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Involvement of Autolysin in Cellular Lysis of *Bacillus subtilis* Induced by Short- and Medium-Chain Fatty Acids

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The addition of saturated C_6, C_8, C_{10}, and C_{12} fatty acids appeared to lyse actively growing cells of *Bacillus subtilis* 168, as judged by a decrease in the optical density of the culture. Of these fatty acids, dodecanoic acid was the most effective, with 50% lysis occurring in about 30 min at a concentration of 0.5 mM. These conditions also decreased the amount of peptidoglycan estimated by the incorporated radioactivity of *N*-acetyl-D-[1-^{14}C]glucosamine. At concentrations above 1 mM, however, bacterial lysis was not extensive. Dodecanoic acid did not affect autolysis of the cell wall. The lytic action of dodecanoic acid was greatly diminished in cells in which protein synthesis was inhibited and in an autolytic enzyme-deficient mutant. The results suggest that fatty acid-induced lysis of *B. subtilis* 168 is due to the induction of autolysis by an autolytic enzyme rather than massive solubilization of the cell membrane by the detergent-like action of the fatty acids.

Fatty acids not only inhibit growth (10, 20, 21, 27), but they also kill (11, 21, 22) and lyse (21) gram-positive and, to a lesser extent, gram-negative bacteria. The bacteriostatic activity of fatty acids has been ascribed to destruction of the proton motive force across the cell membrane (24, 25), leading to the loss of respiratory activity (13, 34, 36), the inhibition of substrate uptake (13, 23, 35, 36), or the decrease in intracellular content of ATP (34, 36).

In contrast, few reports have appeared on the mode of action of bacterial death and lysis caused by fatty acids. In these, the membrane-disturbing or -solubilizing ability of fatty acids as anionic surface-active agents has been suggested to be a possible mechanism (6, 11, 22). However, cellular lysis induced by certain surface-active agents, such as deoxycholate (26), Triton X-100 (3), and cetyltrimethylammonium bromide (4) on gram-positive bacteria, has been found to result from the activity of an autolytic enzyme (autolysin) on the cell wall. It is possible, therefore, that autolysin is similarly involved in bacteriolysis induced by fatty acids.

On the other hand, Carson and Daneo-Moore (1) have reported that the long-chain, saturated fatty acids palmitic acid and stearic acid inhibited autolysis of whole cells and cell walls isolated from *Streptococcus faecalis*. In this respect their effect resembles that of lipoteichoic acid, an endogenous autolysin inhibitor (2, 18, 39). These authors also suggest that the unsaturated fatty acids oleic acid and linoleic acid, in contrast with saturated fatty acids, probably induced lysis by their direct membrane-destabilizing action.

In this study we examine whether short- and medium-chain saturated fatty acids induce autolysis or conversely inhibit autolysis of *Bacillus subtilis* 168, which has a potent autolytic enzyme system.

MATERIALS AND METHODS

Microorganisms. *Bacillus subtilis* 168 *trp* was employed throughout this study. In some experiments, *B. subtilis* FJ2 *trp lyt*, a mutant derived from strain 168 and deficient in the autolytic enzymes *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase (7), were also used. These strains were generous gifts from H. J. Rogers.

Cultural conditions and lysis of whole cells. Spizizen salts medium (37) supplemented with 0.15% sodium glutamate and 20 μg of tryptophan per ml was used. Cells were grown in a 100-ml flask containing 20 ml of the medium at 37°C on a rotary shaker. After 18 h, to observe lysis in the growth medium, portions (0.2 ml) of the culture were transferred into L-shaped tubes with a narrow side tube to circulate the contents. These tubes contained appropriate amounts of fresh medium to give a total volume of 10 ml. The tubes were shaken at 37°C in a Bioscanner (Ohtake Works Ltd.), an automatic growth-recording apparatus (38). When the culture reached an optical density at 650 nm (OD_{650}) of 0.25, a portion (0.1 to 1.0 ml) of fatty acid solution or an identical amount of deionized water was added to each tube. To observe autolysis, which occurred when cells were incubated in a buffer, a portion (2 ml) of the overnight culture was transferred to a 500-ml flask, containing 100 ml of the medium, and shaken at 120 strokes per min at 37°C. At an OD_{650} of 0.25, cells were harvested by centrifugation at 3,000 × *g* for 5 min, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and suspended in 20 ml of fresh buffer. These procedures were performed at 4°C. Portions (1 ml) of the cell suspension were added to the L-shaped in a Bioscanner, containing 9 ml of fresh buffer, at 37°C with or without fatty acid, and then incubated at 37°C. The OD_{650} for each tube was measured at 2-min intervals and automatically recorded. The readings of OD_{650} at higher concentrations of cells (>0.18) were corrected graphically with a calibration curve. The extents of fatty acid-induced lysis and autolysis were expressed by the percent reduction in the OD_{650}.

