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Electron Microscopy and Viability of Lysostaphin-induced Staphylococcal Spheroplasts, Protoplast-like Bodies, and Protoplasts

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The cell walls of a selected isolate of *Staphylococcus aureus* FDA 209P were observed undergoing progressive disintegration when exposed to lysostaphin (1 unit/ml) in 24% NaCl solution. Electron micrographs of ultrathin sections of test cells after exposure to lysostaphin for 2 min showed only superficial evidence of lytic damage. However, an average of 89% of these cells were osmotically fragile, and 21% were damaged beyond their capacity to regenerate cell walls and to grow as normal staphylococci. The 68% (average) of the osmotically fragile cells which retained the capacity to revert to normal staphylococci were designated spheroplasts. Neither perforations of the cell walls nor separation of the cell walls from the plasma membranes were observed in the micrographs of these 2-min spheroplasts. Thus, it appears that the osmotic fragility of these and possibly all lysostaphin-induced staphylococcal spheroplasts results from the hydrolysis of a critical number of the pentapeptide cross-linkages of the murein of the cell wall. Electron micrographs of cells exposed to lysostaphin for 5 to 10 min showed perforations and more extensive damage, including the separation of walls from the plasma membranes and the disintegration of large sections of the walls. Smaller numbers of spheroplasts (21 and 8%) were recovered from these 5- and 10-min preparations; those recovered probably represent cells which were attacked more slowly than the majority by the lytic enzyme. The nonrevertible, osmotically fragile cells that retained segments of cell wall were designated protoplast-like bodies. After 20-min exposure to lysostaphin, all of the cell wall was digested away from most of the cells, and true staphylococcal protoplasts were produced. These lysostaphin-induced, osmotically fragile forms appear to have different osmotic properties from the staphylococcal "protoplasts" reported by other investigators and should serve as the basis for a variety of fundamental investigations.

Schuhardt and Klesius (10) reported a method for the production of staphylococcal spheroplasts by exposing *Staphylococcus aureus* FDA 209P cells to lysostaphin (8, 9) in 24% (w/v) NaCl solution. These studies utilized the same procedures, with the exception that Brain Heart Infusion (BHI) broth and agar were substituted for the Trypticase Soy media, and an isolate of the test organism showing a high degree of susceptibility to lysostaphin was selected. Also, the method of osmotically shocking the test and control preparations used in viability studies was modified to eliminate the necessity for centrifuging and resuspending the cell preparations prior to plating.

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MATERIALS AND METHODS

Viability studies. The selected isolate of *S. aureus* FDA 209P was grown in BHI broth with shaking at 37 C for 18 hr, and the cells were sedimented and resuspended in tris(hydroxymethyl)aminomethane (Tris) buffer to a Klett standardized turbidity of 550 (1.6 mg/ml, dry weight). This cell suspension was diluted 1:9 in a sufficient volume of 30% (w/v) NaCl solution at 37 C to provide the needed number of test and control preparations (9 ml each). At timed intervals and at 37 C, the test preparations received 1 ml of lysostaphin (10 units/ml), and the controls 1 ml of Tris buffer. The treatment times prior to transfer of

the preparations to an ice bath were 2, 5, 10, 20, and occasionally up to 60 min. To establish the effect of osmotic shock upon viability, the primary dilution (1:100) for plating of the test and control preparations was carried out in 99 ml of Tris buffer. For viability counts of nonshocked preparations, the 1:100 dilutions were made in 24% NaCl solution. All subsequent 10-fold dilutions for plating were performed in 24% NaCl solution. Overlay plating of suitable dilutions was done in quadruplicate in 72% sucrose semisolid BHI agar as described (10).

Electron microscope studies. Immediately after making the primary dilutions of the test and control preparations, 4% (v/v) Formalin was added to the remaining 8 ml of the suspensions, and the preparations were returned to the bath (37 C) for 2 hr for Formalin prefixation of the cells (10). The fixed cells were rinsed five times in Veronal acetate buffer at *p*H 7.1, and they were subjected to routine osmium fixation at 4 C for 17 hr (3). After this final fixation, the specimens were dehydrated in alcohol and acetone.

