NOTES

Spheroplast Formation of *Mycobacterium smegmatis* and Morphological Aspects of Their Reversion to the Bacillary Form

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Cell wall-deficient forms (spheroplasts) of *Mycobacterium smegmatis* strain P53 were prepared by combined treatment with glycine, lysozyme, and lytic enzyme no. 2 as the spheroplasting agents. Quantitative mass conversion to spherical forms was effected by pretreatment of the intact cells with 1.2% glycine in nutrient broth, followed by transfer to spheroplasting medium containing the above agents. Two apparent modes of reversion to the bacillary form were observed under electron microscopy. The first one was initiated by budding from the spheroplasts. The buds gradually elongated to become the mycelial form, which showed branching, septation, and fragmentation. The second resulted from the intracellular formation of tiny cells, possibly the elementary bodies, and their release from the spheroplasts.

The bacteria belonging to the genus *Mycobacterium* are resistant to cell wall-destroying enzymes, possibly because these cells possess thick and rigid cell walls containing large amounts of lipid. At present, as described by Rastogi and Venkitasubramanian (4), there are two ways to prepare cell wall-deficient forms of mycobacteria: the first is to treat the cells with inhibitors of cell wall synthesis to make them sensitive to wall-destroying enzymes (1, 2, 5, 8), and the second is to isolate lysozyme-sensitive mutants (7). However, no reports have mentioned the viability and stability of the spheroplasts induced by these methods. In some restricted experiments, for instance, protoplast fusion and regeneration, a great deal of viable protoplasts are required (3). In this paper, we describe highly efficient procedures for preparing stable spheroplasts and some of the interesting morphological events responsible for the process of the reversion of mycobacterial spheroplasts.

An overnight culture of *Mycobacterium smegmatis* P53 (*his arg leu*, ethambutol resistant) was inoculated (5%) into nutrient broth (Difco Laboratories) containing 1% glucose and 0.2% Tween 80 and incubated in an L tube with shaking until the cells reached the midexponential phase of growth. A 20% glycine solution was added to the culture to a final concentration of 1.2%, and the culture was incubated for an additional 16 to 20

h. The cells were harvested by centrifugation at $1,200 \times g$ for 15 min, washed once with SMM (0.5 M sucrose–20 mM $MgCl_2$–0.02 M maleate [pH 6.5]), and resuspended in the original volume of the medium for spheroplast formation (P medium) containing (per liter): sucrose, 120 g; glucose, 10 g; L-glycine, 12 g; $(NH_4)_2SO_4$, 1 g; K_2SO_4, 0.25 g; $MgCl_2 \cdot 6H_2O$, 2.03 g; $CaCl_2 \cdot 2H_2O$, 3.68 g; KH_2PO_4, 0.1 g; 0.025 M [*N*-tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES; pH 7.2); lysozyme (Sigma), 50 mg; lytic enzyme no. 2 (Kyowa Hakko Kogyo Co., Tokyo, Japan), 30 mg; and histidine, arginine, and leucine at final concentrations of 50 µg/ml. The bacterial suspension was incubated at 37°C for 16 to 20 h with gentle shaking. During this time, most of the cells were converted to spherical form. To remove intact cells and aggregated wall ghosts, the culture was centrifuged at $300 \times g$ for 7 min or filtered through cotton wool. The supernatant or filtrate was then centrifuged at $1,200 \times g$ for 15 min, and the pelleted spheroplasts were washed twice with SMM by careful resuspension and centrifuging. The washed spheroplasts were counted with a Petroff-Hausser chamber under a phase-contrast microscope; they were then diluted in distilled water and plated (0.1 ml) onto nutrient agar to assess their resistance to osmotic shock by measuring the reduction in colony formation.

