr*), and the remainder were (*asa*.*cad*.*mer*.*mcr*). All were temperature sensitive.

Discussion.

Uv irradiation of transducing phage gives rise to plasmids that have genes deleted from one end, either *penZ* or *ero* in (PI_{258}), or very rarely both ends (Table 7.2), but not a gene from the middle of the usual linear sequence. When mapping SI[*int*] the only deletion mutants obtained with any ion-sensitivity were of the unusual type (*asa*.*cad* [*mer*] *mcr*). This implies one of the gene sequences:



or



If the plasmid were breaking at the usual site (end), deletions of the type ([asa] cad.mer.mcr) would have been expected, but no plasmids with the mercury-resistance gene deleted should be found. A possible explanation of the map obtained is that when end is deleted, as in SI[int], the plasmid will break at a different site to give a new linear sequence of markers. This new site in SI[int] is either between mcr and mer or between cad and mer,

hence the two sequences of genes given above.

In a double lysogen of phage lambda a complete viral genome can be excised from the chromosome in a cell where all known recombination mechanisms are absent i.e. in a rec⁻ host, with a red⁻, int⁻ phage. (Mousset and Thomas, 1968; Signer, 1968). This excision was attributed to the action of an enzyme - a 'terminase'. It was postulated that terminase is an endonuclease which normally acts on a specific site, end, causing a break in the circular phage genome to give the linear sequence of genes observed when the DNA is within the phage. The excision of a viral genome from the double lysogen is due to terminase acting on end of each lysogen, and so excising a hybrid genome.

It is possible that the map obtained for SI[int] is due to the existence of a similar mechanism. There may be a 'terminase' enzyme which acts on end to give the usual linear sequence in transducing phage, which, when end is deleted acts to break the circular DNA at a different site. Terminase acts preferentially at end but can act elsewhere in the absence of end.

This suggestion is very tentative as the evidence on which it is based is inconclusive. Furthermore many difficulties remain if this hypothesis is correct, for example how does the plasmid form a circle if end, which

presumably represents the 'cohesive ends' of a linear lambda molecule, is deleted? Whether this terminase is host or plasmid determined is not known.

CHAPTER 9

PURIFICATION OF PLASMID DNA

Introduction.

If the deletion in SI[int] very large it is possible that homology between the plasmid and the chromosome is reduced. This could explain the eleven-fold difference in integration frequency between SI and SI[int]. However if the SI[int] deletion is small it is less likely that reduced homology accounts for the decrease in integration frequency. The extent of the SI[int] deletion will be apparent from a comparison of the molecular weights of SI and SI[int]. I therefore attempted to determine the molecular weights of these two plasmids.

There are several ways of obtaining a value of the molecular weights for plasmid DNA but all depend on separation of the plasmid DNA in a pure form. This chapter describes the attempts to obtain such a separation.

Labelling and extraction of plasmid DNA.

The DNA is labelled by growth of the cells in the presence of radioactive thymidine. Thymidine induces an enzyme, thymidine phosphorylase, that converts thymidine to thymine and deoxyribose-1-phosphate (Kachmeler, Gerhart and Kosner, 1961). Since free thymine enters the cell less readily than thymidine (Crawford, 1958), the presence of this enzyme greatly reduces the extent of labelling of DNA by thymidine. In these experiments uridine and deoxyadenosine were included in the medium since these compounds are inhibitors of thymidine phosphorylase (Boyce and Setlow, 1962).

The labelled cells are lysed in conditions inhibitory to nuclease activity. Staphylococcal nuclease requires Ca^{++} (Cunningham et.al., 1956), so the cells are washed, and lysed, in EDTA to chelate these ions. Once the cells are lysed the remaining extraction procedure is essentially an enrichment for plasmid DNA, based on the fact that this DNA is thought to occur in the cells as a covalently closed circle (Rush, et.al., 1969). The circular plasmid DNA is more resistant to the alkaline conditions and heat treatment, to which the cell lysate is subjected, than the linear pieces of DNA derived from the chromosome, so that eventually the plasmid DNA

molecules are the largest DNA species remaining. This allows the plasmid DNA to be separated on the Sephadex column.

