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AN UNUSUAL "PENICILLINASE PLASMID" IN *STAPHYLOCOCCUS AUREUS*; EVIDENCE FOR ITS TRANSFER UNDER NATURAL CONDITIONS

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EXTRACHROMOSOMAL elements that determine production of penicillinase can undergo experimentally a variety of changes within the staphylococcal cell: irreversible loss of the entire element (Novick, 1963) or loss of a fragment from the plasmid (Lacey and Grinsted, 1972), recombination between two of these plasmids (Richmond, 1969), reversible integration of part of the plasmid into the chromosome (Novick, 1967; Richmond and Johnson, 1969), and acquisition of chromosomal genes (Asheshov, 1969). The occurrence of these events under natural conditions, with transfer of the plasmids between cells, could produce re-assortment of many of the available genes; this might occur despite the capacity of transduction to transfer only a small part of the cell genome at any one time. Although the "penicillinase plasmids" may be transferred in the laboratory between cultures of different strains (Novick and Morse, 1967; Lacey, 1972), the extent of this type of transfer between staphylococci in their natural environment is uncertain. Studies on the possible epidemiology of the "penicillinase plasmids" are complicated by the high proportion of strains that produce penicillinase.

In this paper, we describe an uncommon "penicillinase plasmid", designated PF because it also carries the genes for resistance to fusidic acid, that occurs in *Staphylococcus aureus* of phage-groups I, II, and III and in some non-typable strains. Most of these strains have probably acquired the PF plasmid by transfer from other strains.

MATERIALS AND METHODS

Fusidic-acid-resistant Staphylococcus aureus. Strains no. FAR1 to 3 were isolated in Birmingham during 1971 and were supplied by Dr G. A. J. Aylliffe; strains no. FAR7 and 9 (Colindale nos. 1701 and 5487 respectively) were supplied by Dr E. H. Asheshov; strains no. FAR4, 5, 9, and 11 were isolated in Bristol during 1971 and 1972; strains no. FAR24

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to 26 were isolated in Copenhagen by Dr K. Riewerts Eriksen; strains no. FAR6, 8, 10, 12, 13 to 18, and 20 to 23 were isolated during 1971 and 1972 at the Middlesex Hospital and supplied by Dr J. R. Pattison.

Media, determination of antibiotic resistance, phage typing, detection of loss of resistance, and isolation of plasmid DNA were as described previously (Lacey and Grinsted, 1973).

Detection and characterisation of penicillinase. Cultures were maintained on medium containing 10^{-4} M cadmium acetate. Before induction, they were grown in casein-yeast (CY) medium and the induction was performed with methicillin 0.5 µg per ml by a standard

TABLE I

Pigment, antibiotic sensitivity, and phage-sensitivity patterns of strains of Staphylococcus aureus harbouring a plasmid determining resistance to fusidic acid, cadmium ions and penicillin (penicillinase)

Strain number	Colour of pigment*	Sensitivity or resistance to†					Phage-sensitivity pattern at RTD‡
		S	T	E	N	M	
FAR1	O	R	R	R	R	R	77/83A/84
FAR2	B	R	R	R	S	R	42E/47/54/77/83A/84/85
FAR4	O	R	S	R	R	S	3A
FAR5	O	S	S	S	S	S	79/47/53/54/85
FAR6	O	S	S	S	S	S	52
FAR7	W	S	S	S	S	S	52/52A/80
FAR8	B	S	S	S	S	S	NT
FAR9	B	S	S	S	S	S	79
FAR10	B	S	S	S	S	S	29/47/54/75/77/84
FAR11	B	S	R	S	S	S	47/54/75/85
FAR12	B	S	R	S	S	S	NT
FAR13	B	S	S	S	S	S	85
FAR14	Y	S	S	S	S	S	3A/42E/47/54/75/77/81/88
FAR15	B	S	S	S	S	S	85
FAR16	B	S	S	S	S	S	NT
FAR17	O	S	S	S	S	S	52/52A/79/80
FAR18	W	S	S	S	S	S	53/54/75/85
FAR19	Y	S	S	S	S	S	NT

* O = orange; B = buff; W = white; Y = yellow.

