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Cellular Location of Degradative Enzymes in *Staphylococcus aureus*

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Staphylococus aureus, ATCC 6538P, was fractionated into protoplast membranes, mesosomal vesicles, periplasm, and cytoplasm. These fractions and the culture fluid were then assayed for various degradative enzyme activities. They were not restricted to a single fraction nor dispersed homogeneously, but were distributed predominantly (on the basis of specific activity) as follows: nuclease in the culture fluid; alkaline phosphatase, 5'-nucleotidase, and acid phosphatase in the periplasm; adenosine triphosphatase in the protoplast membrane; and protease (low levels) in mesosomal vesicles. No significant esterase nor cell wall hydrolytic activity was found in any fraction. *S. aureus* 80/81 was studied for penicillinase activity after induction with benzyl penicillin; this enzyme was localized in the mesosomal vesicles. Electron microscopy did not reveal any ultrastructural changes associated with secretion of the extracellular fraction. Overall, these studies demonstrate that degradative enzymes are located in several surface compartments and that, therefore, the mesosome does not function as a prototype lysosome in *S. aureus*.

Degradative enzymes, such as proteases, nucleases, and phosphatases, enable bacterial cells to use complex nutrients. Moreover, enzymes with similar activities modulate biosynthetic processes (1, 13). These enzymes require precise regulation to meet changing growth conditions and to prevent excessive autocatabolism. The cellular level of many enzymes varies according to the degree of repression (11). Specific compartmentalization would further facilitate the regulation of degradative enzymes, and the mesosome of gram-positive organisms has been suggested as a possible area for such localization (16). In support of this idea, Owen and Freer found that the mesosome of *Micrococcus lysodeikticus* contains a 15-fold greater concentration of autolytic activity against whole cells than the protoplast membrane (12). Reusch and Burger reported that esterase and acid phosphatase activities are associated with mesosomes in *Bacillus subtilis* and *Bacillus licheniformis*, respectively (16). In an effort to test this hypothesis more completely and to compare the degradative enzyme activities in mesosomal and plasma membranes, we have studied the cellular location of a number of degradative enzymes in *Staphylococcus aureus*. The results indicate that the mesosomal vesicle membrane is qualitatively similar to the plasma membrane in degradative enzyme activities, and that the periplasmic space contains significant quantities of most enzymes surveyed.

MATERIALS AND METHODS

Bacteria and culture conditions. *S. Aureus* ATCC 6538P (strain 209P) and a penicillin-resistant, coagulase-positive strain of *S. aureus*, phage type 80/81, clinical isolate at the National Institutes of Health, were used throughout. Cells were grown in AOAC synthetic broth (Difco) at 37 C on a rotary incubator shaker (250 rpm).

Protoplast formation and cell fractionation. Cells grown to late log phase were harvested by centrifugation, washed once in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, containing 0.02 M $MgSO_4$ (Tris-M), and resuspended in Tris-M containing 3.45 M NaCl, and *S. aureus* strain LS muralytic enzyme (18) exposed for 1 h at 37 C {1 liter of cell (culture optical density at 600 nm [OD_{600}] = 2.0)} representing 600 mg dry weight was resuspended to 40 ml in hypertonic buffer and treated with 10 ml of LS enzyme (8 mg of protein). This reaction mixture was then incubated an additional 15 min in the presence of deoxyribonuclease (DNase) (10 μg/ml), except for those preparations to be used for nuclease assays. The stability and intactness of the protoplasts was routinely monitored by electron microscopy and turbidometric measurements in the presence and absence of hypertonic buffer. Lysis or leakage of the protoplasts was measured by the release of cytoplasmic enzymes (see Table 1) and deoxyribonucleic acid (DNA). Less than 5% of the

total cellular DNA, as measured by diphenylamine-positive material, was ever found in the protoplasting medium.

Protoplasts, intact cells, and cell walls were removed by centrifugation at 10,000 × *g* for 1 h. The supernatant fluid from this pellet contained mesosomal vesicles (14) and substances released from the periplasm. To obtain protoplast membranes, the pelleted protoplasts were resuspended in Tris-M to the original volume of the reaction mixture and allowed to lyse for 15 min at 37 C. Cell debris (whole cells, cell walls, and unlysed protoplasts) was removed by centrifugation at 2,000 × *g* for 10 min. This supernatant fluid contained the protoplast membranes and cytoplasm. Membranes were collected by centrifugation at 20,000 × *g* for 20 min and washed three times in Tris-M. The cytoplasmic fraction was the supernatant fluid obtained after the first centrifugation of the protoplast membrane.

