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Protoplast Formation of Selected *Mycobacterium smegmatis* Mutants by Lysozyme in Combination with Methionine

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Lysozyme-sensitive mutants of *Mycobacterium smegmatis*, isolated by nitrosoguanidine treatment, have been converted into protoplasts in a nutritionally enriched medium containing lysozyme and DL-methionine.

Bacterial protoplasts and spheroplasts are osmotically fragile forms that lack rigid or partially rigid cell walls. A conversion of bacterial cells into protoplasts or spheroplasts may be achieved by the addition of enzymes, such as lysozyme, or antibiotics, such as penicillin, to growth media. The methods for removing the cell wall by lysozyme have been used with success on a variety of bacteria. However, protoplasts of mycobacterial cells were not readily prepared by lysozyme alone, since the complex nature of mycobacterial cell wall makes these organisms relatively resistant to the action of lysozyme (8, 10). Some strains of rapidly growing mycobacteria and of strain BCG, when grown in the presence of lysozyme and glycine, become osmotically sensitive forms believed to be spheroplasts (1, 4-6). There has been no report of the successful removal of the cell wall material from mycobacterial cells to prepare a true protoplast. This report describes a method of preparation of protoplasts from lysozyme-sensitive mutant cells of *Mycobacterium smegmatis* in a medium containing lysozyme and DL-methionine.

M. smegmatis ATCC 607 was grown in a modified Dubos liquid medium (11). The mutants were isolated by exposing the cells for 30 min to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml in 0.9% saline supplemented with 0.2% Tween 80). The suspensions were washed with saline and spread on the surface of an agar medium of the same composition as modified Dubos liquid medium, except that no albumin was present. After incubation at 37°C for 3 days, colonies were picked with sterile toothpicks and plated on the agar medium for selection. Colonies present on the plate containing no lysozyme, but absent on the plate containing lysozyme (200 μg/ml), were picked and streaked on the plates for further testing.

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A tryptone soy broth-L medium, used for protoplast formation, was prepared as follows: tryptone (Difco), 15 g; phyton (BBL), 5 g; K_2HPO_4, 2.5 g; NaCl, 5 g; and Na_2SO_3, 0.2 g; dissolved in 1 liter of distilled water. To 100 ml of this broth, 1 g of activated charcoal was added. After the solution was stirred, 3 ml of packed horse blood cells was added, and the mixture was heated in a boiling-water bath for 20 min. Sediment was removed by filtration through a filter paper, and the medium was sterilized by fractional sterilization. To each 100 ml of the sterilized medium, 40 ml of a sterile solution of 40% sucrose, 10 ml of 0.2 M $MgSO_4$, and 15 ml of horse serum were added. The method selected for routine protoplast production was the addition of 1 mg of lysozyme and 170 μmol of DL-methionine per 5 ml of the tryptone soy broth-L medium. Approximately 5×10^7 cells of the culture grown in the modified Dubos liquid medium were transferred to 5 ml of the medium for protoplast formation. Incubation was carried out at 37°C in a Monod-type incubator with gentle shaking.

The protoplasts were fixed for 2 h in ice-cold 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) containing 0.34 M sucrose, followed by postfixation for 2 h in 1% OsO_4 in the same buffer. The specimens were dehydrated with a graded ethanol series, embedded in Epon 812, and sectioned. The sections were stained with uranyl acetate and lead citrate and viewed in a Hitachi HU 12AS electron microscope.

Five mutants sensitive to lysozyme were isolated by nitrosoguanidine treatment. When the mutants were further tested for sensitivity to DL-methionine using the modified Dubos liquid medium containing 34 mM DL-methionine, the growth of two mutants was inhibited approximately 70% more than the other mutants and the parent strain. These two mutants were examined for conversion to spherical forms in the medium for protoplast formation. The metamorphosis of the cells was followed by phase-

contrast microscopy. The mutants sensitive to lysozyme and DL-methionine produced a higher yield of spherical cells during 2 weeks of incubation, whereas the parent strain showed only a limited formation of spheres. One of these two mutants, L-17, was analyzed in detail. Initially, this mutant exhibited typical mycobacterial morphology (Fig. 1A). The first indication of morphological changes in the cells appeared on day 2 or 3, when the cells were observed to be