† S = streptomycin; T = tetracycline; E = erythromycin; N = neomycin; M = methicillin.

‡ RTD = routine test dilution; NT = non-typable.

procedure (Richmond *et al.*, 1964). The serotype of penicillinase produced by each strain was determined by the method of Richmond (1965). Strain no. 524SC which produces A-type penicillinase and no. 22260 which produces B-type enzyme by the criteria of Richmond (1965) were used as controls; the latter was supplied by Dr E. H. Asheshov.

RESULTS

Linkage of resistance to fusidic acid and cadmium ions with penicillinase production of Staphylococcus aureus

Twenty-six strains of *S. aureus* that were resistant to fusidic acid and cadmium ions, and produced penicillinase, were examined for loss of resistance after incubation overnight at 43°C; at least 3000 colonies from each strain were tested. Findings for five of the strains (nos. FAR1 to 5) have already been reported (Lacey and Grinsted, 1972). From 14 other strains, loss of resistance to fusidic acid and cadmium ions and loss of penicillinase occurred in from

0.2–0.3% of colonies. Loss of the three resistances was always linked in these strains. Two derivatives of each strain that had lost these properties were phage-typed and in each case the patterns resembled those of the wild strains. Table 1 shows the heterogeneity of these strains in respect of pigment production,

TABLE II

MIC of cadmium, arsenate, and mercury ions, and fusidic acid for strains of Staphylococcus aureus resistant to fusidic acid and producing penicillinase

Strain number	Linkage of fusidic-acid resistance to "penicillinase plasmid" **	Minimum inhibitory concentration of:			
		cadmium acetate (M)	sodium arsenate (μg per ml)	mercuric chloride (μg per ml)	sodium fusidate (μg per ml)
FAR1	+	$>10^{-4}$	200	5	16
FAR2	+	$>10^{-4}$	200	2.5	16
FAR4	+	$>10^{-4}$	200	5	16
FAR5	+	$>10^{-4}$	100	2.5	16
FAR6	+	$>10^{-4}$	200	10	8
FAR7	+	$>10^{-4}$	200	5	16
FAR8	+	$>10^{-4}$	200	5	8
FAR9	+	$>10^{-4}$	200	10	20
FAR10	+	$>10^{-4}$	100	5	8
FAR11	+	$>10^{-4}$	200	5	16
FAR12	+	$>10^{-4}$	200	2.5	8
FAR13	+	$>10^{-4}$	200	10	16
FAR14	+	$>10^{-4}$	200	5	8
FAR15	+	$>10^{-4}$	200	5	16
FAR16	+	$>10^{-4}$	200	10	16
FAR17	+	$>10^{-4}$	200	5	16
FAR18	+	$>10^{-4}$	200	5	8
FAR19	+	$>10^{-4}$	200	2.5	8
FAR3	—	$>10^{-4}$	2000	1000	16
FAR20	—	$>10^{-4}$	>2000	20	32
FAR21	—	2×10^{-5}	>2000	200	32
FAR22	—	$>10^{-4}$	100	10	4
FAR23	—	$>10^{-4}$	>2000	2000	8
FAR24	—	1×10^{-5}	200	20	16
FAR25	—	4×10^{-6}	200	10	32
FAR26	—	4×10^{-6}	2000	100	32
"Oxford staphylococcus" (NCTC no. 6571)	—	3.5×10^{-6}	200	5	0.1

* + = linkage present; — = linkage not detected.

and in antibiotic-resistance and phage-typing patterns. However, in eight strains, resistance to cadmium ions was lost at frequencies of 0.3–14%, but resistance to fusidic acid was stable; in five of these strains penicillinase production was also lost.

Each of the strains in which there was linkage of resistance to fusidic acid and to cadmium ions with penicillinase production was also sensitive to arsenate and mercury ions, and the MIC to fusidic acid was about 16 μg per ml (table II). In contrast, strains in which fusidic-acid resistance was not linked

with cadmium-ion resistance showed variable sensitivity to arsenate and mercury ions (table II).

Properties of penicillinase produced by 18 strains of S. aureus in which resistance to fusidic acid was linked to the "penicillinase plasmid"

The basal and induced level of penicillinase production was determined for each culture; from these figures the induction ratio was calculated (table III).

