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Lysis of *Bacillus subtilis* Cells by Glycerol and Sucrose Esters of Fatty Acids

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The lytic action of glycerol and sucrose esters of fatty acids with different carbon chain lengths on the exponentially growing cells of *Bacillus subtilis* 168 was investigated. Of each series of esters, glycerol dodecanoate and sucrose hexadecanoate were the most active. Lysis at 1 h after the addition of 0.1 mM glycerol dodecanoate or 20 μg of sucrose hexadecanoate per ml was 81 or 79%, respectively, as evaluated by the reduction in optical density. During this treatment a great loss of viability occurred that preceded lysis. The results that were obtained suggest that autolysis is induced by these esters. The esters caused morphological changes in the cells, but a seeming adaptation of the cells to esters was seen.

The glycerol and sucrose esters of fatty acids are often added to some processed foods as stabilizing agents or emulsifiers. These esters, as well as fatty acids themselves, also inhibit bacterial growth (1, 2, 6, 7, 11). Glycerol monododecanoate inhibits the germination and outgrowth of bacterial spores (9). Glycerol and sucrose esters of fatty acids also enhance the thermal death of *Escherichia coli* (16) and bacterial spores (17). The lysis of microorganisms by these esters, however, has not been studied extensively.

We have reported that the short- and medium-chain fatty acids lyse actively growing cells of *Bacillus subtilis*, probably by the induction of autolysins (15). Ved et al. (18) found that dodecylglycerol, which has an ether bond instead of an ester bond between glycerol and dodecanoic acid, had a stronger lytic action on *Streptococcus faecium* ATCC 9790 than glycerol dodecanoate; dodecylglycerol lysed at concentrations above 7 μg/ml; and glycerol dodecanoate lysed at above 18 μg/ml. The lysis caused by both compounds was due to autolytic enzymes (18).

This study was undertaken to study the lytic action of glycerol and sucrose esters of fatty acids on *B. subtilis* 168.

MATERIALS AND METHODS

Microorganisms. *B. subtilis* 168 *trp* and its mutant FJ2 *trp lyt*, which is deficient in the autolytic enzymes *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase (4), were employed as described previously (15).

Cultural conditions and cellular lysis. The procedure for cellular lysis was as described elsewhere (15). Cells were grown at 37°C in Spizizen salts (12) medium supplemented with 0.5% glucose–0.15% sodium glutamate–20 μg of tryptophan per ml in an automatic growth-recording apparatus (Bioscanner OT-BS12; Ohtake Works Ltd., Tokyo, Japan). The solution (0.1 to 1 ml) of compounds tested was added to the culture at an optical density at 650 nm (OD_{650}) of 0.25, and lysis was observed.

Viability measurement. A portion (1 ml) of the culture was removed into a test tube containing 9 ml of 10 mM phosphate buffer (pH 7.0) and 5 mM glucose at 4°C. Samples were diluted with fresh glucose-phosphate buffer and plated on nutrient agar. After the plates were incubated for 24 h, the number of colonies was counted.

Chemicals. The glycerol monoesters of C_8, C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} saturated fatty acids and of $C_{18:1}$ unsaturated fatty acid (99% purity) were gifts of Riken Vitamin Co., Ltd. The purity of the sucrose esters of fatty acids, which were gifts of Mitsubishi Chemical Industries, Ltd., has been described previously (17); preparations contained 70 to 88% of the monoester, 12 to 25% of the diester, and 0 to 5% of the triester forms and over 99% of each fatty acid moiety. The stock solutions of glycerol esters in 10% ethanol and sucrose esters in distilled water were kept at −20°C in a freezer. *p*-Chloromercuribenzoic acid (Wako Pure Chemical Industries, Ltd.) and glutaraldehyde (Ohken Shoji Co., Ltd.) were used in aqueous solution.

RESULTS

Cellular lysis induced by esters. Glycerol dodecanoate and sucrose hexadecanoate lysed exponentially growing cells of *B. subtilis* (Fig. 1). The addition of 0.1 mM (27.4 μg/ml) glycerol dodecanoate or 50 μg of sucrose hexadecanoate per ml to the culture caused an 81 or 91% reduction in the OD_{650}, respectively, after 1 h of incubation. Of the glycerol and sucrose esters with different carbon chain lengths tested, glycerol dodecanoate and sucrose hexadecanoate were the most effective (Fig. 2).

