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Osmotic Fragility and Viability of Lysostaphin-induced Staphylococcal Spheroplasts

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When *Staphylococcus aureus* FDA 209P cells were treated with lysostaphin (1 unit/ml) in hypertonic sodium chloride or sucrose environments, viable, osmotically fragile spheroplasts were produced. Turbidimetric studies indicated that 64% (w/v) sucrose or 20 to 28% (w/v) sodium chloride gives maximal protection against lysis of the lysostaphin-treated cells. The NaCl appeared to give greater protection than the sucrose and proved to be much more suitable for viability and related studies. Viability of both shocked and nonshocked treated cells was determined by *S. aureus* colony counts on agar plates overlayered with the test dilution of the cells suspended in 4 ml of semisolid agar containing 72% sucrose. The difference in the counts represented the number of revertible spheroplasts. Under these conditions, 30 to 50% of the test cells were recovered as osmotically fragile, but revertible, spheroplasts after 5 to 10 min of exposure to lysostaphin in 24% NaCl. This re- were obtained after 5 to 10 min of exposure to lysostaphin in 24% NaCl. This recovery rate fell off rapidly with prolonged exposure. In view of residual turbidity of 30- and even 60-min exposure preparations, it appeared probable that most of the osmotically fragile cells were eventually converted to protoplasts by the prolonged lysostaphin treatment. Osmotically fragile cells were converted to osmotic stability by fixation with 4% (v/v) Formalin.

Mitchell and Moyle (7) were the first to report the osmotic properties of *Staphylococcus aureus* "protoplasts" obtained after autolytic changes in a hypertonic environment of 1 M sucrose. Hash et al. (4) described "protoplasts" which were obtained by treating *S. aureus* cells with a fungal *N*-acetylhexosaminidase in 0.5 M sucrose solution. The literature dealing with osmotically fragile bacterial cells has been reviewed by Weibull (10), McQuillen (5), and Martin (6). Such cells have been designated as protoplasts (with or without quotation marks), protoplastlike bodies, spheroplasts, L forms, and other less generally accepted names. Burke and Patee (2) designated osmotically fragile *S. aureus* cells as spheroplasts on the basis of the single criterion of turbidity changes after osmotic shock. The term protoplast has been widely used to designate osmotically fragile cells which, in addition to having lost their cell walls, have lost the ability to regenerate cell walls and to replicate. Consequently, we have chosen to make a distinction between protoplasts and spheroplasts on the basis of the ability of the osmotically fragile cells to rapidly repair or regenerate cell wall material, to revert to osmotic stability, and to replicate as normal staphylococci.

The discovery of the staphylolytic enzyme lysostaphin by Schindler and Schuhardt (9) has provided a means of studying the osmotic consequences of the action of this enzyme on the cell wall of the staphylococcus. Schindler (8) published electron micrographs showing the effect of lysostaphin on isolated mucopeptide fragments of *S. aureus* cell walls. Some of these fragments showed discrete perforations, which suggested the possibility of producing osmotically fragile forms if such perforations could be induced in the walls of osmotically protected, live staphylococcal cells.

MATERIALS AND METHODS

Test organism. S. aureus FDA 209P was used in all experiments reported here, although comparable results were obtained with other coagulase-positive *S. aureus* strains. The culture media used were BBL Trypticase Soy (TS) Broth and Agar (*p*H 7.4). Test cultures in TS Broth were incubated with shaking for 18 hr at 37 C.

Test preparations. The test cells were sedimented and suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (3) containing 0.045 M NaCl at *p*H 7.4 (Tris buffer). The suspension was adjusted to a turbidity of 550 on a Klett-Summerson photoelectric colorimeter having a no. 54 (500 to 570 nm) filter. This standardized *S. aureus* cell suspen-

sion was diluted 1:10 in all test and control preparations. The range of the final concentrations (w/v) of the hypertonic solutions tested was 8 to 28% for NaCl and 16 to 64% for sucrose. Lyophilized lysostaphin was dissolved in Tris buffer and was kept at −20 C until needed. Just prior to use, the lysostaphin was thawed and diluted in Tris buffer to 10 units/ml (9), and was kept in an ice bath until used.

Turbidimetric measurements. The test preparations in optically matched Klett tubes received hypertonic solution, lysostaphin, and the standardized *S. aureus* cell suspension. Controls received Tris buffer in place of lysostaphin. All tests were conducted at 37 C. After the addition of cells to the test and control preparations, Klett readings were recorded at 0, 5, 10, 30, and occasionally at 60 min. These readings were converted to the per cent reduction of the zero-time turbidity (% rt) for plotting graphs.

