Genetic basis, epidemiology, and future significance of antibiotic resistance in *Staphylococcus aureus*: A review

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The extensive use of antibiotics has undoubtedly been responsible for the large numbers of staphylococci that have become resistant to them. This resistance could have arisen entirely by mutation and selection, ie, multiplication of a few cells already resistant which have replaced the existing, predominantly sensitive population. If this has been the only mechanism, then the more antibiotics are used, the greater should be the incidence of resistant strains. Although this may be true overall, there are a number of anomalies; eg, resistance to neomycin did not appear for nine years after its introduction, resistance to fusidic acid is still uncommon (this is particularly significant because fusidic-acid-resistant variants arise at high frequency *in vitro*), and methicillin-resistant isolates are infrequent in the UK and very rare in the USA, although the use of all the penicillins and cephalosporins might be expected to favour their appearance. In contrast, resistance of hospital staphylococci to some antibiotics, eg, tetracycline and penicillin (by the production of penicillinase), may be higher than expected. These observations argue against the development of antibiotic resistance by mutation and selection alone. Transfer of certain resistance genes between strains of *Staphylococcus aureus* could account for some of these discrepancies. But as recently as 1969, the Swann Report considered only the mutation/selection origin for antibiotic resistance in this organism.

This review will describe ways in which staphylococci can acquire antibiotic resistance *in vitro*, and other genetic manipulations that can be performed in the laboratory, and it will attempt to assess the importance of these, particularly transfer of resistance, under natural conditions. The rationale behind, and the execution of some of the genetic techniques that are used, will be discussed. The relationship of antibiotic resistance to virulence will also be examined.

Genetic Organization in *Staphylococcus aureus*

In some bacteria most of the cellular DNA exists as one continuous strand (Hayes, 1968). This DNA comprises the chromosome and because it is not enclosed in a nuclear membrane it can interact with various other DNA particles (plasmids or extra-chromosomal elements). The chromosome provides information essential for the cell to grow on ordinary media. Plasmids are usually much smaller than the chromosome and determine specific and often very potent protection against toxic components which are infrequently present in the organism's environment.

In *Staphylococcus aureus*, although there is conclusive proof for the existence of plasmids, the arrangement of the major part of the cellular DNA is unknown. Calculation of the total amount of DNA per cell can be difficult because of the rather variable clumping of the cells. Estimates of 5.5×10^{-14} – 12×10^{-14} g of DNA per cell have been obtained (Novick and Bouanchaud, 1971; Chopra, Bennett, and Lacey, 1973; Grinsted and Lacey, 1973a). If this DNA comprised one chromosomal element, it would have a molecular weight of about 2.4×10^9 daltons, ie, 100–200 times the size of a 'penicillinase plasmid' (see below). Ignorance about the distribution of the bulk of staphylococcal DNA is partly because conjugation—which can transfer the entire chromosome of *E. coli* between cells—does not occur in this organism, and partly because of the difficulty in isolating large DNA molecules. The term 'chromosome' in *Staphylococcus aureus* will be used as a functional definition, ie, the genome essential for growth on ordinary media; it may or may not be a single entity. There is some evidence for at least two 'chromosomes' in this organism (Altenbern, 1967). There are only two processes whereby the genetic composition of the staphylococcal cell is likely to alter in its natural habitat: (a) mutation which in the broadest sense is any alteration

of existing DNA and (b) new genes introduced by bacteriophage released from another cell. In other organisms two further processes of gene transfer can occur—conjugation and transformation. Conjugation has not been described in *Staphylococcus aureus* and this process seems to be excluded by the rigidity of the cell wall. Transformation (the release from one cell and uptake by another of 'naked' high molecular weight DNA) can occur between staphylococci *in vitro* but is unlikely under natural conditions because of the almost invariable presence of DNase enzymes released by strains of *Staphylococcus aureus* (Catlin and Cunningham, 1958).

Transfer of genes by bacteriophage (transduction) was first described in *Salmonella* (Zinder and Lederberg, 1952) and was so named because of the ability of a phage released from one cell (the donor) to infect and change the properties of another cell (the recipient); the donor and recipient were separated by a bacterial filter, ie, cell-to-cell contact was unnecessary. The term 'transduction' is unfortunate because it implies that liberation of the phage into the supernatant is an essential step in the process. In staphylococci, transducing phages (ie, phages capable of establishing in the recipient a marker from the donor) are rarely found in culture supernatants although transduction can occur at high frequency (Lacey, 1971b).

There are three types of gene transfer mediated by bacteriophage (for a full description see Hayes, 1968).

LYSOGENIC CONVERSION

On becoming established in the recipient (the recipient is now lysogenized) the phage invariably confers a new property on the cell. This probably occurs because the transferred gene(s) is permanently part of the phage genome. This process occurs in *Staphylococcus aureus in vitro*: (a) the Tween 80 reaction is converted from positive to negative or (b) the production of beta haemolysin is blocked (Rosendal, Bülow, and Jessen, 1964; Rosendal and Bülow, 1965; Winkler, de Waart, Grooten, Zegers, Tellier, and Vertregt, 1965). These phages have probably also spread naturally (Jevons, John, and Parker, 1966; Jessen, Rosendal, Bülow, Faber, and Eriksen, 1969). There has been a preliminary report of lysogenic conversion of tetracycline resistance in *S. aureus* (Duncan and Monaghan-Cannon, 1969), but some of the derivatives possessed unusual properties and might be contaminants.

SPECIALIZED TRANSDUCTION

In this process a phage confers a new property (and only one) on the recipient at very high frequency—as many as one in two phage particles may possess

this ability. Strains that contain this type of phage have been manipulated in the laboratory so that the genes transduced are situated adjacent to where the phage integrates (integration of a phage is the stable incorporation of the phage DNA into the chromosome or other genetic element, by covalent linkage; incorporation of phage into a plasmid is usually referred to as recombination because some of the genes are lost but is probably a similar process). When the donor cell undergoes induction—the development and release of mature phage particles—these genes very frequently become included in the phage heads. On entry into the recipient many of these genes will become expressed, ie, the cell will take on a new phenotypic property.