Mesosomal vesicles were collected from the protoplast supernatant fluid by centrifugation at 100,000 × *g* for 2 h and washed once in Tris-M. The supernatant fluid after the first centrifugation represented the periplasm. Sucrose density gradient centrifugation of mesosomal vesicles and protoplast membranes was done as described previously (14, 18).

S. aureus cell walls were prepared by the method of Huff and Silverman (8).

Protein concentration of all cell fractions was determined by the method of Lowry et al. (9) with bovine serum albumin (3 × crystallized) as a standard.

Electron microscopy. Methods have been previously described (14).

Preparation of radioactive S. aureus DNA. *S. aureus* ATCC 6538P was grown in AOAC synthetic media supplemented with 0.4% glucose to OD_{600} = 0.5. [6-H^3]thymidine (New England Nuclear) was added to a final concentration of 1 μCi/ml and incubation was continued until the cells reached OD_{600} = 1.0. Cells were harvested and DNA was extracted by the method of Marmur (10) using LS muralytic enzyme for cell wall lysis (18). The specific activity of the preparation was approximately 2,950 trichloroacetic acid-precipitable counts/min per μg of DNA.

Enzyme assays: (i) nuclease. The assay mixture consisted of 0.1 ml of H^3-labeled DNA in 0.1 M Tris-hydrochloride, pH 8.8, 0.05 M $CaCl_2$ (10 mM), and 0.1-ml suspension of the various cell fractions. The mixture was incubated for 30 min at 37 C, and then undigested DNA was removed by adding 0.1 ml of salmon sperm DNA (4 mg/ml in standard saline citrate) and 0.35 ml of 10% trichloroacetic acid. After 30 min on ice, the resulting precipitate was collected by centrifugation, and 0.5 ml of the supernatant was added to 10 ml of Aquasol (New England Nuclear) and counted in a Packard TriCarb Scintillation counter. Background activity was obtained by incubating the reaction mixture with 0.1 ml of 0.85% saline in place of the cell fraction sample.

(ii) Protease. The assay mixture consisted of 1.0 ml of sample and 1.0 ml of 1% Hammersten Quality Casein (Nutritional Biochemicals Co.) in 0.02 M Tris-hydrochloride, pH 7.8. $CaCl_2$ and freshly prepared cysteine were added to a final concentration of 0.5 mM. The casein solution was heat denatured for 10 min at 100 C before use. The assay mixture was incubated for 30 min at 37 C and then stopped with 5.0 ml of ice cold 10% trichloroacetic acid. Controls consisted of tubes incubated without substrate, to which 1.0 ml of casein solution was added prior to trichloroacetic acid treatment. After 10 min on ice, the resulting precipitate was collected by centrifugation and the supernatant read at 280 nm in the Beckman DU Spectrophotometer against the appropriate complete assay mixture incubated 0 min on ice before trichloroacetic acid addition (2).

Penicillinase was assayed by the method of Sargent (17). Adenosine triphosphatase (ATPase) and 5'-nucleotidase (AMPase) were assayed by the method of Davies and Bragg (4). Acid and alkaline phosphatase activities were determine by use of *p*-nitrophenol phosphate as a substrate (15). Glucose-6-phosphate dehydrogenase was assayed by measuring the reduction of nicotinamide adenine dinucleotide phosphate (5). Hexokinase was measured by following the decrease in OD using cresol red for an acid-base indicator (3).

(iii) Penicillinase induction. *S. aureus* 80/81 was grown to OD_{600} = 1.0 and then induced with benzyl penicillin (Sigma, 30 U/ml of culture).

RESULTS

Initially, the principal objective of these studies was to evaluate the mesosome as the primary cellular location for degradative enzymes and to compare the relative activities in the mesosomal vesicles and protoplast membranes. In addition, the periplasm and culture fluid, which potentially contain enzymes necessary for complex substrate catabolism, were also analyzed. The cytoplasm was included as a check on strictly intracellular degradative enzyme levels. The specific activities of several enzymes in the cell fractions from *S. aureus* ATCC 6538P are summarized in Table 1. Staphylococcal nuclease was found principally in the culture fluid, with a small cell-bound fraction of intermediate specific activity located in the periplasm. Alkaline phosphatase, acid phosphatase, and AMPase were localized primarily in the periplasmic fraction. The protoplast membranes contained the highest specific activity of ATPase. Only low levels of protease activity were found, and these were concentrated in the mesosomal vesicles.