The cells were embedded in a plastic mixture consisting of 70% dodecenyl succinic anhydride, 20% Araldite 6005, and 10% Epon 812 with 1 drop of added accelerator DMP-30 (Rohm & Haas Co., Philadelphia, Pa.) per ml of plastic used. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife. The sections were stained with Reynolds' (7) lead citrate and viewed with a Hitachi HS-7S electron microscope with a double-condenser and 50-kv accelerating voltage. Micrographs were taken on Kodak projector slides, contrast grade.

Release of ultraviolet-absorbing material from lysostaphin-treated cells. Two experiments were performed to determine whether a significant proportion of the nonrevertible cells in the 30- or 60-min lysostaphin-treated preparations in 24% NaCl solution had undergone lysis or had leaked cell constituents. First, the 60-min test and control preparations were sedimented at 7,700 × *g*, and the supernatant fluid 24% NaCl fraction was decanted and saved. The cells were suspended in Tris buffer for osmotic shock, the residual cells and debris in the shocked test preparation were sedimented, and the supernatant fluid was saved. An ultraviolet absorption spectrum was run on each supernatant fluid using a Cary model 14 recording spectrophotometer. Absorption readings were recorded in a scan at wavelengths from 210 to 310 nm at a rate of 30 nm per min.

Release of tritiated deoxyribonucleic acid (DNA) from lysostaphin-treated cells. In a second experiment, *S. aureus* cells which had been grown in BHI broth containing 1 mc of tritiated thymidine per ml were washed in Tris buffer, adjusted to a Klett turbidity of 550, and exposed to lysostaphin in 24% NaCl for 30 min at 37 C, as previously described. The test and control cells were sedimented at 4 C, and the supernatant fluids were saved. The cells were suspended in Tris buffer for osmotic shock, the residual cells and debris were sedimented, and the supernatant fluid was saved. Finally, the sedimented cells and cellular debris were suspended to the original volume in Tris buffer. Radioactivity counts were obtained on 0.1-ml samples dispensed in 15 ml of the scintillation counting fluid of Lin, Mosteller, and Hardesty (4). The samples counted were (i) the supernatant fluid 24% NaCl solution from the test and control preparations, (ii) the supernatant fluid Tris buffer after subjecting the sedimented cells from (i) to osmotic shock, and (iii) the resuspended residual cells and cellular debris. Background radioactivity counts were determined on the 24% NaCl solution and on the Tris buffer. The counts were determined in an Ansitron liquid scintillation counter making duplicate 20-min counts.

RESULTS

Viability studies. Table 1 presents the average yields of the osmotically fragile spheroplasts and other forms from three to five experiments. Invariably our maximal yields of spheroplasts (average 68%) occurred during the first 2 min of treatment with lysostaphin. At this time, although 11% of the test cells were not yet osmotically fragile, 21% were both osmotically fragile and damaged beyond their capacity to revert to normal staphylococci. This capacity of the fragile cells to revert decreased rapidly with increasing time of exposure to the lytic enzyme.

Table 2 presents results of a type occasionally observed. Here, the revertible spheroplast colonies in the 2-min exposure preparation constituted 158% of the colony-forming units counted in the untreated controls. We interpret such results as evidence that one of the early effects of lysostaphin is the separation of pairs or clumps of staphylococci, many of which during the first 2 min developed osmotic fragility but were revertible to normal staphylococci.

Electron microscopy. Figures 1–6 depict the results of exposing *S. aureus* cells to the action of

TABLE 1. *Effect of the time of exposure to lysostaphin upon the recovery of normal staphylococcal colonies*

Exposure time[a]	Per cent of treated cells recovered[b] as:		
	Osmotically stable cells	Osmotically fragile spheroplasts	Not recoverable
min			
2	11	68	21
3.5	3	30	67
5	6	21	73
10	1	8	91
20	0.1	2.6	97.3

[a] Exposed to lysostaphin (1 unit/ml) at 37 C in 24% (w/v) NaCl solution.

[b] Per cent of total cells treated as indicated by control counts. Results are averages of five experiments, except for the 3.5-min exposure which are the averages of three experiments.

