Effect of pH on Inhibition of Plasmid-carrying Cultures of *Staphylococcus aureus* by Lipids

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The inhibition of *Staphylococcus aureus* by fatty acids was pH dependent. The inhibitory properties of myristic acid were greater at pH 5.5 than at pH 7, whereas those of linoleic acid were less at pH 5.5 than at pH 7. Seven clinical penicillin-resistant cultures survived better in the presence of linoleic acid at pH 5.5 than did their sensitive counterparts. The resistance to linoleic acid in most plasmid-carrying cultures at pH 5.5 was probably not due to the production of penicillinase, but to some other determinants carried by the plasmid.

INTRODUCTION

Noble (1977) described variation in the distribution of antibiotic resistance in *Staphylococcus aureus* strains isolated from the skin and nose of patients in a skin hospital. He found that, in several patients, isolates from the skin were more often resistant to penicillin or to tetracycline than were isolates of the same phage type from the same patient's anterior nares. The reverse distribution rarely occurred. This implies selection of antibiotic-resistant variants by factors on the skin, or of sensitive variants in the nose.

Butcher *et al.* (1976) reported that a strain of *S. aureus* (NCTC 8325) became more susceptible to the inhibitory effect of linoleic acid when the 'penicillinase plasmid' PI258 was introduced. Carriage of a plasmid mediating resistance to tetracycline had no effect on sensitivity to linoleic acid. These findings are apparently in conflict with natural events, since linoleic acid, a constituent of skin lipid, should select *sensitive* variants on the skin (Noble, 1977). However, the experiments of Butcher *et al.* (1976) were most probably made at neutral pH, and although the pH of nasal secretions is about 7, that of the skin is about 5.5 (Noble, 1981). Greenway & Dyke (1979) reported that an acid pH could modify the response of *S. aureus* 8325 to fatty acids, confirming the general observations of Altenbern (1977); however, the combined effect of pH and plasmid carriage was not studied. This paper reports studies on the inhibition of *S. aureus* by some components of skin lipid and attempts to give a plausible explanation for the distribution of resistant and sensitive variants.

METHODS

Strains. *Staphylococcus aureus* strains CL2 (non-typable), HO4 (phage type 94/96), J44 (phage type I, III, IV), J66 (phage type 3A/3C), PI (phage type I, IV), HA4 (phage type 96), BU2 (phage type 79/95), Git (phage type 29/79/80/42E/54/85) and AA3 (phage type 75) were isolated from the nose and skin of patients. In addition, strain NCTC 8325 designated 8325N to indicate that it contains no plasmid, 8325pen which contains the penicillinase plasmid PI258, and 8325tet which contains the tetracycline resistance plasmid T1044 were kindly supplied by D. L. A. Greenway. The cadmium-sensitive variant of strain NCTC 9789 (PS80) was obtained after exposure of the strain to sodium dodecyl sulphate (SDS).

Characterization of strains. Antibiotic sensitivity testing was carried out by a modified Kirby–Bauer technique on Oxoid Mueller Hinton agar (CM337) at 37 °C (30 °C for methicillin). Oxoid sensitivity discs contained the

following antibiotics: penicillin, 10 units; tetracycline, 30 µg; neomycin, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; clindamycin, 2 µg; methicillin, 5 µg.

Phage typing was carried out with the International Set of Typing Phages kindly supplied by Mrs M. de Saxe (Central Public Health Laboratories, Colindale).

Minimum inhibitory concentrations (m.i.c.) of cadmium were determined by the plate incorporation method, using cadmium acetate in Oxoid blood agar base (CM55). Cultures were incubated overnight in nutrient broth, diluted 1/100 and spot inoculated on to the cadmium agar plates. The m.i.c. was taken as the lowest concentration which prevented any growth of the organism.

Loss of plasmid-coded resistances from clinical strains was detected by exposure to SDS (Sonstein & Baldwin, 1972). Single plasmids in antibiotic-resistant strains CL2, HO4, J44, J66, PI, HA4, BU2, AA3, 8325 and 9789 were detected by the technique of Clewell & Helinski (1969), modified for staphylococci, and agarose gel electrophoresis (Meyers *et al.*, 1976). Strain Git contained a small cryptic plasmid in addition to a plasmid coding for antibiotic resistance. Each sensitive variant had the same bacteriophage type as that of the parent strain.

