SHORT COMMUNICATION

Preparation of Protoplasts and Whole Cell Ghosts from *Mycobacterium smegmatis*

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Cell wall-deficient forms of *Mycobacterium smegmatis* were produced in growth medium containing D-cycloserine and horse serum. These cells were transformed into protoplasts with EDTA and lysozyme. Subsequent lysis by nucleases followed by osmotic shock produced membrane vesicles (whole cell ghosts).

INTRODUCTION

Bacterial protoplasts are osmotically sensitive forms of bacteria which completely lack a peptidoglycan wall. By controlled lysis, they can easily be converted to intact, unit-membrane bound sacs, essentially devoid of cytoplasmic contents. Spheroplasts are spherical forms of bacteria, which unlike protoplasts might retain wall residues. Though some bacterial walls can be removed by lysozyme alone or by the combined action of lysozyme and EDTA, mycobacterial protoplasts cannot be prepared in this way (Thacore & Willett, 1963; Willett & Thacore, 1967). There have been reports of spheroplast formation in some fast-growing mycobacteria and *Mycobacterium bovis* BCG, when grown in the presence of lysozyme and glycine (Sato *et al.*, 1965, 1966; Adamek *et al.*, 1969). Recently, Winder & MacNaughton (1978) have developed a relatively rapid procedure for preparing lysis-susceptible forms of *M. smegmatis*.

At present there are two reports of protoplast and ghost preparation from mycobacteria, the first utilizing lysozyme and glycine for *M. phlei* (Asano *et al.*, 1973) and the second isolating lysozyme-sensitive mutants of *M. smegmatis* and growing them in a rich medium containing lysozyme and DL-methionine (Yabu & Takahashi, 1977).

Several antibiotics, including D-cycloserine have been shown to induce spheroplast formation in *M. tuberculosis* (Dorozhkova & Volk, 1972). We have developed a method for the preparation of wall-deficient forms of *M. smegmatis* CDC 46, utilizing D-cycloserine and horse serum in the growth medium. These wall-deficient forms were converted to protoplasts by treatment with lysozyme and EDTA, and were subsequently converted to whole cell ghosts, employing the technique of Kaback (1971).

METHODS

Organism and growth. *Mycobacterium smegmatis* CDC 46 (originally obtained from the Center for Disease Control, Atlanta, Georgia, U.S.A.) was used in these studies. It was grown on the medium of Youmans & Karlson (1947), in which magnesium citrate was replaced by citric acid (1.5 g l⁻¹) and MgCO₃ (0.6 g l⁻¹); Tween 80 (0.5%, v/v) was added for cultures grown on a rotary shaker.

Medium for the formation of wall-deficient forms contained, in addition to the above, 15% (v/v) sterile

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horse serum and 90 μg D-cycloserine ml^{-1} . Horse serum and D-cycloserine were added to the autoclaved medium through a 0.45 μm filter.

In both cases cells were grown up to late-exponential phase (36 h) as shake cultures. Normal cells were harvested by centrifuging at 10000 g at 4 °C and subsequently washed with 10 mM-Tris/HCl buffer (pH 8.0) at 0 °C. Wall-deficient forms were harvested by centrifuging at 16000 g at 4 °C and washed with 30 mM-Tris/HCl buffer (pH 8.0) containing 20% (w/v) sucrose.

Preparation of protoplasts and whole cell ghosts. Wall-deficient forms of *M. smegmatis* were suspended (1 g wet wt per 80 ml) in 30 mM-Tris/HCl buffer (pH 8.0), containing 20% (w/v) sucrose. Residual wall material was removed by incubation with 10 mM-potassium EDTA and lysozyme (0.5 mg ml^{-1}) for 60 min with continuous stirring at room temperature (18 °C). During this step, the turbidity of the bacterial suspension, measured at 650 nm, normally decreased significantly and viscosity increased. If it did not, the incubation was continued for another 60 min so as to give at least a 30 to 40% decrease in turbidity. Protoplasts thus obtained were collected by centrifuging at 16000 g for 45 min at 4 °C and resuspended in the minimum volume of 100 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.6), containing 20% (w/v) sucrose and 25 mM- MgSO_4 , with a Teflon and glass homogenizer. DNAase and RNAase (7.5 mg ml^{-1}) were added to facilitate homogenization. Lysis was achieved by pouring the homogenate into 250 vol. 45 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.6) followed by incubation at 37 °C for 30 min on a rotary shaker, after which potassium EDTA (pH 7.0) was added to give 10 mM. The mixture was incubated again at 37 °C for 20 min with swirling; during this time the turbidity of the suspension gradually decreased and viscosity increased. MgSO_4 was then added to 15 mM and incubation was continued for another 20 min at 37 °C. The lysate was centrifuged at 16000 g for 60 min at 4 °C and the pellet obtained was resuspended by vigorous homogenization in 100 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.6), containing 10 mM-EDTA at 0 °C. The sample was centrifuged two or three times at 800 g for 45 min each, to remove the cell debris. The supernatant was carefully decanted and centrifuged at 45000 g for 60 min at 0 °C to give the high-speed membrane pellet. This was washed with 100 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.6) and stored in the same buffer at a concentration of 7 to 10 mg dry wt ml^{-1} below -20 °C in 1 to 2 ml portions.