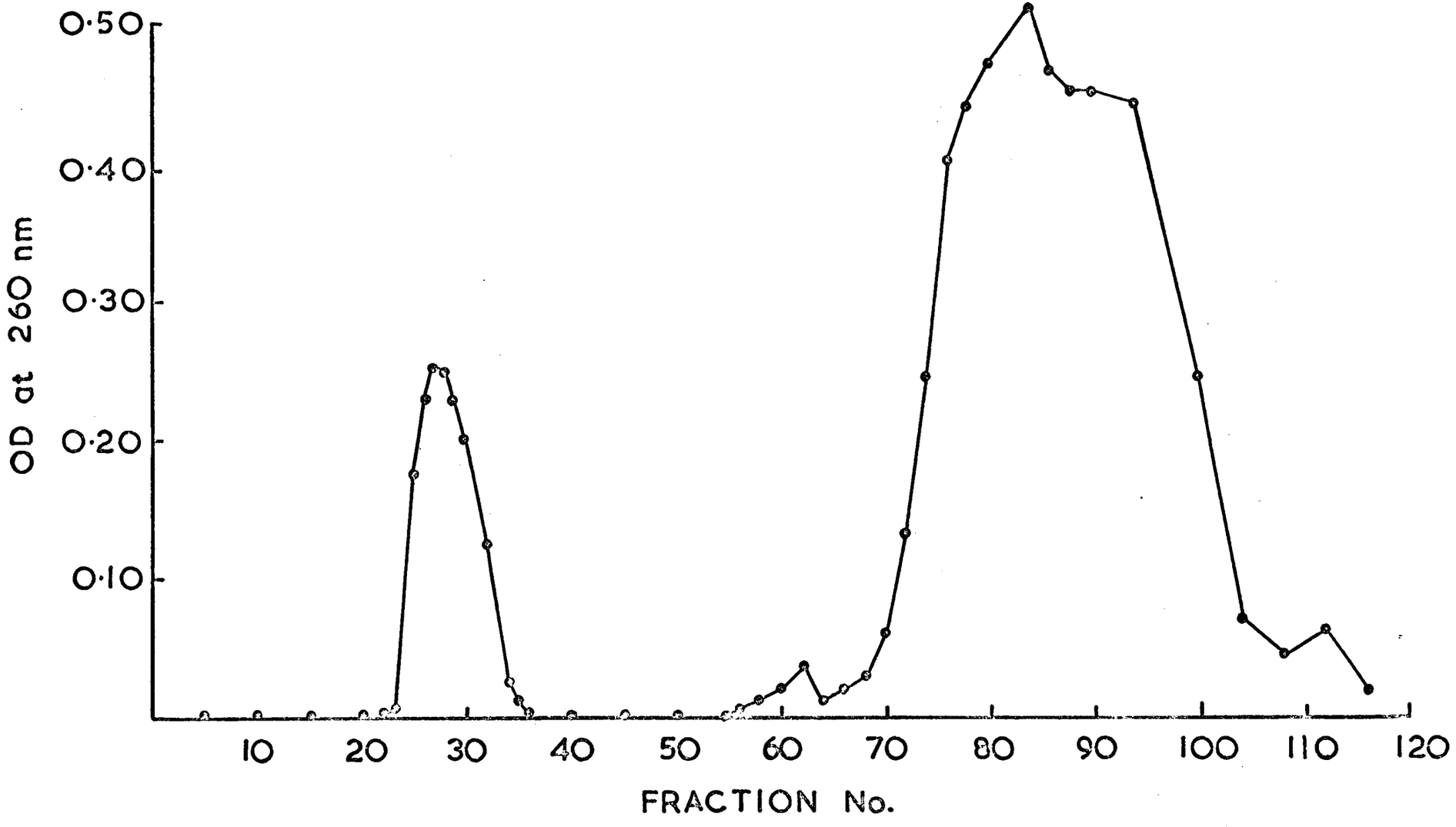
Nitrocellulose adsorbs denatured DNA, and double - stranded pieces with single-stranded regions (Armstrong and Boezi, 1965; Boezi and Armstrong, 1967) so that the DNA obtained after passage through nitrocellulose should be covalently closed, or nicked, DNA.

