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Preparation of Metabolically Active *Staphylococcus aureus* Protoplasts by Use of the *Aeromonas hydrophila* Lytic Enzyme

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Stable, metabolically active protoplasts of *Staphylococcus aureus* have been prepared by the use of a staphylolytic enzyme produced by *Aeromonas hydrophila*. Respiratory and glycolytic rates and synthesis of nucleic acids, protein, and lipid in these protoplasts, stabilized variously in 1.1 M sucrose, 0.37 M sodium succinate, or 0.37 M sodium sulfate, have been shown to be comparable with the same parameters in intact cells under the same conditions.

Since the original studies on protoplasts of *Bacillus megaterium* by Weibull (16), protoplasts of many organisms susceptible to the action of lysozyme have been studied. Due to the intractability of the cell wall of *Staphylococcus aureus* to the action of this enzyme, protoplasts of this organism were not readily prepared until the availability of active staphylolytic enzymes. One of the earliest reports dealing with the metabolic activity of protoplasts of *S. aureus* prepared with enzymes of known specificity was that of Hash et al. (8) who used a fungal *N*-acetylhexosaminidase for removal of the cell wall. These protoplasts were stabilized by 0.5 M sucrose, and the only metabolic activity investigated was the incorporation of ^{14}C-alanine into cold trichloroacetic acid-insoluble material, which proceeded at about 60% of the rate of control cells.

Schuhardt and Klesius (15) obtained osmotically fragile bodies from *S. aureus* using lysostaphin for cell wall removal. The resultant protoplasts required 24% (4 M) NaCl or 64% (1.9 M) sucrose for stabilization, and the only metabolic function studied (10) was respiration in the absence and presence of glucose. Hirachi et al. (9) concentrated their efforts on studying the antigenic properties of *S. aureus* protoplast membranes. No extensive investigations of metabolic capabilities of *S. aureus* protoplasts appear to have been undertaken.

The present report describes a method for the preparation of metabolically active protoplasts from *S. aureus* using the *Aeromonas hydrophila* staphylolytic enzyme (2). The protoplasts formed respire, glycolyze, are active in macromolecular syntheses, and, as reported in a preceding communication (7), are very effective in synthesizing inducible penicillinase.

MATERIALS AND METHODS

Bacterial strains. Two strains of *S. aureus* inducible for penicillinase were used and have been described previously (3, 4, 6) together with their method of cultivation (6). The phage type 80/81 organism originally described has now been designated 2237. Cells were harvested in the early exponential phase and washed three times with 0.02 M tris(hydroxymethyl)aminomethane (Tris)-glycine buffer (pH 8.2 at 37 C).

Cells for the production of protoplasts were resuspended in 1.8 M sucose in 0.02 M Tris-glycine, pH 8.2, to give a concentration of 1 mg dry weight per ml. Sufficient *Aeromonas* lytic enzyme (0.02 to 0.08 ml/ml of cell suspension) was added to produce protoplasts (considered complete when the absorbance was 0.04 at 660 nm on dilution of the suspension with three volumes of distilled water) within 30 min of gentle swirling at 37 C. Four volumes of 1.8 M sucrose containing 10^{-2} M $MgSO_4$ were added as soon as removal of the cell wall was complete as the presence of magnesium in the medium during protoplast formation is known to inhibit severely the action of the lytic enzyme. This protoplast suspension was used in experiments reported below except for measurements of respiration and glycolysis when protoplasts were not diluted with sucrose solution containing Mg^{2+} but were used as such after the addition of solid magnesium sulfate to a final concentration of 7.5×10^{-3} M. In the preparation of protoplasts for some experiments, equiosmolar sodium succinate or sodium sulfate was substituted for sucrose. When comparisons of metabolic activity between cells and protoplasts were made, cell suspensions were treated as above except for exposure to the lytic enzyme.