In *Staphylococcus aureus* one specialized transducing agent has been created artificially (Novick, 1967a) by recombination of a phage and a penicillinase plasmid. The resultant element is a plasmid and can be transferred to another cell at high frequency both *in vitro* and *in vivo* in mice (Novick and Morse, 1967). This could be an attractive model for the evolution of antibiotic resistance in nature, but for (1) another phage (ie, a helper) must be present in the donor for transducing particles to be produced, and (2) these specialized transducing agents do not seem to occur in nature.

GENERALIZED TRANSDUCTION

Many phages can transfer almost any gene, whether plasmid or chromosomally located. The transfer of each gene is at low frequency. In this process the gene in question is accidentally (apparently) incorporated into the phage head during induction. These adventitious genes replace normal phage genome, hence the transducing phage particles are defective and cells infected with them are neither lysed nor lysogenized as they would be by 'normal' phage particles. Many clinical isolates of *Staphylococcus aureus* of phage group I and III possess transducing phages which are of serological group B, eg, typing phages 53 and 80 (Blair and Williams, 1961). If transfer of resistance has occurred to a significant extent naturally, then it has probably occurred via such phages.

Cells that harbour a generalized transducing phage can undergo induction *in vitro*: agents that provoke this change include ultraviolet (UV) light, mitomycin C, and growth at elevated temperature. Occasionally induction occurs spontaneously. Induction is shown by the sudden lysis of the culture two to four hr after exposure to the inducing agent. Resultant preparations (lysates) after careful filtration to remove residual cells can be used to transduce to a recipient a gene(s) which had been present in the induced culture. Lysates can also be obtained by propagation

of an external phage on the donor. Each type of lysate contains about $10^7 - 10^{10}$ phage particles/ml of which about 1 in $10^5 - 10^{10}$ may be able to transduce a certain marker. Plasmid-born genes are often transduced at higher frequencies than chromosomal genes. The frequency of transduction can be defined in several ways, relating the number of recipients that have acquired the new gene (transductants) to either the donor, recipient, or phage. In practice, frequency is usually defined as the number of transductants/number of phage particles added.

In mixed culture experiments, ie, transducing particles are released spontaneously from the donor, the frequency can be conveniently defined as the number of recipients that have acquired resistance per total number of either donors or recipients. The relationship of transfer in mixed cultures to transduction from lysates is not fully clear. Both processes are mediated by generalized transducing phages and certain cofactors (eg, calcium ions) are required for each. There are, however, two important differences. (a) Cell-to-cell contact favours mixed culture transfer. (b) The recipients in mixed culture experiment are little prone to lysis by 'normal' phage released from the donor, whereas in transduction experiments employing cell-free lysates precautions have to be taken to avoid lysis of the recipient. The transfer in mixed culture has great potential for transfer naturally, whilst the lysate technique has not.

Methods for Locating a Gene as Plasmid-borne or Chromosomal

Where possible, two or more parameters are used to locate genes determining antibiotic resistance because any one criterion may not be reliable. The isolation of plasmid DNA corresponding to the presence of the marker provides the single most convincing demonstration of plasmid inheritance for further details see Novick (1969) and Clowes, (1972).

SPONTANEOUS LOSS

The loss of antibiotic resistance by cells grown at 37 or 43°C in ordinary media at high frequency (1 in $10^3 - 10^5$ cells/cell division lose the resistance) is suggestive of a plasmid locus. However some unprovoked chromosomal mutations can also occur at this frequency in several species of bacteria although they have not been described in *Staphylococcus aureus*. 'Curing' agents accelerate the loss of the plasmid from the cell, without producing a mutagenic action. These agents include acridine dyes (Hashimoto, Kono, and Mitsuhashi, 1964), ethidium bromide (Bouanchaud, Scavizzi, and Chabbert, 1968), rifampicin (Johnston and Richmond, 1970), sodium dodecyl sulphate

(Sonstein and Baldwin, 1972), and methicillin (Lacey, Lewis, and Grinsted, 1973). The 'curing' action of the acridine dyes has been disputed (Richmond, 1968). Finally a number of markers have been lost on storage of the culture at room temperature (Dyke, 1969; Ayliffe, 1970; Lacey, 1972a).

Stability of a marker does not exclude a plasmid locus; we have found that in a tetracycline-resistant strain no colony out of more than 20 000 tested had lost the resistance after growth at 43°C, but on the basis of other criteria the resistance was considered plasmid-borne (Lacey and Grinsted, 1973). It may be necessary to examine as many as 10 000 colonies to exclude the occurrence of spontaneous loss. The only feasible way of examining this number of colonies is by replica plating (Lederberg and Lederberg, 1952). In this procedure the culture is diluted and spread over the surface of agar plates to yield 100-200 discrete colonies/plate. These are then contacted lightly onto a flat velvet surface which in turn is touched on media containing the antibiotic. Colonies that fail to grow on the antibiotic-containing medium have lost resistance (the possibility that such colonies are contaminants must first be excluded).

TRANSDUCTION KINETICS

A cell-free transducing lysate is prepared from the donor and aliquots are irradiated with UV light for intervals before addition to the recipient (for details see Novick, 1963; Asheshov, 1966a, 1969). Ultra-violet light generally produces one of two effects—either for small doses to increase the transduction frequency, often by more than 10-fold which is characteristic of chromosomal genes, or to decrease progressively (with increasing exposure) the frequency as occurs with plasmid-borne genes. The viability (as shown by plaque-forming units) of the 'normal' phage in the lysate is also exponentially reduced by UV light. These kinetics were initially established for *E. coli* (Garen and Zinder, 1955; Arber, 1960) but have been successfully applied to staphylococci (Asheshov, 1966a, 1969; Novick, 1969). The stimulation in the transduction frequency of the chromosomal genes is probably due to activation of DNA repair enzymes in the recipient by the incoming UV-damaged DNA. This facilitates the integration of the transduced fragment into the chromosome. The loss of phage viability and the reduction in transduction frequency of plasmid genes is probably simply due to UV-induced damage to the DNA.

