FURTHER EVOLUTION OF A STRAIN OF *STAPHYLOCOCCUS AUREUS* *IN VIVO*: EVIDENCE FOR SIGNIFICANT INACTIVATION OF FLUCLOXACILLIN BY PENICILLINASE

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DURING the past 32 months, we have made an extensive study of a strain of *Staphylococcus aureus* (no. FAR4) isolated at frequent intervals from the sputum of a patient with fibrocystic disease of the lungs (Lacey, Lewis and Grinstead, 1973; Lacey, Lewis and Rosdahl, 1974). Although the isolates of staphylococci from this patient varied in their phenotypic properties, all were considered to be members of one clone. This view was based on a good baseline when the cocci exhibited uniform characters, on some constant properties (e.g. phage-typing pattern 3A and sensitivity to tetracycline), and our ability to reproduce *in vitro* the changes that were thought to have occurred *in vivo* (Lacey *et al.*, 1973 and 1974).

Further variation in isolates of this strain obtained from the patient has occurred. This report describes the changes between May 1973 and July 1974; from these we conclude that: (1) staphylococcal penicillinase inactivates flucloxacillin to a significant extent *in vivo*; (2) mutation to lincomycin resistance gives the cell a slight disadvantage *in vivo*; (3) production of δ -haemolysin may be an important virulence factor for *S. aureus*.

MATERIALS AND METHODS

The media used, the general methods of testing sensitivity to antibiotics, the methods of assaying penicillinase, of identifying δ -haemolysin and of treating cultures with nitrosonoguanidine, and the conditions used to "cure" strains of antibiotic resistance, have been described by Lacey *et al.* (1973 and 1974). Methods of testing sensitivity to particular antibiotics are given in the appropriate places in *Results*.

Maintenance of penicillinase production in isolates. Because of the instability of the plasmid coding for this enzyme (Lacey *et al.*, 1974), these isolates were maintained at 4°C on nutrient-agar plates containing 10^{-4} M cadmium acetate.

RESULTS

Thirty-one variants from one clone of Staphylococcus aureus arising in vivo

Specimens of sputum were received either fortnightly or monthly between May 1973 and July 1974 from the patient (EJR). They were plated on MacConkey's agar; coagulase-positive staphylococci were isolated from all the

Received 20 Sept. 1974; accepted 1 Nov. 1974.

specimens, and between 50 and 200 colonies from most of the specimens were subsequently tested for antibiotic sensitivity. As noted previously (Lacey *et al.*, 1974), the sensitivity of the colonies to cadmium ions, fusidic acid, neomycin, streptomycin and lincomycin varied. Initial disk-sensitivity results were confirmed on a proportion of isolates by MIC determinations. As before (Lacey *et al.*, 1974), the zones around disks containing 1.5 U of benzylpenicillin nearly always showed one of three appearances: (a) a zone similar to that given by a known penicillinase-negative culture, (b) a zone slightly reduced in comparison with this, and with a raised edge to the bacterial growth, characteristic of the production of penicillinase microconstitutively or (c) no zone, indicating the

TABLE I

Phenotypes of strain FAR4 isolated from a patient between Dec. 1971 and July 1974

Isolate		Phenotypic characters* of the stated isolate							
		<i>pen</i>	<i>cad</i>	<i>fus</i>	<i>str</i>	<i>neo</i>	<i>ero</i>	<i>lin</i>	<i>hem</i>
Wild strain		+	R	R	R	R	R	S	+
Variants (in order of first isolation)	1	—	S	S	R	R	R	S	+
	2	+	R	R	R	R	R	S	—
	3	—	S	S	R	R	R	S	—
	4	—	S	S	S	S	R	S	—
	5	—	S	S	R	R	S	S	+
	6	+	R	R	S	S	R	S	+
	7	+	R	R	S	S	S	S	+
	8	+	R	R	S	S	R	S	—
	9	+	R	R	R	R	S	S	+
	10	+	R	R	R	R	S	S	—
	11	—	R	R	R	R	R	S	+
	12	+	R	R	R	R	R	R	+
	13	+	R	R	S	S	R	R	—
	14	(+)	R	R	R	R	R	R	+
	15	(+)	R	R	R	R	R	R	+
	16	+	R	S	R	R	R	S	+
	17	—	S	S	R	R	R	R	+
	18	—	S	S	S	S	R	R	—
	19	+	R	R	S	S	R	R	+
	20	(+)	R	R	S	S	R	R	+
	21	—	S	R	R	R	R	S	+
	22	—	S	R	S	S	R	R	+
	23	—	R	R	R	S	R	R	+
	24	—	S	S	S	S	R	R	+
	25	—	R	R	S	S	R	R	+
	26	—	R	R	R	R	R	S	+
	27	(+)	R	R	S	S	R	R	+
	28	(+)	R	S	R	R	R	R	+
	29	(+)	R	S	R	R	R	S	+
	30	+	R	R	S	R	R	S	+

R = resistant; S = sensitive; + = production of penicillinase macroconstitutively, (+) = microconstitutively and — = no detectable enzyme; also, + = production and — = no production of δ -haemolysin.