Cell concentrations were determined at 660 nm on a Bausch and Lomb Spectronic 20 colorimeter by relating the absorbance to either dry weight or viable cell counts estimated from calibration curves. Viable cell counts were performed in duplicate by spreading 0.1-ml samples of a suitable dilution of cells on the surface of nutrient agar plates and incubating overnight at 37 C. The dry weight or numbers of protoplasts quoted in experiments refer to the dry weight or numbers of cells from which the protoplasts were derived. In this way direct comparisons of the metabolic activities of cells and protoplasts may be made, with no correction necessary for the amount of cell wall material removed.

Reconstituted ^{14}C-protein hydrolysate containing 13 radioactive L-amino acids and ^{3}H-thymidine were obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y., sodium acetate-*1*-^{14}C from International Chemical and Nuclear Corp., City of Industry, California, and uracil-*2*-^{14}C from the Radiochemical Centre, Amersham, England.

A. hydrophila staphylolytic enzyme was prepared by the method of Coles, Gilbo, and Broad (2) with the following modification. Excess triethylammonium acetate was removed from the pooled fractions of the AG 50 W resin eluate by Diaflo membrane (UM 10) ultrafiltration in place of Sephadex G-10 chromatography. This produced a concentrated solution of lytic enzyme with low salt content.

Incubation mixtures for incorporation of radioactive precursors were the same as induction mixtures (7) with the omission of inducer, but with the following additions: for protein synthesis, 0.1 mg of amino acid mixture (4) containing 0.1 μCi of ^{14}C-protein hydrolysate; for nucleic acid synthesis, 0.1 ml of 3 mM ^{14}C-uracil (0.1 μCi/μmol) or 0.1 ml of 3 mM ^{3}H-thymidine (16.7 μCi/μmol); for lipid synthesis, 0.1 ml of 25 mM ^{14}C-sodium acetate (1 μCi/μmol). Radioactive compounds were added after temperature equilibration at 37 C. At the end of the incubation period samples for the measurement of radioactivity incorporated into nucleic acids and proteins were treated as previously described (3, 5) and counted in a Nuclear-Chicago gas-flow counter.

Lipid synthesis was determined by following the incorporation of ^{14}C-acetate into membrane lipids. At the end of the incubation period, protoplasts were lysed with 1.5 vol of water and membranes were sedimented by centrifugation at 33,000 $\times$ *g* for 30 min. The pellet was then extracted by incubating with chloroform:methanol (1:1) for 30 min at 37 C. The chloroform-methanol solution was extracted twice with 0.5 vol of 0.3% aqueous NaCl solution, and a portion of the organic phase was placed on a planchet, allowed to evaporate, and counted in a Nuclear-Chicago gas flow counter. Incubation mixtures containing intact cells were diluted with 1.5 vol of 0.1 M sodium acetate. The cells were sedimented, washed with 0.02 M Tris-glycine buffer, and resuspended in this buffer to an absorbance of 0.5 to 0.8 at 660 nm. They were treated with lytic enzyme at 37 C until the absorbance had fallen below 0.04 and membranes sedimented as for protoplasts. Subsequent lipid extraction was as described above.

Respiration and glycolysis were measured by conventional manometric techniques. For respiration measurement, 2 ml of cells or protoplasts (0.8 to 1 mg/ml) and 0.5 ml of 0.08 M KH_2PO_4 (adjusted to pH 7.4 with KOH) were placed in the main vessel; 0.5 ml of 0.3 M glucose was tipped from the side arm after temperature equilibration. The temperature was 37 C, the atmosphere was air, and 0.2 ml of 20% KOH was in center well. For glycolysis measurement, 2 ml of cells or protoplasts (0.8 to 1 mg/ml), 0.1 ml of 0.04 M KH_2PO_4 (adjusted to pH 7.4 with KOH), 0.2 ml of 0.255 M $NaHCO_3$, and 0.2 ml of water were placed in the main vessel. A 0.5-ml amount of 0.3 M glucose was tipped from the side arm after temperature equilibration at 37 C. The aerobic glycolysis atmosphere was O_2:CO_2 (95:5); anaerobic glycolysis atmosphere N_2:CO_2 (95:5).