In the interpretation of transduction kinetics, it is usually assumed that the gene(s) in question exists in both the donor and recipient at the same type of locus, so that plasmid genes are transduced *in toto*

Fig (a)

Fig Electromicrographs of plasmid DNA¹ from a strain of *Staphylococcus aureus* in covalently closed circular and open circular form, $\times 10\ 700$.
(a) Covalently closed circular form (b) Open circular form

¹Determines neomycin resistance, isolated from strain no. 649 after transduction from strain no. 609 (Lacey, 1971a). This plasmid has a molecular weight of about 5.9×10^6 daltons and is present in about 15 copies per cell (Chopra *et al*, 1973).

Fig (b)

and do not become integrated into the recipient's chromosome. But on rare occasions plasmid genes can become integrated into the chromosome (Novick, 1967b) or become incorporated into an existing plasmid (Rubin and Rosenblum, 1971); the latter was stimulated by UV light. It is therefore possible for a plasmid gene to give chromosomal type transduction kinetics. There is as yet no evidence for the establishment of genes which are chromosomal in the donor as a plasmid in the recipient. Interpretation of these transduction experiments must be made with caution: UV stimulation probably indicates a chromosomal locus, but not necessarily so; an exponential decline in the frequency probably denotes a plasmid gene.

ISOLATION OF PLASMID DNA

Plasmid DNA from several bacterial species can be separated from chromosomal DNA on account of differences in the physico-chemical properties of the two types (see Clowes, 1972). A procedure frequently adopted for the isolation of staphylococcal plasmids is as follows (for details see Novick and Bouanchaud, 1971; Lacey and Grinsted, 1972). Cultures are grown in media containing radioactive thymidine which becomes incorporated into plasmid and chromosomal DNA; the cells are then treated with the enzyme lysostaphin, which very rapidly lyses the cell wall (Schindler and Shuhardt, 1964). Any intact protoplasts are lysed by the addition of detergent in the presence of EDTA. The latter chelates calcium ions in order to inactivate DNase (Catlin and Cunningham, 1958) and so prevents the breakdown of the extracted plasmid DNA. RNA is deliberately hydrolysed by the addition of RNase and the extract now contains linear fragments of chromosomal DNA and plasmid DNA present in the covalently closed circular (CCC) form (see fig). This type of plasmid DNA is highly coiled and binds much less ethidium bromide than the linear chromosome fragments and therefore becomes denser. The two different DNA types can now be separated by high-speed centrifugation in caesium chloride. The plasmid DNA forms a satellite band (detected by its radioactivity) on the dense side of the main chromosomal band. The plasmid DNA is then collected and after removal of ethidium bromide and caesium chloride consists of relatively pure covalently closed circular DNA. The molecular weight of this can be estimated by sucrose gradient analysis. The sucrose gradient consists of a linear gradation of high to low concentrations of sucrose in a tube. The DNA is placed on the surface of the low concentration and on high-speed centrifugation its rate of sedimentation is approximately linear with respect to time. By comparing the rate of sedimentation with a

marker of known sedimentation (S) value, the S value of the plasmid DNA can be obtained. However covalently closed circular DNA is unstable, tending to break down to the open circular form (see fig). This transition occurs on storage and is accelerated by freeze-thawing and treatment with minute amounts of DNase. Thus the sedimentation of most types of plasmid DNA produces two S values corresponding to the open circular and covalently closed circular form. After storage of the preparation, the amount of the open circular form will have increased at the expense of the covalently closed circular form. Because the S value of the open circular form is probably more reliable than that of the closed form, the former is used for calculation of the molecular weight (see Studier, 1965; Vinograd, Lebowitz, Radloff, Watson, and Laipis, 1965; Clowes, 1972).

Correlation of plasmid DNA with a known phenotypic marker can be difficult. Some staphylococcal plasmids (judged to be plasmids by other criteria) have not been isolated as covalently closed circular DNA (Novick and Bouanchaud, 1971; Grinsted and Lacey, 1973a, b; Lacey and Grinsted, 1973). Reasons for the failure to detect these elements as covalently closed circular DNA are uncertain, but could be because of their large size ($MW > 150 \times 10^6$ —see Clowes, 1972) or their presence as DNA/protein complexes (Clewell and Helinski, 1969). To prove that the isolated plasmid DNA does correspond to the marker in question it is desirable to isolate the DNA after transduction of the marker to a recipient which is known to contain no plasmid DNA rather than to analyse plasmid-positive and negative derivatives of the same strain.

USE OF RECOMBINATION-DEFICIENT (REC-) MUTANTS

These mutants (usually denoted Rec-) are mutant strains isolated *in vitro* which are unable to effect the integration of incoming DNA into their chromosome on account of abnormalities in the relevant enzymes. But plasmids can become established in these cells. Rec-mutants have been extensively studied in *E. coli* but much less so in *Staphylococcus aureus*, probably because of the difficulty in isolating them. One such mutant has been used in attempting to locate the genes for erythromycin resistance (Novick and Bouanchaud, 1971).

Plasmid or Chromosomal Locus of Various Resistance Genes in *Staphylococcus aureus*

These are summarized in the table.

PENICILLINASE PRODUCTION

Spontaneous and irreversible loss of penicillinase

Resistance	Locus of Gene(s)	Mechanism of Resistance (Reference)
1 Penicillin (penicillinase)	Nearly always plasmid	Hydrolysis of β -lactam bond (many)
2 Streptomycin	More often chromosomal than plasmid	Ribosomal modification when gene chromosomal (Lacey and Chopra, 1972). Inactivation by adenylation when gene plasmid borne (Mitsuhashi, Oshima, Kawaharada, and Hashimoto, 1972).
3 Tetracycline	Nearly always plasmid	Reduced permeability (Sompolsky, Zaidenzaig, Zeigler-Schlomowitz, and Abramova, 1970)
4 Neomycin/kanamycin	Plasmid	Inactivation by phosphorylation (Doi, Miyamoto, Tanaka, and Umezawa (1968)
5 Chloramphenicol	Plasmid	Inactivation by acetylation (Shaw and Brodsky, 1968)
6 Erythromycin/lincomycin	Plasmid	Mainly ribosome modification (Waisblum, 1971)
7 Methicillin	Not established, more likely to be plasmid than chromosomal	Not known
8 Fusidic acid	More often plasmid than chromosomal	Not known
9 Trimethoprim	Chromosomal	Not known
10 Sulphonamides	Not known	Not known
11 Novobiocin	Chromosomal	Not known