Isolation of plasmid DNA.

The elution pattern of DNA extracted from PS80.d.(SI) obtained after chromatography on a Sephadex G-100 column is given in Figure 9.1. This pattern is reproduc-ible. When the fractions comprising the first peak are collected, concentrated by the use of polyethylene glycol, and applied to a nitrocellulose column, between 50 and 60% passes through the column. An equivalent amount of DNA from the second peak is retained entirely by the nitrocellulose. Since nitrocellulose binds denatured DNA, the first peak should contain the plasmid DNA fraction.

However, DNA extracted from PS80.d., not containing a penicillinase plasmid, gives exactly the same elution pattern; furthermore, the amount of putative plasmid DNA

Figure 9.1 The elution pattern, from a Sephadex G-100 column, of the DNA extracted from PS80.d.(SI).


0·50
0·40
0·30
0·20
0·10
OD at 260 nm
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION No.

obtained from PS80.d.(SI) was some 50 fold more than that reported by Rush, et.al. Strain PS80 may therefore contain plasmids in addition to the penicillinase plasmid. Two methods were used to eliminate any such cryptic plasmids:

(a). Cells of PS80.d. were grown in TSB containing 10^{-5}M ethidium bromide, subcultured daily into fresh TSB and ethidium bromide and shaken at 37 C. This was repeated for 15 subcultures.

(b). Cells of PS80.d. were irradiated with uv light. Exponentially growing cells, suspended in TSB, were irradiated to give almost complete killing (on average there were 100 survivors from approximately 2 x 10^8 cells irradiated). The irradiated cells were spread on CY-agar, and some of the colonies that appeared were treated again. This was repeated 10 times.

DNA was extracted from cells treated as in (a) and in (b), and chromatographed on Sephadex. In each case there appeared to be a decrease in the size of the first peak (up to 50% reduction). However this decrease may not be significant because the DNA extraction procedure is not quantitatively reproducible.

In a further attempt to eliminate the plasmid-like DNA from PS80.d. , the cells that had been grown for 15 subcultures in ethidium bromide were irradiated with

uv light, twenty times. DNA from these cells was chromatographed on Sephadex, but the first peak remained, approximately the same size as after growth in ethidium bromide. However the phage typing pattern was changed, before irradiation the cells were lysed only by phages 80 and 81, but after irradiation also by phages 52 and 52A. This change in phage-typing pattern suggests a loss of the temperate phages known to be carried by PS80 (Asheshov and Winkler, 1966).

Ultracentrifugation.

The SI-plasmid was transduced into the strain of PS80.d. that had been grown in ethidium bromide and then irradiated 20 times. This strain, it was hoped, would contain fewer, other, cryptic plasmids. That fraction of the DNA, extracted from this strain, passing directly through the nitrocellulose column, was collected, concentrated using polyethylene glycol and sedimented on a neutral, 5 - 20% sucrose density gradient (Figure 9.2). The pattern obtained is reproducible and was repeated several times.

DNA extracted from PS80.d., without SI, was also sedimented (Figure 9.3).

Figure 9.2 Sucrose density gradient analysis of the plasmid-like DNA extracted from PS80.d.(SI).

