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Protoplast Formation in *Escherichia coli*

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A procedure for protoplast formation in *Escherichia coli* is described. Removal of the cell wall was confirmed by examination of cells in thin-section preparations.

During the past few years there has been increased interest in the structure and function of bacterial cell membranes. The methods used for preparing such membranes (2) include the formation of osmotically sensitive forms (protoplasts and spheroplasts) and osmotic lysis.

The term protoplasts as presently used refers to a gram-positive organism after complete removal of the cell wall; spheroplast denotes an osmotically sensitive gram-negative organism, retaining part of the membranous lipopolysaccharide layer.

The present paper reports a method in which protoplasts lacking a cell wall can be readily formed in *Escherichia coli*. The methods used for protoplast formation are as follows. *E. coli* strain ML30 was grown in sucrose minimal salts (1) to an optical density at 450 nm of 0.9 to 1.0 at 37°C. The cells were harvested by centrifugation at 4°C at 10,000 × *g* for 5 min. The bacteria were washed twice at 23°C with 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8). The pellet was suspended by pipetting using the following formula according to the conventions of Osborn et al. (3): 500-ml culture × 0.9 = 450 optical density units, with a suspension of the cells to 10 optical density units/ml performed thereafter. Suspension in 45 ml of 0.1 M Tris, pH 8, containing 20% (weight/weight) sucrose was done at 37°C directly in the centrifuge tube. Cells were then transferred into a small flask, and the temperature was adjusted to 37°C. Within 1 min 2.25 ml of a 2 mg/ml solution of lysozyme in double-distilled water was added for a final concentration of 100 μg of lysozyme per ml. During the addition of lysozyme, the suspension of cells was stirred with a magnetic stirrer. After the addition the temperature was adjusted to 37°C. Incubation was carried out with stirring for 12 min at 37°C, after which ethylenediaminetetraacetic acid was added by slow dilution using 0.1 M dipotassium ethylenediaminetetraacetic acid (pH 7) (1: 10, vol/vol, ethylenediaminetetraacetic acid/cell) with prewarmed (37°C) ethylenediaminetetraacetic acid, added slowly over 2.5 min with continuous stirring to avoid lysis. Disodium ethylenediaminetetraacetic acid, pH 8, may be substituted. The temperature will drop during this addition, and it should be adjusted back to 37°C. Within 8 to 10 min more than 99% of the cells become spherical; they can be checked for roundness in the phase microscope.

For electron microscopic examination all samples were fixed at 4°C in glutaraldehyde and osmium buffered with Veronal at pH 6.1. Dehydration in a graded acetone series was followed by embedding in Spurr's (4) epoxy resin. Micrographs were taken with a Philips 300 microscope at 60 kV.

The cell envelope of *E. coli* is most directly visualized in fixed and sectioned cells. Figure 1 illustrates the features of the envelope: the outer membrane, the rigid layer, and the cell membrane. An unusual feature of the cell illustrated here is the cytoplasm that contains numerous clear areas. This feature is consistently observed in strain ML30, although not in other ML strains. Figure 2 illustrates the morphology of protoplasts at low magnification. Here the outer membrane has been removed from 95% of the cells, forming small wall fragments (Fig. 2, inset). When cells are examined at higher magnification as in Fig. 3, the cytoplasmic membrane is clearly observed to form the outermost cell border, thus confirming cell wall removal. Contaminating wall fragments were seen on some cells (Fig. 3, arrow).

Protoplasts such as these can be centrifuged and suspended without extensive lysis. It is, thus, reasonable to expect that membranes derived from such cells by osmotic lysis may be purified and used for further biochemical analysis. Because it has been established in the literature that gram-negative organisms give spheroplasts, it is important to consider why this procedure gives protoplasts. Three ways in