Table Site of various resistance genes in clinical strains of *Staphylococcus aureus* and the mechanism of resistance where known

production in *Staphylococcus aureus* was first described by Barber (1949). Transduction techniques for staphylococci were not described until 1958 (Ritz and Baldwin, 1958; Morse, 1959; Pattee and Baldwin, 1961, 1962). Employing these transduction methods, Novick (1963) provided good evidence that the genes determining both the synthesis of penicillinase, ie, 'structural genes', and the control of its production, ie, 'control genes', were carried by one plasmid. Some years later penicillinase plasmid DNA was isolated (Rush, Gordon, Novick, and Warner, 1969) and established beyond doubt that penicillinase production was plasmid-mediated. In the great majority of strains, genes for penicillinase production have since been found to be plasmid-borne (eg, Dyke, Parker, and Richmond, 1970). There are, however, four strains in which the genes for penicillinase production are chromosomal (Asheshov, 1966a; Harmon, Baldwin, Tien, and Critz, 1966; Poston, 1966; Sweeney and Cohen, 1968).

TETRACYCLINE RESISTANCE

In many strains there is clear-cut evidence for plasmid inheritance of tetracycline resistance (May, Houghton, and Perrett, 1964; Asheshov, 1966b; Novick and Bouanchaud, 1971; Lacey, 1971b, 1972a). But Kayser, Wüst, and Corrodi (1972) consider that in some strains the locus is chromosomal on account of the stability of the resistance and the type of transduction kinetics.

NEOMYCIN RESISTANCE

Genes determining neomycin resistance have been found to be plasmid-borne in all strains examined (Chabbert, Baudens, and Gerbaud, 1964; Ayliffe, 1970; Lacey, 1971a; Bulow, 1971; Kayser *et al*,

1972; Lacey and Grinsted, 1973). Neomycin resistance is unstable under most cultural conditions

ERYTHROMYCIN RESISTANCE

There is no doubt that the genes for erythromycin resistance are plasmid-borne when they form part of a penicillinase plasmid (Rush *et al*, 1969). In other strains resistance to erythromycin or resistance to both lincomycin and erythromycin are probably also plasmid-mediated (Novick and Bouanchaud, 1971; Lacey, 1972a; Lacey, Lewis, and Rosdahl, 1974).

STREPTOMYCIN RESISTANCE

Extrachromosomal inheritance occurs in some strains (Ayliffe, 1970; Grubb and Annear, 1972b; Grubb O'Reilly, and May, 1972), but streptomycin resistance is chromosomal in others (Poston, 1966; Lacey, 1972a; Lacey and Chopra, 1972). In 74 streptomycin-resistant strains isolated since 1967 in Bristol about 70% showed high-level resistance to streptomycin which is characteristic of the chromosomal type; 30% were resistant to low levels which usually denotes an extrachromosomal inheritance (Grinsted and Lacey, 1973b).

CHLORAMPHENICOL RESISTANCE

There are several reports of an extrachromosomal locus for these genes (Chabbert *et al*, 1964; Novick and Bouanchaud, 1971; Kayser, Felix, and Wüst, 1972; Lacey and Grinsted, 1973).

FUSIDIC ACID RESISTANCE

Evans and Waterworth (1966) found that the resistance was unstable in two strains, being linked to penicillinase production in one. This linkage has been confirmed in 18 out of 26 fusidic-acid-resistant strains recently isolated (Lacey and Grinsted, 1972).

Lacey and Rosdahl, 1973). In the other eight strains the genes were probably chromosomal.

METHICILLIN RESISTANCE

Both chromosomal and extrachromosomal inheritance has been suggested for this resistance. Methicillin resistance can be lost spontaneously *in vitro* (Lacey, 1972a; Al Salihiy and James, 1972; Grubb and Annear, 1972a; Annear and Grubb, 1973) and after treatment with acridine dyes (Dornbusch, Hallander, and Löfquist, 1969). But Cohen and Sweeney (1970) and Kayser *et al* (1972) have found that the resistance is stable and that the transduction frequency is stimulated by low doses of UV light, ie, findings suggestive of a chromosomal locus. These apparently conflicting findings are disconcerting because in other properties (the nature of their resistance to methicillin, concomitant resistance to other antibiotics, orange pigment, and similar survival—see Lacey and Grinsted, 1973) methicillin-resistant strains are all similar and could have evolved from a single origin. It is possible that the genes for methicillin resistance do exist at different loci in different strains; alternatively these findings can all be accounted for by the supposition that in each strain the genes coding for methicillin-resistance are borne by an extrachromosomal element: stable under some conditions *in vitro*, and too large to be transduced *in toto* (see Lacey and Grinsted, 1973). Transduction of a region of an extrachromosomal element would give a chromosomal type response with UV light if the genes became integrated into the recipient's chromosome.

OTHER ANTIBIOTIC RESISTANCES

Resistance to novobiocin is chromosomal in two strains (Novick, 1966; Lacey and Grinsted, 1973). Resistance to trimethoprim is chromosomal in one strain (Lewis and Lacey, 1973). Although resistance to sulphonamides is common (Nakhla, 1972), surprisingly little is known about its genetic basis.

Manipulation of Staphylococcal Genes *in vitro*

For results of laboratory experiments to be related to events occurring naturally, the conditions of the experiment should resemble as far as possible those of the natural environment. The artificiality of the transduction experiments performed *in vitro* in which a cell-free, high-titre phage preparation is used as donor has little relevance to the natural environment. These types of experiment have however been valuable in (a) establishing genetic linkage and (b) demonstrating the possibility of various interactions between these elements.