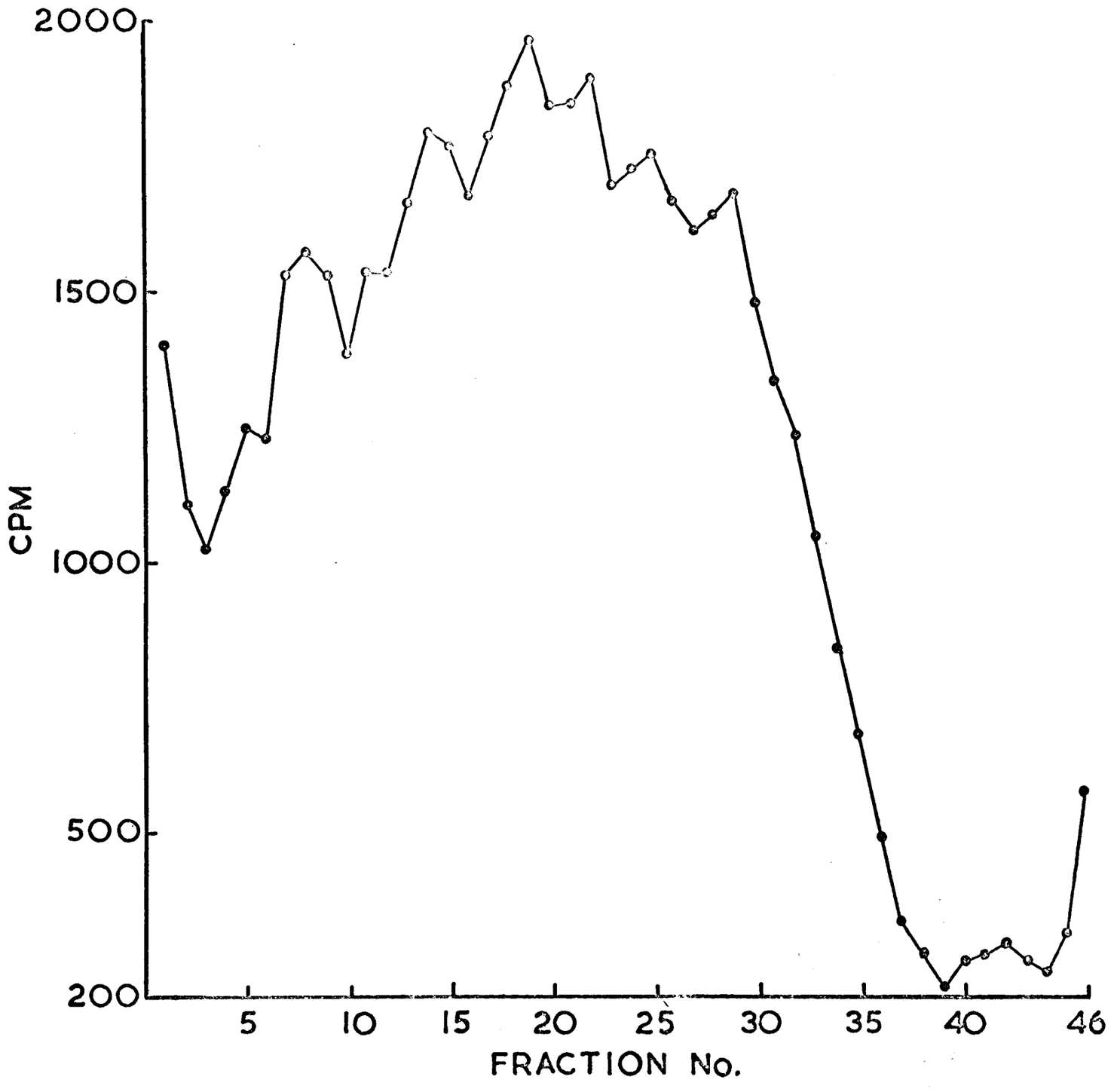
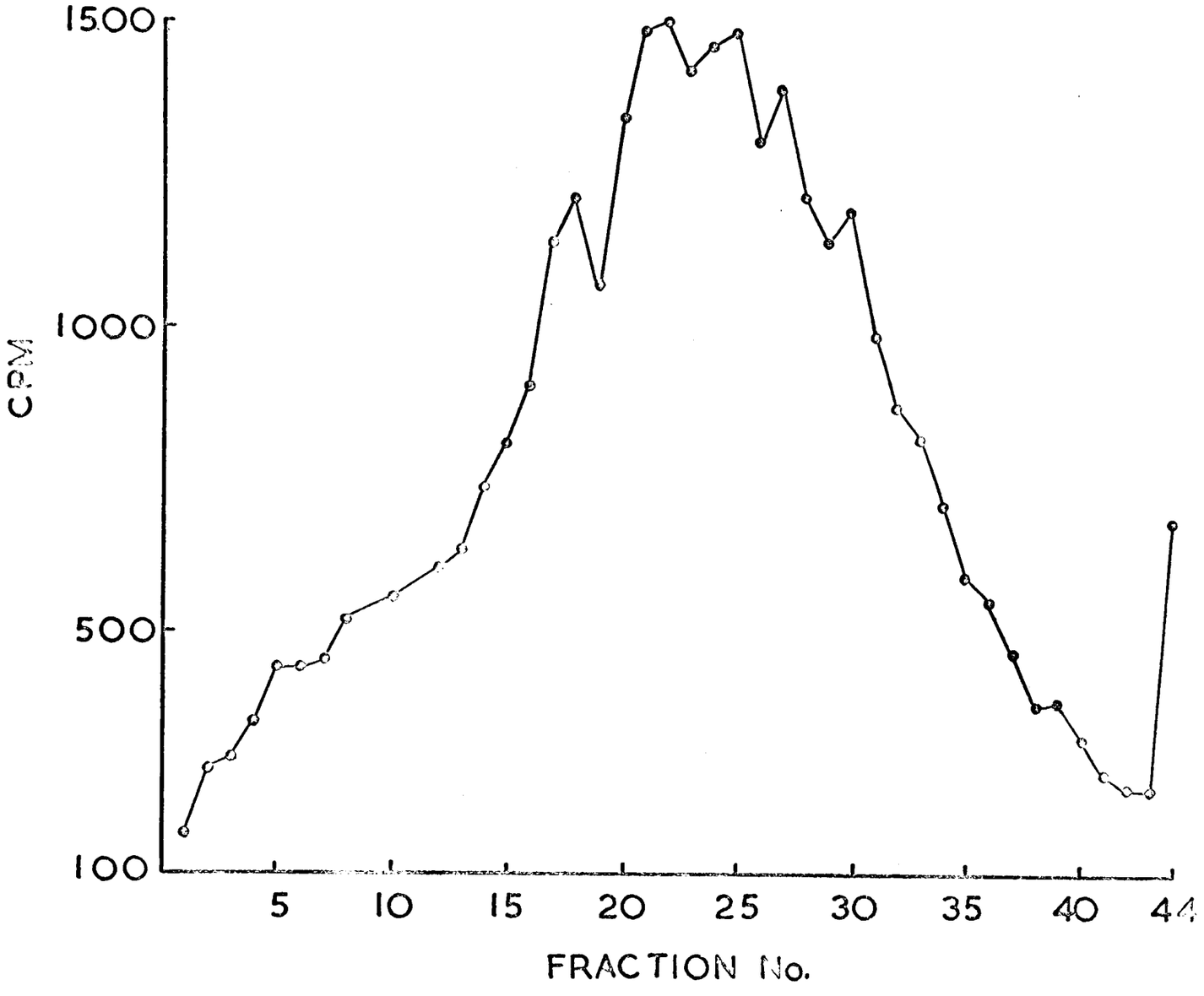

