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Effects of Fatty Acids on Lysis of *Streptococcus faecalis*

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Palmitic, stearic, oleic, and linoleic acids at concentrations of 200 nmol/ml all inhibited autolysin activity 80% or more in whole cells or cell-free extracts. This concentration of the saturated fatty acids palmitic acid and stearic acid had little or no effect on the growth of whole cells or protoplasts. However, the unsaturated fatty acids oleic acid and linoleic acid induced lysis in both situations. This lytic effect is apparently not related to any uncoupling activity or inhibition of energy catabolism by unsaturated fatty acids. It is concluded that unsaturated fatty acids induce cell and protoplast lysis by acting as more potent membrane destabilizers than saturated fatty acids.

It has been suggested (12, 17, 22) that bacterial growth and division processes are, at least in part, influenced or directed by the activity of peptidoglycan hydrolases (autolysins). Regulation of autolysin activity then would also be a critical factor in such growth and division processes. It appears that the autolysin of *Streptococcus faecalis* (23) can be inhibited by certain lipids and lipoteichoic acid (2, 4). Although there are some clear differences in the structures of these compounds (e.g., the nature of the polar head groups), one common feature is the presence of covalently linked fatty acid residues. Furthermore, compounds containing a larger molar ratio of fatty acid residues (i.e., lipoteichoic acid and diphosphatidylglycerol) are substantially more effective in inhibiting autolysin activity than are less acylated compounds (2, 4) (e.g., phosphatidylglycerol). Chemical removal of fatty acids from isolated lipids or lipoteichoic acid (2, 4) also destroys the inhibitory effects of these compounds on autolysin activity in vitro. It seemed worthwhile, then, to determine how effective fatty acids are in inhibiting autolysin activity. In other words, can the inhibitory effects of lipids and lipoteichoic acids be accounted for by the additive effects of their fatty acid residues? In trying to answer this question, we attempted to differentiate between the direct effects of free fatty acids on autolysin activity and other, perhaps indirectly related effects (1, 6, 11, 15, 16, 20). Additionally, we compared the effects of fatty acids on autolysis in vitro with their effects on cell and protoplast growth.

MATERIALS AND METHODS

Preparation and use of fatty acids. Palmitic acid, stearic acid, oleic acid, and linoleic acid were purchased from Calbiochem La Jolla, Calif., and stored at −20°C. Fatty acids were converted to their ammonium salts by adding 0.1 ml of concentrated NH_4OH to 10 mg of fatty acid in a conical glass tube. The excess ammonia was evaporated under nitrogen, and 1 ml of 95% (vol/vol) ethanol was added to the fatty acid residue. This solution was used for all additions of fatty acids. In all assays, the ethanol concentration was adjusted to 0.45% (vol/vol). The absorbance contributed by each fatty acid in suspension was subtracted in spectrophotometric determinations.

Growth of cells. The group D enterococcus *S. faecalis* ATCC 9790 was grown routinely in a chemically defined medium (21) at 37°C. Cultures were frequently checked for purity by plating on blood agar and by microscopic observation. Growth was followed turbidimetrically at 675 nm with a Coleman model 14 spectrophotometer (Coleman Instrument Corp., Maywood, Ill.). Absorbance was corrected for deviations from Beer's Law (24), yielding adjusted optical density units; adjusted optical density units were related to cellular dry weight per milliliter based on previous direct dry weight determinations (P. Lancy, Ph.D. thesis, Temple University, Phialdelphia, Pa., 1976).

Where indicated, portions of the following stock solutions were added to cultures: fosfomycin (4 mg/ml, aqueous solution; Merck, Sharp & Dohme, Rahway, N.J.) and sodium fluoride (5 mg/ml, aqueous solution). All stock solutions were stored at −20°C.