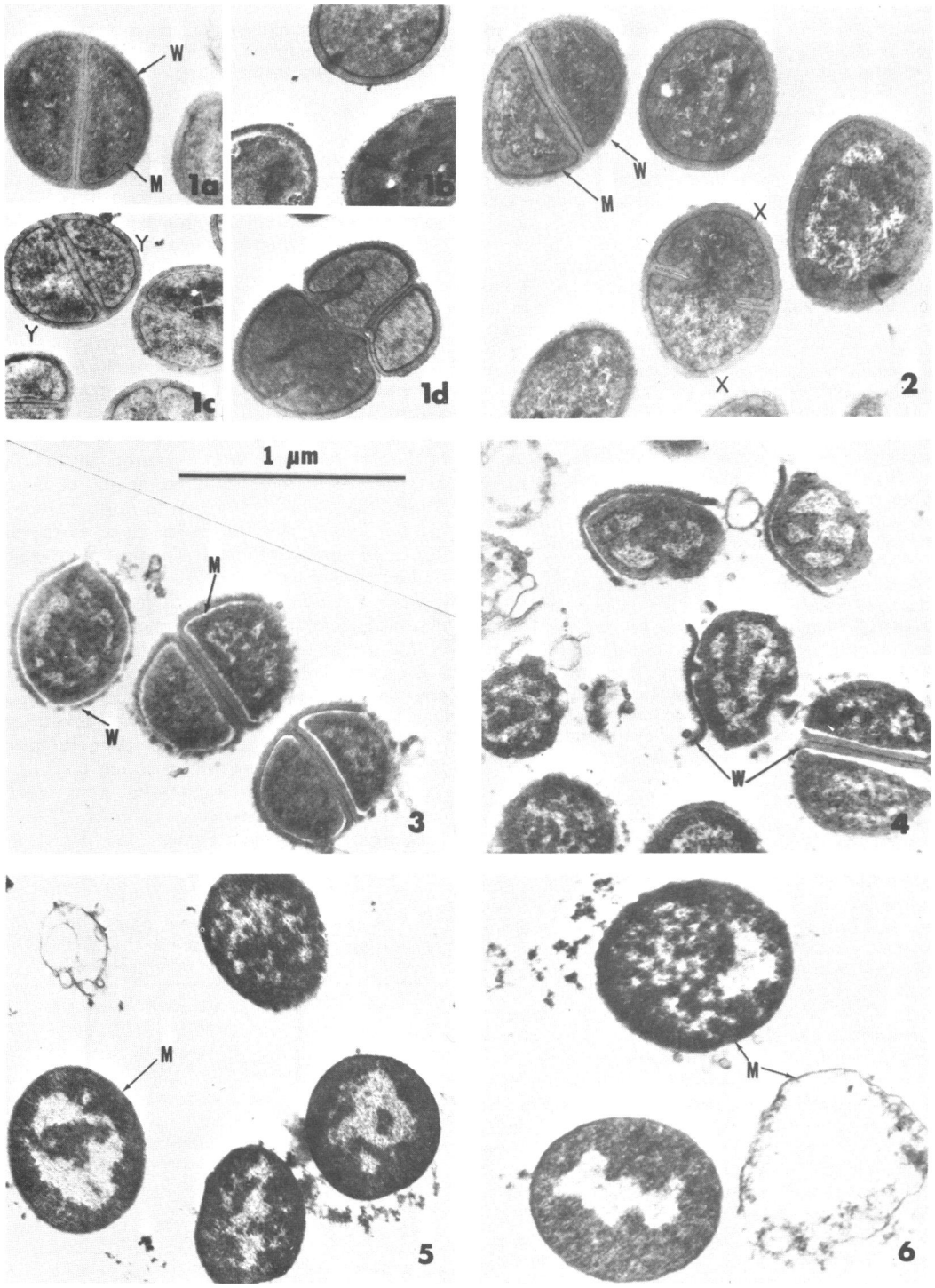

FIG. 1–6. *Thin-section electron micrographs of lysostaphin-treated test and control staphylococcal cells. Fig. 1a, 1b, 1c, and 1d are 2-, 10-, 20-, and 60-min controls, respectively (no lysostaphin). Figures 2, 3, 4, 5, and 6 are 2-, 5-, 10-, and 60-min test preparations, respectively. W, cell wall; M, plasma membrane; X-X and Y-Y, areas enlarged in Fig. 7.*