* *pen* = Penicillinase production. *cad* = Resistance to cadmium ions; *fus* to fusidic acid; *str* to streptomycin; *neo* to neomycin; *ero* to erythromycin; *lin* to lincomycin. *hem* = Production of δ -haemolysin. All isolates had the phage-typing pattern 3A at routine test dilution.

production of enzyme macroconstitutively. Very few cultures gave intermediate zones; on subculture, such isolates were found to consist of a mixture of penicillinase-negative and "macroconstitutive" cocci, a probable consequence of the extreme instability of the macroconstitutive type (Lacey *et al.*, 1974; see below). Although the isolates obtained between May 1973 and July 1974 varied in the above properties, all of them, unlike those obtained earlier, were resistant to erythromycin and produced δ -haemolysin. At least one example of every phenotype identified from each sputum sample was phage typed; as before, all were typed by phage 3A and by no other phage at routine test dilution. Altogether, 31 phenotypes were identified (table I).

Relation of flucloxacillin therapy to penicillinase production by isolates

The early isolates of strain FAR4 (between Dec. 1971 and Apr. 1972) were all resistant to penicillin (Lacey *et al.*, 1973). From Dec. 1971 to Feb. 1972, the patient was treated with ampicillin and cloxacillin (and with fusidic acid for the

FIG. 1.—Proportion of isolates of strain FAR4 obtained from the patient between Jan. 1972 and Dec. 1973 that produced penicillinase; ●—● = production of penicillinase in large amount (macroconstitutive); ○—○ = production of penicillinase in small amount (microconstitutive). During Jan. and Feb. 1972 the patient was treated with ampicillin and flucloxacillin, from Mar. to Aug. 1972 with cloxacillin alone, from Aug. 1972 to May 1973 flucloxacillin, Clindamycin and fusidic acid. No antibiotics were received from the end of May 1973 to Nov. 1973.

first 10 days). Ampicillin was discontinued in Feb. 1972, but cloxacillin (125 mg four times daily) was continued until Aug. 1972. Despite the instability of penicillinase in this strain *in vitro*, most (>80%) of the organisms isolated up to Aug. 1972 were penicillin resistant (Lacey *et al.*, 1974).

Between Aug. 1972 and May 1973, flucoxacin, clindamycin and fusidic acid were prescribed successively, and most of the isolates were still penicillin resistant (see Lacey *et al.*, 1974). From May 1973 to 12 Nov. 1973 all antibiotic therapy ceased, and during this interval the proportion of isolates producing penicillinase macroconstitutively and the proportion producing it microconstitutively fell (fig. 1). The macroconstitutive and microconstitutive types were in most cases deduced from characteristics of inhibition zones; a few β -lactamase assays were performed to confirm these conclusions. The *in-vitro* instability of the penicillinase-fucidin-resistance (PF) plasmid in strain FAR4 appeared to be reflected by loss of the whole or part of the plasmid *in vivo* in the absence of selection pressure, since the majority of the penicillinase-negative isolates had become also sensitive to fusidic acid and cadmium ions.

FIG. 2.—Proportion of isolates of strain FAR4 obtained from the patient from Oct. 1973 to July 1974 that produced penicillinase; ●—● = production of penicillinase macroconstitutively; ○—○ = production of penicillinase microconstitutively. During Oct. and Nov. 1973 no antibiotics were received by the patient; from Dec. 1973 to July 1974 flucloxacillin 250 mg thrice daily was prescribed (cross-hatched area).

Because of an increase in the purulence of the sputum, flucloxacillin treatment was recommenced on 12 Nov. 1973; this was followed by a progressive rise in the proportion of cocci producing penicillinase microconstitutively and then by an increase in the proportion of "macroconstitutive" type (fig. 2). By June 1974, all of fifty colonies examined were "macroconstitutive". Thus during the last 13 months that this strain has been monitored, there is a close correlation between both qualitative and quantitative production of penicillinase by this staphylococcus and flucloxacillin therapy. It is also conceivable that cloxacillin therapy during 1971 and 1972 had played a part in the selection of penicillinase-positive variants of this strain *in vivo*.