Electron microscopy. Protoplasts were prefixed in 0.3% glutaraldehyde, centrifuged, fixed by the method of Kellenberger, Ryter, and Séchaud (12), dehydrated in acetone series, embedded in Araldite, and sectioned with an LKB Ultramicrotome. Sections were stained with lead citrate (14) for 5 min and viewed in a Siemens Elmiskop I Microscope. Photographs were taken using Agfa-Gevaert Scientia 23D56 film.

RESULTS

The majority of the experiments reported below were carried out using both strains of organism, and, although the results are generally reported for one strain only, the conclusions drawn are equally applicable to both strains.

The conditions determined by Coles, Gilbo, and Broad (2) to be optimal for the removal of cell walls of *S. aureus* were assumed to be unsuitable for the formation of metabolically active protoplasts because of the high temperature (45 C) involved. A pH of 8.0 to 8.5 at 37 C was selected as being the optimum for the lytic enzyme and unlikely to be detrimental to the protoplasts. The above conditions were found to produce stable, metabolically active protoplasts as shown by the following criteria.

Morphology. Many photomicrographs such as those shown in Fig. 1 show the complete absence of residual cell wall material adhering to the outside membrane of the great majority of staphylococcal cells treated as above. This fact, together with their osmotic fragility and an inability to resynthesize visible cell wall after incubation in nutrient media (Fig. 1d) prompt us to refer to these structures as protoplasts. Viable counts in many representative experiments showed the number of cells resistant to the effect of lytic enzyme to be between 0.003 and 0.01%. It can be noted from Fig. 1 that the large number of mesosomes known to exist in intact cells (unpublished results; Beaton [1] published such data for the penicillinase con-

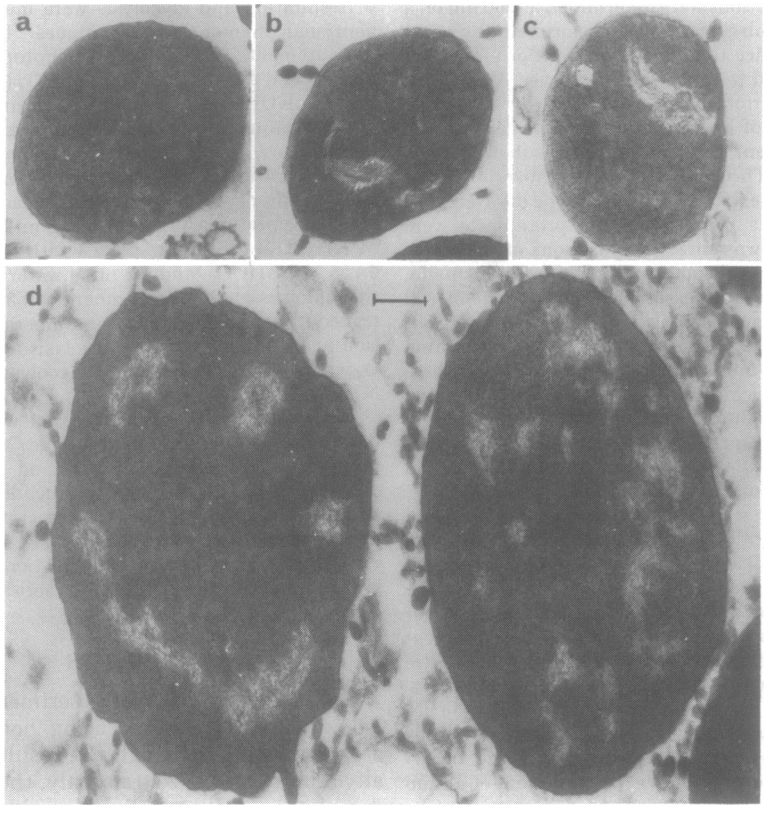

FIG. 1. *Electron micrographs of protoplasts of S. aureus stabilized in 1.1 M sucrose containing 0.017 M $NaHCO_3$ and 5 × 10^{-3} M $MgSO_4$. Samples were fixed immediately on preparation (a), after 90 min at 37 C (b), after incubation with 0.02 M glucose for 90 min at 37 C (c), and after incubation with 0.02 M glucose and 0.27% tryptose for 90 min at 37 C (d). Same magnification throughout, bar represents 200 nm.*

stitutive mutant 8325$\alpha i^{-}p^{+}$) are apparently lost, and small vesicles accumulate in the surrounding medium.