Involvement of autolysins in the lysis. To determine whether autolysins play a role in ester-induced lysis, as they do in the lysis induced by fatty acids (15), the following experiments were done. When a mutant (FJ2) deficient in autolytic enzymes was exposed to 0.1 mM glycerol dodecanoate or 50 μg of sucrose hexadecanoate per ml, the mutant lysed much more slowly than did the wild type. Lysis at 1 h after the addition was only 36 or 40%, respectively. In addition, 1 mM *p*-chloromercuribenzoate or 0.025% glutaraldehyde inhibited the ester-induced lysis of the wild type, with lysis being 16 and 0%, respectively, 2 h after the addition of 0.1 mM glycerol dodecanoate. Similar results were obtained with sucrose hexadecanoate (data not shown).

We also examined the effect of inhibition of protein synthesis on lytic action. After 2 h of incubation with 100 μg of erythromycin per ml, which was added to the logarithmically growing culture, 0.1 mM glycerol dodecanoate or 50 μg of sucrose hexadecanoate per ml was added. The lysis of

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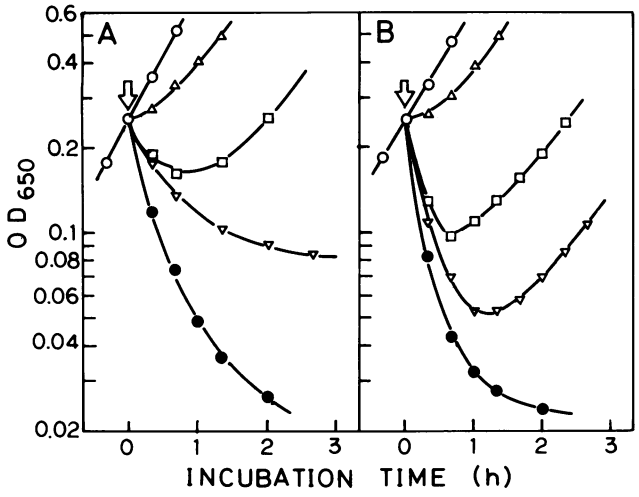

FIG. 1. Cellular lysis of *B. subtilis* 168 induced by glycerol dodecanoate and sucrose hexadecanoate. The esters were added at the time indicated by arrow at concentrations of 0.05 (△), 0.07 (□), 0.08 (▽), and 0.1 (●) mM for glycerol dodecanoate (A) and 10 (△), 15 (□), 20 (▽), or 50 (●) μg/ml for sucrose hexadecanoate (B). Control cells are also indicated (○).

cells inhibited with erythromycin was greatly reduced (Fig. 3).

These results suggest that autolysins are involved in the lysis of *B. subtilis* 168 cells induced by esters, as in the lysis by short- and medium-chain fatty acids described previously (15).

Cell viability. We were also interested in the relationship between the killing effect of esters and lytic action. Cell death occurred before lysis when glycerol dodecanoate was added at 0.1 mM or when sucrose hexadecanoate was added at 50 μg/ml. The loss of viability was more than a four or a three log-cycle decrease, respectively, less than 1 h after the addition (Fig. 4).

Adaptation of cells that survived treatment with the esters.