2000
1500
1000
500
200
CPM
5
10
15
20
25
30
35
40
46
FRACTION No.

Figure 9.3 Sucrose density gradient analysis of the plasmid-like DNA extracted from PS80.d.


1500
1000
500
100
CPM
5
10
15
20
25
30
35
40
44
FRACTION No.

Discussion.

The results obtained indicate the presence of circular DNA, possibly covalently closed or nicked. DNA from the first peak of Figure 9.1 passes directly through nitrocellulose, while an equivalent amount from the second peak is retained. Furthermore the distance sedimented in the linear sucrose gradient used, is compatible with the known molecular weight of the penicillinase plasmid - 18.6×10^6 daltons (Rush, et.al, 1969). In identical conditions, but sedimenting for only 2 hours, a resistance transfer factor of molecular weight 50×10^6 had sedimented to approximately the same position (Silver and Falkow, 1970).

From Figure 9.2 there appear to be some 6 or 7 different molecular species present. Though these are not well differentiated, they appeared in all gradients obtained with DNA extracted from PS90.d.(SI), in approximately the same positions. Some of the lesser peaks in Figure 9.2 were better accentuated in other gradients, and vice versa. A comparison of Figures 9.2 and 9.3 indicate that the penicillinase plasmid DNA is probably present in one of the heavier peaks of Figure 9.2, as these peaks seem to be absent in DNA extracted from PS80.d. In general a much smaller proportion of the DNA is found in the fast sedimenting fractions when the source of DNA

is PS80.d. than when the source is PS80.d.(SI).