GENETIC LINKAGE

Although frequent in the Enterobacteriaceae, linkage of resistance to two or more antibiotics is relatively rare in *Staphylococcus aureus*. Where such linkage has been established in staphylococci the sets of genes are borne by a single plasmid. To establish linkage, cotransduction of the two markers should if possible be demonstrated in addition to observing simultaneous loss of the two resistances. The latter is suggestive of linkage but cannot in itself be taken as proof, as (a) a single event might cause two separate plasmids to be lost simultaneously from the cell, and (b) a cell may harbour two plasmids and one plasmid may be lost at very much greater frequency than the other; eg, assume 0.1% of cells of a culture have lost the plasmid for neomycin resistance and 99% have lost that for tetracycline resistance. All the neomycin-sensitive colonies will also show sensitivity to tetracycline, and if only neomycin-sensitive colonies are tested there will be apparent linkage of the two resistances.

The following combinations of resistance genes are probably carried on single plasmids: (1) fusidic acid resistance and penicillinase production (Evans and Waterworth, 1966; Lacey and Grinsted, 1972; Lacey and Rosdahl, 1973); (2) erythromycin resistance and penicillinase production (Mitsuhashi, Hashimoto, Kono, and Morimura, 1965); (3) kanamycin/neomycin resistance and penicillinase production (Annear and Grubb, 1972); (4) streptomycin and neomycin/kanamycin resistance (Ayliffe, 1970; Grubb and Annear, 1972b; Grinsted and Lacey, 1973b); (5) erythromycin and spectinomycin resistance (Grinsted and Lacey, 1973a); (6) resistance to neomycin, erythromycin, and lincomycin (Annear and Grubb, 1969).

REVERSIBLE INTEGRATION OF PLASMIDS INTO THE CHROMOSOME

There is no report of an entire plasmid becoming integrated into the chromosome. Genes for erythromycin resistance, initially part of a penicillinase plasmid, have once become integrated into the chromosome after prolonged UV irradiation to the transducing lysate (Novick, 1967b). The genes for penicillinase production can also be integrated into the chromosome, at a situation distinct to that for the integration of erythromycin resistance (Wyman quoted by Novick and Bouanchaud, 1971).

Using the strain of Novick's which contained chromosomally located genes for erythromycin resistance, Richmond and Johnston (1969) have reversed this process by recombination of these genes with a penicillinase plasmid newly introduced into the cell. After introduction of the plasmid, cells were isolated that contained neither plasmid nor

resistance to erythromycin. The extent to which these phenomena occur naturally is difficult to assess. Since the great majority of penicillinase-producing strains of *Staphylococcus aureus* harbour the genes for penicillinase on a plasmid, transition of the plasmid genes to chromosome does not seem common.

RECOMBINATION OF CHROMOSOMAL WITH PLASMID GENES

Propagating strain 80 (PS 80) contains chromosomal genes for penicillinase production and a plasmid containing the genes for resistance to cadmium, arsenic, and mercury ions (how these resistance genes benefit the cell is unknown); on storage of the culture, a reduplication of the chromosomal penicillinase genes seems to occur, one copy becoming integrated into the plasmid (Asheshov, 1966a, 1969). These findings are particularly significant as they occurred progressively and spontaneously. The type of penicillinase determined by the chromosomal genes in PS 80 is extremely similar, if not identical, to that produced by another strain in which the genes are plasmid-borne (Richmond, 1972). It is reasonable to assume that the two sets of genes are similar. This observation is certainly compatible with the occurrence of chromosome → plasmid transition naturally.

INCOMPATIBILITY AND RECOMBINATION BETWEEN STAPHYLOCOCCAL PLASMIDS

The occurrence of multiresistant strains of *Staphylococcus aureus* possessing several plasmids suggests that incompatibility does not occur between many. It is possible to construct strains possessing several plasmids *in vitro* (eg, Lacey, 1972c). The only well documented instance of incompatibility *in vitro* occurs between certain penicillinase plasmids. These plasmids have been classified into two groups: those of group I can stably coexist in the same cell with those of group II, but different plasmids of the same group cannot coexist unless a relatively rare recombination event occurs (Richmond, 1967). Recombination between plasmids (in conjunction with transfer between cells) certainly provides theoretical potential for gene reassessment (Richmond, 1969).

It will be difficult to detect the occurrence of this process naturally. The plasmid that determines production of penicillinase and resistance to fusidic acid and cadmium ions may have resulted from such a recombination event (Lacey and Grinsted 1972). This plasmid seems to have gained a gene(s), ie, resistance to fusidic acid, and lost at least one, ie, resistance to arsenate ions. Other penicillinase plasmids if resistant to cadmium ions are also

resistant to arsenate ions (Dyke *et al*, 1970). Thus the projected changes in this plasmid could have resulted from recombination, although they could also have occurred by successive mutations.

LOSS OF FRAGMENTS OF DNA FROM STAPHYLOCOCCAL PLASMIDS

We have found that part of a 'penicillinase plasmid'—this includes the genes for fusidic acid resistance—can be lost after growth at 42°C *in vitro* (Lacey and Grinsted, 1972). The loss of fragments from this plasmid probably also occurs naturally (Lacey and Rosdahl, 1973; Lacey *et al*, 1974). Whether this type of fragmentation occurs in other plasmids under natural conditions is unknown; if it did, it could be an important mechanism in the spread of antibiotic resistance since the smaller the plasmid the greater its frequency of transfer between cells (Chopra *et al*, 1973).

Transfer of Plasmids between Strains of *Staphylococcus aureus* in Mixed Cultures

Naturally occurring plasmids determining neomycin, tetracycline, or erythromycin resistance or penicillinase production may be transferred between cultures of *Staphylococcus aureus* at frequencies of 10^{-3} to 10^{-8} (Lacey, 1971a,b, 1972a). Although many strains cannot act as donors in this type of experiment—presumably because they do not possess a transducing phage—a single donor can transfer a plasmid to a variety of recipient strains. The transfer frequency is very much affected by the nature of the recipient and the plasmid in question.