Cell wall labeling and measurement of wall degradation. Cells were grown in medium containing *N*-acetyl-D-[1-^{14}C]glucosamine (0.25 μCi/ml, 20 μM) to label the cell walls. At an OD_{650} of 0.25, they were harvested, washed with Spizizen medium, and suspended in fresh medium. After the addition of dodecanoic acid, portions (0.5 ml) were put in Eppendorf tubes containing 0.5 ml of 10% trichloroacetic acid at 4°C and kept overnight. The cell wall fraction was prepared from this sample as described by Rogers et al. (33). The samples were heated at 90°C for 20 min in a water bath and filtered through Whatman GF/C filters. These filters

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were then washed 2 times with 1 ml of deionized water, 10 times with 3 ml of 75% ethanol, and 4 times with 5 ml of 0.1 M potassium sodium phosphate buffer at pH 8.0. The filters were dipped into 2 ml of trypsin solution (1 mg/ml) of the fresh phosphate buffer described above in vials and incubated at 35°C for 4 h. Then, the solutions were filtered through new Whatman filters, and the filters were washed twice with 1 ml of deionized water. Dried filters were put into vials containing 10 ml of Aquasol-2 (New England Nuclear Corp.), and the radioactivity was measured in a Beckman LS-7500 scintillation counter.

Preparation of cell walls and protoplasts and their lysis. Cells from exponentially growing cultures (an OD_{650} of 0.25 to 0.30) were harvested by centrifugation (3,000 × *g* for 5 min) at 4°C. For the preparation of cell walls, sedimented cells were suspended in 5 ml of deionized water and disrupted ultrasonically with a sonifier (20 kHz; Kaijo Denki Co., Ltd.) at maximum power, while keeping the vessel containing the cell suspension immersed in an ice-water mixture. Residual whole cells were removed by low-speed centrifugation (1,000 × *g* for 5 min). The walls in the resultant fluid were pelleted by centrifugation at 27,000 × *g* for 10 min. After the supernatant fluid was discarded, wall lysis was begun by suspending the precipitates in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) with or without 0.5 mM dodecanoic acid which had been warmed to 37°C. This suspension was rapidly transferred into test tubes and its OD_{450} read directly at intervals with a spectrophotometer (model 100-10; Hitachi). For the preparation of protoplasts, harvested cells were washed twice with Tris-magnesium buffer at pH 8.0, suspended in 10 mM Tris buffer containing 50 μg of lysozyme per ml plus 0.5 M sucrose, and incubated at 37°C for 30 min. After centrifugation (3,000 × *g* for 5 min), the cells were suspended in Tris buffer (pH 7.2) containing 0.5 M sucrose and incubated in a Bioscanner tube for 10 min. After that, dodecanoic acid was added and the OD decrease was measured.

Chemicals. All fatty acids were used as sodium salts (>99% purity; Nakarai Chemicals, Ltd). Solutions of fatty acid were stored at −20°C in a freezer. *p*-Chloromercuribenzoic acid (Wako Pure Chemical Industries, Ltd.) was dissolved in 0.5 N NaOH at a concentration of 0.01 M. Erythromycin (Shionogi & Co., Ltd.) was kept as a stock solution in 50% ethanol. Glutaraldehyde (Ohken Shoji Co., Ltd.) and bovine serum albumin (Sigma Chemical Co.) were used in aqueous solution. *N*-Acetyl-D-[1-^{14}C]glucosamine (57.9 mCi/mmol) was purchased from Amersham Corp., England.