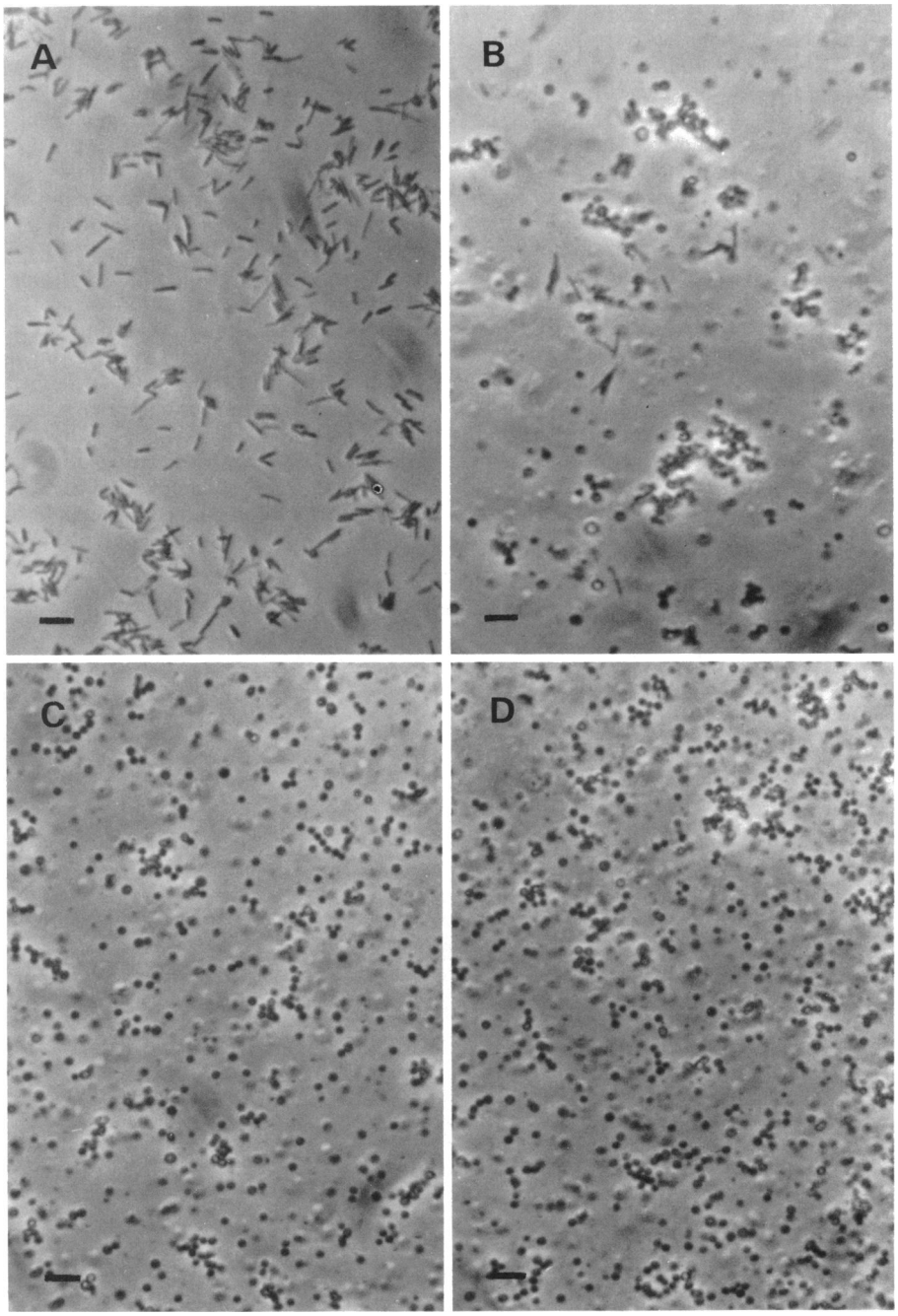

FIG. 1. *Changes in morphology of the lysozyme-sensitive mutant (L-17) in the medium for protoplast formation. Bars, 5 μm. (A) Control; (B) 3 days; (C) 9 days; (D) 12 days.*

