Sensitivity of *Staphylococcus aureus* to Unsaturated Fatty Acids

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SUMMARY

Some unsaturated fatty acids were found to inhibit the growth of *Staphylococcus aureus*. Their effectiveness was related both to the degree of unsaturation and to the configuration of the molecule about the double bonds. Both linoleic acid and linolenic acid increased the proportion of plasmid-negative bacteria in a growing culture of bacteria containing a penicillinase plasmid. This was not due to a 'curing' effect of the fatty acids but was the result of greater sensitivity of the growth of bacteria containing penicillinase plasmid to inhibition by the unsaturated fatty acids. The presence of a plasmid conferring resistance to tetracycline, however, did not make the bacterium more sensitive to inhibition by linoleic or linolenic acids.

INTRODUCTION

The growth of Gram-positive organisms can be inhibited by unsaturated fatty acids (UFAs) at concentrations that do not affect many Gram-negative organisms (see Nieman, 1954). Following the observation of Strong & Carpenter (1942) that linoleic acid inhibits the production of lactic acid by cultures of *Lactobacillus helveticus*, Kodicek & Worden (1945) studied the relative effectiveness of several fatty acids at inhibiting acid production in this organism. The general conclusions (Kodicek, 1956) were that, the greater the inhibition the more unsaturated the fatty acid; *cis* unsaturated fatty acids are more effective than their *trans* isomers; and esterification of the unsaturated fatty acids destroys their effectiveness. Low concentrations of UFAs are bacteriostatic but higher concentrations cause lysis of the bacteria (Kodicek, 1958).

The aims of our work were to find out, firstly, whether Kodicek's conclusions were also true for *Staphylococcus aureus* and, secondly, whether those UFAs that inhibited growth of *S. aureus* interfered with the replication or inheritance of plasmids present in the bacteria.

METHODS

Bacteria. *Staphylococcus aureus* NCTC8325 was the parent strain used throughout and was designated 8325N to indicate that it does not contain a known plasmid. *Staphylococcus aureus* 8325 (PI₂₅₈) was constructed by Dr R. P. Novick by transduction of erythromycin resistance from *S. aureus* 258 (PI₂₅₈) (Mitsuhashi *et al.*, 1965) to *S. aureus* 8325N. *Staphylococcus aureus* 8325 (T₁₀₄₁) was constructed by transduction of tetracycline-resistance from *S. aureus* 1044 (T₁₀₄₁) (Dyke & Richmond, 1967) into 8325N. *Staphylococcus aureus* 8325 rif^R was isolated as a colony growing on 20 µg rifamycin/ml after mutagenesis of 8325N

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with ethyl methanesulphonate by the method of Novick (1963). The derivative 8325 *rif*^R (PI₂₅₈) was constructed by transduction of erythromycin resistance from 8325 (PI₂₅₈) into this mutant. The transductant is resistant to cadmium ions and produces penicillinase; it is assumed to carry the entire plasmid.

Growth of bacteria. CY medium (Novick, 1963) was used and growth was followed by measuring the extinction at 675 nm in a Unicam SP600 spectrophotometer.

In all experiments in which the possible inhibition of growth of bacteria was being studied the following procedure was used. An overnight culture in CY medium (0.25 ml, containing about 10⁸ colony forming units) was added to CY medium (100 ml) in a sterile 250 ml Erlenmeyer flask. This was shaken at 30 or 37 °C until *E*_{675 nm} reached 0.08. Portions of the culture were then transferred to sterile 50 ml flasks and used for subsequent experiments.

Solid medium was CY with added 1.5 % (w/v) agar (Difco Bacto). Strains carrying plasmid PI₂₅₈ were maintained on CY-agar containing 13.3 µg cadmium acetate/ml (CY-cad-agar) and those carrying plasmid T₁₀₄₄ were maintained on CY-agar containing 20 µg tetracycline/ml (CY-tet-agar).

Transduction. Phage 53 of the International (Typing) Phages was used. Procedures for the propagation of bacteriophage and for transduction were as previously described (Johnston & Dyke, 1971). Selection was carried out on CY-agar with added erythromycin (20 µg/ml) or tetracycline (20 µg/ml).

Plate assay for penicillinase. A modification of the starch-iodine method of Perret was used (Dyke, Jevons & Parker, 1966).

