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NOTES

Electron Microscopy of *Staphylococcus aureus* Cells and Cell Walls After Treatment with Lysozyme *Chalaropsis*

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Lysozyme *Chalaropsis*, a β-1,4-*N*,6-O-diacetylmuramidase, attacks the cell wall murein of *Staphylococcus aureus* H in a random manner. In hypertonic sucrose solutions, true protoplasts are produced and, in the process, mesosomal tubules are extruded into the medium.

Lysozyme *Chalaropsis* (Ch) is a bacteriolytic enzyme, produced by *Chalaropsis* species (1), that has been obtained in crystalline form (2). Lysozyme Ch has β-1,4-*N*-acetylmuramidase activity identical to that of egg white lysozyme and β-1,4-*N*,6-*O*-diacetylmuramidase activity, a property that is not shared by egg white lysozyme. The latter activity enables lysozyme Ch to attack the cell walls of *Staphylococcus aureus*, which is in marked contrast to the activity of egg white lysozyme. In analogy to the action of lysozyme on *Bacillus megaterium* in hypertonic sucrose (9), lysozyme Ch converts *S. aureus* to osmotically fragile spheres (3). Electron microscopy has now been used to monitor this conversion.

S. aureus H was grown in brain-heart infusion broth at 37 C in shaken cultures. Cells were harvested, washed twice, and suspended in 0.04 M acetate buffer at pH 5.8. The concentration of cells was adjusted so that a 1:20 (vol/vol) dilution gave a suspension that had an absorbance of 0.500 at 500 nm in a 1-cm light path. An equal volume of sucrose (1 M) in 0.04 M acetate buffer at pH 5.8 was added. Twice-crystallized lysozyme Ch was added twice to a concentration of 50 μg/ml, and the cells were incubated at 25 C. To monitor the course of the reaction, 0.1-ml portions of the cell suspensions were diluted with 0.9 ml of water to test for osmotic fragility. At various time intervals during the conversions, samples of the suspension were mixed with an equal volume of cold 0.5 M sucrose in 0.15 M tris(hydroxymethyl)aminomethane-hydrochloride buffer at pH 7.5. The combination of pH, temperature, and dilution was sufficient to stop lytic action. Control cells or cell walls incubated at pH 7.5 and 0 C with 20 μg of enzyme per ml were completely unaffected as determined by electron microscopy and by turbidity measurements (1). The cells were fixed at 0 C for 2 h in 2.5% glutaraldehyde (or 5% formaldehyde) in 0.5 M sucrose at pH 7.2. The fixed cells were treated with 1% osmium tetroxide in 0.1 M Veronal buffer at pH 7.4 for 30 min, were dehydrated in graded ethanol solutions, and were embedded in Araldite. Sections were stained with uranium acetate followed by lead citrate (5), and examined with a Siemens Elmiskop I electron microscope.

Staphylococcal cell walls were prepared by standard procedures (7). Cell wall suspensions, in 0.01 M ammonium acetate buffer at pH 5.8, were incubated with lysozyme Ch at an enzyme concentration of 0.50 μg/ml. At appropriate intervals samples were taken by streaking a carbon-coated grid over the surface of the reaction mixture. The grids were shadowed with platinum at a height-to-length ratio of 1:5.

In hypertonic sucrose solution lysozyme Ch rapidly attacks the cell wall murein and completely removes it (Fig. 1). Control cells showed no change in their cell walls during the exposure period (Fig. 1A). The cells undergoing conversion to protoplasts show a progressive removal of cell wall (Fig. 1B through 1D). The cells selected for this series of micrographs represent an average of what was observed at each time interval. Even in the shortest interval some cells were observed that were completely devoid of cell wall, while others showed no evidence of attack. The majority of cells, however, indicated the removal of some parts of the cell wall. By the end of the conversion, at which time the