Assays of autolytic activity. Samples (5 to 10 ml) from growing cultures were pipetted onto membrane filters (type DA; pore size, 0.65 μm; Millipore Corp., Bedford, Mass.), vacuum filtered, and washed twice with 3-ml volumes of ice-cold double-distilled water. The filters were immediately placed into 50-ml plastic centrifuge tubes containing 6 ml of prewarmed 0.3 M phosphate buffer (pH 6.7 to 6.8) and vigorously mixed. The resulting suspensions were transferred to cuvettes (18 by 150 mm), with care being taken to avoid transfer of any filter fragments. Assays of cellular autolytic activity were performed as described previously (19) in 0.3 M phosphate buffer (pH 6.7 to 6.8) at 37°C. Autolysis assays of cell wall preparations were

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performed as described previously (14) in 0.01 M phosphate buffer (pH 6.7 to 6.8) at 37°C. Samples of lyophilized wall preparations were suspended in 0.01 M phosphate buffer (pH 6.7 to 6.8) at a concentration sufficient to give an optical density measurement of 0.3 at 450 nm (approximately 0.3 mg [dry weight] per ml).

Assays of RNA and protein syntheses. Incorporation of isotopically labeled leucine and uracil into 10% (wt/vol) ice-cold trichloroacetic acid-precipitable material was used to index protein and RNA syntheses, respectively, as described previously (18). The following isotope specific activities were used (per milliliter of medium): L-[2-^{14}C]leucine, 0.15 μCi/0.15 μmol; and [5-^{3}H]uracil, 0.5 μCi/0.18 μmol. All isotopes were purchased from New England Nuclear Corp., Boston, Mass.

Growth of protoplasts. Protoplasts were prepared by the procedure of Roth et al. (18). Briefly, 100 ml of an exponentially growing culture at 1,000 adjusted optical density units was harvested by centrifugation at 15,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the cell pellet was suspended in 10 ml of 0.3 M phosphate (pH 6.7 to 6.8) containing 1.25 mg of lysozyme per ml (protoplast buffer) and centrifuged at 15,000 × *g* for 10 min at 4°C. The cell pellet was suspended in 0.3 ml of protoplast buffer containing 0.5 M sucrose and incubated at 37°C for 30 min; 25 μl of this protoplast suspension was carefully pipetted into 10 ml of prewarmed (37°C) chemically defined medium (21) containing 0.25 M sucrose. Growth was followed turbidimetrically at 675 nm as described above.

RESULTS

Effects of fatty acids on cells. Exponentially growing cultures of *S. faecalis* were treated with 200 nmol of palmitic, stearic, oleic, or linoleic acid per ml (Fig. 1). Stearic acid had little or no effect, whereas palmitic acid transiently retarded the increase in culture turbidity. Microscopic observations of cultures treated with these two saturated fatty acids (SFA) revealed extensive chaining and clumping of cells. On the other hand, the unsaturated fatty acids (UFA) oleic acid and linoleic acid induced 27 and 68% losses, respectively, of optical density over a 2-h period. Microscopic observations of these cultures revealed extensive cell lysis.

The effects of these fatty acids on the incorporation of a leucine or uracil isotope into trichloroacetic acid-precipitable material are shown in Fig. 2. The results were similar to the effects on culture turbidity discussed above. The observation that UFA induced a decrease in trichloroacetic acid-precipitable counts representing protein and RNA suggests that degradation of these macromolecules occurred during the 90-min period studied.

Effects of fatty acids on autolysis. We investigated the possibility that the fatty acid

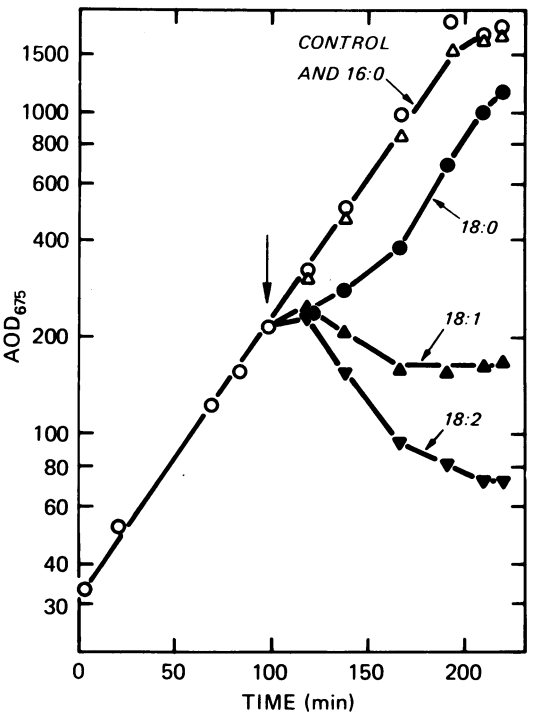

FIG. 1. *Effect of various fatty acids on the growth of whole cells. Portions of exponentially growing cultures of S. faecalis were treated with 200 nmol of fatty acid per ml at the time indicated by the arrow. All cultures contained a final concentration of 0.45% ethanol. Symbols: ○, untreated control; ●, palmitic acid (16:0); △, stearic acid (18:0); ▲, oleic acid (18:1); ▼, linoleic acid (18:2). AOD_{675}, Adjusted optical density at 675 nm.*