Plasmid-borne genes can usually be transferred at higher frequencies than chromosomal (Lacey, 1972b) although methicillin resistance has not been transferred. Tetracycline resistance has proved the easiest plasmid to transfer in mixed culture; it can also be transduced to a wide range of recipients from cell-free lysates (Mitsuhashi, Oshima, Kawaharada, and Hashimoto, 1965; McDonald, 1966). It seems that mixed culture transfer and transfer from lysates are different ways of achieving the same results. The relatively high frequency of transfer that can be attained in mixed cultures is probably in part due to the continuous nature of the process and partly because there are very many more transducing particles present in the culture than is shown by examination of the culture supernatant. The relatively few phage particles detected in supernatants has previously cast doubts about the ability of generalized transduction to be important in the spread of antibiotic resistance among staphylococci (Richmond, 1969).

Evidence for Transfer of Plasmids between *Staphylococci* under Natural Conditions

Detection of transfer of plasmids between strains in mice (Novick and Morse, 1967) and experimentally on the human skin (Lacey, 1971a; Lacey and Richmond, 1974) demonstrates the feasibility of transfer occurring naturally. However this process has not yet been demonstrated outside the laboratory. The main evidence for transfer is therefore epidemiological. The emergence of neomycin-resistance strains probably provides the strongest evidence for transfer because there is an extremely good baseline; from 1950 to 1959 neomycin resistance was virtually unknown (Lowbury, 1955; Rountree, Heseltine, Rheuben, and Shearman, 1956; Bynoe, Elder, and Comtois, 1956; Blair and Carr, 1958; Waisbren, 1958; Waisbren and Strelitzer, 1959; Petersdorf, Rose, Minchen, Keene, and Bennett, 1960; Cason and Lowbury, 1960; Finland, Hirsch, and Wallmark, 1960).

Neomycin-resistant clinical strains were first reported from several centres in the USA (Quie, Collin, and Cardle, 1960; Griffith, Ostrander, Smith, and Beswick, 1961; Tisdale, Fenster, and Klatskin, 1960; Finegold and Gaylor, 1960). These strains were resistant to several antibiotics and were usually phage type 54 or non-typable. During the next few years these organisms were reported widely, particularly from the UK (Robertson, 1963; Jacobs, Willis, Ludlam, and Goodburn, 1963; Jevons *et al*, 1966). Their sudden dissemination often followed the topical use of neomycin (Lowbury, Babb, Brown, and Collins, 1964; Rountree and Beard, 1965; Alder and Gillespie, 1967). But in 1967 and 1968, neomycin resistance appeared in a variety of 'new' strains as judged by their phage-typing and sensitivity patterns (eg, Ayliffe, 1970; Lacey, 1971a; Bülow, 1971).

Neomycin-resistant mutants obtained *in vitro* are defective and show a cross resistance to gentamicin (Lacey, 1969; 1971a; Garred and O'Grady, 1971). Therefore the resistance in clinical strains (which are normal growing and sensitive to gentamicin) is not likely to have arisen by mutation.

In many strains the genes for neomycin resistance have been found to be plasmid-borne (see above). There are two possible explanations for the epidemiology of neomycin resistance. (1) Either the plasmid has formed *de novo* in many strains over a very short period of time or (2) one plasmid has spread amongst the staphylococcal population. It is impossible to envisage how a plasmid could have formed so rapidly in so many cells (what advantage would a partly formed non-functional plasmid confer

on the cell and would it not be rapidly lost?). Consistent with the supposition that transfer of one plasmid has occurred to a variety of strains is the similar level of resistance to neomycin and the cross-resistance to kanamycin and paramomycin that the strains exhibit (Chabbert, 1967; Lacey, 1971a). It therefore seems highly likely that the 'neomycin-resistance plasmid' has spread from one or a very few strains to a variety.

The delay in the appearance of the resistance after the introduction of neomycin can be accounted for by the requirement of the following conditions to be met.

- 1 The presence of the genes for neomycin resistance as part of a plasmid sufficiently small for inter-cell transfer. (Possibly relevant to this is that neomycin resistance may be linked to streptomycin resistance and that fragmentation of plasmids can occur.)

- 2 The presence of a transducing phage in the same cell as the 'neomycin resistance plasmid'. Phages have probably spread epidemically amongst the staphylococcal population under natural conditions (Jevons and Parker, 1964; Jevons *et al*, 1966; Jessen *et al*, 1969) and could well lysogenize a cell containing the 'neomycin resistance plasmid'.

Once the transducing phage and plasmid are present in the same cell there is opportunity for spread of the plasmid to other strains.

A similar argument applies to tetracycline resistance. Mutation to tetracycline resistance (of the sort found in clinical strains) has not been achieved *in vitro*. For a year or two after the introduction of tetracycline resistance to it was rare (Birnstingl, Shooter, and Hunt, 1952; Elek, 1959) to be followed by the appearance of the resistance in many strains (Mitsuhashi *et al*, 1965). Indeed, tetracycline resistance has been considered the hallmark of the hospital staphylococcus. Transfer has probably also played an important role in the emergence of this resistance.

Study of the penicillinase plasmid is complicated by the large variety of plasmid types that have been identified and uncertainty about the incidence of penicillinase-producing strains before the use of penicillin. Although a few strains produced penicillinase at the time of and before the introduction of penicillin (Citri and Pollock, 1966), these were probably rare. Spink (1956) found none out of 67 strains and Rammelkamp and Maxon (1942) found that none out of 29 strains isolated before 1942 were resistant to penicillin. North and Christie (1945) found that none out of 128 strains isolated in 1944 were resistant. During the late 1940s the incidence of penicillin resistance rapidly increased (eg, Barber,

1947; Barber and Rozadowska-Dowzenko, 1948; Barber, Hayhoe, and Whitehead, 1949; Barber and Whitehead, 1949). The majority of hospital staphylococci of every phage pattern now produce penicillinase. Penicillin resistance of the type found in clinical strains cannot be produced *in vitro* by mutation, such mutants being defective and unstable (North and Christie, 1946; Blair, Carr, and Buchmann, 1946; Spink and Ferris, 1947).