To demonstrate osmotic fragility, sedimented test cells in one of two duplicate tubes were suspended in Tris buffer for osmotic shock, whereas those in the second tube were suspended in the hypertonic diluent.

Test for viability of the osmotically fragile cells. After the desired incubation time at 37 C, the test and corresponding control preparations were transferred to an ice bath. This chilling was omitted in the preliminary testing when sucrose was used as one of the hypertonic solutions. After centrifugation of duplicate 5-ml preparations at 4 C for 30 min, the supernatant fluid was decanted and the cells in one test preparation and the cells in the corresponding control were suspended in 5 ml of Tris buffer for osmotic shock. The cells in the second test preparation were suspended in 5 ml of the hypertonic solution. Each preparation was diluted in hypertonic diluent for plating.

Duplicate 0.5-ml samples of appropriate dilutions of the test and control preparations were pipetted into tubes [3 by $\frac{5}{8}$ inch (7.62 by 1.58 cm)] containing 4 ml of melted (50 C) 0.8% TS Agar in 80% sucrose solution, and the mixture was poured onto the surface of a 1.5% TS Agar plate. The plates were incubated at 37 C until well-developed colonies appeared (48 to 72 hr). Plates containing between 30 and 300 colonies were counted with the aid of a Quebec colony counter. The number of revertible osmotically fragile cells (spheroplasts) was determined by subtracting the count of the osmotically shocked test preparation (residual stable cells) from the count of the osmotically protected test preparation.

Formalin fixation of osmotically fragile cells. Triplicate test and control preparations in 24% NaCl were incubated at 37 C for 30 min. One test and one control preparation received 4% (v/v) Formalin, whereas the other test and control preparations received an equivalent amount of Tris buffer. After 2 hr at 37 C, Klett readings were recorded and the preparations were centrifuged. The cells in the Formalin-fixed test preparation and in one unfixed test preparation and the cells in their controls were suspended in Tris buffer to determine the effect of osmotic shock. The cells in the other unfixed test preparation and in its control were suspended in 24% NaCl. Final Klett readings were taken on all preparations.

RESULTS

Turbidimetric studies. The results of efforts to determine the concentrations of NaCl or sucrose necessary for osmotic protection are illustrated in Fig. 1. In this figure, the change in optical density (% rt) at the end of the 30-min exposure to lysostaphin was plotted for each NaCl and sucrose concentration tested. Although considerable protection was obtained with 12% NaCl and with 30% sucrose, maximal protection was observed with 20 to 28% NaCl and with 64% sucrose.

The curves on the left of Fig. 2 illustrate the % rt for lysostaphin-treated cells and controls in Tris buffer and in 24% (w/v) NaCl. The columns on the right show the results of resuspending sedimented cells from duplicate 30-min, 24% NaCl test preparations in Tris buffer for osmotic shock and in 24% NaCl. Comparable results were obtained with 64% sucrose. These results indicated the osmotic fragility of the lysostaphin-treated cells, but did not provide any conclusive evidence concerning the extent of residual cell wall material or the growth potential of these fragile cells.

Viability studies. In the preliminary viability studies, we used both 24% NaCl and 64% sucrose test and control preparations. The test cells were exposed to lysostaphin for 30 min, centrifuged at room temperature, resuspended, and diluted for plating. Under these conditions, we

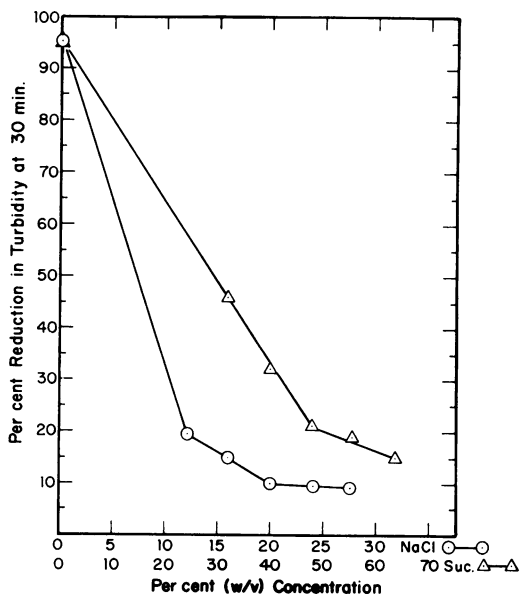

FIG. 1. *Effect of various NaCl and sucrose concentrations on the change in optical density of S. aureus suspensions treated with lysostaphin (1 unit/ml) for 30 min.*

recovered 1.2×10^7 (ca. 4.2%) spheroplasts from the sucrose preparation and 3.6×10^7 (ca. 10.6%) spheroplasts from the NaCl preparation.

