SHORT COMMUNICATION

Isolation and Properties of a Linoleic Acid-resistant Mutant of *Staphylococcus aureus*

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A linoleic acid-resistant mutant (Lar-2) of *Staphylococcus aureus* NCTC 8325 has been isolated and partially characterized. The resistance of the mutant may be due to an increased capacity of the bacterial membrane to incorporate linoleic acid.

INTRODUCTION

The mechanism of the inhibitory action of long-chain fatty acids on bacteria is not known (Galbraith & Miller, 1973*a, b, c*; Sheu & Freese, 1973; Freese *et al.*, 1973; Sheu *et al.*, 1975). Previous studies in this laboratory suggest that inhibition by linoleic acid results from an increase in the permeability of the plasma membrane of *Staphylococcus aureus* (Butcher *et al.*, 1976; Greenway & Dyke, 1979). These studies also suggested that (i) the free unsaturated fatty acid itself was inhibitory, and not the neutral lipid into which linoleic acid had become incorporated and (ii) the enhancement of inhibition due to linoleic acid by a plasmid conferring resistance to penicillin (PC plasmid, pI₂₅₈blaI⁻) was perhaps due to increased sensitivity of the membranes of PC plasmid-bearing bacteria to permeability induced by linoleic acid.

To investigate further the mechanism of linoleic acid growth inhibition of *S. aureus*, a linoleic acid-resistant mutant was isolated and partially characterized. Such mutants have been isolated previously but not fully characterized (Lacey, 1969). This paper describes a resistant mutant whose membrane has an increased capacity to incorporate linoleic acid.

METHODS

Linoleic, linolenic and arachidonic acids were obtained from Sigma. [1-¹⁴C]Linoleic acid was obtained from The Radiochemical Centre, Amersham. Phospholipid standards were obtained from Koch-Light. All other chemicals were the best grades commercially available.

Staphylococcus aureus NCTC 8325 was the wild-type parent strain used in all these studies and it is designated here as 8325(N) to indicate that it contains no known plasmid. A linoleic acid-resistant variant of *S. aureus* 8325(N) was obtained after treatment with ethyl methanesulphonate (Novick, 1963), selecting on agar containing linoleate (Lacey, 1969), and was designated 8325-Lar-2(N). It was indistinguishable from strain 8325(N) in its phage sensitivity and antibiotic sensitivity (Multodisk no. 11–14C, Oxoid).

Growth inhibition, uptake of [1-¹⁴C]linoleic acid, thin-layer chromatography (t.l.c.) and the detection of leakage of 260 nm-absorbing material have been described previously (Greenway & Dyke, 1979). Lipids were extracted by the method of White & Fremman (1967).

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Fig. 1. Determination of the minimum inhibitory concentrations of linoleic acid for *S. aureus* strains 8325(N) (○) and 8325-Lar-2(N) (●). Inhibition of growth by linoleic acid at various concentrations was determined as follows. Stationary phase cultures grown in CY at 30 °C were diluted into fresh warm CY to an A_{675} of 0.1 and grown for three generations. The cultures were then diluted with fresh warm CY and added to flasks containing linoleic acid such that the linoleic acid was at its desired final concentration and the A_{675} was 0.1. Growth was followed at 30 °C. Linoleic acid was added in a small volume of ethanol [present at 1% (v/v) final concentration in all flasks]. Growth inhibition was calculated from the mean generation time of the treated cultures (see Greenway & Dyke, 1979).

RESULTS

Isolation of mutants resistant to inhibition by linoleic acid

From 10 independent treatments with mutagen, 5×10^9 surviving bacteria were spread on selective plates (10^7 per plate). Colonies that grew were restreaked for single colonies on selective plates. Seven possible mutants which grew well on selective plates were tested for sensitivity to antibiotics and to phage 53 and examined for doubling time in CY medium. Only one of the colonies [8325-Lar-2(N)] had a phenotype that was indistinguishable from the parent apart from its ability to grow on linoleate.

Although the mutant was more resistant to linoleic acid than the parent there was no concentration of linoleic acid that completely inhibited the parent but had no effect on the mutant (Fig. 1). The solubility of linoleic acid in water was too low for higher concentrations to be tested.