Effect of penicillinase production by strain FAR4 on sensitivity to flucloxacillin in vitro

Six cultures of strain FAR4 that produced penicillinase microconstitutively, three that produced it macroconstitutively and three that were penicillinase negative were grown overnight in nutrient broth at 37°C, both with and without the addition of methicillin 0.25 µg per ml; incubation with methicillin ensured

FIG. 3.—Relationship of diameter of inhibition zone and antibiotic concentration for three variants of strain FAR4; ■—■ = a penicillinase-negative variant; ○—○ = a variant producing penicillinase microconstitutively; ●—● = a variant producing penicillinase macroconstitutively. The top three curves are for flucloxacillin, and the bottom three for tetracycline.

full induction of penicillinase production. The cultures were then diluted 1 in 10^2 and 1 in 10^4 with physiological saline, and c. 0.01 ml of each dilution, together with the neat culture, was applied in duplicate to the surface of sets of nutrient-agar plates containing 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.35 μg of flucloxacillin per ml. The sets of plates were incubated at 30° or 37°C and the end-point was read as no visible growth after 24 h. In all cases, the MIC was between 0.1 and 0.2 μg flucloxacillin per ml and was unaffected by the penicillinase phenotype, the temperature of incubation, or pre-incubation with methicillin. A slight inoculum effect was observed, in that the MIC with neat cultures was usually 0.2 μg of flucloxacillin per ml and with diluted cultures 0.1–0.15 μg flucloxacillin per ml.

In other experiments, an overnight culture of each of the three penicillinase phenotypes—the two penicillinase-positive cultures having been grown in the presence of 10^{-4}M cadmium acetate to select plasmid-positive cells—were diluted 1 in 10 in fresh warmed nutrient broth containing 0, 0.1, 0.15, 0.2, 0.25, or 0.5 μg flucloxacillin per ml, and incubated at 37°C. Samples were

TABLE II

Instability of the PF plasmid after growth overnight at 42°C in isolates of strain FAR4 obtained after flucloxacillin therapy

Isolate	Number of colonies examined	Number (and percentage) of colonies sensitive to cadmium ions
(P)*	11,500	8 (0.1)
P-1	1,236	1,209 (98)
P-2	1,045	780 (75)
P-3	1,012	796 (80)

* (P) denotes microconstitutive production of penicillinase; P, macroconstitutive.

TABLE III

Doubling time of lincomycin-resistant variants of strain FAR4 relative to that of sensitive counterparts in mixed culture

Cultures*	Origin of lincomycin resistance	Doubling time† of lincomycin-resistant relative to that of lincomycin-sensitive culture
S, R1	<i>in vitro</i>	100 105
S, R2	<i>in vitro</i>	103 104
S, R1	<i>in vivo</i>	102 102
S, R2	<i>in vivo</i>	101 110

* Pairs of cultures isogenic except in lincomycin resistance; S = sensitive, R1 and R2 = two resistant variants.

† Results of duplicate experiments.

removed after 0, 2, 4, 6 and 24 h and plated on nutrient agar to assess viability. No differences in the rate of destruction of these cultures were found.

These results suggest that the staphylococci cell is similarly sensitive to flucloxacillin irrespective of whether or not it produces penicillinase. This is not surprising because, even if penicillinase does hydrolyse flucloxacillin *in vitro* or *in vivo*, little of the penicillinase synthesised by strain FAR4 is cell bound (Rosdahl, 1973). Thus, this type of sensitivity testing may not accurately represent conditions *in vivo*. Diffusion testing may resemble natural conditions more closely because, as *in vivo*, a gradient of antibiotic concentration confronts an actively growing organism. Although disk-sensitivity testing at 37°C with cloxacillin (and by implication flucloxacillin) is generally considered difficult to interpret, preliminary experiments indicated that cultures of strain FAR4 that produced large amounts of penicillinase gave smaller zones of inhibition around disks containing cloxacillin or flucloxacillin at 37°C than did penicillinase-negative derivatives. This was verified by a cup-diffusion-assay procedure as follows. Individual overnight cultures of the three different "penicillinase phenotypes" of strain FAR4 were seeded on to the surface of nutrient-agar plates. Solutions (replicates of six) of flucloxacillin and of tetracycline (as control) were placed in assay cups and the diameters of the zones of inhibition

FIG. 4.—Proportion of isolates of strain FAR4 that were resistant to lincomycin between Jan. 1973 and July 1974. Lincomycin therapy ceased on 10 Jan. 1973.