lysostaphin (1 unit/ml) for various times in 24% (w/v) NaCl solution. Figures 1a, 1b, 1c, and 1d are representative of control cells (no lysostaphin) exposed to the 24% NaCl solution for 2, 10, 20 and 60 min, respectively. Figure 2 shows the appearance of osmotically protected cells after exposure to lysostaphin for 2 min. The cell walls were essentially intact and were in intimate association with the plasma membranes. Careful scrutiny, however, particularly of the enlargements of sites X–X and Y–Y of Fig. 2 and 1c as presented in Fig. 7, reveals evidence of diffuse disintegration of the outer surface of the test cells (X–X). These are the cells which, although osmotically fragile, were revertible to normal, osmotically stable staphylococci (*see* Tables 1 and 2). They are the forms we call spheroplasts. The 21% of the 2-min preparations, which on the average failed to revert (*see* Table 1), probably passed through the spheroplast stage more rapidly than the 68% which reverted to normal cells.

Figures 3 and 4 are cells which had been exposed to lysostaphin for 5 and 10 min, respectively. The progressive disintegration of the cell walls and the separation of these from the plasma membranes are obvious. Even with extensive destruction of the cell walls, dividing staphylococcal cells did not immediately assume spherical configurations. These 5- and 10-min test preparations yielded averages of 21 and 8% spheroplasts, respectively. Probably these represented cells which were more like those shown in Fig. 2 rather than the ones shown in Fig. 3 and 4. Since many, if not all, of the nonrevertible forms of Fig. 3 and 4 had residual cell wall attached, they could not be designated protoplasts, but they were called protoplast-like bodies.

Figures 5 and 6 are cells which were exposed to

TABLE 2. *Type of results obtained on occasional individual experiments*

Exposure time[a]	Staphylococcal colony counts		
	Osmotically shocked cells[b]	Not shocked	Spheroplasts
min			%
0 (controls)	1.1×10^9	1.2×10^9	
2	9.6×10^7	2×10^9	158
5	2.5×10^7	2.5×10^8	19
10	8.4×10^6	4.7×10^7	3
20	1.4×10^6	3×10^7	2

[a] See Table 1.

[b] Primary dilution was 1:100 in 99 ml of Tris buffer.