Since the results are expressed in specific activities, the total activity in each fraction can be calculated from the total protein found in each fraction. The protein content for each fraction after a typical fractionation of 1 liter of *S. aureus* culture is shown in Table 1. Except for the ATPase activity, it can be concluded that a fraction with a high specific activity of a particular enzyme also contains a significant percent-

TABLE 1. *Degradative enzyme activity in various fractions of Staphylococcus aureus ATCC 6538P*

Fraction	Protein culture (mg/liter)	Enzymatic activity[a]							
		Nuclease	Alkaline phosphatase	Acid phosphatase	AMPase	ATPase	Protease	Hexokinase	Glucose-6-phosphate dehydrogenase
Culture fluid	30	2.83×10^6	0.64	4.18	*	*	<0.001	*	*
Cytoplasm	126	136	0.15	0.10	0.11	0.49	0.022	0.25	0.12
Periplasm	41.4	980	4.95	18.00	1.51	1.22	0.001	0.10	0.04
Protoplast membranes	30	94	0.11[b] 0.12 (0.14)[c]	0.96 1.22 (1.20)	0.05 0.10 (0.14)	1.92 1.86 (2.96)	0.022	<0.01	<0.01
Mesosomal vesicles	1.4	97	0.48 0.43 (0.17)	3.58 2.91 (0.92)	0.51 0.21 (0.10)	0.34 0.22 (0.39)	0.130	<0.01	<0.01

[a] Enzyme activities expressed as: nuclease, acid soluble counts per minute released per milligram of protein; alkaline and acid phosphatase, μmoles of p-nitrophenol released per milligram of protein; AMPase and ATPase, μmoles of Pi released per milligram of protein; protease, Δ OD_{280} per milligram of protein; hexokinase and glucose-6-phosphate dehydrogenase, units/minute per milligram protein. *, Not done.

[b] Assayed immediately.

[c] Fractions stored overnight at 4 C and assayed concurrently with sucrose gradient material shown in parentheses.

TABLE 2. *Time study of penicillinase distribution after induction with 30 U of benzyl penicillin per ml in S. aureus 80/81*

Fraction	Time after induction (min)[a]			
	30	60	90	120
Culture fluid	4.15	3.7	5.4	4.75
Whole cells	2.2	2.7	2.8	2.9
Cytoplasm	0.38	0.34	0.60	0.63
Periplasm	3.04	2.91	2.99	3.06
Protoplast membranes	1.53	2.1	2.50	2.10
Mesosomal vesicles	8.2	11.8	11.7	12.9

[a] Results are expressed as Δ OD_{490} per milligram of protein.

age of the total cellular enzyme. This conclusion is less certain with enzymes localized primarily in the mesosomal vesicle, since this organelle contained only a small part of the total cellular protein. However, as suggested by the mesosomal data on the penicillinase assays (Table 2), a high specific activity may reflect an important physiological role even if the number of units of activity is a small fraction of the total cellular pool.

Four enzymes were assayed before and after purification on sucrose gradients of a single lot of protoplast membranes and mesosomal vesicles. The values given in parentheses (Table 1) indicate that alkaline phosphatase, acid phosphatase, and AMPase activities in mesosomal vesicles decreased some 60 to 70% after this step; whereas there was either no significant change or a moderate increase (less than twofold) in specific activity after gradient centrifugation of the protoplast membrane fraction. These data indicate that these particular enzymes were not merely inactivated by additional manipulation (storage overnight at 4 C or overnight gradient centrifugation) and suggest that they may be loosely bound to mesosomal vesicles. Only the ATPase activity in mesosomal vesicles increased after gradient centrifugation, suggesting a slight purification. Hexokinase and glucose-6-phosphate dehydrogenase were included as cytoplasmic marker controls and were absent from both membranes and mesosomal vesicles (Table 1).

In addition to the tests tabulated, lytic activity against purified *S. aureus* cell walls and esterase activity on *N*-benzoyl-L-tyrosine ethyl ester were investigated; no significant activity was detected in any fraction. The LS muralytic enzyme preparation was tested in the various assays listed in Tables 1 and 2 and did not contain detectable levels of any enzyme activity.