were measured after incubation for 24 h at 37°C. The reproducibility of this assay procedure was demonstrated by performing replicate experiments on different occasions. Whilst all three derivatives gave the same assay curves for tetracycline, the zones around flucloxacillin were much reduced for the "macroconstitutive" culture compared with those for either the "microconstitutive" or the penicillinase-negative culture (fig. 3). Calculations based on this assay indicate that flucloxacillin is only about one-fifth as active for the "macroconstitutive" type as for the other two variants. Although it is not possible to predict with certainty the outcome of flucloxacillin therapy from experiments performed *in vitro*, these data provide an explanation for the close association of the administration of flucloxacillin to the patient with penicillinase production by the staphylococci isolated from his sputum. It is unlikely that some factor other than penicillinase production was responsible for the inactivation of flucloxacillin, because the change from "macroconstitutive" to "microconstitutive" type is associated with a minute genetic change (Lacey *et al.*, 1974) in otherwise isogenic cells. That some of the "penicillinase-resistant" penicillins are slowly hydrolysed by staphylococcal penicillinase *in vitro* (Novick, 1962; Richmond, 1963) provides further support for this conclusion, although values for flucloxacillin itself are not available.

One factor that possibly contributed to the high proportion of recent isolates that produced penicillinase macroconstitutively could be the integration of genes into the chromosome coding for the enzyme that were initially plasmid-located. Such integration has probably occurred for tetracycline resistance under natural conditions (Chopra, Lacey and Connolly, 1974). This possibility was investigated by examining three "macroconstitutive" and one "microconstitutive" variants isolated in May 1974. After purification on agar containing 10^{-4} M cadmium acetate, each derivative was incubated overnight at 42°C and examined by replica plating for loss of markers. Resistance to penicillin, cadmium ions and fusidic acid were coincidentally lost at high frequency from each of the three "macroconstitutive" types, and one variant was observed that lost resistance to fusidic acid alone (table II). These results imply that the locus for these genes is on a plasmid rather than chromosomal. The markers in the "microconstitutive" variant were much more "stable" (table II), presumably because of the "normal" growth rate of the cell producing penicillinase microconstitutively (Lacey *et al.*, 1974).

Disappearance of lincomycin-resistant mutants after the cessation of clindamycin therapy

When clindamycin therapy ceased in Dec. 1972, *c.* 50% of the cocci of strain FAR4 had probably acquired resistance to lincomycin by mutation (Lacey *et al.*, 1974). During the subsequent 18 months, in which no lincomycin or clindamycin was prescribed, the proportion of cells resistant to lincomycin progressively declined, so that by June 1974 none was detected (fig. 4). This was surprising, because the doubling times of lincomycin-resistant and lincomycin-sensitive derivatives of strain FAR4 are similar (Lacey *et al.*, 1974). However,

assessment of the growth rate of individual cultures may not be sufficiently sensitive to detect slight variations between different strains, because some environmental variables cannot be controlled. Accordingly, mixed cultures, comprising one lincomycin-resistant and one lincomycin-sensitive organism were monitored by the method of Lacey and Chopra (1974). In some experiments we used pairs of cultures, obtained directly from the patient, that were similar phenotypically except in lincomycin sensitivity. In other experiments, one-step mutants (see Lacey *et al.*, 1974) resistant to lincomycin were monitored with their lincomycin-sensitive counterparts. The relative doubling time of the lincomycin-resistant cells was marginally longer than that of the lincomycin-sensitive cells, the time for the resistant cell being 100–110% longer (mean 103%) than for the sensitive (table III). Thus, mutation to lincomycin resistance appears to give the cell a possible slight disadvantage *in vitro*, and this probably accounts for the progressive decline in the proportion of lincomycin-resistant cells *in vivo* after the cessation of clindamycin therapy.

Possible role of δ -haemolysin as a virulence factor for Staphylococcus aureus

Although several of the early isolates of strain FAR4 were either δ -haemolysin negative or erythromycin sensitive, every isolate obtained after May 1973 was both erythromycin resistant and δ -haemolysin positive. In contrast, the proportion of cocci resistant to streptomycin and neomycin fluctuated between 30 and 70%, and the frequency of penicillinase-producing strains fell progressively—together with the frequency of resistance to fusidic acid and cadmium ions—between May and Nov. 1973, a period during which no antibiotic was prescribed (see above).