The absorbance of a suspension of protoplasts prepared as above in 1.8 M sucrose was maintained constant at 37 C over a period of 1 h (longest time tested). This osmotic stability was also maintained when the concentration of sucrose used was as low as 0.9 M. Electron micrographs of protoplasts in various sucrose media taken at the beginning and end of this period showed no morphological changes in structural appearance or size. It was found, however (7), that 1.1 M sucrose was the lowest concentration to give consistently reproducible levels of induced penicillinase formation in protoplasts. Two other compounds considered as potential osmotic stabilizers, sodium succinate and sodium sulfate, were also tested at this osmolarity (0.37 M) for their effect on the morphology of protoplasts, with similar results. No decrease in stability or changes in morphology of *S. aureus* protoplasts were noted on incubation in 1.1 M sucrose containing 0.01 M glucose at 37 C for 90 min. On the other hand, marked changes in size but not stability occurred when *S. aureus* protoplasts were incubated in 1.1 M sucrose containing peptone (0.27% Fisher Tryptose) and 0.02 M glucose. As the density of the cytoplasm and structures such as polysomes has not diminished and there has been a great increase in the amount of nuclear material (light areas in Figs. 1b, c, and d), it is evident (Fig. 1) that the increase in the size of the protoplasts supplied with nutrients has been brought about by synthesis of protoplasm rather than a mere swelling of the initial body. Replacement of sucrose with either sodium succinate or sodium sulfate as stabilizer in the above nutrient medium resulted in similar rates of growth of the protoplasts.

Respiration and glycolysis. The respiratory and glycolytic rates of whole cells and prepared protoplasts were compared, and the results are presented in Tables 1 and 2. Respiratory rates of protoplasts did not vary in the different

TABLE 1. *Respiration of cells and protoplasts in the presence of various osmotic stabilizers*

Stabilizer	Final concn (M)	Q_{O_2} (μliters of O_2 per h per mg [dry wt] of cells)	
		Cells	Protoplasts
Sucrose	1.1	65	77
Succinate	0.37	95	80
Sulfate	0.37	79	77

TABLE 2. *Glycolysis of cells and protoplasts in the presence of various osmotic stabilizers*

Stabilizer	Final concn (M)	$O_2(Q_{CO_2})^a$		$N_2(Q_{CO_2})^a$	
		Cells	Protoplasts	Cells	Protoplasts
Sucrose	1.1	51	48	96	83
Succinate	0.37	40	29	79	66
Sulfate	0.37	54	41	84	54

[a] Q_{CO_2} = μliters of CO_2 per h per mg [dry wt] of cells.

stabilizing media, whereas respiratory activity of intact cells was markedly influenced by the medium. In general, aerobic and anaerobic glycolytic rates of protoplasts were somewhat less than those of whole cells.

Protein synthesis. Figure 2 shows that protoplasts in either sucrose- or succinate-stabilizing medium are capable of incorporating ^{14}C-amino acids into hot trichloroacetic acid-insoluble material. In sucrose medium, comparable rates between protoplasts and intact cells are observed for about 60 min when cells rapidly increase their metabolic activity, whereas, in succinate-stabilizing medium, protoplasts and cells retain comparable incorporating ability.

Nucleic acid synthesis. Comparison of nucleic acid synthesis between protoplasts and cells was made using as a basis the incorporation of ^{14}C-uracil and ^{3}H-thymidine into cold trichloroacetic acid-insoluble material. Figures 3 and 4 compare the incorporation, respectively, of thymidine and uracil by cells and protoplasts. It can be seen that protoplasts actively incorporate both isotopes at rates initially only slightly less than those of whole cells.