Ultrastructural studies. The high-speed membrane pellets, normal cells, wall-deficient forms and protoplasts were fixed for 6 h in a solution containing 2% (w/v) formaldehyde and 1.25% (w/v) glutaraldehyde (Sigma) in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (100 mM, pH 7.1), followed by post-fixation in 2% (w/v) OsO_4 in 200 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.1) for 2 h. The specimens were dehydrated in a graded ethanol series and propylene oxide and embedded in an Epon 812/araldite mixture (Ladd Research Laboratories, U.S.A.). Sections (40 nm thick) were cut with a LKB Ultratome III, using a diamond knife, mounted on copper grids, stained in uranyl acetate and lead citrate and observed in a Siemens 101 electron microscope.

RESULTS AND DISCUSSION

When normal *M. smegmatis* cells were treated with lysozyme and EDTA by the method of Kaback (1971), hardly any change in the cell morphology was observed. Rarely, spherical structures were seen but a very thick cell envelope always remained. When mycobacteria were grown in a medium containing D-cycloserine and horse serum, nearly 90% of the cells were converted to spherical wall-deficient forms, as observed by phase contrast microscopy. These forms appeared to be neither like L-forms, since they multiplied fast, nor true spheroplasts, since they grew in a medium devoid of sucrose. We believe that these coccoid forms are deficient in their wall material because they can be converted more readily to protoplasts by lysozyme and EDTA than normal bacteria; hence we describe them as wall-deficient forms, a term introduced by Mattman (1970) for the coccoid forms of mycobacteria resembling

Fig. 1. Electron micrograph of a thin section of protoplasts formed by the action of lysozyme and EDTA on wall-deficient forms of *Mycobacterium smegmatis* obtained in a medium containing D-cycloserine (90 μg ml^{-1}) and 15% (v/v) horse serum. Protoplasts are recognized by their spherical structure, complete absence of cell wall and their sensitivity to osmotic shock. Bar marker represents 0.5 μm .

Fig. 2. Electron micrograph showing whole cell ghosts formed by osmotic shock treatment of mycobacterial protoplasts. These structures are recognized by a complete lack of intracellular contents, which are replaced by the isotonic buffer, and the existence of an intact unit membrane. Bar marker represents 0.33 μm .

ling L-forms or spheroplasts. These forms were transformed to osmotically sensitive true protoplasts, spherical in shape and devoid of any wall material by the action of potassium EDTA and lysozyme (Fig. 1). Controlled lysis by nucleases followed by osmotic shock gave rise to cytoplasmic membrane vesicles (whole cell ghosts; Fig. 2). When stained with Ziehl-Neelsen stain, these structures were not acid-fast.

Changes in the turbidity of the bacterial suspension during osmotic swelling of protoplasts are presumably due to changes in the size and shape of bacteria since their dimensions are of the same order as the wavelength of the light used for turbidity measurements (650 nm) and a change in shape of bacterial particles is known to have a distinct effect on light scattering (Kock, 1961; Wyatt, 1973).

Various antibiotics other than cycloserine were tested. Penicillin showed no effects. Isoniazid, which interferes with the formation of mycolic acid in the cell envelope (Winder & Collins, 1970), did not produce wall-deficient forms in *M. smegmatis*. D-Cycloserine from 15 to 150 $\mu\text{g ml}^{-1}$ was used with horse serum at 5 to 25% (v/v). A mixture of 90 μg D-cycloserine ml^{-1} and 15% (v/v) horse serum was found to be optimal for the formation of wall-deficient forms of *M. smegmatis*. D-Cycloserine when used alone at more than 100 $\mu\text{g ml}^{-1}$ was largely bactericidal. Horse serum alone did not produce significant numbers of wall-deficient forms.

D-Cycloserine appears to block the final stages in the synthesis of UDP-*N*-acetylmuramoyl-tripeptidyl-D-alanyl-D-alanine, an intermediate in wall synthesis (Neuhaus & Lynch, 1964; David *et al.*, 1970). It also inhibits the synthesis of wax-D heteropolymer of the H₃₇Ra strain of *M. tuberculosis* (David *et al.*, 1970), which is chemically related to the mycobacterial cell wall. Horse serum may act through its content of lysozyme (Hankiewicz & Swierczek, 1974). Although the serum used here had no antimycobacterial antibodies it may be of some advantage to use serum from a horse infected with tubercle bacilli where an antibody complement system might then act on specific sites at the cell surface.

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