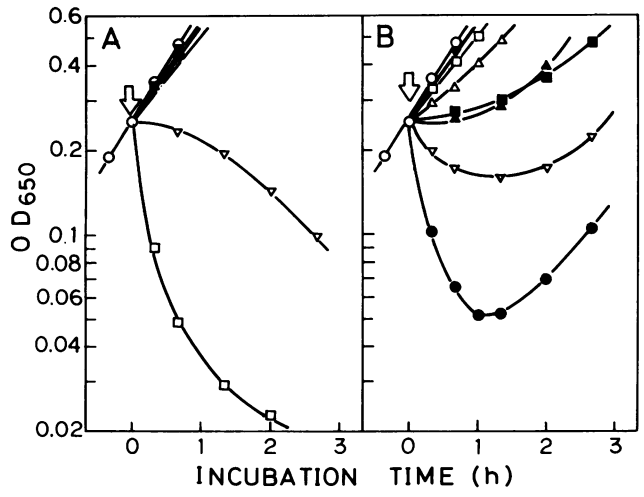

FIG. 2. Lytic action of glycerol and sucrose esters with different carbon chain lengths of the fatty acid moiety. Glycerol (A) and sucrose esters (B) with C_8 (▼), C_{10} (△), C_{12} (□), C_{14} (▽), C_{16} (●), C_{18} (▲), and $C_{18:1}$ (■) were added at concentrations of 0.18 mM for glycerol esters and 20 μg/ml for sucrose esters. Control cells are also indicated (○).

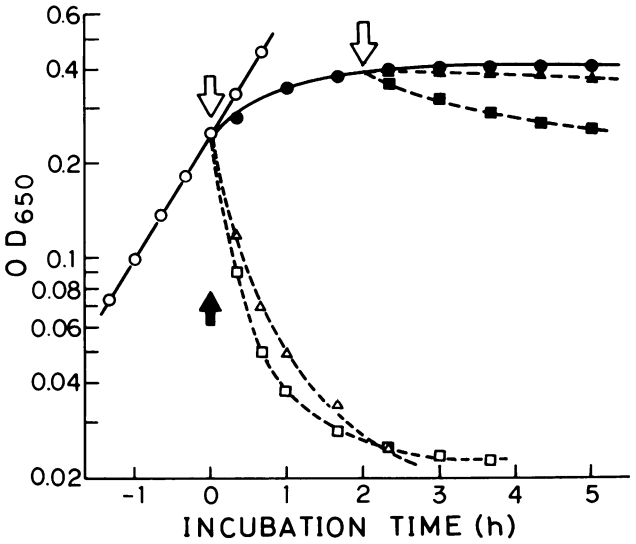

FIG. 3. Effect of erythromycin on cell lysis induced by glycerol dodecanoate or sucrose hexadecanoate. Erythromycin (100 μg/ml; ●) was added at time zero (black arrow). Glycerol dodecanoate at 0.1 mM (△, ■) or sucrose hexadecanoate at 50 μg/ml (□, ▲) was added at the times (0 or 2 h) indicated by the open arrows. Control cells are also indicated (○).

Cells that survived the treatment with 0.07 mM glycerol dodecanoate or 15 or 20 μg of sucrose hexadecanoate per ml grew again (Fig. 1). Even at higher concentrations of esters, cells could grow after prolonged incubation: about 20 or 10 h after the addition of 0.1 mM glycerol dodecanoate or 200 μg of sucrose hexadecanoate per ml, respectively. The newly grown cells apparently were tolerant to the addition again of 0.1 mM glycerol dodecanoate or 20 μg of sucrose hexadecanoate per ml and grew more, although slightly more slowly. When cells grown after treatment with 0.07 mM glycerol dodecanoate were inoculated in fresh medium with-

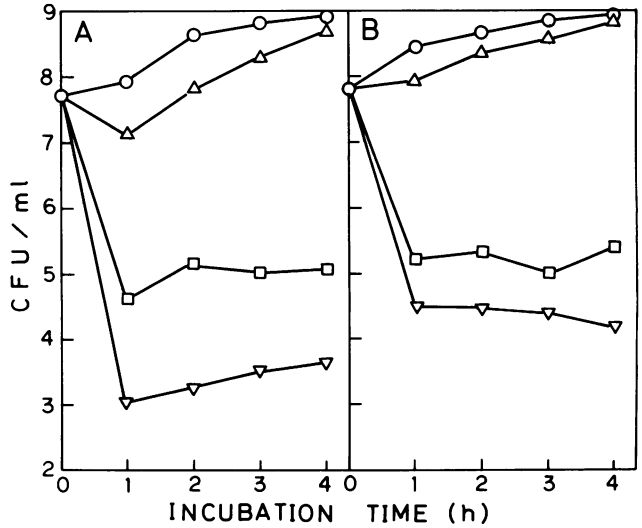

FIG. 4. Loss of viability during treatment with glycerol dodecanoate (A) or sucrose hexadenoate (B). Glycerol dodecanoate at 0.05 (△), 0.08 (□), or 0.1 (▽) mM and sucrose hexadecanoate at 10 (△), 20 (□), or 50 (▽) μg/ml were added to exponentially growing cells at time zero. Control cells are also indicated (○).