RESULTS

Fatty acid-induced lysis of *B. subtilis* 168. Exponentially growing cells of *B. subtilis* 168 in Spizizen salts medium were rapidly lysed by adding dodecanoic acid to the culture at a concentration of 0.25 or 0.5 mM (Fig. 1). The OD_{650} of the culture treated with 0.5 mM dodecanoic acid decreased by 68% after 1 h of incubation and by 86% after 3 h. At 1 and 2 mM, the OD_{650} decreased only slightly. A similar lytic response was evoked by other saturated fatty acids, including hexanoic, octanoic, and decanoic acids, although the lytic effect diminished considerably with decreasing carbon chain length of the fatty acid (data not shown). Of the fatty acids tested, dodecanoic acid was the most effective.

For fatty acid-induced lysis of *B. subtilis* 168, two mechanisms may be proposed: massive solubilization of the cell membrane by the detergent-like action of fatty acids and wall degradation resulting from the induction of an active autolytic enzyme system. In this study, we examined these possibilities mainly with dodecanoic acid.

The lytic action of dodecanoic acid was examined as a function of pH. Cultures were treated with 0.5 mM dodecanoic acid at pH 7.0 for 15 min (15% lysis) and then shifted to different pHs by adding HCl or NaOH solution (1 ml) at different concentrations. The resulting lysis at 45 min after the shift was 67, 75, 80, and 67% at pHs 6.8, 7.5, 8.4, and 9.6, respectively; that is, lysis was marked at alkaline pH between 8 and 9. This condition was very close to the optimum (pH 8.5) for autolytic activity of cells in a buffer. In the following experiments, autolysis was examined with potassium phosphate buffer at pH 7.0 for comparison with fatty acid-induced lysis.

Effects of inhibitors. If the lytic process induced by fatty acids depends on cellular processes, it should be affected by metabolic inhibitors. In fact, 1 mM *p*-chloromercuribenzoic acid partly (73%) and 0.025% glutaraldehyde completely prevented dodecanoic acid-induced lysis of *B. subtilis* 168. In addition, heat treatment of a growing culture at 70°C for 15 min before the addition of dodecanoic acid completely suppressed lysis. Correspondingly, these treatments also inhibited cellular autolysis in phosphate buffer (data not shown). Similar results were obtained with hexanoic, octanoic, and decanoic acids (data not shown).

Bovine serum albumin can interact with and remove fatty acids bound to the cell membrane (15, 24). Therefore, we were interested in investigating the effect of bovine serum albumin on fatty acid-induced lysis of *B. subtilis* 168. The albumin was first added at different concentrations to growing cultures of *B. subtilis* 15 min before the addition of dodecanoic acid (0.5 mM). The results (Table 1) indicate that bovine serum albumin added at a concentration of 0.25 or 0.5% overcame the lytic action of dodecanoic acid, although it still allowed the inhibition of cell growth. A lower concentration (0.125%) of the albumin had little effect on dodecanoic acid-induced lysis. In contrast to the above data, when

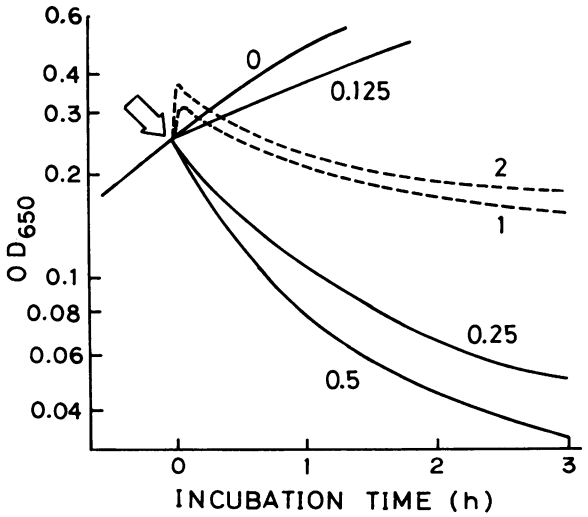

FIG. 1. Time course of dodecanoic acid-induced lysis of *B. subtilis* 168. Cells grown at 37°C for 18 h in Spizizen salts medium were inoculated into fresh medium. When the culture reached an OD_{650} of 0.25 (at the time indicated by the arrow), dodecanoic acid was added at concentrations shown by the numbers. The change in the OD_{650} was recorded automatically at 2-min intervals in a Bioscanner.

the albumin was added after the induction of lysis, no inhibition of bacterial lysis could be detected with 0.25 and 0.5% albumin (Table 1). Bovine serum albumin had no effect on cellular autolysis in phosphate buffer (data not shown).