Before the molecular weight of the SI-plasmid can be determined it will be necessary to obtain better identification and separation of the penicillinase plasmid DNA. The behaviour of the DNA preparations on ethidium bromide-caesium chloride gradients centrifuged to equilibrium should provide further evidence as to the nature and conformation of the DNA extracted from the two types of cells.

When the DNA of the SI and SI[int] plasmids has been identified and separated it should be possible to determine their molecular weights from velocity sedimentation data and also from contour length measurement of electron micrographs of the DNA preparations prepared by the Kleinschmidt technique.

Since it has proved difficult to remove from PS80.d. the plasmid-like DNA, it is possible that this DNA represents plasmids that are essential to survival of the cell. Do such plasmids constitute chromosomes?

CHAPTER 10.

DISCUSSION

The study of the effect of various agents on the stability of plasmids has added little to our understanding of the molecular processes involved in this stability. These agents have, however, been useful aids to the genetic studies on stability - in particular in facilitating the isolation of plasmid-negative variants.

The experiments reported in Chapter 3 recorded that there is a genetic determinant for ethidium bromide resistance present on the penicillinase plasmid of a strain of S.aureus isolated in a London hospital. What is the advantage to the cell to possess such a resistance? If this particular resistance proved to be more common, then it would merit further investigation in the context of survival of strains in the hospital environment.

The analysis of mutants affected in the stability of the penicillinase plasmid has proved more informative

than the study of effects of various agents on the stability. On the basis of the replicon hypothesis of Jacob et.al, a possible interpretation of the known instability mutants is as follows. Host-determined mutations causing instability of a penicillinase plasmid could be located in the genes determining the membrane attacment site. The postulation of two separate sites, which could be proteins specific for each compatibility group, would explain those host mutations causing instability in only one of the two compatibility groups, (Novick, 1967a). In addition, the S2-mutation described here implies that the maintenance of penicillinase plasmids requires a component which is common to the two compatibility groups, but not required by the tetracycline plasmid or by the chromosome. One possibility is that a particular species of membrane sub-unit is used as a base for the attachment of penicillinaseplasmids, and that the tetracycline plasmid and the chromosome are attached to different sub-units. Mutations causing instability and located on the plasmid itself could be an alteration in DNA base sequence that normally recognises the attachment site, so that there is less efficient attachment (though it is unlikely that such a mutation would be temperature sensitive). Or it could be a mutation in a protein, if there is a plasmid determined protein involved in the

attachment.

This interpretation of the instability mutants is likely to be an over simplification. There are a number of proteins involved in replication of a replicon, e.g. initiating proteins, DNA polymerase, and a lesion in one of these might reduce the efficiency of that protein and cause a reduced rate of replication of the plasmid. This could lead to segregation of the plasmid from some of the daughters of a division, an imbalance that would be apparent as instability. Replication therefore probably has a direct bearing on stability. However it is not known whether a penicillinase plasmid codes for some or all it's own replicating proteins or whether it uses those of the host cell.

The probable advantage to a cell in having genes on a plasmid is that this aids the rapid and widespread of these genes through a population e.g. conjugation, and the increased frequency of transduction of episomal markers (Arber, 1960). The disadvantage is that plasmid genes are unstable and may be lost from the cell at rates greater than those at which gene function is lost by normal mutation rates. This may be the reason why genetic material essential for survival of the cell is not found to any extent on extrachromosomal elements, but genes that are required only in extreme conditions are most often

extrachromosomal. If the plasmid is lost from a cell, then under most conditions the cell will survive and have a chance of subsequently acquiring the plasmid from another member of the population. In view of the instability, a mechanism whereby a plasmid could integrate into the chromosome and have increased stability (Table 5.1) would be an advantage. This would help to ensure that the plasmid genes were retained in a population, even in absence of positive selection for these genes. A return to the extrachromosomal state by excision from the chromosome completes the cycle. It is possible that the integration and excision functions identified on the penicillinase plasmid evolved for these reasons. The replicon functions of the plasmid may have, for example, been derived from a chromosome, and the integration and excision functions evolved as a means of increasing the stability of the plasmid without losing the advantages of autonomous existence.