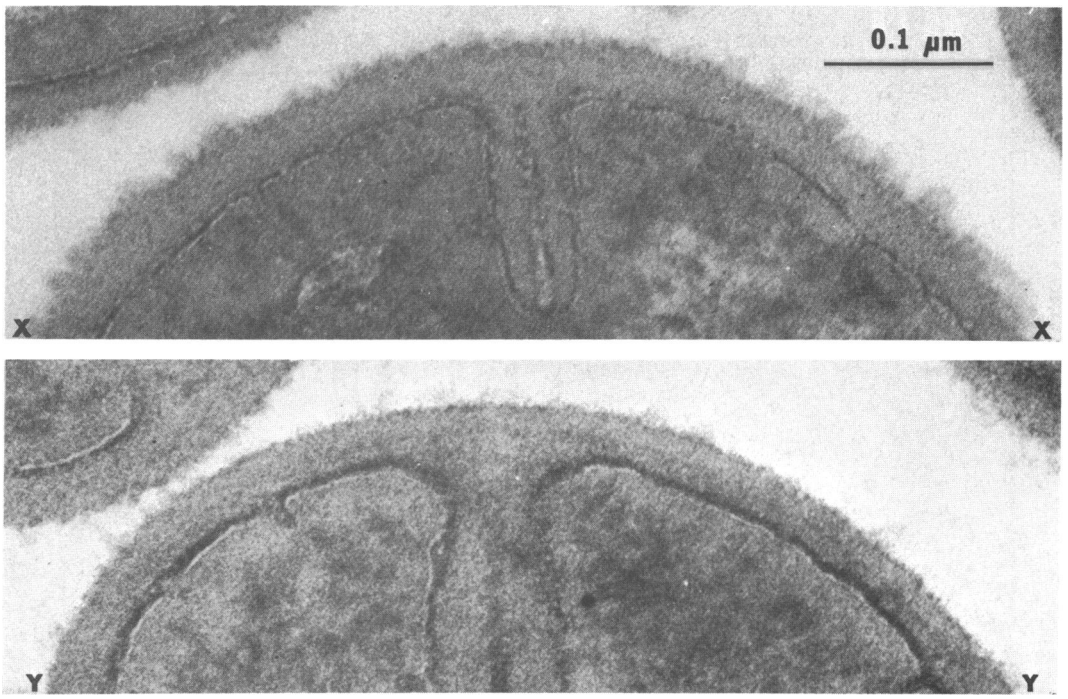

FIG. 7. *Enlargements of Fig. 2 (X-X) and 1c (Y-Y) illustrating the diffuse disintegration of the outer surface of the 2-min test cells (X-X), which although osmotically fragile, are revertible to normal staphylococci.*

lysostaphin for 20 and 60 min, respectively. The cell walls of these typical protoplasts have been completely eliminated. We recovered about 2% spheroplasts from cells treated with lysostaphin for 20 min (Tables 1 and 2). There is little liklihood that the cells (Fig. 5 and 6) were spheroplasts, but were representative of the 98% nonrevertible forms.

The empty membranes (Fig. 5 and 6) suggested the possibility that a significant number of the test cells were undergoing rupture at some time during or subsequent to the lysostaphin treatment. If this phenomenon occurred during treatment, it could be a factor in our low recovery of revertible cells after the 5- to 10 min-exposures to the enzyme. Consequently, we designed two experiments to determine whether significant rupturing of the cells occurred during the exposure to lysostaphin in the 24% NaCl solution.

Release of 210- to 310-nm absorbing material from lysostaphin-treated cells. Figure 8 presents evidence which indicates that little, if any, ultraviolet-absorbing materials were released into the 24% NaCl solution during the 60-min exposure of the *S. aureus* cells to lysostaphin. Osmotic shocking of the 60-min exposure cells, however, resulted in the release of such material.

Release of tritiated DNA from lysostaphin-treated cells. Figure 9 presents evidence that indicates that very little radioactive DNA was liberated into the 24% NaCl solution during a 30-min exposure of tritium-labeled *S. aureus* cells to lysostaphin. This activity was released by exposing these cells to osmotic shock.

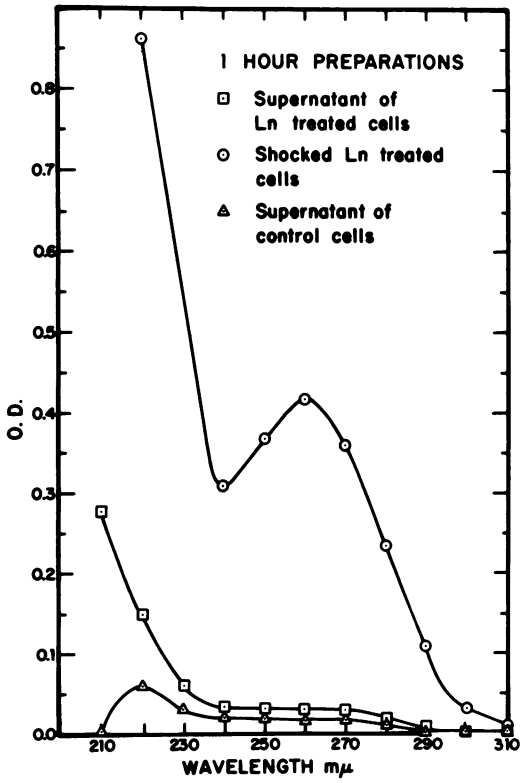

FIG. 8. *Release of 210- to 310-um ultraviolet-absorbing material by cells exposed to lysostaphin (Ln) for 60 min in 24% NaCl solution.*

DISCUSSION

In preliminary viability studies on lysostaphin-induced staphylococcal spheroplasts, Schuhardt and Klesius (10) obtained their maximal yields after 5- to 10-min exposure to lysostaphin. These investigators did not test for yields at less than 5-min treatment time. The fact that in these studies our maximal yields of spheroplasts occurred after only 2-min exposure to lysostaphin is attributed, in part, at least to our use of a carefully selected, highly susceptible isolate of *S. aureus* FDA 209P. In limited studies with less susceptible staphylococci using 1 unit of lysostaphin per ml and in studies with the highly susceptible staphylococci using concentrations of lysostaphin below 0.5 unit/ml, we observed a tendency toward later yields of spheroplast maxima. Whether the BHI broth and agar used in these experiments was a factor in the rate of spheroplast formation was not determined.