effects were related to alterations in cellular autolytic activity. Fatty acids at concentrations ranging from 4 to 800 nmol/ml were added to suspensions of cells in phosphate buffer (19), and lysis was followed turbidimetrically (Fig. 3A). All fatty acids appeared to inhibit autolytic activity to the same extent. Cellular autolytic activity appeared to be inhibited 50% relative to untreated controls by fatty acid concentrations of approximately 200 nmol/μg of cellular dry weight.

To observe directly the effects of fatty acids on autolysin activity (14), we performed similar autolysis assays, using isolated cell wall preparations. In this case, wall lysis seems to be related to the single, detectable autolytic enzyme activity of this organism (23). As Fig. 3B shows, there was little difference among the four fatty acids tested. All appeared to inhibit wall autolysis at a concentration of approximately 40 nmol/μg of wall dry weight.

Effects of fatty acids on protoplast

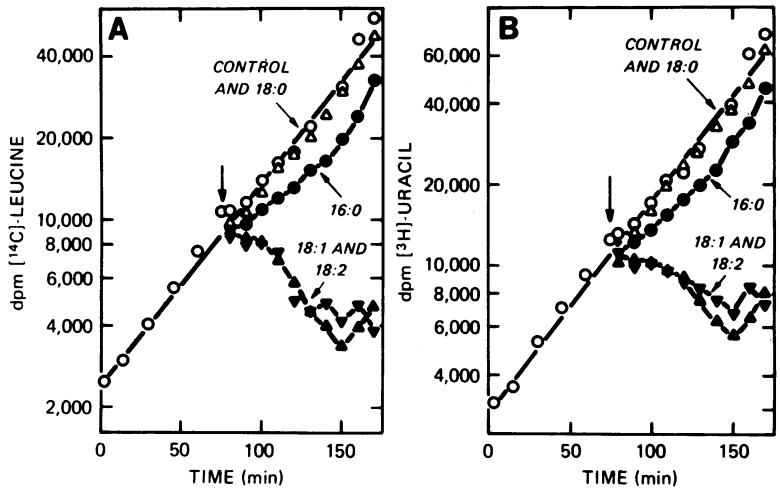

FIG. 2. *Effects of various fatty acids on RNA and protein syntheses in whole cells. Cultures were grown for five to seven generations in the presence of isotope before sampling. At the times indicated, 0.5-ml samples were taken for estimation of isotope incorporation into cold trichloroacetic acid-insoluble material, as described in the text. At the times indicated by the arrows, portions of exponentially growing cultures were treated with 200 nmol of the indicated fatty acid per ml. All cultures contained a final concentration of 0.45% ethanol. Symbols are as described in the legend to Fig. 1. (A) Protein synthesis. (B) RNA synthesis.*

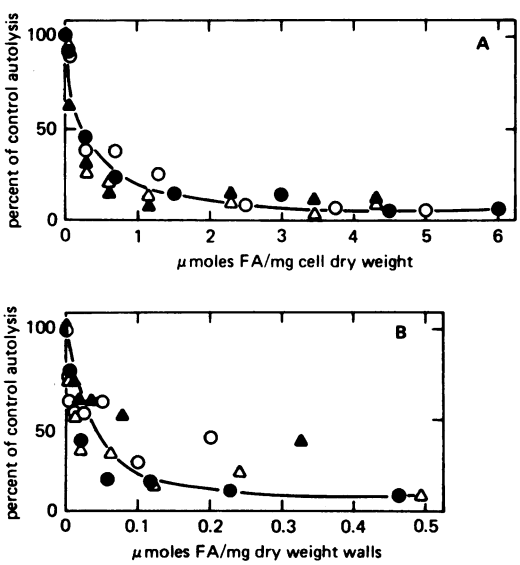

FIG. 3. *Effects of various fatty acids (FA) on autolytic activity. Autolysis assays were performed as described in the text. Fatty acids were added as their ammonium salts. All assays contained a final concentration of 0.45% ethanol. Symbols: ○, 16:0; ●, 18:0; ▲, 18:1; △, 18:2. (A) Whole cell autolysis in 0.3 M phosphate buffer, pH 6.7 to 6.8. The control autolytic rate was −2.0/h. (B) Wall autolysis in 0.01 M phosphate buffer, pH 6.7 to 6.8. The control autolytic activity was 62 U. All assays were performed at 37°C.*