TABLE III

Amount and serotype of penicillinase produced by staphylococci in which genes specifying resistance to fusidic acid were part of the penicillinase plasmid

Strain number	Amount of induced penicillinase (units per mg*)	Induction ratio†	Percentage stimulation of activity when 12 units of penicillinase were incubated with antiserum diluted 1 in 10 in indicated amounts			Penicillinase type
			0.012 ml	0.050 ml	0.120 ml	
FAR1	106	60	127	150	225	D
FAR2	78	29	124	170	241	D
FAR4	72	1.1	133	164	223	D
FAR5	81	93	126	151	241	D
FAR6	46	115	123	157	227	D
FAR7	108	111	125	150	240	D
FAR8	51	83	123	148	229	D
FAR9	90	130	124	167	233	D
FAR10	75	64	119	140	230	D
FAR11	75	175	118	162	238	D
FAR12	56	115	125	163	222	D
FAR13	1.51	2.3	135	158	220	D
FAR14	85	1.9	122	142	230	D
FAR15	79	226	130	167	228	D
FAR16	76	130	115	152	225	D
FAR17	48	133	133	151	225	D
FAR18	81	166	126	161	220	D
FAR19	52	14	128	152	†	D
524SC	350	50	258	400	360	A
22260	86	41	105	117	127	B

* Dry weight of bacteria.

† Ratio of penicillinase produced after induction with 0.5 µg methicillin per ml to basal level, determined under identical conditions except for the omission of methicillin.

‡ Enzyme unstable with 0.120 ml serum; with 0.500 or 0.062 ml, percentage stimulation was 202.

The enzyme activity produced by strains no. FAR4 and FAR14 was high under basal and under induced conditions (macroconstitutive); that produced by strain no. FAR13 was low under both conditions (microconstitutive; Rosdahl, 1973). The other 15 strains produced inducible enzyme (table III). In each culture more than 50% of the lactamase produced was extracellular, apart from that from strains no. FAR1 and FAR19 which was 23% and 40% respectively.

In all 18 strains the serological type of the enzyme was the same, having a stimulation coefficient intermediate between that of type A and B after incubation with type-A antiserum (table III). This is a new type and has been called D (Rosdahl, 1973).

Molecular weight of covalently closed circular (CCC) DNA of plasmids isolated from seven strains resistant to fusidic acid

Seven strains that had different phage-sensitivity patterns and were not resistant except to fusidic acid, cadmium ions, and penicillin were selected for analysis by ethidium bromide/CsCl-gradient centrifugation. Lysates of each wild strain contained a satellite band of DNA on the dense side of the main chromosomal band, which represented CCC-DNA. Because lysates of the corresponding antibiotic-sensitive derivatives did not contain a satellite band (data not shown), it was assumed that the CCC-DNA in the satellite band represented the plasmid or plasmids that carry the genes coding for these resistances.

The CCC-DNA prepared from ethidium bromide/CsCl gradients was analysed on sucrose gradients. Tritium-labelled DNA of each plasmid—other than that from strain no. FAR5—was mixed with ^{14}C -labelled DNA from strain no. FAR5 and analysed by sucrose-gradient centrifugation. Each preparation sedimented as two bands; fresh preparations consisted of a major faster-moving band with a minor slow-moving one. Analysis of the DNA after storage showed an increase of the slower-moving band at the expense of the faster, indicating that the two bands represented the CCC and open form respectively of the same plasmid. The *S* values (44 and 31.2) of the plasmid DNA from strain no. FAR5 have been previously obtained (Lacey and Grinstead, 1972). Five of the other plasmids sedimented exactly at the same rate as that of strain no. FAR5, and we can confidently assign *S* values of 44 and 31.2 to each of these DNA species and a molecular weight of 16 million daltons (table IV). But the DNA from strain no. FAR13 sedimented more slowly than that from strain no. FAR5 (38.4 and 29.0 *S*); a molecular weight of 13 million daltons is calculated for this plasmid.

Thus six out of the seven plasmids isolated are identical in size (molecular weight 16 million daltons) and one, from strain no. FAR13, is somewhat smaller (13 million daltons).