Chemicals. Saturated and unsaturated fatty acids (Sigma) were used without further purification.

RESULTS

Effect of fatty acids on the growth of S. aureus 8325N

Several fatty acids were added at either 100 µg ml⁻¹ or 10 µg ml⁻¹ to cultures of 8325N at 37 °C. The presence or absence of inhibition was determined by measuring *E*_{675 nm} after 2½ h. Measurements were made at only one point in time since only qualitative results were needed at this stage. The results (Table 1) were consistent with the generalizations of Kodicek (1956). The most effective inhibitors were linolenic, linoleic and arachidonic acids; further studies were undertaken with linoleic and linolenic acid.

Effect on loss of plasmids

Kodicek (1956) suggested that UFAs exert their bacteriostatic effect by a mechanism involving a functional disruption at the bacterial membrane. The effect of the anionic detergent sodium dodecyl sulphate in causing loss of plasmids (Sonstein & Baldwin, 1972; Inuzuka *et al.*, 1969) can be interpreted in terms of a disruption of this general type. It therefore was of interest to see whether UFAs promoted loss of plasmids. This was investigated by a plating technique with strain 8325 (PI₂₅₈).

The results (Fig. 1) showed that the inclusion of a UFA in the culture medium led to an increase in the proportion of penicillinase-negative colonies. The penicillinase-negative variants were sensitive to cadmium ions and to erythromycin so we assume they lost the entire plasmid. The size of the effect was roughly related to the degree of growth inhibition up to a limit of about 16 µg linoleic acid/ml. No significant increase in the proportion of plasmid-negative colonies was observed if the test culture grew at the same rate as a control culture without any UFA. The results could be explained, partly or wholly, by a greater sensitivity of the plasmid-bearing bacteria to the UFAs.

Table 1. *Inhibition of growth of S. aureus 8325N at 37 °C by fatty acids*

Extinctions at 675 nm were measured after 2½ h growth in media containing added fatty acid at initial concentrations of 100 µg ml⁻¹ or 10 µg ml⁻¹.

Fatty acid	$E_{675 \text{ nm}}^*$	
	Fatty acid concentration	
	100 µg ml ⁻¹	10 µg ml ⁻¹
Saturated		
Palmitic (C ₁₆)	1·8	1·5
Stearic (C ₁₈)	1·7	1·6
DL-12-hydroxystearic (C ₁₈)	1·25	1·3
One double bond		
Elaidic (C ₁₈)	1·9	1·6
Cis-vaccenic (C ₁₈)	1·5	1·5
Two double bonds		
Linoleic (C ₁₈)	0·175	0·61
Linoelaidic (C ₁₈)	1·12	1·9
Three double bonds		
Linolenic (C ₁₈)	0·17	0·49
γ-Linolenic (C ₁₈)	0·135	1·55
Four double bonds		
Arachidonic	—	0·235
Esters		
Ethyl linoleate	1·9	2·0
Ethyl linolenate	1·8	2·0

* $E_{675 \text{ nm}}$ at start, 0·076; $E_{675 \text{ nm}}$ after 2½ h (no fatty acid added), 1·9.

Fig. 1. The effect of linoleic acid on growth and on the proportion of penicillinase-negative variants. An exponentially growing culture of *S. aureus* 8325 (PI₂₅₈) in CY medium with an $E_{675 \text{ nm}}$ of 0·07 was divided into 10 ml portions, to which different concentrations of linoleic acid were added. These subcultures were grown at 30 °C for 3½ h and samples of the initial culture, the control culture and experimental cultures were serially diluted and plated on starch-CY-agar plates. After incubation for 18 h at 30 °C, colonies were tested for production of penicillinase. At least 1000 colonies were screened for each of the samples. $E_{675 \text{ nm}}$ was also measured for each sample after 3½ h growth. ○, Percentage of penicillinase-negative colonies; ●, $E_{675 \text{ nm}}$.

Fig. 2

Fig. 3

Fig. 2. Growth of *S. aureus* 8325N(a) and 8325 (PI₂₅₈) in the presence of linoleic acid. Exponential cultures of the two strains were grown at 30 °C in CY medium until $E_{675\text{ nm}}$ was 0.085. Ten ml portions were then transferred to 50 ml Erlenmeyer flasks and, where appropriate, linoleic acid was added to give final concentrations ($\mu\text{g ml}^{-1}$) of: ●, 0; ○, 4; □, 8. Incubation was continued at 30 °C with aeration. The solid line represents the results with strain 8325N and the dotted line those with 8325 (PI₂₅₈).