The mutant showed increased resistance to arachidonic acid but not to linolenic acid. For example, $10 \mu\text{g}$ arachidonic acid ml^{-1} increased the doubling time of the mutant in CY medium at 30 °C by less than 20% but completely prevented growth of the parent.

Uptake of linoleic acid by S. aureus 8325-Lar-2(N)

The mutant might be resistant to the inhibitory effects of linoleic acid because of increased or reduced uptake of the acid. The initial rates of uptake of linoleic acid over a wide range of concentrations were measured and kinetic parameters were determined by the method of Eisenthal & Cornish-Bowden (1974). The maximum rate of uptake (V_{max}) was $0.17 \mu\text{g}$ linoleic acid min^{-1} (mg bacterial dry wt) $^{-1}$ (median of 18 points) for the parent compared with $0.20 \mu\text{g}$ linoleic acid min^{-1} (mg bacterial dry wt) $^{-1}$ (median of 14 points) for the mutant. In addition to finding that the mutant may take up the acid at a slightly faster rate than the parent, the affinity of the uptake system of the mutant (K_m $0.72 \mu\text{M}$; median of 14 points) was higher than that of the parent (K_m $2.34 \mu\text{M}$, median of 18 points).

The proportion of bacterial dry weight that comprises linoleic acid is known to be constant at a given concentration of linoleic acid even when the concentration is partly inhibitory (Greenway & Dyke, 1979). A limiting value is reached at high concentrations of linoleic acid. For the mutant, this limit was 1.05% of the dry weight compared with 0.85% for the parent strain.

Fate of the incorporated linoleic acid

It is possible that the resistant mutant can convert linoleic acid to non-inhibitory compounds. To test for this, *S. aureus* strains 8325(N) and 8325-Lar-2(N) were grown in the presence of [^{14}C]linoleic acid [$10\ \mu\text{g ml}^{-1}$; $0.5\ \mu\text{Ci ml}^{-1}$ ($18.5\ \text{kBq ml}^{-1}$)] for one generation and then the lipid was extracted. Some 91% of the incorporated radioactivity was recovered in the total lipid fraction from the mutant. Thin-layer chromatography on silica gel G plates of this lipid fraction gave separation into six components all of which contained radioactivity. There were no differences in the relative distribution in these fractions between the lipid from the mutant and parent. Further, following esterification of the lipid of both mutant and parent by acid-catalysed methanolysis, 98% of the radioactivity migrated in t.l.c. with methyl linoleate. Thus, resistance of 8325-Lar-2(N) was probably not due to an unusual metabolism of linoleic acid itself, although it may be esterified to different hydroxyl groups.

Permeability induced by linoleic acid

Linoleic acid increases the permeability of the membrane of *S. aureus* 8325(N) (Greenway & Dyke, 1979). This permeability was measured as release of 260 nm-absorbing material for both parent and mutant but no significant difference was found.

DISCUSSION

The resistant mutant has an increased ability to incorporate linoleic acid into neutral lipid. Because the pool of free fatty acids present in *S. aureus* is less than 0.12% of the total fatty acid content (White & Frerman, 1968) it is unlikely that an expanded pool could explain the resistance. Alternatively, one of the proteins incorporating linoleic acid and arachidonic acids into phospholipid might be altered. For example, if the acyltransferase that incorporates linoleic acid into lipid became more efficient in the mutant than in the parent the concentration of free linoleic acid might be reduced in the mutant. Free linoleic acid is probably the growth inhibitory substance (Greenway & Dyke, 1979). A consequence of this might be to enable more linoleic acid to be incorporated into phospholipid, so lessening its inhibitory action.

The mutant has increased resistance to arachidonic acid but not to linolenic acid. This can be explained if the mutant protein has altered its affinity for arachidonic acid but is either not involved in linolenic acid incorporation or is unaltered in that function.

Study of the acyltransferases of this organism with particular reference to their specificities for unsaturated fatty acids is needed to confirm this hypothesis. It will also be important to investigate other mutants that are resistant to the effects of unsaturated fatty acids.

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