Inhibition of *S. aureus* by fatty acids. Fatty acids tested were linoleic acid (*cis,cis*), myristic acid, lauric acid, oleic acid (*cis*), palmitic acid and stearic acid. Strains were grown overnight on agar supplemented with penicillin (10 µg ml⁻¹) or tetracycline (10 µg ml⁻¹) or on nutrient agar. Pairs of resistant and sensitive variants of the same strain were used for each experiment. Culture pairs were each suspended in CY medium (Novick, 1963) and brought to a turbidity of about 0.4 (approx. 10⁵ colony-forming units ml⁻¹). One ml of each culture was inoculated into 50 ml prewarmed CY broth in 250 ml Erlenmeyer flasks. After incubation with shaking at 37 °C for 1.5 h, the temperature was reduced to 33 °C (approx. skin temperature) and the medium was brought to the required pH by adding 1 M-NaOH or 2 M-HCl (the required volumes were determined by adjusting a sample using a direct reading pH meter). The appropriate fatty acid dissolved in alcohol (5 mg ml⁻¹) was added to give a final concentration of 4 or 5 µg ml⁻¹; this is approximately the maximum level found on skin (µg cm⁻²) (Noble, 1981). An equal volume of alcohol alone was added to control flasks. Growth was then followed by measuring the turbidity at hourly intervals on a SP1300 Pye Unicam photometer at 370–515 nm.

Similarly, cholesterol, cholesterol esters (cholesterol linoleate, oleate, stearate and palmitate), squalene and skin steroids (5-dihydrotestosterone, 5-androstan-3β,17β-diol, androsterone and dihydroepiandrosterone) were dissolved in alcohol and added to cultures to give the appropriate concentrations. Equal volumes of alcohol alone were always added to control cultures.

Fatty acids, cholesterol, cholesterol esters, squalene, steroids and SDS were from Sigma.

RESULTS

Inhibition of S. aureus by fatty acids

Linoleic acid. At pH 7 the inhibitory effect of linoleic acid on strain 8325 (Fig. 1 *a*) was as described by Butcher *et al.* (1976); that is, the strain bearing the penicillinase plasmid PI258 was more strongly inhibited by linoleic acid (5 µg ml⁻¹) than was the antibiotic-sensitive strain. At pH 5.5, however, the inhibitory effect of the fatty acid was less and there was very little difference in growth between the strain bearing the penicillinase plasmid and the plasmid-free strain (Fig. 1 *b*).

Fig. 1. Effect of linoleic acid at pH 7 (*a*) and pH 5.5 (*b*) on *S. aureus* strain 8325 resistant to penicillin (●, 0; ○, 5 µg linoleic acid ml⁻¹) and sensitive to penicillin (▲, 0; △, 5 µg linoleic acid ml⁻¹).

Table 1. Turbidity of *S. aureus* cultures after 4 h growth at pH 5.5 and pH 7 with and without linoleic acid (4 $\mu\text{g ml}^{-1}$)

Strain	Resistance*	Cadmium m.i.c. ($\mu\text{g ml}^{-1}$)	pH 5.5		pH 7	
			Control	+ Linoleic acid	Control	+ Linoleic acid
8325N	S/All	16	0.58	0.51	0.69	0.61
8325pen	R/P,E	128	0.50	0.50	0.62	0.29
8325tet	R/T	16	0.51	0.57	0.65	0.70
CL2	S/All	16	0.58	0.04	0.80	0.01
CL2	R/P	64	0.56	0.32	0.75	0.08
CL2	S/All	16	0.51	0.10	0.71	0.02
CL2	R/P	64	0.45	0.37	0.80	0.07
CL2	S/All	16	0.52	0.13	0.68	0.02
CL2	R/P	64	0.60	0.57	0.70	0.06
HO4	S/All	16	0.56	0.15	0.68	0.02
HO4	R/P	64	0.47	0.30	0.75	0.01
J44	S/All	16	0.25	0.03	0.63	0.03
J44	R/P	64	0.44	0.37	0.77	0.03
J66	S/All	16	0.45	0.30	0.69	0.21
J66	R/P	128	0.45	0.40	0.78	0.20
PI	S/All	16	0.42	0.22	0.73	0.20
PI	R/P	64	0.47	0.44	0.77	0.20
HA4	S/All	16	0.45	0.22	0.51	0.20
HA4	R/P	64	0.45	0.43	0.54	0.20
BU2	S/All	16	0.40	0.37	0.80	0.02
BU2	R/P	64	0.57	0.52	0.88	0.03
Git	S/All	64	0.55	0.42	0.72	0.15
Git	R/P	128	0.58	0.40	0.80	0.13
9789	R/P	8	0.55	0.06	0.80	0.02
9789	R/P	128	0.72	0.58	0.80	0.05
AA3	S/All	16	0.50	0.10	0.78	0.01
AA3	R/T	16	0.38	0.08	0.73	0.01

* S/All, sensitive to all tested antibiotics (see Methods); R/P,T,E, resistant to penicillin, tetracycline and erythromycin, respectively.