'Penicillinase plasmids' identified during the last decade have been found to carry a variety of other markers: resistance to erythromycin, kanamycin or fusidic acid (see above), or to ethidium bromide (Johnston and Dyke, 1969), to cadmium, arsenate, arsenite, bismuth, lead, or mercury ions (Peyru, Wexler, and Novick, 1969). Four different serotypes of penicillinase have been identified (Richmond, 1965; Rosdahl, 1973) and strains show at least three types of enzyme control (Rosdahl, 1973; Lacey and Rosdahl, 1973). The size of the plasmid varies from about 20×10^6 to 12×10^6 daltons (Rush, Gordon, Novick, and Warner, 1969; Smith and Novick, 1972; Lacey and Grinsted, 1972; Lacey and Rosdahl, 1973; Lacey *et al.*, 1974).

If selection alone had caused the appearance of 'penicillinase plasmids' in so many strains, then each of the plasmid types must have evolved *de novo*. It is difficult to envisage how this could have happened. It seems much more plausible that two other processes have also occurred. (1) Natural variation in what may be one or a few primordial 'penicillinase plasmids'; we have seen rapid evolution of such a plasmid *in vivo* (Lacey *et al.*, 1974). The most likely origin for the penicillinase plasmid is the mechanism described by Asheshov (1969), although the problem of the formation of the plasmid genes for metal ion resistance is not solved. (2) Inter-cell transfer.

Further evidence for the spread of plasmids in *Staphylococcus aureus* is presented elsewhere (Lacey and Grinsted, 1973; Lacey and Rosdahl, 1973). In summary, there is epidemiological evidence for inter-cell transfer of plasmids determining production of penicillinase or resistance to neomycin, tetracycline, fusidic acid, erythromycin, and chloramphenicol.

Mutation to Antibiotic Resistance in *Staphylococcus aureus*

There are two ways in which mutation can affect antimicrobial therapy. (1) Resistant mutants can arise during treatment and prevent eradication of the organism. (2) These resistant mutants can become disseminated and disrupt therapy in other patients. The latter process seems to be rare in staphylococci;

this is well illustrated by the study of fusidic acid resistance.

Resistance to fusidic acid develops rapidly *in vitro* (Hilson, 1962; Newman, Bhat, Hackney, Robinson, and Stewart, 1962; Taylor and Bloor, 1962; Jensen and Kiær, 1964) and arises during treatment with the drug (Lowbury, Cason, Jackson, and Miller, 1962; Wright and Harper, 1970). But the overall incidence of fusidic acid resistance is still very low (Parker and Hewitt, 1970; Pattison and Mansell, 1973). The reason for this is probably the reduced growth rates of fusidic-acid-resistant mutants (Pattison and Mansell, 1973) which will presumably be (in the absence of fusidic acid) at a disadvantage compared to most fusidic-acid sensitive strains.

In contrast, most of the fusidic-acid-resistant strains isolated in 1971 and 1972 are resistant by possession of plasmid genes (as part of the penicillinase plasmid—see above). The growth rates of these strains is normal (R. W. Lacey, unpublished observations).

Staphylococci may also develop resistance to lincomycin during therapy (Duncan, 1968; McGhee, Barrett, and Finland, 1969; Wright and Harper, 1970; Lacey *et al.*, 1974). At present there are insufficient data to ascertain whether lincomycin-resistant mutants will be important epidemiologically.

Apart from resistance to fusidic acid and lincomycin, staphylococci can rapidly develop resistance *in vitro* to streptomycin, novobiocin, and rifampicin (Garrod and O'Grady, 1971). Of these only resistance to streptomycin has become common. In many strains this resistance is of the ribosomal/chromosomal type (see above) and has probably arisen by mutation.

It has been traditional in testing a new antimicrobial agent to predict the likelihood of resistance developing *in vivo* by passage of the organism *in vitro* in the presence of the antibiotic. The correlation of resistance *in vitro* and *in vivo* seems poor and only when high-level resistance develops rapidly *in vitro* and those derivatives are normal growing (as with resistance to streptomycin) should the development of much resistance by mutation in the natural population be predicted.

The pathogenicity of resistant mutants that have arisen during therapy has rarely been determined and would seem to merit further investigation.

Significance of Methicillin-resistant *Staphylococci*

Much has been published about the incidence, ways of detecting the resistance, and the genetic basis of methicillin resistance over the last few years. The

genetic basis has already been discussed here; suffice to add that there is no evidence for transfer of methicillin resistance *in vitro* or *in vivo* (Lacey, 1972a; Lacey and Grinsted, 1973).

The practical significance of methicillin-resistant strains lies in (a) their resistance to all the cephalosporins and penicillins *in vitro* and (b) their resistance to many other antibiotics (Parker and Hewitt, 1970). The chief uncertainty about methicillin-resistant strains is whether they can successfully be treated with methicillin or analogues *in vivo*. This problem results from the nature of the resistance which is only fully expressed at 30°C or below, or in the presence of 5% (w/v) NaCl (Annear, 1968; Dyke, 1969; Parker and Hewitt, 1970). At temperatures above 30°C, only a minority of cells (about 1 in 10⁵ at 37°C) are resistant; resistant clones have reduced growth rates in the presence of methicillin (Sutherland and Rolinson, 1964). These slow-growing variants are not mutants (Seligman, 1966) in the usual sense as subculture of such a clone in methicillin-free medium yields immediately a population of cells once more predominantly sensitive (Dyke, 1969). Methicillin might well be able to eliminate such cells *in vivo*. However these strains seem fully virulent (Benner and Kayser, 1968; Hallander and Laurell, 1971) and Chabbert and his colleagues have found in a small number of patients that methicillin-resistant staphylococci were not eliminated by methicillin or cephalothin as effectively as by other antibiotics (Chabbert, Baudens, Acar, and Gerbaud, 1965; Acar, Couvalin, and Chabbert, 1971). These findings have been criticized recently by Bulger, Feigl, and Nielson (1972): 'In the absence of a controlled clinical trial of alternate programs of treatment, clinical experience permits the competent observers, in matters like this, only educated guesses rather than scientific conclusions.' These authors have found that in rats experimentally infected with methicillin-resistant staphylococci, cephalothin was as effective as kanamycin in treating the infection and both drugs were better than no treatment. However either vancomycin alone or a combination of cephalothin and kanamycin were even better. It would have been interesting to know how effective the drugs were against methicillin-sensitive organisms!