Lipid synthesis. ^{14}C-acetate incorporation into membrane lipids was always more extensive with protoplasts than with intact cells in both sucrose- and succinate-stabilizing media (Table 3). Results expressed in Table 3 were derived from separate experiments and therefore do not permit a direct comparison of the relative effectiveness of these two stabilizers in promoting lipid synthesis.

DISCUSSION

The data presented indicate that protoplasts of *S. aureus*, prepared using the *Aeromonas* staphylolytic enzyme under the conditions de-

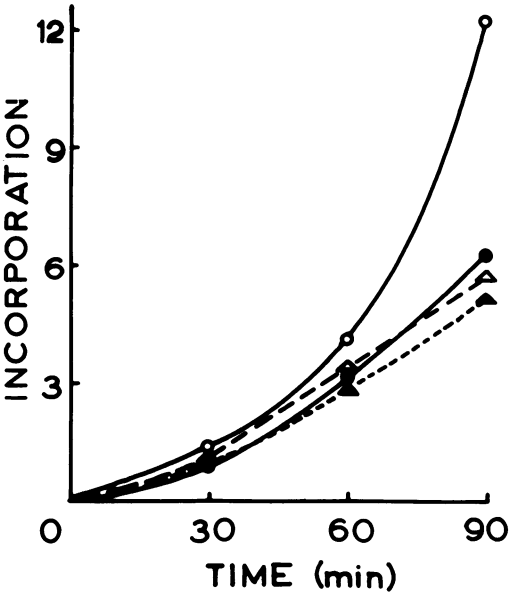

FIG. 2. *Comparison of incorporation of ^{14}C-amino acids by cells and protoplasts of S. aureus in two stabilizer media. Symbols:* O——O, *cells in 1.1 M sucrose;* Δ- - -Δ, *protoplasts in 1.1 M sucrose;* ●——●, *cells in 0.37 M sodium succinate; and* ▲- - - -▲, *protoplasts in 0.37 M sodium succinate. Incorporation measured in counts per minute per 10^6 cells or protoplasts.*

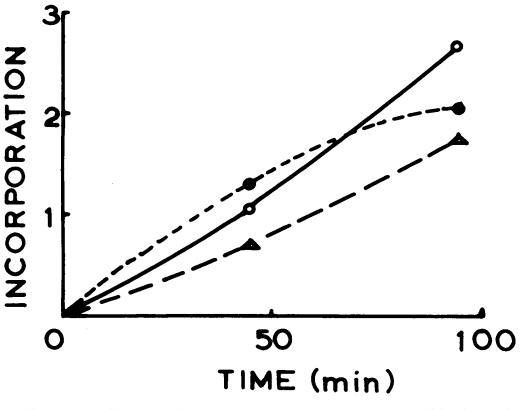

FIG. 3. *Comparison of incorporation of ^{3}H-thymidine by cells and protoplasts of S. aureus in two stabilizer media. Symbols:* O——O, *cells in 1.1 M sucrose;* Δ- -Δ, *protoplasts in 1.1 M sucrose or 0.37 M sodium succinate; and* ●- - - -●, *cells in 0.37 M sodium succinate. Incorporation measured in counts per minute per 10^6 cells or protoplasts.*