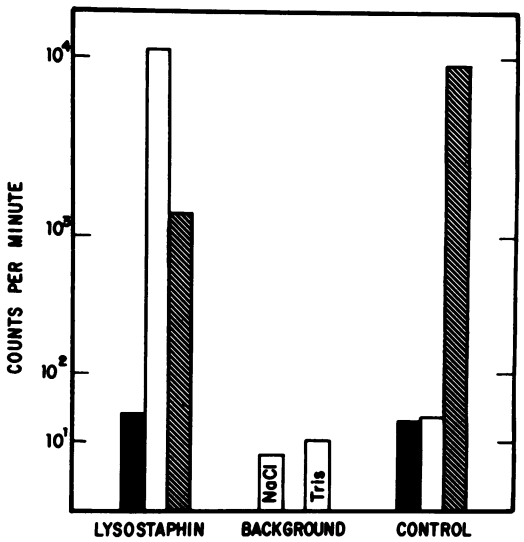

FIG. 9. *Release of tritium-labeled DNA into supernatant fluid 24% NaCl solution by 30-min test (lysostaphin) and control preparations. Solid bars, supernatant fluid NaCl solution; open bars, supernatant fluid of osmotically shocked cells; hatched bars, resuspended shocked cell sediment.*

Mitchell and Moyle (6) reported that their "protoplasts," produced from *S. aureus* cells by an autolytic process, were osmotically stabilized by 1.2 M sucrose or NaCl. Hash et al. (2) were able to stabilize their *N*-acetylhexoseaminidase-induced staphylococcal "protoplasts" with 0.5 M sucrose solution. Our lysostaphin-induced, osmotically fragile *S. aureus* cells required much higher NaCl (24% w/v or ca. 4 M) and sucrose (10) concentrations for maximal protection. Possibly the "protoplasts" described by the above investigators were more closely related to staphylococcal L forms than our osmotically fragile cells. We were unable to demonstrate significant L-form growth with our test preparations.

Our electron micrographs indicated that lysostaphin (1 unit/ml) in 24% NaCl solution rapidly attacks the surface of highly susceptible cell walls, probably hydrolyzing a sufficient number of the pentapeptide cross-linkages (1) to render most of the cells osmotically fragile within 2-min exposure to the enzyme. We found no evidence of discrete perforations in the cell walls of our 2-min revertible spheroplasts. With increasing time (5 to 10 min) of exposure to lysostaphin, perforations and more extensive damage to the cell walls were observed. These osmotically fragile cells continued to show the presence of fragments of cell wall, but most, if not all, of them were not revertible to normal staphylococci. We designated these nonrevertible, osmotically fragile cells, which retained attached cell wall material, protoplast-like bodies. After 20 min of exposure to lysostaphin, the cell walls of most of the staphylococci were completely removed, and true protoplasts were produced.

Limited metabolic studies indicated that our protoplasts respired as actively as normal staphylococci. However, they did not produce normal staphylococcal nor L-form colonies in our testing procedures. Our results indicated that very few of our test cells ruptured during 30- or 60-min exposures to lysostaphin in the 24% NaCl solution. Therefore, our osmotically fragile staphylococci should provide tools for a variety of fundamental studies. The spheroplasts can be used in cell wall repair and related studies. Comparative studies of spheroplasts and protoplast-like bodies, coupled with studies of the type reported by Malamy and Horecker (5) for their *Escherichia coli* spheroplasts, should yield information on the factor(s) that controls the revertibility phenomenon. The protoplasts can be used for metabolic and other plasma membrane related studies. Finally, all three forms and their supernatant fluids can be used for immunological studies.

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