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Lysostaphin-Induced, Osmotically Fragile *Staphylococcus aureus* Cells

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Staphylococcus aureus FDA 209P cells when suspended in 24% (w/v) NaCl were rendered osmotically fragile by exposure to lysostaphin for time intervals ranging from 2 to 60 min. Such cells were analyzed chemically and serologically for evidence of residual cell wall material, were plated in hypertonic sucrose medium to determine revertibility to normal, and were subjected to manometric studies to determine metabolic capabilities. Most of the cells (95%) which were exposed to lysostaphin (0.5 or 1.0 unit/ml) for 2 min, although osmotically fragile, retained their cell wall hexosamine and were capable of reverting to osmotically normal cells when plated in hypertonic medium. Cells exposed to lysostaphin for 5 and 10 min also retained much of their cell wall hexosamine, but lost their ability to revert to normal staphylococci. Cells exposed to lysostaphin for 2 to 10 min continued to react with staphylococcus anti-k antiserum. Complete removal of cell wall hexosamine was attained only after exposure to lysostaphin for 20 min or more; these cells failed to react with k antiserum. Lysostaphin-induced L-type colonies were extremely rare in our experiments, even if incubation times and media were optimal for their detection. Lysostaphin-induced staphylococcal protoplasts were as active metabolically in manometric studies as were untreated staphylococci.

The formation of osmotically fragile *Staphylococcus aureus* cells due to exposure to lysostaphin (7, 8) was described by Schuhardt and Klesius (10) and by Schuhardt, Huber, and Pope (9). Further observations on the biochemical, serological, and physiological properties of these osmotically fragile *S. aureus* cells are presented in this paper.

MATERIALS AND METHODS

Organism and culture media. *S. aureus* FDA 209P (9) was grown in Brain Heart Infusion (BHI; Difco) for 18 hr at 37 C with shake aeration. Hypertonic, semisolid BHI Agar for osmotically protected overlay plating of the fragile cells was prepared as previously described (10). *S. aureus* L-form 160 was obtained from J. H. Marston, Baylor University College of Medicine, Houston, Tex., and was grown in BHI containing 5% NaCl with or without 5% inactivated horse serum.

Lysostaphin treatment of cells and viability studies. Solutions of lysostaphin, buffered 30% (w/v) NaCl, and 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer were prepared as previously described (9). Approximately 10^9 cells/ml were exposed to lysostaphin in buffered hypertonic NaCl for timed intervals. Treated cell suspensions were assayed for revertible viability and for osmotic sensitivity by comparing the colony counts of osmotically protected and osmotically shocked preparations as described (9). In these, as in prior studies, a number of revertant spheroplasts colonies from the osmotically protected platings were transferred to BHI broth and agar to test for osmotic stability and for parental strain characteristics of the reverted cells.

Cell wall extraction, hydrolysis, and hexosamine determination. Approximately 1.3 mg (dry weight) of cells was extracted for peptidoglycans by the hot trichloroacetic acid method of Park and Hancock (6). The peptidoglycan preparations were hydrolyzed in sealed ampoules with 4 N HCl at 100 C for 4 hr. The hydrolysates of the peptidoglycans were evaporated to dryness, dissolved in 1 ml of deionized water, and analyzed for hexosamine by the method of Belcher, Nutten, and Sambrook (1).

Serological typing. Cells treated with lysostaphin, cells from colonies of reverted spheroplasts, and untreated control cells were fixed in 4% Formalin (10), washed in Tris buffer, and serotyped by the procedure of Cohen and Smith (2).

Metabolic activity of protoplasts. Protoplasts were formed by exposing *S. aureus* cells (3×10^{10}/ml) in buffered 24% NaCl to 50 μg of lysostaphin per ml for 1 hr at 37 C. Controls consisted of (i) 3×10^{10} cells/ml in buffered 24% NaCl and (ii) 3×10^{10} cells/ml lysed by exposure to lysostaphin in Tris buffer after which NaCl was added to a 24% concentration. The

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equivalent of 14 mg of dry whole cells was added to each manometric flask used for testing the metabolic activity of the protoplast, whole cell, or lysate preparations. Endogenous and glucose-supported (3.3 μg/ml) respiratory rates at 30 C were determined for each preparation, by use of a Warburg apparatus.

Lysostaphin treatment of S. aureus L-form 160. Approximately 7 $\times$ 10^8 *S. aureus* L-forms/ml were exposed in buffered 5% NaCl to 1 unit of lysostaphin per ml for 1 hr at 37 C. A control sample was incubated with Tris buffer instead of lysostaphin. Turbidimetric readings were taken at zero time and at 10 min intervals after the addition of lysostaphin. After lysostaphin treatment, viable *S. aureus* L-forms were enumerated by overlay plating of triplicate 0.5-ml amounts of the appropriate dilutions of the test and control suspensions in 4 ml of BHI soft agar onto BHI Agar (1.5%) plates. The BHI soft agar consisted of 0.8% agar containing 5% (w/v) NaCl and 5% (v/v) sterile, inactivated horse-serum.