It has been reported that the autolytic activity of cells is decreased by stopping protein synthesis (15, 17, 30). A similar effect has been shown by the lysis of gram-positive bacteria induced by penicillin (31) and other wall-inhibiting antibiotics (32) and NaCl- (29), and sucrose- (28) induced lysis of *Clostridium saccharoperbutylacetonicum*, all of which involve an autolytic enzyme. We investigated whether dodecanoic acid-induced lysis is affected by the presence of an inhibitor of protein synthesis. The lytic action of dodecanoic acid gradually decreased with an increase in the contact time of cells with erythromycin (100 μg/ml) before the addition of 0.5 mM dodecanoic acid (Fig. 2). The extent of lysis induced by dodecanoic acid after 1 h was 82, 62, 42, and 29% at 0, 0.5, 1, and 2 h, respectively, after the addition of erythromycin. The lysis of erythromycin-untreated cells 1 h after the addition of dodecanoic acid amounted to 82, 75, and 55% at ODs of 0.250 (0 h), 0.415 (0.5 h), and 0.532 (1 h), respectively. A similar trend could be observed for autolysis in phosphate buffer of cells incubated with erythromycin (data not shown).

Lysis of autolysin-deficient mutant. We tested the action of fatty acids on the growing cultures of an autolysin-deficient mutant, *B. subtilis* FJ2. The extent of cellular lysis induced by dodecanoic acid was 71 and 17% for strains 168 and FJ2, respectively. Other saturated fatty acids, hexanoic, octanoic, and decanoic acids, also caused much less lysis than on the wild-type strain (data not shown). Autolysis of strain FJ2 in phosphate buffer was 13% 1 h after incubation, whereas that of the parent was 55%.

Cell wall degradation. We examined the in vivo degradation of peptidoglycan. The amount of peptidoglycan labeled with *N*-acetyl-D-[1-^{14}C]glucosamine was found to decrease with the duration of incubation with 0.5 mM dodecanoic acid (Fig. 3). At a higher concentration (2 mM) of dodecanoic acid, no clear degradation of peptidoglycan was observed.

The finding that fatty acid-induced lysis was more extensive than autolysis at pH 7.0 suggests that dodecanoic acid might enhance the lysis of *B. subtilis* 168 cell walls. To examine this, cell walls were prepared ultrasonically from exponentially growing cells and incubated in 50 mM potassium phosphate buffer (pH 7.0) at 37°C, with or without 0.5 mM dodecanoic acid. However, no difference in the degree of autolysis was observed between the untreated cell wall preparation and the preparation treated with dodecanoic acid, being 24, 58, and 75% for the former preparation and 25, 58, and 76% for the latter after incubation for 20, 60, and 120 min, respectively.

Protoplast lysis. Protoplasts prepared from *B. subtilis* 168 lysed at a concentration of 0.125 mM dodecanoic acid and most of the protoplasts were lysed at 0.5 and 2.0 mM, with the extent of lysis 1 h after incubation being 30, 87, and 81%, respectively. Protoplasts of strain FJ2 also were solubilized by the fatty acid at these concentrations (data not shown).

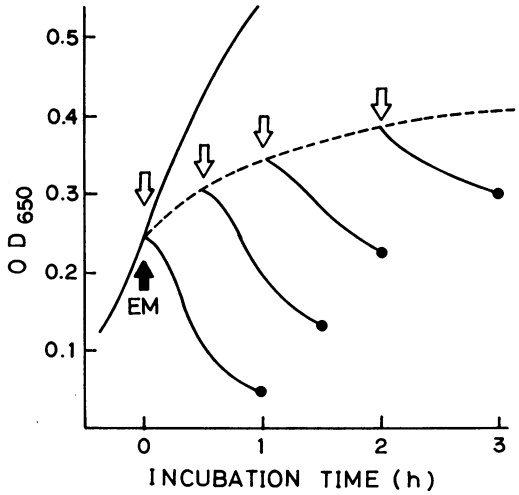

FIG. 2. Effect of erythromycin on cellular lysis of *B. subtilis* 168 induced by dodecanoic acid. Erythromycin (EM; 100 μg/ml) was added to the exponentially growing culture at zero time. Thereafter, dodecanoic acid (0.5 mM) was added at the times (0, 0.5, 1, or 2 h) indicated by the open arrows.