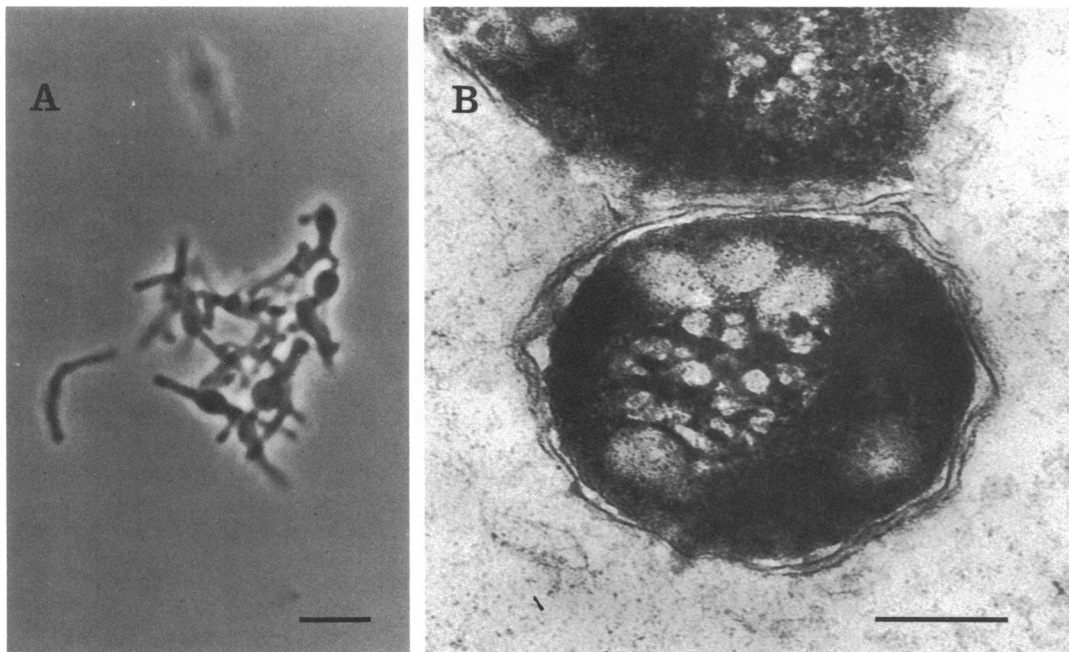To regenerate the wall of spheroplasts, the

FIG. 1. Morphological change in *M. smegmatis* cells treated with glycine (1.2%) for 16 h in nutrient broth. (A) Phase-contrast micrograph; bar, 5 μm. (B) Electron micrograph; bar, 0.2 μm.

washed spheroplasts were inoculated in a liquid medium for regeneration (R medium) consisting of (per liter): sucrose, 120 g; glycerin, 20 ml; (NH$_4$)$_2$SO$_4$, 1 g; K$_2$SO$_4$, 0.25 g; MgCl$_2$·6H$_2$O, 10.12 g; CaCl$_2$·2H$_2$O, 2.95 g; KH$_2$PO$_4$, 0.1 g; 0.025 M TES; and histidine, arginine, and leucine at final concentrations of 50 μg/ml. Alternatively, spheroplasts were plated on R agar (R medium containing 1.5% agar).

To prepare specimens for electron microscopy, glycine-treated cells, spheroplasts, or regenerating spheroplasts were washed with 0.2% cacodylate buffer (pH 6.5) containing 0.5 M sucrose, fixed with 2% formaldehyde–4% glutaraldehyde in ice water for 30 min, and then fixed with 0.5% osmium tetroxide in a refrigerator for 16 h. The fixed sample was embedded in 2% agar, cut into small pieces, dehydrated in a series of ethanol dilutions, and embedded in Epon 812. Sections were cut with an ultramicrotome and examined with a JEM-100CX electron microscope. In preparing cells for scanning electron microscopy, the osmium tetroxide-fixed samples were first washed thoroughly in 0.03 M Veronal acetate buffer (pH 7.2), suspended in distilled water, and then poured together with a piece of glass slide into filter paper bags approximately 2 cm square. The bags were dehydrated in a series of acetone dilutions,
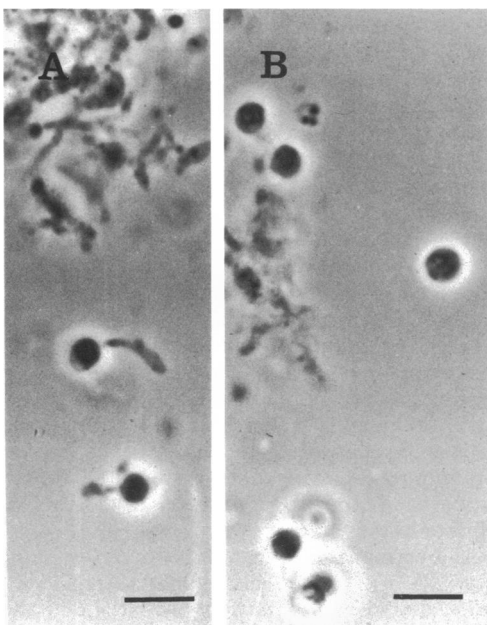