RESULTS

Effect of lysostaphin concentration upon the formation of osmotically fragile staphylococci. The yields of osmotically fragile forms of *S. aureus* FDA 209P resulting from exposure of approximately 10^9 cells/ml to 1.0, 0.5, or 0.2 units (5, 2.5, and 1 μg/ml) of lysostaphin per ml are presented in Table 1. There was no significant difference in the yields of viable (revertible) and nonviable cells from preparations treated with lysostaphin concentrations of 1 and 0.5 units/ml. However, the preparation exposed to 0.2 units of lysostaphin per ml showed a significant decrease in the rate of formation of osmotically fragile spheroplasts. Here, the maximal yield (83%) was reached after 5 min of exposure to lysostaphin as contrasted to the maximal yields at 2 min with the higher concentrations. The percentage of spheroplasts in these samples was calculated with respect to the colony counts of the preparations treated for 2 min and the osmotically protected preparations rather than the untreated control counts. This was done because the former counts frequently were higher than the latter, owing to the early clump-dispersing properties of the lysostaphin (9). All reverted "spheroplast" colony cells tested grew on ordinary BHI culture media and were osmotically stable. They demonstrated the biochemical and phage-type characteristics of the parent strain. The number of osmotically fragile cells which became nonviable with respect to normal *S. aureus* colony formation increased rapidly with the time of lysostaphin treatment.

Serological typing. The *S. aureus* FDA 209P cells, when grown for 4 to 6 hr under optimal conditions for the expression of serotype antigens and tested with international *S. aureus* typing antisera, demonstrated serotype antigens a, b, h, and k. When reverted spheroplast colony isolates were comparably tested, they revealed the same serotype antigens. In 18-hr cultures of the untreated cells, the a and b antigens were blocked. Serotyping of Formalin-fixed, lysostaphin-treated, 18-hr culture cells yielded 1+ or greater agglutination with anti-k when cells exposed to lysostaphin for 10 min or less were used. Cells treated for 20 min or longer gave no evidence of agglutination in this antiserum.

Lysostaphin removal of cell wall hexosamine. Figure 1 depicts the effect of exposure to lysostaphin on the cell wall hexosamine content. About half the hexosamine present in untreated cells was still detectable on the osmotically fragile cells after 10 min of exposure to lysostaphin. Digestion of the cell wall into soluble fragments (not sedimentable at 4,000 $\times$ *g*) was essentially complete at 60 min.

Metabolic activity of protoplasts. The metabolic activities of protoplasts, whole cells, and cell lysates in 24% NaCl is illustrated in Fig. 2. Cell

TABLE 1. *Effect of lysostaphin concentration upon the recovery of viable staphylococci*

Exposure time[a] (min)	Percentage of treated cells recovered[b] as								
	Osmotically stable cells			Osmotically fragile "spheroplasts"			Not recoverable		
	1[c]	0.5	0.2	1	0.5	0.2	1	0.5	0.2
2	5	6	37	95	94	63	—	—	—
5	2	3	17	20	16	83	78	81	0
10	0.5	1	8	3.5	3.5	4	96	95.5	88
20	0.1	0.4	1.2	2.6	3.1	2.8	97.3	96.5	96

[a] Exposed to lysostaphin at 37 C in 24% (w/v) NaCl in Tris buffer (ca. 10^9 cells/ml).

[b] Percentage of total cells treated as indicated by the colony counts on the 2-min exposure preparations diluted in 24% NaCl. These counts frequently exceed the untreated counts because of the rapid clump-dispersing effect of lysostaphin.

[c] Lysostaphin concentration (units per milliliter).

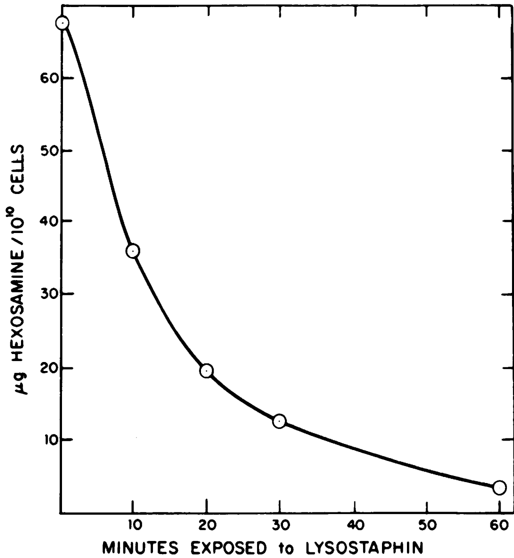

FIG. 1. *Effect of the time of exposure of S. aureus FDA 209P cells to lysostaphin (5 μg/ml) upon the cell wall hexosamine content.*

lysates failed to take up oxygen at a measurable rate. Protoplasts respired as well as, if not better than, whole cells in the presence of glucose. No cell wall hexosamine was detectable chemically in these protoplast preparations.