TABLE 1. Effect of bovine serum albumin on dodecanoic acid-induced lysis of *B. subtilis* 168[a]

Cells	% of initial OD_{650}[b]
Untreated	
−BSA	244
+BSA (0.5%)	264
Treated with dodecanoic acid (0.5 mM)	
−BSA	21
+BSA[c]	
0.125	25
0.25	126
0.5	236
+BSA (0.5%)[d]	
0.5	25
5	20
15	21

[a] Cells were grown to an OD_{650} of 0.25. Then, 0.5 ml of bovine serum albumin (BSA) solution or the same amount of deionized water was added to the culture 15 min before, or at the indicated times after, treatment with dodecanoic acid.

[b] After 1 h of incubation.

[c] Added before treatment at the indicated concentrations (%).

[d] Added at the indicated times (min) afte exposure to dodecanoic acid.

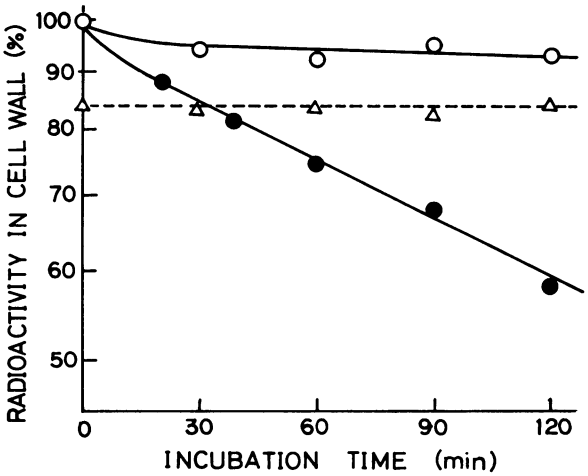

FIG. 3. Degradation of peptidoglycan during dodecanoic acid-induced lysis. Cells were labeled with *N*-acetyl-[1-^{14}C]glucosamine, washed, and exposed to dodecanoic acid at 0 (○), 0.5 (●), or 2 mM (△).

DISCUSSION

The results of this study suggest that fatty acid-induced cellular lysis, evaluated by OD decrease of the culture, of *B. subtilis* 168 is due to the action of autolytic enzymes, not to the massive membrane-solubilizing property of fatty acids. A correlation of fatty acid-induced lysis with cellular autolysis was obtained from experiments conducted under different conditions. The addition of glutaradehyde or *p*-chloromercuribenzoic acid and heat treatment of the culture, all of which inactivate the autolytic enzyme system (15, 17, 28, 41), suppressed fatty acid-induced lysis. In addition, bovine serum albumin was found to have no inhibitory effect on autolysis or on lysis triggered by dodecanoic acid, in spite of its ability to interact with fatty acids. Bovine serum albumin is reported to remove fatty acid molecules adsorbed to bacterial membranes and to restore amino acid transport by the membrane (14, 24). It has been known also that the lytic activity of dodecanoic acid toward bacterial protoplasts is antagonized by bovine serum albumin (12). However, albumin failed to stop dodecanoic acid-induced lysis when added after the addition of the fatty acid, although it prevented this lysis when added before fatty acid addition. This finding might suggest that the interaction of the fatty acid with the membrane is not in itself sufficient to cause lysis, although the role of bovine serum albumin should be clarified further. This may be compatible with the idea that, once induced by dodecanoic acid, the activity of autolysins is not affected by bovine serum albumin.

The hypothesis that autolysin has a role in fatty acid-induced lysis of *B. subtilis* may explain the findings that lysis by fatty acids was greatly decreased by prolonged inhibition of protein synthesis of cells and that the autolytic enzyme-deficient mutant was much less lysed by fatty acids than was the wild-type strain. These results might be interpreted as a loss in the activity of functional cellular autolysin similar to that in lysis induced by wall-inhibiting antibiotics (7) and Triton X-100 (3), although the possibility of alteration of composition or amount of membrane lipids and lipoteichoic acid content in the wild-type culture inhibited by the protein synthesis inhibitor and the autolysin-deficient mutant cannot be ruled out. Also, in fact, the radioactivity in the peptidoglycan fraction from cells labeled with *N*-acetyl-D-[1-^{14}C]glucosamine was diminished in the course of incubation with dodecanoic acid.