swollen and transparent (Fig. 1B). During the next 7 days there was an increase in the number of spherical cells, and from day 9 a rapidly increasing number of spheres was observed (Fig. 1C). During the period between days 10 and 13, the maximum numbers of spheres was seen (Fig. 1D). Greater than 99% of the cells were converted into osmotically fragile forms, which lysed when collected and suspended to the original volume in distilled water. Electron micrographs of the spherical cells taken between days 12 and 14 showed the absence of residual cell wall material adhering to the outside of the cytoplasmic membrane, the disappearance of the mesosomes known to exist in the intact cells, and the accumulation of small vesicles, as usually found in L-forms or protoplasts of other bacterial species (Fig. 2). These facts, together with their osmotic fragility, prompted us to refer to these structures as protoplasts.

Concentrations of lysozyme from 50 to 400 μg/ml and DL-methionine from 10 to 50 mM were tried for the conversion into protoplasts. Optimal results were obtained with a concentration of 200 μg of lysozyme per ml and 34 mM DL-methionine. With the addition of L-methionine at the same concentration in place of 34 mM DL-methionine, no obvious formation of protoplast was observed. D-Methionine at concentrations of 17 and 34 mM was less effective than 34 mM DL-methionine. This indicates that D- and L-methionine are necessary for the conversion of the cells into protoplasts. D-Methionine, as well as glycine and D-serine, when present in a growth medium at high concentrations, inhibits the growth of various bacteria by incorporating into the cell wall, presumably in place of some normal constituents (3, 7, 9, 11). It seems that the incorporation of D-methionine into the cell wall may occur and render the cell more susceptible to the action of lysozyme. However, the exact role of the L-isomer of DL-methionine in the formation of protoplasts is not explained, although two mutants sensitive to DL-methionine resulted in better formation of protoplasts.

To ascertain whether the protoplasts formed by a prolonged incubation, such as over 10 days, are active in macromolecular synthesis, the incorporation of radioactive thymidine and uracil into cold trichloroacetic acid-insoluble

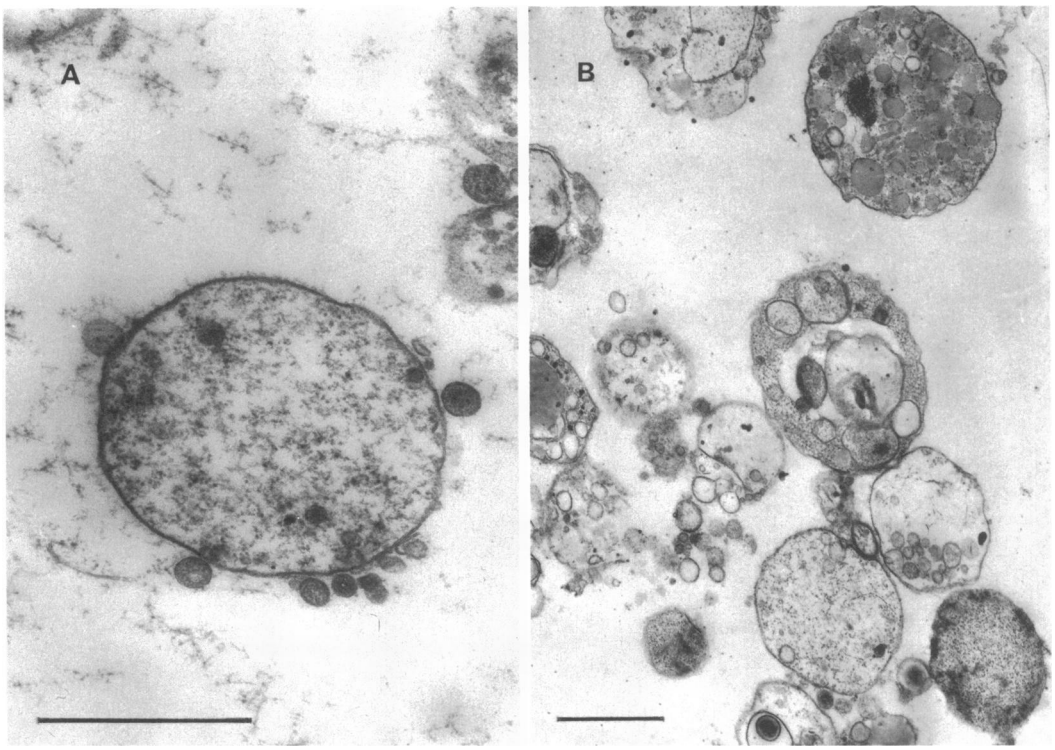

FIG. 2. *Electron photomicrographs of thin sections of protoplasts. Bars, 1.0 μm. (A) 12 days; (B) 14 days.*