Lysostaphin treatment of L-forms. *S. aureus* L-forms, which possessed less than 0.3 μg of cell wall hexosamine per 10^{10} cells, were exposed to lysostaphin (1 unit/ml) to determine whether the enzyme might contain ingredients deleterious to a cell component other than the cell wall. Both turbidity measurements and colony plating indicated no significant effect of lysostaphin treatment on the staphylococcus L-forms. Lysostaphin-treated and untreated *S. aureus* L-forms were stable in 5% NaCl but rapidly lost turbidity and viability when suspended in hypotonic solutions.

DISCUSSION

In a prior paper (9), we described three types of lysostaphin-induced, osmotically fragile staphylococci: spheroplasts which could revert to osmotically normal cells, protoplast-like bodies which could not revert but retained cell wall, and protoplasts which neither possessed cell wall nor were revertible. L-forms constitute still another class of osmotically fragile staphylococcal cells. These were rarely detected in our osmotic fragility experiments. Efforts to determine the frequency of lysostaphin-induced L-forms of *S. aureus* FDA 209P indicated that L-forms arose from these staphylococcal cells at a frequency of only 10^{-6} to 10^{-7}. Our results are consistent with those of Dienes (3), who used other L-form inducing agents. However, they are lower than the maximal yields of staphylococcal L-colonies (0.002%) reported by Watanokunokorn et al. (11), who used graded doses of lysostaphin ranging from 1 to 600 μg/ml incorporated in the agar medium. These workers reported no L-colonies in preparations from both extreme concentrations of the enzyme.

The results of serological analysis for surface antigens and chemical analysis for cell wall hexosamine were consistent with our electron microscopic detection of cell walls (9). Chemical analysis detected more cell wall material in treated cells than did electron microscopic and serological analyses, because cell-free fragments of cell walls were more likely to be detectable by the chemical test.

The fact that *S. aureus* cells which were treated with lysostaphin for 5 or 10 min retained much of their cell wall as indicated microscopically (9) and chemically, but were unable to revert to osmotic normalcy, indicates that this residual staphylococcal cell wall was not capable of functioning as the "indispensable primer" (5, 12) for murein synthesis and reversion to osmotic normalcy as demonstrated for *Escherichia coli*. Since our staphylococcal protoplasts were found to be metabolically active, a number of experiments were directed toward attempts to induce reversion of our protoplasts and protoplast-like cells to osmotic normalcy. Revertibility in osmotically protected media was not increased in the presence of sodium citrate or other chelating agents, by sodium thioglycolate, nor by the

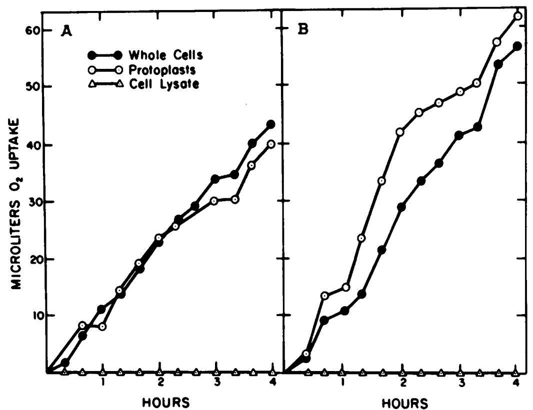

FIG. 2. *Metabolic activity of S. aureus FDA 209P protoplasts, whole cells, and cell lysates in 24% NaCl at 30 C. (A) Endogenous respiration. (B) Glucose-supported (3.3 μg/ml) respiration.*

presence of antilysostaphin antibody. Revertibility was not improved by plating in hard agar as reported by Landman and Halle (4), by incorporation of 10^9 autoclaved *S. aureus* cells per ml, nor by a trichloroacetic acid extract of 10^9 cells per ml into the agar plating media.

Since lysostaphin-induced protoplast-like bodies and protoplasts appear to be damaged only by the loss of part or all of the cell wall, elucidation of the factors which are lacking in these cells but are present in the revertible staphylococcal spheroplasts is a problem which warrants further investigation.

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