out the ester and then cultivated to the exponential phase, they lysed on exposure to the ester at 0.1 mM, as did control cells (data not shown), suggesting that regrowth is not due to the selection of a resistant variant. Also, cells lysed on the addition of 0.1 mM glycerol dodecanoate, even in fresh medium containing an autolysate obtained from the culture at the same cell concentration, although to a lesser extent (data not shown). The cells that were grown again to the exponential phase grew further, even after they were washed and then suspended in fresh medium containing 0.1 mM glycerol dodecanoate (data not shown). These results suggest that regrowth is not due to the inactivation of the ester molecules remaining in the culture by the exudate from lysed cells but to the adaptation of cells to esters.

Morphology of cells. Cellular lysis was also observed by phase-contrast microscopy (data not shown). In addition, we found that cells exposed to sublytic concentrations of esters showed characteristic changes in their morphology. In the presence of 0.05 to 0.07 mM glycerol dodecanoate or, to a lesser extent, 10 to 15 μg of sucrose hexadecanoate per ml, many cells became twisted or bended (Fig. 5). This change in morphology was also observed in cells exposed to 70 μg of Triton X-100 per ml or 0.22 mM dodecanoic acid, both of which induce autolysis of cells (3, 14, 15).

DISCUSSION

Glycerol dodecanoate and sucrose hexadecanoate induced the lysis of *B. subtilis* cells at concentrations lower than the concentrations of dodecanoic acid with the same effect (15). The sucrose hexadecanoate preparation that was used contained small amounts of di- and triester forms, so we could not evaluate how the ester forms contribute to the overall activity of the preparation. The concentrations of sucrose hexadecanoate preparation that caused cell lysis (15 to 50 μg/ml) corresponded to 0.025 to 0.086 mM, provided that the ester preparation only consisted of the monoester. With this assumption, sucrose hexadecanoate should cause cell lysis more effectively than glycerol dodecanoate. The MIC of sucrose hexadecanoate for growth determined 24 h after incubation was above 1 mg/ml, whereas that for glycerol dodecanoate was 0.1 mM, which was relatively close to that which induced cell lysis effectively. The high MIC of the sucrose ester may be due to the relatively rapid adaptation of the cells. Ved et al. (18) reported that at a concentration of 0.0656 mM (18 μg/ml) glycerol monododecanoate completely inhibited the growth of *S. faecium* for 3 h. This concentration was relatively close to that used for *B. subtilis* in our study.

As with short- and medium-chain fatty acids (15), the lysis of *B. subtilis* cells induced with glycerol dodecanoate and sucrose hexadecanoate may be due to the action of autolytic enzymes and not to the direct action of solubilization of the bacterial membrane. The results obtained with inhibitors such as *p*-chloromercuribenzoic acid, glutaraldehyde, and erythromycin and with an autolysin-deficient mutant support the hypothesis presented above, as discussed previously for lysis induced by fatty acids (15).

After lysis the surviving cells grew. These cells seemed to adapt to esters, as indicated by their renewed growth and tolerance to esters if they were added again. Tilby (14) reported that *B. subtilis* 168 cells grown in the presence of 0.006% Triton X-100 adapted to concentrations of 0.08 to 0.25% of the surfactant. Similar adaptation to Triton X-100 was also observed with *Staphylococcus aureus* H (10). We found that *B. subtilis* 168 cells adapted after a prolonged lag