Deletion analysis in an “early” isolate of strain FAR4 suggested that the genes coding for the following phenotypic properties were linked in the order: erythromycin resistance–streptomycin resistance–neomycin resistance– δ -haemolysin production (Grinsted and Lacey, 1973). However, the invariable association of δ -haemolysin production and erythromycin resistance in isolates obtained since May 1973 suggests that these two characters may be more closely linked than was originally thought. This relationship can be explained by postulating that the above genes are on a circular plasmid; there is no description of a plasmid that is not circular at some time in its life cycle. If this is so, mutants showing coincidental loss of the markers for both δ -haemolysin production and erythromycin resistance should arise. This was in fact demonstrated as follows. A derivative of strain FAR4 with all the above markers, isolated in Aug. 1973, was treated with nitrosoguanidine and examined for loss of δ -haemolysin by plating on agar containing human RBC, and for loss of erythromycin resistance by replica plating. From 4200 colonies surviving nitrosoguanidine treatment, 18 were sensitive to erythromycin but still produced δ -haemolysin, 69 were δ -haemolysin negative and erythromycin resistant, and five were sensitive to erythromycin and δ -haemolysin-negative; all retained resistance to neomycin and streptomycin and had the phage-typing pattern 3A. Confirmation of this gene order has not been possible, mainly because of the inability to isolate the

plasmid physically (and hence employ it in transformation experiments) or to transduce markers from strain FAR4.

Because δ -haemolysin has potent leucocidal activity *in vitro* (Gladstone and van Heyningen, 1957), its production may have selected *in vivo* variants that were δ -haemolysin positive and therefore, because of the linkage of the two characters, also resistant to erythromycin. However it is impossible to exclude the possibility that a third as yet unidentified factor has caused the selection of cocci showing both production of δ -haemolysin and resistance to erythromycin.

DISCUSSION

The repeated isolation of the same strain of *S. aureus* from a patient for 30 months has enabled us to observe changes that have occurred in this organism in its natural habitat. Initially, the organisms were uniformly resistant to several antibiotics but they have evolved into more than 30 different phenotypes; presumably a more extensive examination would have revealed further diversification in properties. Such evolution has been permitted by, and probably accelerated by, inadequate antibacterial therapy (Lacey *et al.*, 1973 and 1974). All these changes have probably occurred at plasmid loci. If this type of change has occurred in other strains of *S. aureus*, in conjunction with transfer of plasmids between cells, it could provide an explanation for the speed with which "hospital staphylococci" have altered during the last three decades.

If, as we suggest, the penicillinase of the staphylococcus inactivated flucloxacillin (and possibly also cloxacillin) sufficiently *in vivo* to render treatment with the antibiotic ineffective, this may be of practical importance. The extent to which this inactivation might occur in other patients, and with other strains, is uncertain. However, in some situations (such as bronchial secretions) where high levels of some antibiotics, including the penicillins, are difficult to achieve, caution should be exercised before relying on any penicillin to eradicate a strain of *S. aureus* that produces penicillinase. In these situations, the release of penicillinase may reduce the level of antibiotic sufficiently to render it ineffective, even if that drug is "penicillinase-insensitive".

Lincomycin resistance in this strain arose by mutation *in vivo*, yet the mutants, in the absence of selection pressure from the relevant antibiotics, gradually became replaced by sensitive cells. This illustrates (cf. mutants resistant to fusidic acid) why few cultures of *S. aureus* obtained from clinical material show resistance to antibiotics that has arisen by mutation (Lacey, 1973), although mutation to antibiotic resistance may occur at high frequency during therapy.

SUMMARY

A strain of *Staphylococcus aureus* (no. FAR4) has been isolated at intervals, for 32 months, from the sputum of a patient with cystic fibrosis of the lung. Changes in the properties of isolates of this strain over the first 18 months have been reported previously (Lacey *et al.*, 1973 and 1974). During the last 14 months (May 1973 to July 1974), further evolution has occurred to produce a total of 31 distinct phenotypes. Recent changes are as follows.

1. The ability of isolates to produce penicillinase *in vitro* was closely correlated with flucloxacillin therapy. Inactivation of flucloxacillin by penicillinase was demonstrated by diffusion testing (but not MIC determination) *in vitro* and may have occurred to a significant extent *in vivo*.

2. Lincomycin-resistant mutants slowly disappeared from the sputum after the termination of clindamycin therapy.

3. All of the recent isolates were resistant to erythromycin, possibly because of the linkage of the genes coding for erythromycin resistance with those coding for the production of δ -haemolysin; δ -haemolysin may be an important "virulence factor".

We thank Professor W. A. Gillespie for helpful comment, Dr V. G. Alder for phage typing, and the Cystic Fibrosis Research Trust for financial support.

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