FIG. 2. Phase-contrast micrographs of *M. smegmatis* cells undergoing spheroplast formation. The glycine-treated cells shown in Fig. 1 were transferred to P medium and incubated for (A) 6 through (B) 16 h. Bar, 5 μm.
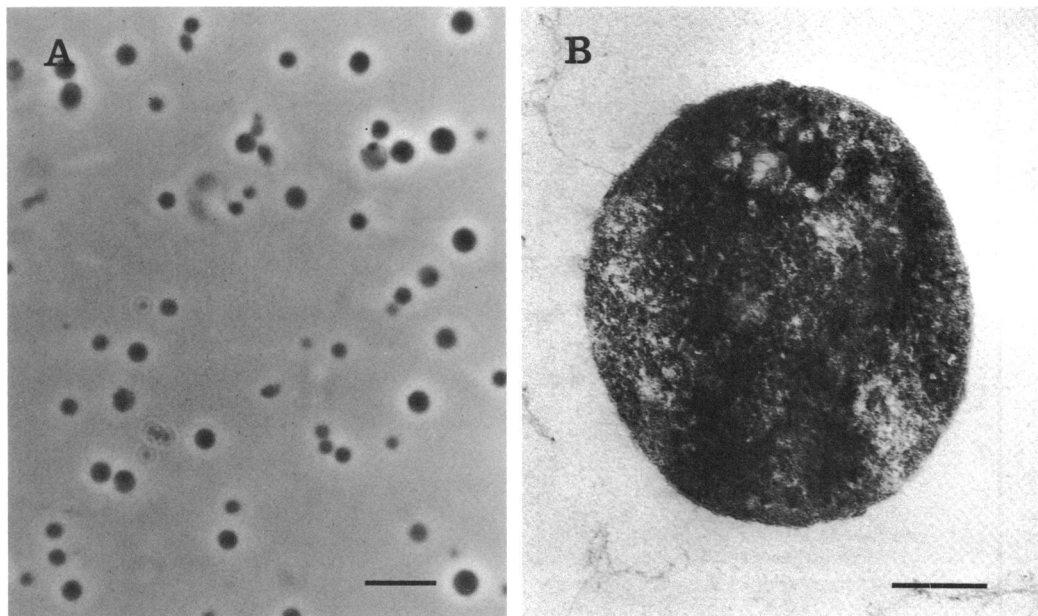
FIG. 3. Phase-contrast (A) and electron (B) micrographs of the spheroplasts induced from *M. smegmatis* in P medium. The cell shown in (B) appears to be devoid of cell wall materials and is bound by a unit membrane. Bars, (A) 5 μm and (B) 0.5 μm.

critical-point dried, and cut into pieces approximately 5 mm square. The filter paper pieces or glass pieces were coated with gold-palladium and then observed with a Hitachi S-700 scanning electron microscope.

The optimum concentrations of glycine added to the cultures during the midexponential phase of growth varied with the strain tested. In nutrient broth, *M. smegmatis* P53 was efficiently converted to spherical forms by concentrations between 0.75 and 1.2%; the addition of glycine at concentrations of more than 1.5% greatly decreased the viability of the cells (less than 6%). The percentage of remaining osmotically stable cells after transfer to P medium was 5.4, 0.28, 4.1, and 3% for cells grown with 0.5, 1.0, 1.5, and 2% glycine, respectively.