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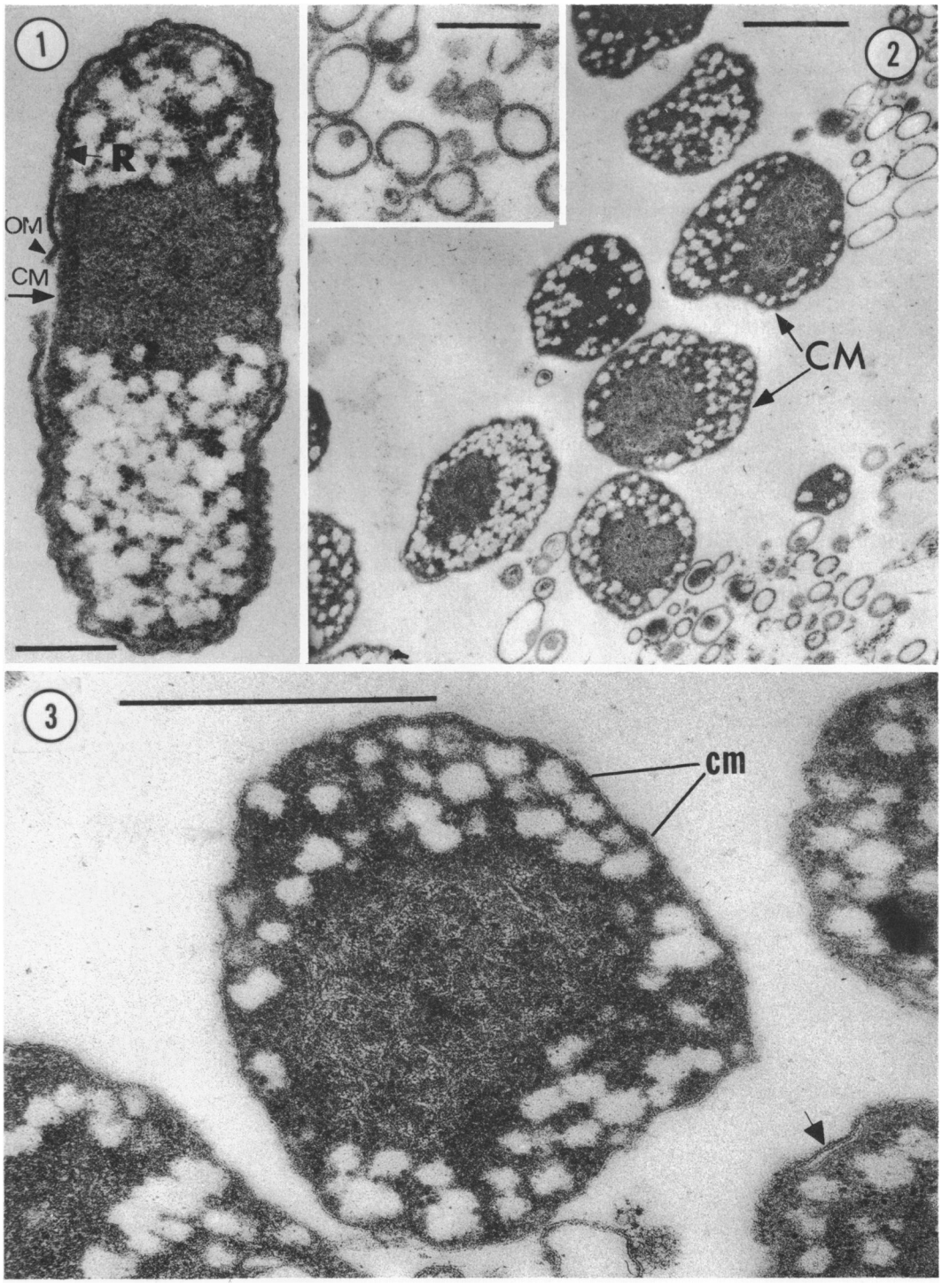

FIG. 1. *Control cell. The cell envelope appears as the outer membrane (OM). The rigid layer (R) and the cytoplasmic membrane (CM). Bar represents 0.25 μm.*

FIG. 2. *Protoplasts of E. coli. The outer membrane and rigid layer are removed. The cell membrane (CM) is seen around the cell. Bar represents 0.5 μm. Inset illustrates cell wall fragments; bar represents 0.25 μm.*

FIG. 3. *Protoplasts of E. coli. The cytoplasmic membrane (CM) forms the outer boundary of the cell. Arrow marks contaminating cell wall fragment. Bar represents 0.5 μm.*

which this could occur may be listed as: (i) the enzyme-substrate ratio may be more favorable for wall removal; (ii) the constant temperature or ionic conditions used for cell wall lysis may ensure more complete degradation; or (iii) the mild osmotic changes during introduction of ethylenediaminetetraacetic acid may "open up" the cell wall.

It is important to consider that the loss of the cell wall depends not only on enzyme access to the substrate but also on the activity of the enzyme at that site. Lysozyme is apparently sufficiently active under the usual methods (2), since gram-positive organisms readily form protoplasts. Therefore, the simplest explanation for cell wall lysis in *E. coli* is to postulate that osmotic stress used with this method allows for more complete access of lysozyme, perhaps due to the release of loosely bound enzymes.

One final consideration is whether protoplasts may be derived from other strains such as B or K-12 that yield less clean Kaback vesicles. With the Kaback method (2) strain ML30 forms spheroplasts rather than protoplasts. It is, therefore, likely that this method will work with other strains of *E. coli*.

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