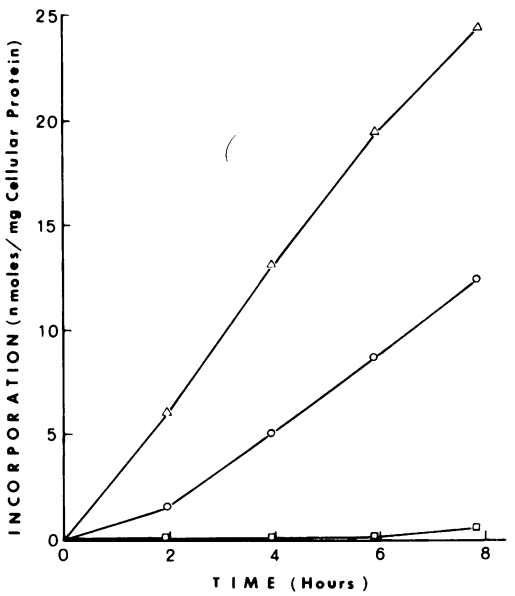

FIG. 3. *Incorporation of L-[^{14}C]leucine (○) into hot trichloroacetic acid-insoluble material and [^{14}C]thymidine (□) and [^{14}C]uracil (△) into cold trichloroacetic acid-insoluble material by protoplasts. The protoplasts were obtained by incubating the cells for 11 days and aspirating the upper layer of cultures with a pipette after spontaneous sedimentation to remove a cell clump. To each 1 ml of this protoplast suspension was added L-[^{14}C]leucine (2 μCi/ml), [^{14}C]thymidine (4 μCi/ml), or [^{14}C]uracil (2 μCi/ml) at a final concentration of 0.5 mM. Radioactive compounds were added at the beginning of the incubation at 37°C. The incorporation of L-[^{14}C]leucine into hot trichloroacetic acid precipitates was used as a measure of protein synthesis. At varying times after the addition of the radioactive compound, a sample of the protoplasts was immediately added to an equal volume of cold 10% trichloroacetic acid containing non-radioactive L-leucine (1 mM). Samples were heated in a boiling-water bath for 10 min. The precipitates were collected on a 0.45-μm-porosity membrane filter and washed three times with 5 ml of cold trichloroacetic acid containing the non-radioactive amino acid. The filters were dried, placed on a planchet, and counted in a gas flow counter. For measuring nucleic acid synthesis, the incorporation of [^{14}C]uracil or [^{14}C]thymidine into trichloroacetic acid precipitates was used as an indication of ribonucleic acid or deoxyribonucleic acid synthesis. Samples were removed at intervals and immediately added to an equal volume of ice-cold trichloroacetic acid containing non-radioactive uracil or thymidine (1 mM). The samples were stored at 0°C for 2 h, and the precipitates were collected on a membrane filter and washed three times with ice-cold trichloroacetic acid containing non-radioactive uracil or thymidine. The filters were dried and counted for radioactivity.*

material and of radioactive L-leucine into hot trichloroacetic acid-insoluble material was measured. The protoplasts actively incorporated uracil and L-leucine. In contrast, only a slight increase in incorporation of thymidine was obtained during 8 h of incubation (Fig. 3).

The present investigation showed that a rather long period of incubation is needed for a high yield of protoplasts. Electron micrographs of the protoplasts taken at earlier stages of incubation revealed that the cytoplasmic membrane was dissociated from the ruptured cell wall and the protoplasts were released into the extracellular environment. At the end of incubation, the accumulation of a number of protoplasts was seen (Fig. 2B). In *M. smegmatis* cells grown in the presence of D-cycloserine, protoplast formation was found to occur during cell division (2). Prolonged incubation may be necessary to lead to cell division and loss of the cell wall, which result in protoplast formation.

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