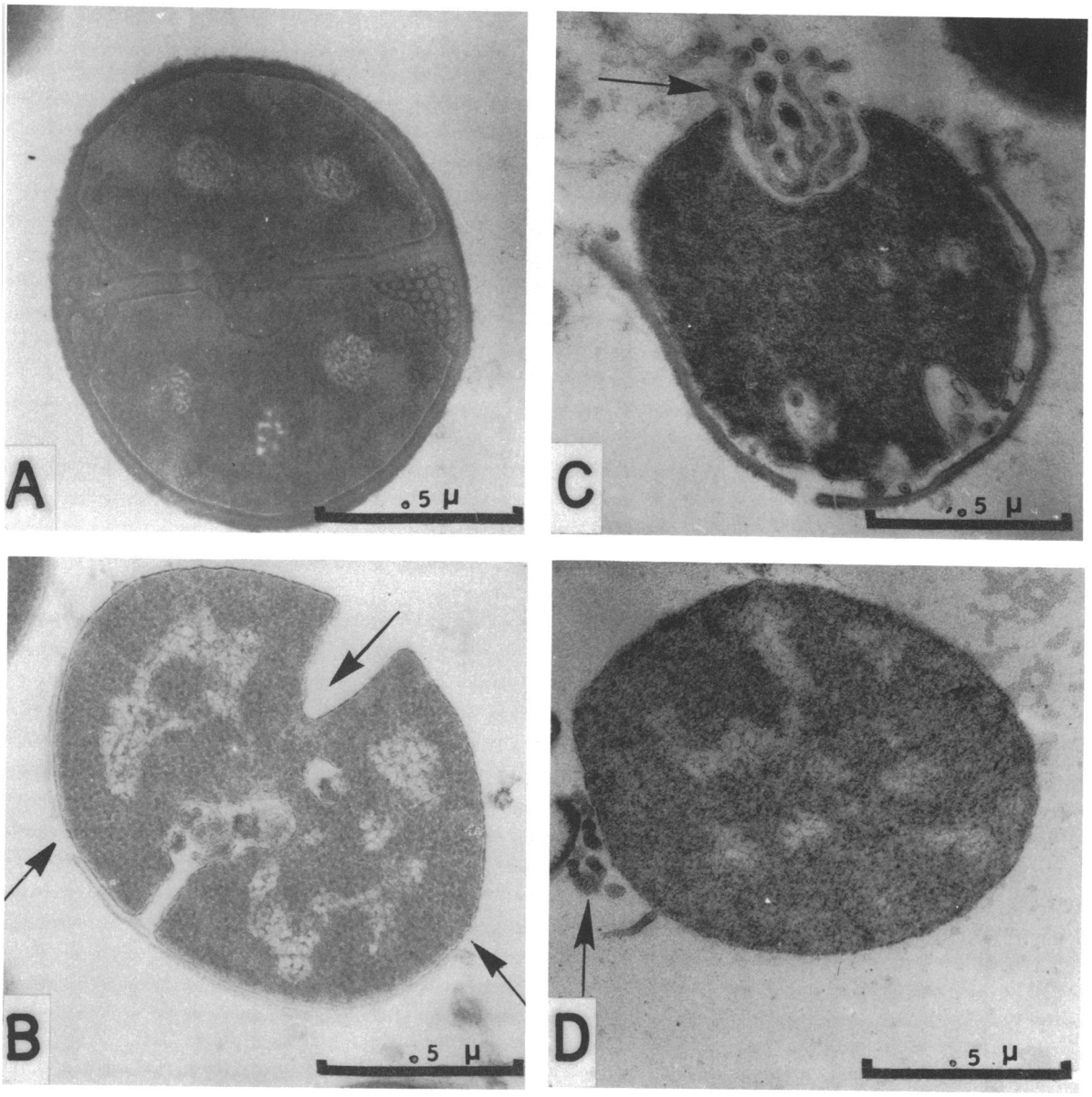

FIG. 1. *Conversion of S. aureus to protoplasts with lysozyme Ch. Fig. 1A represents a control cell that was not exposed to the enzyme. Fig. 1B was from a time sample that showed a 35% reduction in turbidity on dilution with water. Fig. 1C was from a sample that showed a 60% reduction in turbidity, and Fig. 1D shows a protoplast in which the cell wall is completely removed. This time sample showed a 90% reduction in turbidity on dilution with water. The arrows in Fig. 2B point to multiple breaks in the cell wall murein and to the complete removal of the septum separating the dividing cells. The arrows in Fig. 2C and 2D point to the mesosomal tubules that are extruded into the medium when the wall is removed.*