Exogenous enzymes used during protoplast formation. Although the most stringent conditions were used to prepare the various cell fractions and their purity was routinely monitored throughout, one possible source of error is contamination of the various fractions with enzymes (such as the LS enzyme). This is particularly important with degradative enzyme studies. For example, we found large differences in nuclease activity when, as a test, we compared membranes and mesosomes prepared in the presence and absence of pancreatic DNase (data not shown). We also investigated this type of problem by adding lysozyme (250 μg/ml) to an *S. aureus* 6538P protoplasting

mixture. When the membranes and mesosomal vesicles were subsequently purified and tested against *M. lysodeikticus* whole cells, those samples prepared in the presence of lysozyme had definite lytic activity. This activity corresponded to 2 μg of lysozyme per ml (dry weight) of mesosomal vesicles and 1 μg/ml (dry weight) of membranes. Protoplasts prepared in the absence of lysozyme did not contain lytic activity against either *S. aureus* 6538P or *M. lysodeikticus* whole cells. These results illustrate that at least two exogenous enzymes (DNase and lysozyme) can bind to particulate cell fractions during purification.

Penicillinase induction. *S. aureus* 80/81 produced an inducible extracellular penicillinase. The results in Table 2 show the distribution of penicillinase with time after induction. At each time interval, the highest specific activity was in the mesosomal fraction; the mesosome-membrane ratio ranged from 4.7 to 6.1. Additional purification with sucrose gradients did not change the specific activities in either fraction.

Ghosh, Sargent, and Lampen demonstrated that penicillinase induction and secretion in *B. licheniformis* was associated with the formation of periplasmic tubules and vesicles (6). Similar structures are found in constitutive mutants and these organelles were thought to represent a secretory apparatus. Consequently, it was of interest to examine *S. aureus* 80/81 for ultrastructural changes associated with penicillinase induction and secretion. We found no differences in morphology between the 0-min sample and after induction at 30, 60, 90, and 120 min. This serial examination failed to demonstrate the formation of an elaborate secretory apparatus for the secretion of the extracellular fraction of penicillinase in *S. aureus*. Ideally, the mesosomal site would be the most strategic intracellular location of penicillinase activity, since these organelles arise from the membranous septum and precede the formation of crosswall.

DISCUSSION

These studies were designed to answer two related questions: (i) is the bulk of degradative enzyme activity in *S. aureus* concentrated in any one cell fraction? (ii) Are all the activities detected in the mesosomal vesicles also present in the protoplast membrane? The second question stresses current problems in determining the primary function of the mesosome. Many of the functions attributed to this organelle probably relate more to its origin as an invagination of the protoplast membrane system than to its primary cellular function. Therefore, establishing which activities (degradative or otherwise) are localized only in the mesosome will direct research efforts toward its more important functional roles. Furthermore, the level of degradative enzyme activity in this organnelle will be relevant to assessing the hypothesis that the mesosome is a lysosome-like body in bacteria (16).

The results of our studies in *S. aureus* 6538P and 80/81 are summarized in Tables 1 and 2. In brief, all degradative enzymes studied can be detected in most cell fractions, but each particular activity is concentrated in one of several distinct compartments. However, no one compartment contains the bulk of each activity, and the data fail to support the hypothesis that the mesosome functions as a prototype lysosome in bacteria. Furthermore, all activities detected in the mesosomal vesicles are also present in the protoplast membrane.

The quantities of alkaline phosphatase, acid phosphatase, and AMPase in the staphylococcal periplasm demonstrate the necessity of complete cell fractionation when one is attempting to localize a particular activity to either protoplast membrane or mesosomal membrane. For example, Reusch and Burger found high levels of acid phosphatase in mesosomal vesicles from *B. licheniformis* (16). However, without assaying the periplasm, one cannot conclude that the mesosome is the principal source of cellular activity; and conceivably this mesosomal activity could be relatively insignificant. Information from gram-negative organisms (principally *Escherichia coli*) also emphasizes the importance of the periplasm. In these bacteria the phosphatases, ribonuclease I, and DNase I are released by osmotic shock or spheroplast formation (7). Therefore the periplasm constitutes an important metabolic compartment, in both gram-negative and gram-positive organisms, for hydrolytic activities and uptake of potential carbon and phosphorous sources.

Reusch and Burger (16) have stressed the utility of membrane markers for differentiating protoplast and mesosomal membranes to routinely monitor preparation purity. Unfortunately, none of the degradative enzymes studied in this paper are suitable for this purpose (Table 1). However, other work in this laboratory has demonstrated an absolute chemical difference in glycerol teichoic acid content between protoplast membranes and mesosomal vesicles (E. Huff, R. M. Cole, and T. S. Theodore, Abstr.

Annu. Meet. Amer. Soc. Microbiol., p. 169, 1974). This difference may provide a better method for determining purity of membranes than previously used markers.

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