Without chilling, the 64% sucrose preparations were extremely viscous, and with the ice-bath treatment they were impossible to handle. Consequently, all subsequent viability and other studies were done with 24% NaCl as the hypertonic solution. The results of a maximal spheroplast yield experiment (ca. 52%) are given in Table 1. In other experiments, we obtained higher spheroplast yields from the 5-min exposure to lysostaphin than from the 10-min exposure. In all instances, however, the yield of viable spheroplasts fell off rapidly after the maximum had been reached.

Formalin fixation of osmotically fragile cells. After Formalin fixation, the osmotically fragile *S. aureus* cells showed practically no (1%) reduction in turbidity when suspended in Tris buffer; however, the unfixed cells showed an 81% rt under the same osmotic shock conditions. When the unfixed test cells were suspended in 24% NaCl, they showed practically no reduction in turbidity; the same was true of the unfixed control cells suspended in Tris buffer. Formalin fixation of the osmotically fragile *S. aureus* cells has provided a means by which electron micrographic and serological studies can be reported.

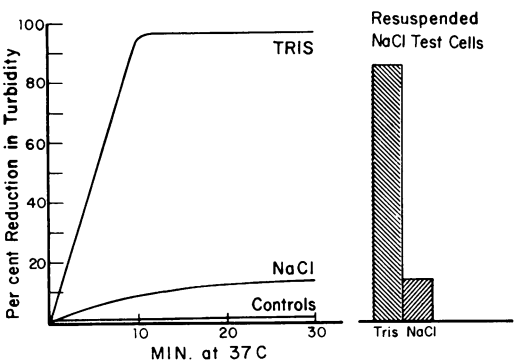

FIG. 2. *Effect of 24% (w/v) NaCl on the change in optical density of S. aureus cells treated with lysostaphin (1 unit/ml).*

DISCUSSION

Our results indicate that lysostaphin attacks the cell wall of *S. aureus* in the presence of hypertonic environments of sucrose (64%, w/v) or NaCl (24%, w/v), and that these hypertonic environments protect many, if not all, of the attacked cells from the osmotic disintegration which occurs in the absence of osmotic protection. The hypertonic concentrations necessary for maximal osmotic protection were markedly higher than those previously reported for staphylococcal protoplasts (4, 7) and for L forms (5% NaCl). Whether the osmotic fragility of the attacked cells is due to (i) discrete perforations of the cell wall, (ii) weakening of the cell wall as a result of hydrolysis of the polyglycine bridges cross-linking the mucopeptide polymers (1), or (iii) some other form of damage to the cell wall remains to be determined. We have repeatedly observed spheroplast counts ranging from 30 to 50% or higher during the first 10 min of lysostaphin treatment as well as a uniform tendency of these counts to fall off rapidly after the observed maxima. This tendency of the revertible spheroplasts to decrease after 5 to 10 min of treatment, without comparable decrease in turbidity, raises the question of the nature of the residual cells. Protoplasts are probably produced by prolonged lysostaphin treatment. However, electron micrographs of ultrathin sections and chemical and serological studies are necessary for the elucidation of the exact nature of both the

TABLE 1. *Effect of the time of exposure to lysostaphin upon the recovery of viable spheroplasts from 24% (w/v) NaCl preparations*

Lysostaphin exposure time[a]	Control colony count	Avg colony counts/ml[b]			
		Nonshocked count	Shocked count	Spheroplasts[c]	
				Count	Per cent
min					
5	1.8×10^8	5.4×10^7	2.0×10^6	5.2×10^7	28.9
10	1.5×10^8	7.8×10^7	1.6×10^5	7.78×10^7	51.8
30	1.3×10^8	2.0×10^7	6.8×10^4	1.99×10^7	15.3

[a] In 24% NaCl.
[b] After the cells were centrifuged and suspended in 24% NaCl (nonshocked) or in Tris (shocked).
[c] Count equals the difference between nonshocked and shocked (residual osmotically stable) cell counts.

revertible spheroplasts and the nonrevertible, osmotically fragile forms observed in this study.

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