Compatibility of the "penicillinase-fusidic-acid-resistance (PF) plasmids" with a plasmid of compatibility-group I

The "penicillinase plasmids" have been classified into two compatibility groups: those of group I can exist in the same cell with those of group II, but different plasmids of the same group cannot coexist stably unless a relatively rare recombination occurs (Richmond, 1969). We have not tested the compatibility of the PF plasmid with plasmids of group II because of the absence of suitable markers to detect transductants.

However, compatibility of the PF plasmids with a group-I plasmid has been investigated. The group-I plasmid determines resistance to erythromycin, cadmium, arsenate, and mercury ions in addition to penicillinase; this plasmid is harboured by strain 8325 and was supplied by Dr Joan Ballans. Portions of mitomycin C-induced lysates of this culture were added to strains harbouring the PF plasmid and to their antibiotic-sensitive derivatives; transductants were selected on media containing erythromycin 10 μg per ml. In each of the eight

pairs of strains examined, the transduction frequency was similar whether or not the recipient contained the PF plasmid (table V). To exclude the possibility that recombination between resident and incoming plasmid was occurring at high frequency, three cultures that apparently harboured both plasmids were

TABLE IV

Molecular weight of seven plasmids determining resistance to fusidic acid, cadmium ions, and penicillin (penicillinase)

Strain number	Phage group	Molecular weight (million daltons)
FAR5	III	16
FAR7	I	16
FAR9	I	16
FAR10	I, III	16
FAR13	III	13
FAR14	II, III	16
FAR18	III	16

induced with mitomycin C and the resultant lysate added to strain 8325; transductants were selected with 10^{-4} M cadmium acetate and were found to be phenotypically characteristic of either the PF plasmid or the plasmid of compatibility group I.

TABLE V

Effect of a resident plasmid (PF) in the recipient on the transduction of a plasmid of compatibility group I to that cell

Strain number of recipient	Transduction frequency* when	
	recipient contains a PF plasmid	recipient does not contain a PF plasmid
FAR5	2.5×10^{-6}	3.9×10^{-6}
FAR7	1.0×10^{-6}	6.5×10^{-7}
FAR9	7.5×10^{-7}	3.8×10^{-7}
FAR10	7.9×10^{-8}	6.7×10^{-8}
FAR11	8.4×10^{-8}	1.0×10^{-7}
FAR14	4.0×10^{-7}	1.5×10^{-8}
FAR16	$c. 1 \times 10^{-9}$	$c. 2 \times 10^{-9}$
FAR18	4.4×10^{-8}	7.0×10^{-8}

* Expressed as the number of transductants per plaque-forming unit (on strain no. 6936).

We interpret these findings as indicating compatibility of PF plasmids in eight strains with a group-I plasmid.

DISCUSSION

The gene or genes coding for fusidic acid resistance were found to be part of a "penicillinase-fusidic-acid" (PF) plasmid in 18 out of 26 strains of *S. aureus*

examined. In the other eight strains, fusidic-acid resistance was not linked to the "penicillinase plasmid". In these, the loci of the gene or genes specifying resistance to fusidic acid are probably chromosomal because the resistance was stable; mutants—presumably chromosomal—resistant to fusidic acid occur at high frequency *in vitro* (Evans and Waterworth, 1966), and the same type of resistance probably arises during treatment of staphylococcal infections (Wright and Harper, 1970).