Fig. 3. Growth of 8325N and 8325 (T₁₀₄₄) in the presence of linoleic acid. Method as for Fig. 2 except that the concentrations of linoleic acid were ($\mu\text{g ml}^{-1}$): ●, 0; □, 3; ○, 6. The solid line represents growth of 8325N and the dotted line that of 8325 (T₁₀₄₄).

Effect of unsaturated fatty acids on the growth of S. aureus 8325 (PI₂₅₈) and 8325N

Since it was possible that the stock 8325N might differ from 8325 (PI₂₅₈) in more ways than just the absence of plasmid, a new 8325N was picked as a penicillinase-negative colony from a culture of 8325 (PI₂₅₈) spread on a CY-agar plate. This clone, designated 8325N(a), had the same efficiency of plating for phage 53 as 8325N and 8325 (PI₂₅₈).

The growth of 8325 (PI₂₅₈) and its derivative 8325N(a) was followed in CY medium with various concentrations of linoleic acid. Great care was taken to ensure that the same concentrations of linoleic acid were present for the two variants and that the initial bacterial densities were the same. The results of one such experiment are shown in Fig. 2. Clearly, the plasmid-bearing bacteria were more sensitive, and the effect was most striking at 4 μg linoleic acid/ml, where the rate of growth of 8325N(a) was reduced to 70 % of the control and 8325 (PI₂₅₈) was inhibited completely.

Very similar results were obtained using a second plasmid-negative variant derived from 8325 (PI₂₅₈); and qualitatively similar results were obtained using linolenic acid in place of linoleic acid. It is concluded that possession of the PI₂₅₈ plasmid renders strain 8325 more sensitive to the effects of these two UFAs at least.

The question arises of whether this is a phenotype associated with one of the genes on the PI₂₅₈ plasmid, or whether it is a general phenomenon resulting from the possession of any plasmid. To investigate this the growths of 8325 (T₁₀₄₄) and 8325N in the presence of linoleic acid were compared (Fig. 3). The presence of a tetracycline plasmid did not make this strain more sensitive to linoleic acid.

Effect of linoleic acid on loss of the plasmid from 8325 rif^R (PI₂₅₈)

Despite the differential sensitivity of 8325 (PI₂₅₈) to linoleic acid, the possibility that the fatty acid also acts as a plasmid 'curing' agent had not been excluded, and so an experiment was devised to test this. The rifamycin-resistant mutant of 8325 was used since the gene

Table 2. *Effect of linoleic acid on loss of plasmid*

Extinctions at 675 nm were measured after 2½ h growth in media containing added fatty acid at initial concentrations of 100 µg ml⁻¹ or 10 µg ml⁻¹.

	Control	Linoleic acid absent	Linoleic acid added
Linoleic acid (µg ml ⁻¹)	0	0	3
Time of growth (h)	0	3	3
Total colonies counted	2314	2312	3617
Percentage of colonies that were:			
<i>rif^R pen⁺</i>	66.4	66.6	60.7
<i>rif^S pen⁻</i>	33.6	33.4	39.2
<i>rif^R pen⁻</i>	0	0.004	0
Ratios of colony types			
<i>rif^R pen⁺/rif^S pen⁻</i>	1.97	1.99	1.55
<i>rif^R pen⁻/rif^R pen⁺</i>	—	0.007	—

conferring rifamycin-resistance is likely to be chromosomal (rifamycin-resistance is not lost at high frequency at 42 °C) and so could serve as an internal marker. Strains 8325N and 8325 *rif^RN* were equally sensitive to inhibition of growth by linoleic acid. The principle of the experiment was to add linoleic acid to a culture consisting of a mixture of 8325 *rif^R* (PI₂₅₈) and 8325 *rif^SN* and, after a period of growth, to determine the proportions of 8325 *rif^R* (PI₂₅₈), 8325 *rif^RN* and 8325 *rif^SN* present. The ratio of 8325 *rif^R* (PI₂₅₈) to 8325 *rif^SN* should indicate the effect on growth of plasmid-positive and plasmid-negative strains, and the ratio of 8325 *rif^RN* to 8325 *rif^R* (PI₂₅₈), the amount of plasmid loss that has occurred.