Figure 1 shows the morphological changes in the cells after 16 h of incubation in 1.2% glycine. The center or one end of the cells became swollen (Fig. 1A), suggesting that the inhibition of cell wall synthesis was limited to cell growing points. The electron micrograph shows that the cell wall had exfoliated from the cytoplasmic membrane (Fig. 1B). These cells were easily converted to spherical shape in P medium. The cells pretreated with glycine were subsequently incubated for 16 h in P medium with gentle shaking, and the changes in cell morphology were monitored at intervals by phase-contrast microscopy. Spheroplasts were gradually re-

leased from one end of the rods, apparently casting off the wall ghosts (Fig. 2A), and finally 90 to 95% of the cells were converted to spherical forms (Fig. 2B). Suspensions consisting of more than 99% spherical cells were obtained after purification by repeated centrifugation and washing (Fig. 3A). The surface of these cells appeared to be a unit membrane structure; no detectable wall structures were seen on the surface (Fig. 3B). However, we describe the spherical cells as spheroplasts, since we have no chemical or immunological evidence that they totally lacked wall material.

Scanning electron microscopy indicated that the following phenomena are involved in the reversion process. The first step of regeneration is budding from the spheroplasts after incubation for 20 to 48 h (Fig. 4A). At this stage, the surface of the spheroplast was smooth, but it became wrinkled along with the elongation of the filamentous cell (Fig. 4B). During subsequent incubation for 3 to 5 days, the elongated sprouts exhibited mycelium-like growth accompanied by branching (Fig. 4C), and normal bacillary cells which reverted to nearly the parental shape (presumably produced by division of the filaments) were seen by phase-contrast microscopy (Fig. 5). These mycelial cells were never observed under normal culture conditions and were not acid fast or only weakly so, suggesting that some of the cell wall components are lack-
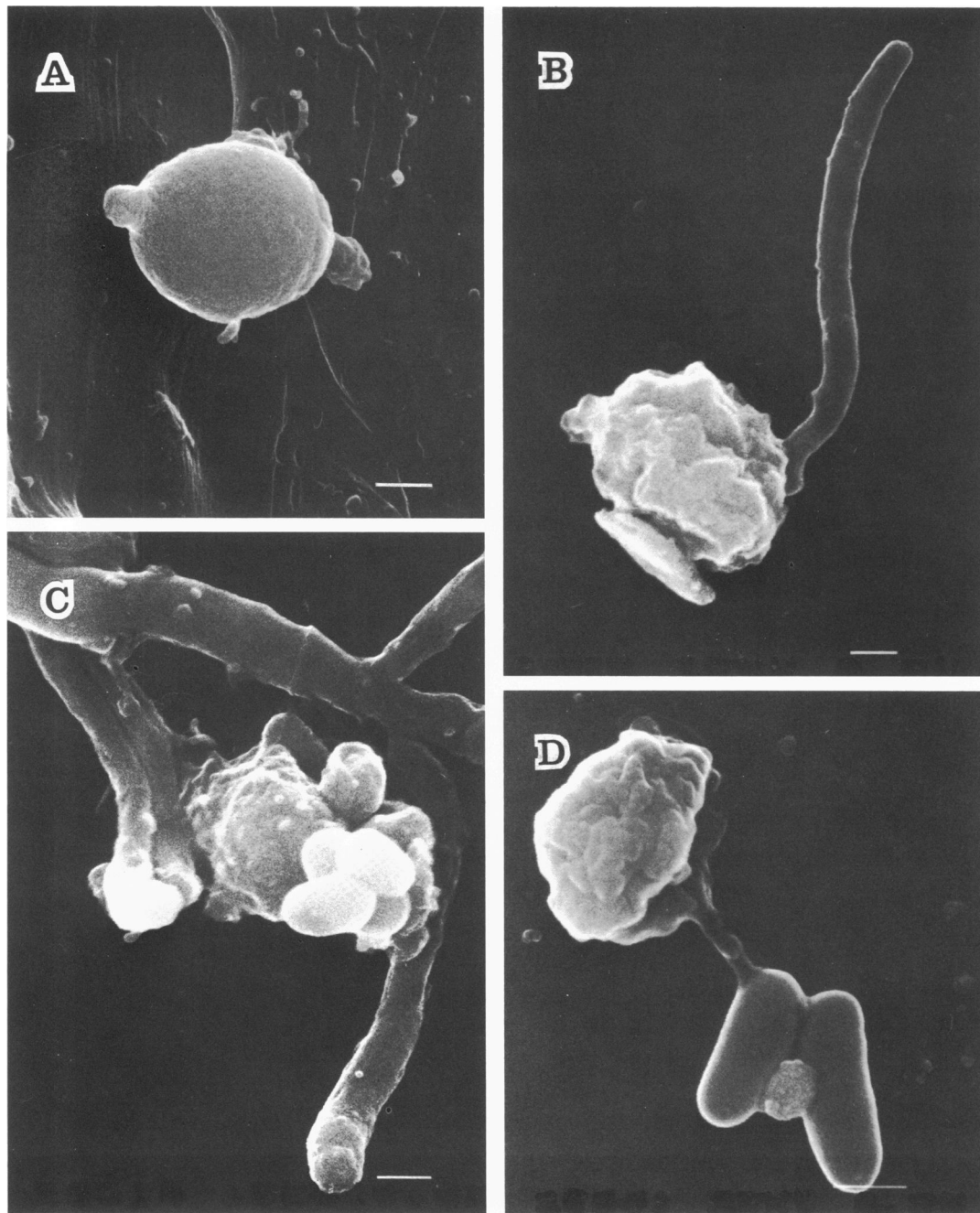
FIG. 4. Scanning electron micrographs of regenerating *M. smegmatis* spheroplasts engaged in a type of budding (A and B), elongation with branching (C), and production of tiny cells (C and D). The spheroplasts were incubated in R medium for (A, B, and D) 48 through (C) 72 h. Bar, 0.5 μm.