In *B. subtilis* two autolysins are known, *N*-acetylmuramyl-L-alanine amidase and *N*-acetylglucosaminidase (5). The former is the major lytic enzyme in bacilli (8, 41). The autolysin that may be involved in fatty acid-induced lysis seems to be the amidase, because the optimal pH for this enzyme is about 8.0 (5, 16), which corresponds to that for fatty acid-induced lysis, whereas that for glucosaminidase is between 5 and 6 (5). Furthermore, we observed the release of L-alanine residues, although no substantial release of reducing groups has been detected by using cells grown in glycerol instead of glucose as a carbon source (unpublished data). This supports the idea that the amidase may be involved in the fatty acid-induced lysis.

It is unclear at present how fatty acids induce autolysis. We found that lysis induced by fatty acids is more marked than autolysis of whole cells of *B. subtilis* 168 at pH 7.0 and that fatty acids enhanced the autolytic activity of whole cells (data not shown) but not cell walls. Therefore, it is unlikely that fatty acid directly activates autolytic enzymes in the cell wall. Alternatively, the primary target of action of fatty acids may be the cell membrane, as suggested for Triton X-100 (3). Forsberg and Ward (9) have suggested that *B. licheniformis* *N*-acetylmuramyl-L-alanine amidase was located on the membrane surface in latent form in close contact with the cell wall before migration to the wall, the site of enzyme action, and they demonstrated that this enzyme was released by Triton X-100 from its L-form membrane. Similarly, one explanation for the induction of autolysis by fatty acids is that the fatty acids solubilize autolysins from the membrane at relatively low concentrations. Such an action would bring about the enhancement of autolysis as described above. The results of experiments on protoplasts seem to support this idea. In addition, the dependency of the lytic action of fatty acid on the carbon chain length of the molecule suggests that the surface-active action of the acid might participate in the dissociation of autolysins from the membrane.

Jolliffe et al. (19) have reported that various agents that dissipate an energized state of the membrane could induce autolysis of *B. subtilis* cells and have suggested that there is a close relationship between cellular lysis and energized membranes. Because fatty acids also have an uncoupling action on oxidative phosphorylation (24, 25), it cannot be ruled out that they induce autolysis by their uncoupling action. For penicillin-induced lysis of pneumococci (40) and Triton X-100-induced lysis of *S. faecalis* (2), the possibility that these chemicals bind an endogenous inhibitor, such as some lipid or lipoteichoic acid (2, 18), of autolytic enzymes located in or near the cell membrane and inactivate or release it from the cell into the menstruum has also been proposed. However, this is unlikely for fatty acids, because long-carbon-chain fatty acids have been reported to behave as autolysin inhibitors (1).

Autolysins are reported to be inactivated by anionic detergents such as sodium dodecyl sulfate (9). In addition, Carson and Daneo-Moore (1) have found that the long chain, saturated fatty acids palmitic acid and stearic acid inhibit the autolysis of whole cells and cell walls of *S. faecalis* at 0.2 mM. This inhibitory effect is suggested to be essentially identical to that of lipoteichoic acids, because fatty acid esters are necessary for the inhibition (2). Also in our study, the OD loss (for all fatty acids tested) and the degradation of peptidoglycan (for dodecanoic acid) were reduced by high concentrations. Therefore, the fatty acids tested in this study seem to have a dual effect on cellular lysis, depending on their concentration, with the action on the cell membrane leading to the induction of autolysis being detectable at relatively low concentrations, and the association with the autolytic enzyme itself being strong at relatively high concentrations.

The mechanism of lytic action of fatty acids seems to be different from that of their bactericidal action. We observed a greater than four log-cycle decrease in cell viability after treatment with 0.5 mM dodecanoic acid. At 2 mM dodecanoic acid, the loss of colony-forming ability was greater, in spite of increased cellular resistance to the lytic action of dodecanoic acid. Cell death may be due to the direct interaction of fatty acids with the cell membrane.

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