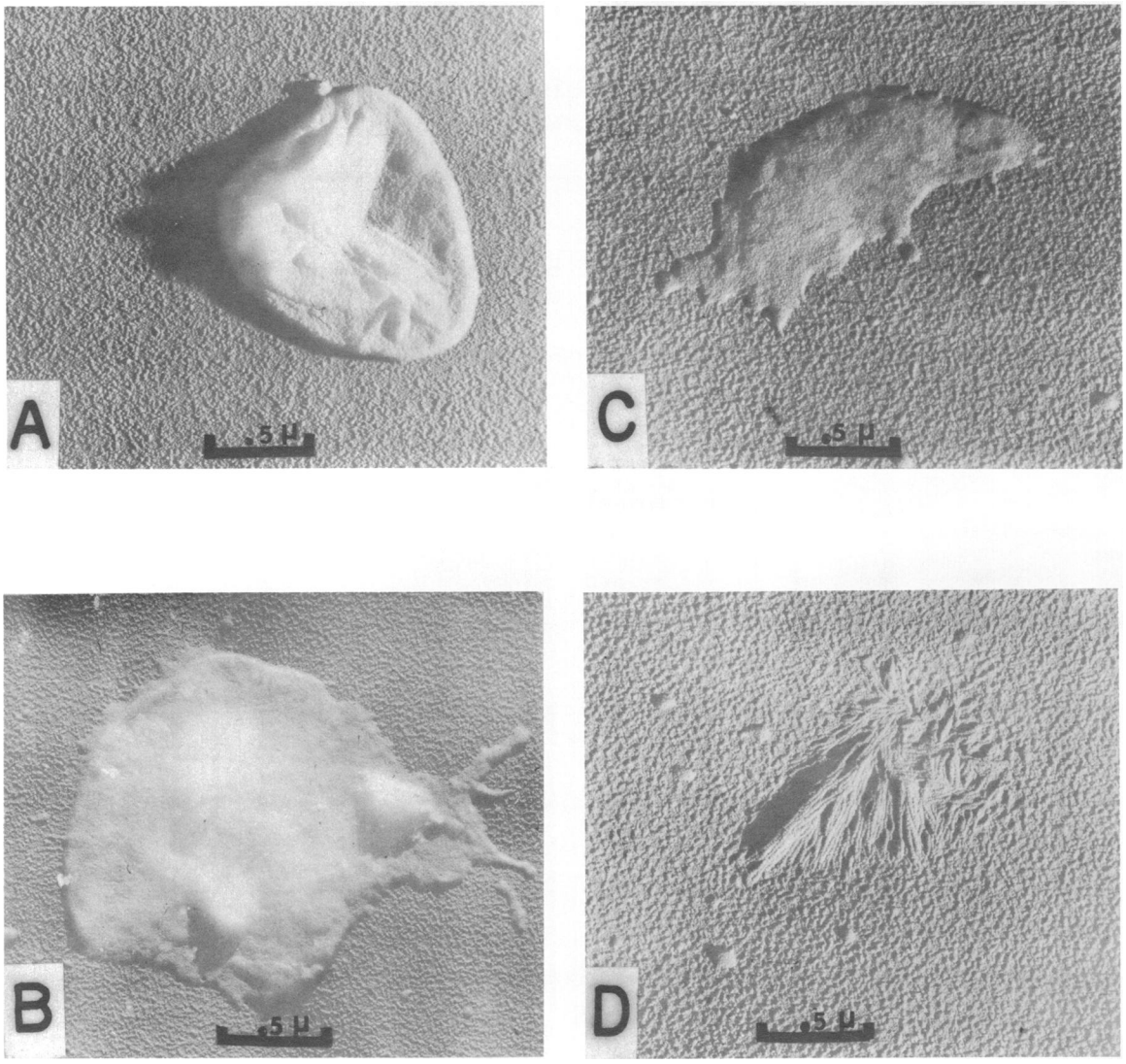

FIG. 2. *Dissolution of S. aureus cell walls by lysozyme Ch. Fig. 2A illustrates a control cell wall that was not exposed to the enzyme. Fig. 2B illustrates intermediate stages of breakdown and was from a sample showing 30% reduction in turbidity. Fig. 2C represents the final stages of breakdown and was from a sample showing a 70% reduction in turbidity. Fig. 2D illustrates fibrillar structures that were occasionally observed, and this sample was taken when the reduction in turbidity was 60%.*

cells were all osmotically sensitive, there was no cell wall murein left on any cell (Fig. 1D).

The attack of the enzyme on the murein appears to be generalized rather than localized. Multiple breaks in the murein are evident in many cells (Fig. 1B and 1C). The most prominent structures visualized in the control cells are the mesosomes attached to the cytoplasmic membrane adjacent to cross septae of dividing cells. There is an expansion of the mesosomes which is apparent in Fig. 1B and 1C. The latter ultimately appear as tubular structures outside the cytoplasmic membrane of the protoplast and are indicated by the arrows in Fig. 1C and 1D. Other mesosomes are observed in the space between the cell wall and the cytoplasmic membrane as the wall is separated from the protoplast. As the wall is completely removed, the mesosomal tubules are extruded into the surrounding medium (Fig. 1C and 1D) in the same manner as that observed for *Bacillus subtilis* by Ryter and Landman (6). These tubular structures are similar to those observed by other workers while purifying mesosomal vesicles and protoplasts of *S. aureus* (4). The wall murein is stripped cleanly off (Fig. 1D), and the cytoplasmic membrane of the cell appears to be intact wherever the cell wall has been removed.

Lysozyme Ch readily dissolved *S. aureus* cell walls in 0.5 M sucrose medium, as well as in aqueous buffers at pH 5.0. Figure 2 illustrates the dissolution of cell walls in 0.01 M acetate buffer at pH 5.0 from the intact wall in frame A to the fragments in frames B and C. Samples taken at later times than that shown in frame C contained no detectable fragments. Control preparations showed no changes in the absence of enzyme during the same period of time. Some fibrillar structures were occasionally observed during the reaction (Fig. 2D). These structures were not observed in the intact cell wall or the clear solution after complete dissolution of the walls. They probably represent some intermediate stage in the breakdown of the cell walls.

Schuhardt et al. (8) suggested that the lysozyme Ch protoplasts (3) were more closely related to L-forms than to protoplasts. Lysozyme Ch protoplasts are unable to regenerate cell walls and form colonies, and we have never observed the formation of L type colonies from lysozyme Ch protoplasts. These protoplasts swell slightly in 0.5 M sucrose (Fig. 1B), suggesting that this concentration is slightly hypotonic, but the membranes remain intact as evidenced by no loss in 260 nm-absorbing material (3). Therefore, the structures appear to be true protoplasts.

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