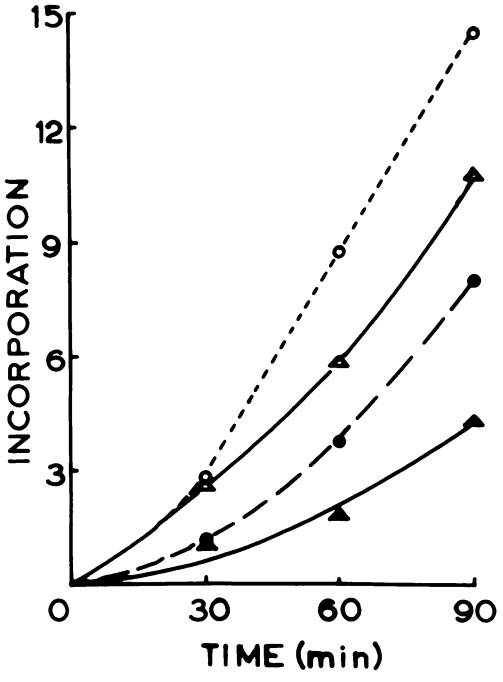

FIG. 4. *Comparison of incorporation of ^{14}C-uracil by cells and protoplasts of S. aureus in two stabilizer media. Symbols: O- - -O, cells in 1.1 M sucrose; Δ——Δ, protoplasts in 1.1 M sucrose; ●- -●, cells in 0.37 M sodium succinate; and ▲——▲, protoplasts in 0.37 M sodium succinate. Incorporation measured in counts per minute per 10^6 cells or protoplasts.*

TABLE 3. *The incorporation of sodium ^{14}C-acetate into membrane lipids by intact cells and protoplasts of S. aureus*[a]

Stabilizer	Expt 1 (1.1 M sucrose)	Expt 2 (0.37 M succinate)
Cells	28,300	34,120
Protoplasts	35,460	48,610

[a] Measured in counts per minute per milligram (dry wt) of cells, corrected for zero time incorporation (280 counts per min per mg).

scribed and devoid of most of their mesosomal structures, are metabolically active and that their anabolic and catabolic activities are comparable to those of intact cells.

The initial studies of Weibull (16) showed that protoplasts having similar respiratory values to intact cells could be prepared from *B. megaterium*. Our own results and those of Mitchell and Moyle (13) and Huber and Schuhardt (10) showed that protoplasts having respiratory rates similar to those of intact cells could also be prepared from *S. aureus*. However, marked differences in absolute Q_{O_2} values exist between our results and, for example, those of Huber and Schuhardt, who report Q_{O_2} values about 1.2% of those determined on our preparations. This is probably due to the inhibitory effect on the respiration of both cells and protoplasts of the high concentration of NaCl (4 M) necessary to stabilize protoplasts prepared with lysostaphin. The concentrations of sucrose needed to stabilize the protoplasts produced with the *Aeromonas* lytic enzyme were morphologically 0.9 M and functionally (as measured by induction of penicillinase [7]) 1.1 M, concentrations similar to those reported by others (8, 13).

It is interesting to note the higher respiration of cells in sodium succinate than in sodium sulfate or sucrose, whereas protoplasts show no differences in the different stabilizing media. If this result is an indication of a greater rate of transport of succinate in cells than in protoplasts, then the cell wall may contribute to this effect, possibly by confining some aspect of the transport mechanism to the periplasm of the cell.

Although the possibility of a slight swelling of our protoplasts in stabilizer medium supplemented with glucose and peptone cannot be excluded, the great increase in volume occurring under these conditions is compatible with normal growth without cell division. The observed synthetic capabilities of the protoplasts are consistent with this electron microscope evidence. In Fig. 1, the examples depicted were chosen by eye from lower magnification pictures to portray as far as possible sections that revealed the maximum diameter of the protoplasts. The great increase in size of protoplasts incubated with glucose and peptone is immediately obvious. From the electron microscope evidence alone, the extent of the increase cannot be stated with certainty because it is not proven that the sections shown are of maximum diameter. An estimated increase of four- to fivefold would probably not be in great error.

In all cases where comparisons of metabolic activities between cells and protoplasts have been made, the cells were subjected to the same conditions as the protoplasts except for exposure to lytic enzyme. It should be pointed out here that, in all stabilizers tested, the activities of the cells tend to be less than those of the same number of cells in the absence of stabilizer. In all parameters measured except the incorporation of acetate into membrane lipids, the activi-

ties of protoplasts have been less than those of the corresponding number of intact cells. The exception, where lipid synthesis by protoplasts exceeded that of whole cells, may be a reflection of rapid membrane synthesis now uncoupled from restrictive cell wall synthesis.

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