An alternative origin of the integration and excision functions, and for the penicillinase plasmid, is that they were derived from a defective temperate phage. The similarities between the integration and excision activities of the penicillinase plasmid and the lysogenic response of a temperate phage, such as lambda, are striking. The penicillinase plasmid is

normally extrachromosomal and replicates autonomously. It may then be regarded as analogous to the autonomous replication of phage in the lytic cycle. Occasionally the plasmid integrates into the chromosome, probably by a Campbell (1962) type integration event, mediated by a protein produced by the *int* gene (Figure 7.2). There is a single site of action for this protein (att_p) and probably many sites for integration on the chromosome. Once integrated the plasmid replicates as part of the chromosome - as does the prophage of a lysogenic phage. Excision of the plasmid genome by the protein product of gene *xis* acting on the hybrid att_p and chromosomal integration sites, returns the plasmid to the autonomous state.

Although many questions remain regarding genetic control of these events - for example what determines the frequencies of integration and excision, and why does a superinfecting plasmid excise an integrated plasmid with such high frequency - in broad outline the similarities between at least some penicillinase plasmids and temperate phage are very close. The major differences with phage lambda, for example, are that in lambda *xis* function depends on the presence of an active *int* product (Gottesman and Yarmolinsky, 1968a,b; Guarneros and Echols, 1970); this is probably also true for the

Salmonella typhimurium temperate phage, P 22, where correct excision seems to require an active integration function (Smith, 1968), while *int* and *xis* are independent in penicillinase plasmids (SI[*int*] excises itself from the chromosome). The other difference is that phage lambda has only one site for integration on the *E.coli* chromosome. The similarities extend to the postulated termination function - though evidence for this, both in lambda and penicillinase plasmids, is inconclusive. In view of the similarities I suggest that the probable origin of staphylococcal penicillinase plasmids was a defective temperate phage, lacking functions necessary for cell lysis (e.g. the strain of lambda with an amber mutation in the N gene, which persists and replicates as a plasmid in the cytoplasm of the host (Signer,1969)), but possessing the integration and excision functions.

The integartion frequency of SI[*int*] is only eleven-fold less than that of SI. It is possible that the residual integration, not promoted by *int*, is catalysed by host recombination systems. This could be studied by determining the frequency of integration of SI in mutants of strain PS80 that are defective in recombination. No such mutants are available in this strain, however the integration of plasmids in other species is affected by host recombination systems. For