Overnight broth cultures of strain 8325 *rif^R* (PI₂₅₈) and 8325 *rif^SN* were diluted 100-fold with fresh CY and grown for more than two generation times at 30 °C. They were then diluted with warm CY and mixed so that $E_{675\text{ nm}}$ was 0.07 and there were twice as many 8325 *rif^R* (PI₂₅₈) as 8325 *rif^SN*. This mixed culture was divided and linoleic acid, to a final concentration of 3 µg ml⁻¹, was added to one half. Incubation of both portions was continued for a further 3 h at 30 °C by which time $E_{675\text{ nm}}$ was 0.61 for the control flask and 0.53 for the flask containing linoleic acid. The cultures, together with a zero time control, were diluted and spread on CY-agar to give 200 colonies per plate. The plates were incubated for 20 h at 30 °C, then replica plated to CY-rif-agar, and both master and replica were incubated for a further 20 h at 30 °C. The master plates were developed with penicillin-iodine indicator and penicillinase-producing colonies were marked. Rifamycin-sensitive colonies were also scored.

The results are shown in Table 2. The proportion of penicillinase-producing bacteria was reduced by growth in linoleic acid but there was no evidence for loss of plasmid. Inhibition of growth as measured by $E_{675\text{ nm}}$ was used to calculate the proportion of *rif^R pen⁺* to *rif^S pen⁻* bacteria and the result agreed with that found by measurement of colony type. This experiment was repeated four times, and although there was some variability in the exact amount of inhibition of growth, in no case was there evidence for loss of plasmid induced by growth in the presence of linoleic acid.

DISCUSSION

Effects of various fatty acids on the growth of S. aureus 8325N. The results agree with and extend those of Kodicek & Worden (1945, 1946). *Cis* isomers of UFAs were far more effective inhibitors of growth than the corresponding *trans* isomers, e.g. oleic acid was much more effective than elaidic acid. Effectiveness in a series of compounds with the same carbon-chain length increased with increasing unsaturation: stearic < linoleic < linolenic. Two other UFAs, arachidonic and γ -linolenic acid, were also shown to be effective. Since the ethyl esters of linoleic and linolenic acids did not inhibit growth, it seems that a free carboxyl group is essential for the effect.

Effects of linoleic and linolenic acids on S. aureus 8325 (PI₂₆₈). Dulaney (1971) screened more than 12500 compounds for possible elimination of a penicillinase plasmid from *S. aureus* 8325. He did not find any curing agents, but found a number of compounds that acted selectively against bacteria carrying the plasmid. To this extent our experiments resemble his. However, the active compounds he found were all fairly complex and included phenols, bisphenols, salicylanilides and polyhaloacids. Understandably the whole list of compounds tested is not published; the only UFA mentioned is ricinoleic acid, which was inactive. Dulaney (1971) also found no increased sensitivity with a plasmid conferring resistance to tetracycline.

It is likely that UFAs represent a class of compounds which should be added to those named by Dulaney as selectively inhibiting plasmid-bearing bacteria. If the effect of all these compounds has the same basis, then studying UFAs, which have the simplest chemical structures in the group, may facilitate theoretical interpretation.

The mechanism of action of UFAs remains to be elucidated and until this is known it is difficult to understand how the presence of a penicillinase plasmid affects the sensitivity of the bacteria to UFAs. Unsaturated fatty acids may become incorporated into the membrane during its synthesis and so affect its 'fluidity' (Singer & Nicolson, 1972). When grown at low temperature many poikilothermic organisms incorporate greater quantities of UFAs into their membranes than at higher temperatures (Fraenkel & Hoff, 1940). This is believed to arise from a homeostatic mechanism which maintains the 'tumbling time' of transport proteins. When applied externally, UFAs might enter membranes and upset those systems that are sensitive to the 'fluidity', 'phase' or 'micelle:lipid bilayer ratio' of the membrane. Thus they may act in a similar way to certain membrane-fusing agents such as lysolecithin (Lucy, 1970). The greater sensitivity of the plasmid-containing strain could be the result of either a direct physical interaction of the plasmid DNA with the membrane or the action of some gene product specified by the plasmid DNA.

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