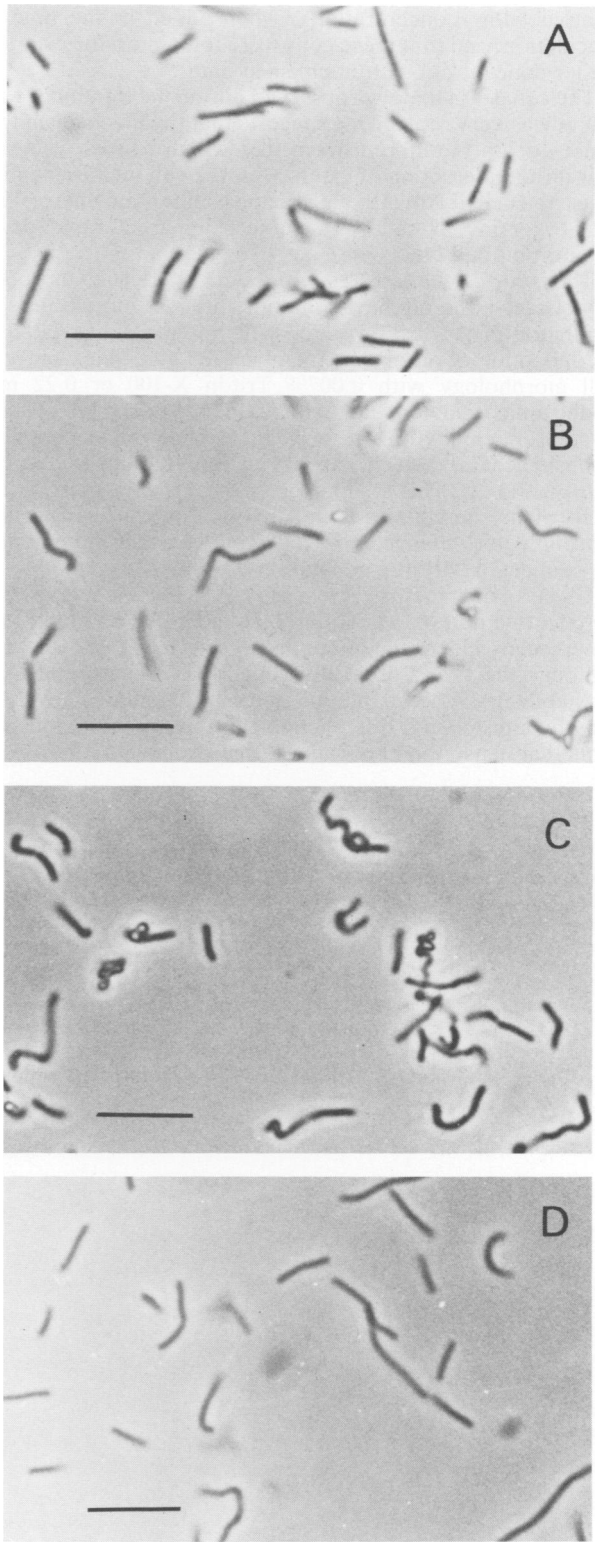

FIG. 5. Morphological changes of cells treated with glycerol dodecanoate or sucrose hexadecanoate. Logarithmically growing cells were incubated with glycerol dodecanoate at 0.05 (B) or 0.07 (C) mM or with sucrose hexadecanoate at 20 (D) μg/ml for 4 h and then observed by phase-contrast microscopy. Control cells are also indicated (A). Bars, 10 μm.

period to glycerol dodecanoate or sucrose hexadecanoate at relatively low concentrations, when added at the time of inoculation, and that these cells were tolerant to the esters at higher concentrations (unpublished data).

The cell death that was observed during the treatment with esters was very rapid. This suggests that the mechanism that causes death is different from that which causes lysis and that direct interaction of esters with the cell membrane may cause the cell death, although the possibility that an irreversible triggering of cell lysis induces apparent rapid death cannot be ruled out.

The fact that cells treated with esters altered cell morphology suggests the inhibition of some process of synthesis or regulation of the cell envelope which is possibly related to the induction of autolysis. We observed a similar change in cell morphology with 0.007% Triton X-100 or 0.22 mM dodecanoic acid, but not with 0.07 mM carbonyl cyanide *m*-chlorophenylhydrazone or 10 mM potassium cyanide (unpublished data), which have been reported to induce cell autolysis (3, 5). This result suggests that the de-energization itself of the membrane is not involved in the induction of morphological change. The change in cell morphology may be connected with the adaptation described above.

Glycerol and sucrose esters of fatty acids are supposed to be effective nontoxic antimicrobial agents; and in fact, they have been added to some processed foods (8, 13, 19), although they were originally added to foods as emulsifiers or stabilizers. The adaptation seen in this study, however, may be a problem if this phenomenon exists in microorganisms that cause food spoilage or that produce toxins.

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