The strains in which fusidic-acid resistance was linked to the PF plasmid showed an unusual and uniform reaction to cadmium ions (resistant), arsenate (sensitive), mercury (sensitive), and fusidic acid (MIC about 16 μg per ml). Each culture produced D-serotype penicillinase. In all eight of the strains tested, the PF plasmid could exist with a plasmid of group-I compatibility; the molecular weight of the PF plasmid from six out of the seven strains examined was the same (16 million daltons). But in one strain (no. FAR13), the plasmid is smaller (molecular weight 13 million daltons), although in most phenotypic properties it resembles the other PF plasmids. Because we propose that one PF plasmid has spread amongst the staphylococcal population (see below), how can we account for the apparent discrepancy in the size of the plasmid in strain no. FAR13 with those in other strains? A possible explanation for the smallness of the PF plasmid in strain no. FAR13 is that the plasmid has lost a fragment. In another strain, no. FAR5, a fragment of the PF plasmid may be lost *in vitro* (Lacey and Grinstead, 1972). Consistent with this proposal is the finding that although strain no. FAR13 produces D-type enzyme—i.e., the genes for structural enzyme seem to be intact—it produces very small amounts of basal and induced enzymes; we interpret this as an abnormality in the loci controlling enzyme production—at least two such loci have been identified (Richmond, 1967). It is improbable that the production of defective penicillinase is due to abnormalities in the host chromosome rather than in the PF plasmid, because the same amount of basal and induced enzyme is produced after transduction of the plasmid to another strain (no. 649). The abnormality in production of penicillinase in strain no. FAR13 might have resulted from loss of a part of the plasmid that contains the loci for control of penicillinase production. Similarly, the plasmids in strains no. FAR4 and FAR14 that determine the enzyme constitutively but in "normal" amounts may have undergone mutation at the locus for inducibility. We therefore consider that each PF plasmid in these strains is essentially the same. Apart from one strain reported by Evans and Waterworth (1966) in which resistance to fusidic acid was associated with penicillinase production, plasmids expressing these properties—type-D penicillinase, resistance to cadmium ions and fusidic acid, with concurrent sensitivity to arsenate and mercury ions—have not been detected before; Richmond (1965) found that the serotype of penicillinase produced by 56 strains of *S. aureus* was either A, B, or C, and Dyke, Parker and Richmond (1970) found that of about 100 strains producing plasmid-determined penicillinase, none was simultaneously resistant to cadmium and sensitive to arsenate ions.

The presence of this unusual plasmid in a variety of strains isolated during

1971 and 1972 has three possible explanations or combinations of them: (a) the plasmid has arisen *de novo* in most strains, (b) a single clone harbouring this plasmid has evolved to produce the diversity in the properties of strains now containing the plasmid, (c) the plasmid has been transferred between strains. We can immediately discount (a) on the grounds of improbability. The single-clone origin (b) is improbable for the following reasons: the properties of the strains harbouring the plasmid seem to be too variable (table I) to be accounted for by evolution from a single clone, in particular their variety of phage-typing patterns. The susceptibility of staphylococci to lysis by phages can be altered by changes in prophage carriage, *in vitro* and probably under natural conditions (Lowbury and Hood, 1953; Rountree, 1959; Asheshov and Rippon, 1959; Rosendal and Bülow, 1965; Jevons, John and Parker, 1966; Jessen *et al.*, 1969). These changes can be summarised as either a restriction or widening in the phage susceptibility within phage-group I or a progressive restriction in the susceptibility within group III. There is no evidence for conversion between the three phage groups. Another argument against the single-clone hypothesis is previous failure to detect the PF plasmid in strains (Richmond, 1965; Dyke *et al.*, 1970). Finally, the presence of the PF plasmid in two methicillin-resistant strains (nos. FAR1 and FAR2) is difficult to explain on this basis. Methicillin-resistant strains have probably evolved from a single clone (Lacey and Grinsted, 1973); if the strains harbouring the PF plasmid were also derived from one clone, most methicillin-resistant strains would be expected to harbour the PF plasmid. This is certainly not so, resistance to fusidic acid being rare in methicillin-resistant strains (Parker and Hewitt, 1970). For these reasons we consider that the evolution of strains containing this plasmid from a single clone is extremely unlikely.

Because transfer of penicillinase plasmids can occur between cells *in vitro*, we consider that the probable explanation for these findings is that the plasmid has spread between cells under natural conditions.

SUMMARY

A plasmid (PF) has been identified in 18 strains of *Staphylococcus aureus* that belong to phage-groups I, II, and III or are non-typable. This plasmid is characterised by: (a) carrying the genes for resistance to fusidic acid and cadmium ions, but not to arsenate or mercury ions, and for the production of D-serotype penicillinase, (b) a molecular weight of 16 million daltons, (c) the ability to exist with a "penicillinase plasmid" of compatibility-group I, and (d) a tendency to fragment. The PF plasmid has probably spread among the natural population of staphylococci.

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