ing in the mycelium-like cells. Extremely short rod cells could also be observed together with mycelial cells (Fig. 4C). Perhaps these tiny cells were produced in the spheroplasts and then released (Fig. 4D). It is not clear whether these tiny cells are analogous to the elementary bodies produced in the cytoplasm of L-phase variants derived from *Bacillus subtilis* and *B. lichenifor-*
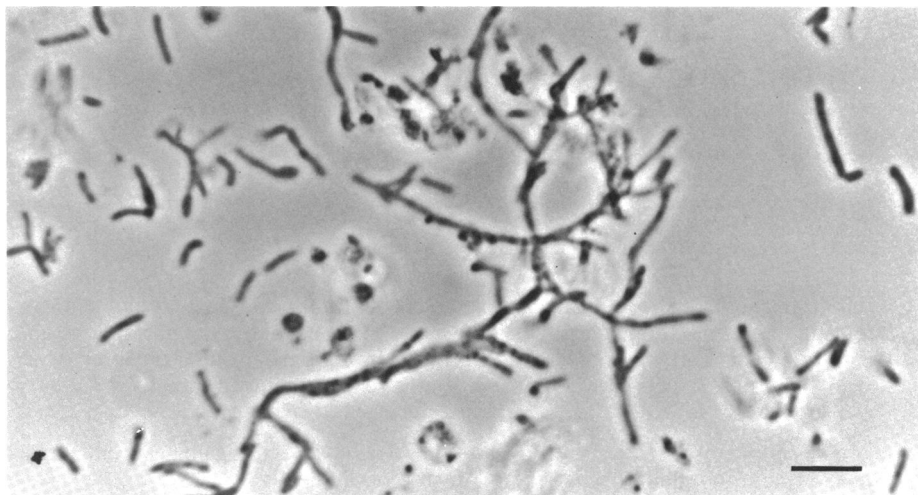
FIG. 5. Phase-contrast micrograph of the regenerated bacillary forms originating from *M. smegmatis* spheroplasts. The spheroplasts were incubated for 72 h in R medium. The cells that reverted to the parental shape are seen together with mycelium-like growth. Bar, 5 μm.

mis (6). However, this process may play a role, as the second event other than budding, in the spheroplast regeneration of this organism.

The reversion frequencies calculated from the ratio of colony-forming units on R plates to the number of input spheroplasts varied with the experiments, but were always within the range of 0.1 to 20%.

Our experiments show that stable spheroplasts can easily be formed from *M. smegmatis* with high efficiency and that they are capable of converting again to bacillary form. The availability of the methods provides a basis upon which genetic manipulation systems may be developed in *Mycobacterium*.

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