With the clinical isolates slightly different results were obtained. Table 1 shows the turbidity of the cultures after 4 h growth with 4 μg linoleic acid ml^{-1} . All cultures except strain 8325 were inhibited more at pH 7 than pH 5.5 by linoleic acid. The penicillin-resistant and penicillin-sensitive isolates of each clinical strain were inhibited equally at pH 7, but at pH 5.5 the penicillin-sensitive cultures were inhibited more than resistant cultures. This is illustrated for strain CL2 (Fig. 2). Experiments were repeated using different batches of media, different preparations of fatty acids and subcultures of strains. The results for strain CL2 studied on three separate occasions are included in Table 1. Although growth of the strain as a whole differed slightly on the different occasions, the penicillin-resistant culture consistently grew better than the sensitive culture in the presence of linoleic acid at pH 5.5. This also occurred with the other clinical strains. No effect of plasmid carriage was seen with strains bearing the tetracycline resistance plasmid (Table 1).

Results for strain Git were different from the others in that there was very little difference in growth between the penicillin-resistant and penicillin-sensitive isolates in the presence of the fatty acid at pH 5.5. This apparent anomaly was studied further. Both the penicillin-resistant and penicillin-sensitive isolates of this strain were resistant to cadmium (Table 1). Loss of

Fig. 2. Effect of linoleic acid on the growth of the penicillin-sensitive (a) and penicillin-resistant (b) variants of *S. aureus* strain CL2 at pH 7 (●, 0; ○, 4 µg linoleic acid ml⁻¹) and pH 5.5 (▲, 0; △, 4 µg linoleic acid ml⁻¹).

Fig. 3. Effect of linoleic acid on the growth of the cadmium-sensitive (a) and cadmium-resistant (b) variants of *S. aureus* strain 9789 at pH 7 (●, 0; ○, 4 µg linoleic acid ml⁻¹) and pH 5.5 (▲, 0; △, 4 µg linoleic acid ml⁻¹).

penicillinase production from this strain was not accompanied by loss of cadmium resistance as in the other strains. This suggested that it was not the penicillinase genes that were responsible for the increased survival in linoleic acid but some other determinant on the plasmid, e.g. metal ion resistance. To study this further, strain NCTC 9789 was used which has chromosomal penicillin resistance and a separate plasmid which bears the genes for resistance to metal ions including cadmium (Asheshov, 1966, 1969). The variant of 9789 which had become sensitive to metal ions was sensitive to the inhibitory effects of linoleic acid at pH 5.5 while the parent was not (Fig. 3), although both variants were still penicillinase producers.

Other fatty acids. Both myristic and lauric acids were more inhibitory at pH 5.5 than at pH 7 (Table 2), and lauric acid was slightly more inhibitory to the penicillin-resistant than to the sensitive variant of strain CL2 at pH 5.5. Oleic, palmitic and stearic acids were not inhibitory at the concentration used at either pH to strains bearing plasmids or their sensitive counterparts. No effect of the tetracycline resistance plasmid in the clinical strain AA3 was seen with any fatty acid.

Inhibition of S. aureus by free cholesterol and cholesterol esters

Cholesterol alone had no significant effect on the growth of either variant of strain CL2 at either pH. However, when 10 µg cholesterol ml⁻¹ was added to medium containing 4 µg

Table 2. Turbidity of *S. aureus* cultures after 4 h growth at pH 5.5 and pH 7 with and without different fatty acids ($4 \mu\text{g ml}^{-1}$)

Fatty acid	Strain	Resistance*	pH 5.5		pH 7	
			Control	+ Fatty acid	Control	+ Fatty acid
Myristic	CL2	S/All	0.59	0.08	0.85	0.77
	CL2	R/P	0.67	0.04	0.85	0.75
	AA3	S/All	0.75	0.07	0.85	0.82
	AA3	R/T	0.71	0.10	0.79	0.76
Lauric	CL2	S/All	0.59	0.25	0.78	0.85
	CL2	R/P	0.57	0.15	0.78	0.81
	AA3	S/All	0.56	0.34	0.65	0.72
	AA3	R/T	0.52	0.40	0.76	0.74
Oleic	CL2	S/All	0.67	0.60	0.87	0.80
	CL2	R/P	0.75	0.64	0.80	0.87
	AA3	S/All	0.54	0.51	0.70	0.77
	AA3	R/T	0.45	0.52	0.75	0.76
Palmitic	CL2	S/All	0.51	0.46	0.70	0.67
	CL2	R/P	0.53	0.56	0.66	0.72
	AA3	S/All	0.43	0.41	0.66	0.70
	AA3	R/T	0.42	0.38	0.61	0.71
Stearic	CL2	S/All	0.50	0.46	0.68	0.63
	CL2	R/P	0.54	0.56	0.68	0.70
	AA3	S/All	0.52	0.45	0.84	0.82
	AA3	R/T	0.50	0.54	0.87	0.84