growth. In whole cells, lysis may reflect a combination of events associated with disruption of the integrity of cell walls and membranes. To investigate the effects of fatty acids on cellular lytic processes not related to wall-associated autolysin activity, we added fatty acids to suspensions of growing protoplasts. Figure 4 shows the results obtained when protoplast growth was followed turbidimetrically in the presence of 200 nmol of fatty acid per ml. SFA had little or no effect on protoplast growth, whereas both UFA induced a rapid and profound lysis of the protoplasts. Sodium fluoride (500 µg/ml), a metabolic poison (25) which induces autolysis in whole cells of *S. faecalis* (E. Ternove-Hinks, Ph.D. thesis, Temple University, Philadelphia, Pa., 1978), inhibited growth of protoplasts but did not induce substantial lysis. Fosfomycin (400 µg/ml), an inhibitor of cell wall biosynthesis (9), also had no lasting effect on protoplast growth.

DISCUSSION

Several studies (2–4) have indicated that certain lipids and lipoteichoic acid are potent inhibitors of autolytic activity in *S. faecalis*. Some of these studies (2, 4) demonstrated that fatty acids are essential constituents of these inhibitors. The work described here indicates that free fatty acids can inhibit autolytic activity both in whole cells and in cell-free extracts. Palmitic, stearic, oleic, and linoleic acids all have similar activities in autolysis assays. The 50% effective inhibitory concentrations were approximately 200 nmol/mg of cellular dry weight for the whole cell assays and 40 nmol/mg (dry weight) for the isolated wall assays. The free fatty acid concentrations effective in the autolysis assays, how-

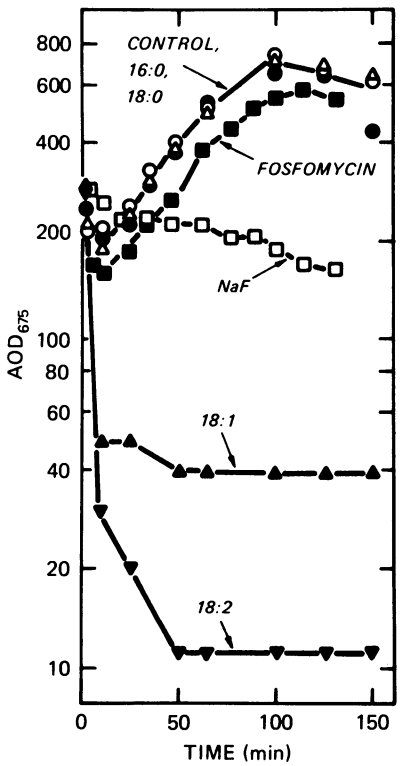

FIG. 4. *Effects of fatty acids, sodium fluoride, and fosfomycin on protoplast growth. Protoplasts were prepared and grown as described in the text. Ammonium salts of fatty acids (200 nmol/ml), sodium fluoride (500 μg/ml), and fosfomycin (400 μg/ml) were added at zero time. All tubes contained a final concentration of 0.45% ethanol. Symbols: ○, untreated control; △, 16:0; ●, 18:0; ▲, 18:1; ▼, 18:2; □, NaF; ■, fosfomycin. AOD_{675}, Adjusted optical density at 675 nm.*