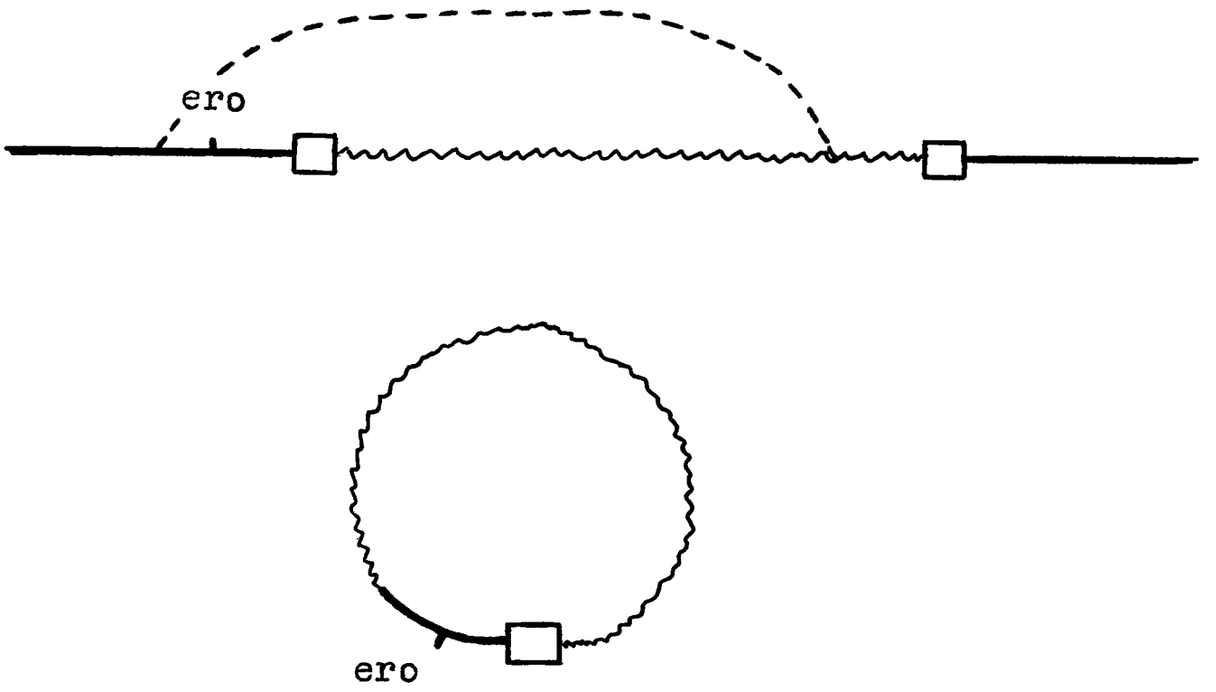
example, the residual integration of *int*$^-$, *red*$^-$ mutants of phage lambda requires an active bacterial *recA* gene (Gottesman and Yarmolinsky, 1968b), and there is a large reduction in the integration frequency of a F' episome in *rec*$^-$ strains of *E.coli* (De Vries and Maas, 1971).

Certain of the genes on the penicillinase plasmid, such as *penZ* and some of the ion resistance genes, are also found on the chromosome (Asheshov, 1966b; Novick and Roth, 1968; Dyke, Parker and Richmond, 1970). Genes like these may have been incorporated into the defective phage genome by a series of integration and excision events. An intermediate stage in the formation of penicillinase plasmids may have been a high frequency transducing phage for some resistance gene, which would have aided the rapid and widespread distribution of these genes e.g. Pllde (Novick, 1967).

The incorporation of chromosomal genes from the chromosome into the plasmid may have occurred in one of two ways:

1). As shown in Figure 7.2.

2). As in formation of λ dg (Guerrini, 1969),



The distinction between these is that 2) is a faulty excision event, resulting in some plasmid DNA remaining on the chromosome, and the incorporation of some chromosomal DNA into the plasmid. In 1) association between the chromosomal gene and the plasmid is by a phage in lytic cycle picking up all the genes on a single

piece of DNA and then transducing them to another cell where they form an enlarged plasmid.

Which of these methods is chiefly responsible for incorporation of resistance genes onto the plasmid is not known but 1) is believed to be the manner in which the chromosomal ero was linked to SI. It is interesting to note that the four SI-ero linked plasmids would afterwards be expected to have a double integration function, and they do in fact revert with a frequency much higher than SI in PS80.d.ero (Chapter 5). The SI-ero plasmid that showed 0.6% cotransduction with the chromosomal ero integrates with greater frequency than that which showed 14.6% cotransduction. The difference in frequency of cotransduction is possibly indicative of the proximity of association of the integrated plasmid with the ero - the plasmid cotransducing ero with low frequency would be less closely linked than those cotransducing with higher frequency. The SI-ero plasmid derived from a revertant cotransducing ero with low frequency will therefore possess more chromosomal DNA (Figure 7.2) and hence will have greater homology with the bacterial chromosome. This increased homology may allow more frequent integrations mediated by the normal host recombination system.

However the integration and excision functions

evolved - whether for reasons of increased stability or whether they were derived from a temperate phage - they are likely to be only a subsidiary to the main mechanisms of stability. These mechanisms probably are: maintenance (attachment to the host membrane) and replication. It is possible that defects in any of the proteins involved in these systems may lead to instability. In fact the system of maintenance and replication may be the same, if, for example, the attachment protein is DNA polymerase and replication of the plasmid is by rotation about this site. This is pure speculation but the fact remains that a true understanding of stability requires elucidation of plasmid replication.

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