It is debatable whether such controlled trials in human subjects are ethically acceptable. But in view of the still relative rarity of these isolates in the UK (Parker and Hewitt, 1970; Ridley, Barrie, Lynn, and Stead, 1970), analogues of methicillin must still be considered important drugs in treating hospital staphylococci before their sensitivity is known, and possibly even after they are found to be 'methicillin-resistant'.

Relationship of Antibiotic Resistance to Virulence in *Staphylococcus aureus*

Organisms trained to antibiotic resistance *in vitro* often have reduced growth rates and diminished virulence (Garrod and O'Grady, 1971). But are naturally occurring resistant derivatives more or less virulent? Some resistant strains have been considered to show enhanced virulence (eg, Rountree and Freeman, 1955; Temple and Blackburn, 1963). However, virulence is extremely difficult to measure and results from a complex interaction of host and parasite (Elek, 1959). Thus, although there have been reports (eg, Watt and Okubadejo, 1967) of the increasing incidence of staphylococcal bacteraemia, these authors consider that this could be a reflection on changes in the type of hospital patient rather than on the pathogen, since deaths from staphylococcal bacteraemia were confined to patients with severe underlying disease. Prophylactic measures aimed at preventing staphylococcal cross-infection must also alter the incidence and type of staphylococcal disease. It therefore seems impossible to detect changes in virulence by study of the incidence and associated mortality and morbidity of staphylococcal infections.

However the properties of the multiresistant organisms *in vitro* and *in vivo* can give some indication as to the expected virulence of the pathogen.

1 In the absence of antibiotics, resistant strains tend to be replaced by sensitive (Lepper, Moulton, Dowling, Jackson, and Kofman, 1954; Forfar, MacCabe, Gould, and Bain, 1966; Bulger and Sherris, 1968; Ridley *et al.*, 1970). There is also evidence that certain specific strains lose their resistance (plasmid-mediated) *in vivo* (Ayliffe and Collins, 1967; Annear and Grubb, 1969, 1972; Noble, 1972; Lacey *et al.*, 1973). Loss of a plasmid from the cell can be associated with increased growth rate *in vitro* (Grinsted and Lacey, 1973a; Lacey *et al.*, 1974). These findings suggest that antibiotic-sensitive derivatives will generally have an advantage over the antibiotic-resistant *in vivo*.

2 In organisms other than staphylococci, plasmid DNA may account for as much as 20% of the total cellular DNA because the plasmid, although of small size, is present in multiple copies in each cell (Clowes, 1972). In *Staphylococcus aureus*, individual plasmids that determine antibiotic resistance may account for 3 to 8% of the cellular DNA (Chopra *et al.*, 1973). Thus the size of the plasmid determining tetracycline resistance is probably equivalent to about 0.1% of the total cellular DNA; but this plasmid is present in about 30 to 50 copies per cell (Novick and Bouanchaud, 1971; Chopra *et al.*, 1973) so that it comprises about 3 to 5% of the total DNA.

Presumably the maintenance of this additional DNA reduces other capacities of the cell. It is therefore not surprising that the cell seems to lose progressively plasmids in the absence of the antibiotic. Sometimes a plasmid appears to be stable, eg, that determining tetracycline resistance in methicillin-resistant strains (Lacey and Grinsted, 1973) and this may explain why methicillin-resistant strains are almost universally resistant to tetracycline.

3 Few 'virulence factors' are genetically linked to antibiotic resistance. In a few methicillin-resistant strains enterotoxin B may be linked to resistance to methicillin (Dornbusch *et al*, 1969).

4 Antibiotic-resistant derivatives survive on glass as well as those antibiotic sensitive (Lacey, 1972c). The important property of the organism associated with survival is pigment (Grinsted and Lacey, 1973a). If these findings are applicable to natural conditions, we expect some, at least, of multi-resistant staphylococci to persist in the environment.

In conclusion, there is no satisfactory method of assessing virulence, but antibiotic resistance tends to give the organism a disadvantage *in vitro* and probably *in vivo* (except in the presence of the antibiotic) and this could be associated with decreased virulence *in vivo*. Multiresistant strains are not expected to be eliminated entirely from the environment.

Conclusions

The organization of most of the DNA in the staphylococcal cell is unknown. The chromosome is a useful functional term, ie, the genome necessary for growth on ordinary media. Many staphylococci harbour small elements distinct from the chromosome (plasmids). It is sometimes difficult to establish whether a certain gene is plasmid-borne or chromosomal.

Most naturally occurring antibiotic resistance is determined by genes carried on plasmids. Use of antibiotics has selected these strains at the expense of plasmid-negative organisms.

The potential for modification of the staphylococcal genome seems confined to (a) mutation, (b) alterations in prophage carriage, and (c) acquisition of new genes mediated by bacteriophage (transduction).

In vitro, plasmids can usually be transferred by transduction at higher frequencies than chromosomal genes.

Many plasmids have probably spread amongst the staphylococcal population by generalized transduction. The phages that have effected this transfer have also spread epidemically amongst staphylococci.

Incompatibility between plasmids is rare, being confined to certain 'penicillinase plasmids'. The

variation in these plasmids has probably occurred by evolution from one or a few types. Strains are expected to acquire additional plasmids, once that plasmid has evolved.

Plasmids tend to be lost from the cell in the absence of selection pressure by antibiotics.

Few strains show antibiotic resistance that has arisen by mutation (except to streptomycin). Although mutants can arise during treatment, they infrequently produce disease in other patients.

Policies of withholding certain antibiotics have been followed by increasing numbers of antibiotic-sensitive organisms. There is ample scope for the extension of these policies.

Methicillin resistance is not expected to become widespread in strains in the UK, at least in the near future.

It is not known whether methicillin-resistant strains can be treated successfully with penicillinase-resistant penicillins.

Naturally occurring multiresistant strains are probably less virulent than the corresponding sensitive derivatives.

I thank Dr Peter Bennett for the electron micrographs and members of the department for useful discussions.

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