* For definitions, see Table 1.

Fig. 4. Effect of linoleic acid and cholesterol on the growth of the cadmium-sensitive (a) and cadmium-resistant (b) variants of *S. aureus* strain 9789 at pH 7 (●, $4 \mu\text{g}$ linoleic acid ml^{-1} and $10 \mu\text{g}$ cholesterol ml^{-1} ; ○, $4 \mu\text{g}$ linoleic acid ml^{-1} only) and pH 5.5 (▲, $4 \mu\text{g}$ linoleic acid ml^{-1} and $10 \mu\text{g}$ cholesterol ml^{-1} ; △, $4 \mu\text{g}$ linoleic acid ml^{-1} only).

linoleic acid ml^{-1} , the inhibitory effect of the fatty acid was reduced. This was also true of strain 9789 (Fig. 4). But even in the presence of cholesterol the advantageous effect of the penicillinase and cadmium resistance plasmids against the inhibitory action of linoleic acid at pH 5.5 was still apparent. Cholesterol was also able to reduce the inhibitory effect of myristic acid on strain CL2, but in strain 9789 it reduced inhibition of the cadmium-resistant variant only.

In strain AA3, cholesterol reduced the inhibitory activity of linoleic acid at both pH 5.5 and pH 7 and of myristic acid at pH 5.5. However, there was no differential effect on the plasmid-carrying or plasmid-free variant.

The various cholesterol esters had no effect on growth of sensitive and resistant variants at either pH value.

Inhibition of S. aureus by squalene and skin steroids

There appeared to be no significant effect of squalene on the growth of isolates with or without plasmids at either pH. The general effect of steroids seemed to be stimulatory at $10\text{ }\mu\text{g ml}^{-1}$ but inhibitory at $100\text{ }\mu\text{g ml}^{-1}$. There was no special effect on strains bearing penicillinase plasmids or tetracycline resistance plasmids.

DISCUSSION

Although *S. aureus* is rarely found on normal human skin, it is often present on diseased skin even on areas regarded as clinically normal (Noble, 1981). Fatty acids have often been included amongst the factors thought likely to suppress organisms on skin, and this may indeed be true for the streptococci. However, Heczko & Kasprowicz (1976) have shown that staphylococci have higher m.i.c. values for various fatty acids including linoleic acid than have propionibacteria, the latter being very common inhabitants of both normal and diseased skins. Some other mechanism presumably governs the rarity of *S. aureus* on normal skin.

In many patients with skin diseases the proportion of antibiotic-resistant *S. aureus* cells is often similar in the nose and on the skin surface, but when differences do occur, there are usually more resistant variants on the skin than in the nose (Noble, 1977; Naidoo, 1980). The experiments reported here show that, although many components of skin lipid have no differential effect on isolates bearing plasmids, linoleic acid is selective for cells bearing the penicillinase plasmid at pH 5.5 but not at pH 7. The effect persisted even in the presence of cholesterol which reduced the inhibitory effect of linoleic and myristic acids.

The genes governing the resistance to inhibition by linoleic acid in the clinical strain Git and strain NCTC 9789 are apparently not those specifying the production of penicillinase, since the same effect is obtained with cultures carrying the cadmium resistance plasmid. The mechanism of resistance has not been determined, but the genes which specify metal ion resistance might also be responsible since resistance, for example, to cadmium involves an alteration in the cell membrane leading to exclusion of the metal ion (Chopra, 1975). Exclusion of the fatty acid may be mediated in the same way. However, it is possible that a separate gene mediates fatty acid resistance.

The conditions used in these experiments may not be directly relevant to those in vivo. The alcohol used to dissolve the lipids may have affected their physical dispersion and their effect on the cells in a manner not encountered in vivo. Lipid on skin is a complex mixture of a variety of substances including the free fatty acids. However, as yet, no suitable model for studying the effects of lipids on plasmid-carrying bacteria in vivo is available.

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