TABLE 1. *Apparent lipid concentration producing 50% inhibition of autolysis*

Lipid	Lipid concn		
	Whole cells (nmol/mg [dry wt])	Wall enzyme complex (nmol/mg [dry wt])	Amt (nmol/mg of cellular dry wt) found in vivo[a]
Diphosphatidylglycerol (bovine)	5–10[b]	1–3[c]	
Diphosphatidylglycerol (*S. faecalis*)	20[b]	15[c]	4.6
Phosphatidylglycerol (*S. faecalis*)	50[b]	40–80[c]	8.5
Amino acid esters of phosphatidylglycerol (*S. faecalis*)	30[b]	40–80[c]	8.6
Diglyceride (*S. faecalis*)	60[a]	<160[c]	2.0
Diglucosyldiglyceride (*S. faecalis*)	60[a]	140[a]	7.5
Fatty acids (16:0, 18:0, 18:1, 18:2)	200	40	0.84
Lipoteichoic acid	—[d]	1[c]	1.2

[a] See D. D. Carson, Ph.D. thesis, Temple University, in preparation.
[b] See reference 2.
[c] See reference 4.
[d] —, Maximum inhibition achieved was 20% at approximately 15 nmol of lipoteichoic acid per mg of cellular dry weight.

ever, appear to be inordinately high when compared with either the levels of free fatty acids normally found in bacteria (9) or the effective concentrations of other lipid or lipoteichoic acid inhibitors of autolytic activity (Table 1). It is concluded that free fatty acids are unlikely effectors of autolytic activity in vivo.

Numerous observations indicate that fatty acids may have either stimulatory or inhibitory (8, 13, 20) effects on growth and survival of gram-positive bacteria. In *S. faecalis*, turbidimetric measurements indicated that supplementation with either of the SFA (stearic acid or palmitic acid) had little or no effect on cell growth. Supplementation with the UFA (oleic acid or linoleic acid), however, induced rapid cell lysis. These studies were supported by microscopic observations, as well as by estimates of macromolecular syntheses in cultures treated with either an SFA or a UFA. Cells treated with an SFA, however, appeared to grow in long chains. This apparent inhibition of cell separation is consistent with an inhibition of autolysin activity (12, 17, 22). We also found (data not shown) that three treatments known to inhibit profoundly autolytic activity in *S. faecalis*, namely addition of 20 μg of chloramphenicol per ml (19), addition of 1.7 nmol of diphosphatidylglycerol per ml (2), and addition of 200 nmol of palmitic acid per ml, do not prevent UFA-induced cell lysis. It appears that although all fatty acids tested inhibit lysis of whole cells in medium incapable of supporting their growth (i.e., phosphate buffer), UFAs actually induce lysis of whole cells in growth media. One explanation of these seemingly disparate results is that in a growing situation fatty acids may be taken up from the medium and incorporated into the membrane structure. Excess UFA incorporation might be expected to enhance cell lysis by membrane destabilization.

To test further the possibility that UFA produced their lytic effects by disrupting membrane structure, protoplasts were grown in the presence of various fatty acids. In this situation, interpretations are not complicated by considerations of effects on wall dissolution. Although SFA once again had little effect on cell growth, UFA induced rapid and profound protoplast lysis. This lysis occurred too rapidly to reflect

any substantial incorporation of the UFA into membrane phospholipids. Furthermore, the UFA-induced lysis is not likely to be related to any uncoupling activity of UFA on membrane processes (1, 15, 16) since Harold et al. (7) have reported that the uncoupler dicyclohexylcarbodiimide does not cause protoplast lysis in *S. faecalis*, whereas it does inhibit adenosine triphosphatase activity. Furthermore, our studies show that sodium fluoride, an inhibitor of glycolysis (25) which also induces lysis in whole cells of *S. faecalis* (Ternove-Hinks, Ph.D. thesis), does not induce protoplast lysis. Fosfomycin, an inhibitor of cell wall biosynthesis (9) bearing structural analogy to the glycolytic intermediate phosphoenolpyruvate, also had no effect on protoplast growth at a concentration which induces lysis in whole cells of *S. faecalis* (5). We suggest that the UFA-induced lysis is caused by a direct membrane-destabilizing effect of the UFA themselves.

The above studies demonstrate that free fatty acids can inhibit autolysin activity at physiologically high concentrations and that this inhibition is not fatty acid species specific. It also does not appear that the enhanced inhibitory activities of diphosphatidylglycerol and lipoteichoic acid compared with other lipids in autolysis assays can be explained simply by their higher molar ratios of fatty acids. Finally, UFA, but not SFA, exert lethal effects on growing cells, probably by a direct disruption of membrane structure.

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