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Staphylococcus aureus H Autolytic Activity: General Properties

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Staphylococcus aureus strain H has an autolytic activity which can be found in the cell wall but is most easily obtained from high-speed supernatant fractions of broken-cell preparations. As measured either turbidimetrically or radioactively, this activity is much greater on murein extracted from penicillin-treated cells than on murein extracted from normal cells.

Many and perhaps all bacteria produce one or more murein depolymerases (6, 16). Although the function of these depolymerases is unknown, such murolysins could prove useful to the cell (i) in the biosynthesis of murein, (ii) in the possible turnover of murein (1), (iii) in the division of the murein sacculus (20) into two sacculi during cell division, (iv) in sporulation, (v) in transformation competence (22), or (vi) in extracellular digestion of exogenous murein to make assimilatable nutrients. Murolysins may also be important in the mechanism of final cell death as caused by some antibacterial agents as, for example, penicillin (13), especially when such death is accompanied by lysis. We discovered one of these depolymerases, an autolysin, in *Staphylococcus aureus* strain H several years ago during attempts to demonstrate an effect of penicillin on murein biosynthesis in a *S. aureus* cell-free system. This enzyme very readily attacked *S. aureus* H murein substrate prepared from cells grown in the presence of penicillin. We will denote such a substrate "penicillin murein." This enzyme was much less effective in dissolving normal *S. aureus* H murein.

The structure of a completed murein in any bacterium can be viewed as either a large polymer composed of relatively short glycan chains cross-linked by peptides or, alternatively, as short peptide chains cross-linked by glycan chains. Attack on either the glycan chains or the peptide chains (or on both) results in the formation of soluble fragments from the original murein sacculus. Penicillin murein is more readily attacked by lytic enzymes in general because the shorter peptide chains, resulting from penicillin inhibition of murein transpeptidase (19, 21), yield a more open structure. This allows not only a more rapid attack on penicillin murein as judged by faster solubilization of substrate (measured either turbidimetrically or radiochemically), but also a faster rate of attack on a per bond broken basis, presumably because of less steric hindrance. The bond hydrolyzed by the present enzyme was shown to be the *N*-acetyl muramyl-L-alanine amide linkage, as will be shown in a subsequent report.

Murolysins of various specificities have also been reported from other bacteriophage types of *S. aureus* (7, 8, 19) and from other species of *Staphylococcus* (18).

MATERIALS AND METHODS

Cell strain. *S. aureus* H, bacteriophage type 52A, 79,80, was used throughout this study.

Culture method. Cells were grown overnight at 37 C with shaking in "5-5-3" medium. This medium contained 0.5% Yeast Extract (Difco), 0.5% Phytone (Difco), and 0.3% K_2HPO_4. The medium was adjusted to *p*H 7.3, autoclaved, and then 0.1% glucose was added. In the morning, a 2% inoculum of overnight culture was added to fresh 5-5-3 medium containing 0.2% glucose. The cells, in late-exponential phase at 0.3 mg (dry weight) per ml [(OD) = 1.5 for a 1-cm light path at 582 nm], were harvested by centrifugation and washed with a large volume of cold 0.03 M potassium phosphate buffer (*p*H 6.8).

Enzyme preparation. Cells grown, collected, and washed as above, were suspended to give 50 to 70 mg (dry weight) of cells per ml in 0.03 M potassium phosphate buffer (*p*H 6.8). The cells were broken by shaking for 3 min in a Braun disintegrator with an equal volume of HCl-washed glass beads (no. 12, Ballotini). The suspension of broken cells and beads was treated with deoxyribonuclease and ribonuclease (each at 2 μg/ml; both crystalline preparations from Worthington Biochemical Corp., Freehold, N.J.).

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The beads were removed by decantation and low-speed centrifugation, and the supernatant fraction was centrifuged at 10,000 × *g* for 10 min. The 10,000 × *g* supernatant fraction was centrifuged at 35,000 × *g* for 30 min, and then at 100,000 × *g* for 1 hr. Where noted, the extract (100,000 × *g* supernatant fraction) was dialyzed against buffer overnight. All operations above were at 0 to 3 C. The extract was stored at −20 C.

Substrate preparation. Cells were grown and washed as described above and were resuspended in 0.03 M phosphate buffer (*p*H 6.8) to give 5 to 9 mg/ml. One part cell suspension was added to four parts of a medium containing amino acids, vitamins, and salts. The final concentrations were 0.01 M glucose, 0.001 M L-glutamic acid, 0.001 M glycine, 0.0005 M DL-alanine, 0.0002 M L-lysine, 10 μg of uracil/ml, 1 μg of nicotinamide/ml, 1 μg of thiamine/ml, 0.001 M $MgCl_2$, 0.0001 M $MnCl_2$, and 0.08 M potassium phosphate buffer (*p*H 7.3). This medium is called 4AA. Penicillin G and radioactive amino acids were added as required. Triple-strength 4AA medium had three times the above concentrations of buffer, salts, amino acids, and vitamins, and ten times the glucose concentration.

The cells were incubated in 4AA or triple-strength 4AA at 37 C for various lengths of time as specified in the figures. Cells used to make routine substrate were incubated with penicillin and radioactive amino acids for 6 hr in triple-strength 4AA, as in Fig. 3. The cells were then broken with glass beads, and walls were separated by differential centrifugation and treated with the Park and Hancock procedure (10) to purify them further. Final preparations of walls were freeze-dried and were then suspended in water or buffer and frozen prior to use.

Radioactive assay. A 0.025-ml amount of ^{3}H-L-lysine-labeled murein (2 mg/ml) preparation was added to a 4-ml polycarbonate tube and prewarmed at 37 C. A 0.025-ml amount of enzyme was then added, and the tubes were incubated at 37 C. At various times, tubes were removed and boiled for 1 min. Distilled water (0.20 ml) was added, and the tubes were centrifuged at 35,000 × *g* for 10 min. Then 0.20 ml of the supernatant fraction was counted in scintillation counting equipment.

Radioactivity determination. Samples in up to 0.5 ml of water were counted in 10 ml of scintillation counting fluid A: 1,930 ml of dioxane, 400 g of recrystallized naphthalene, 14 g of PPO (2,5 diphenyloxazole), and 0.6 g of POPOP [*p*-bis-2-(5-phenyloxazolyl)-1-benzene]. In later experiments, 0.2 ml of aqueous samples were counted in 10 ml of counting fluid B: 2,000 ml of toluene, 1,000 ml of ethylene glycol monoethylether, 12 g of PPO, and 0.3 g of POPOP.

RESULTS

Autolysis of broken cell preparations. *S. aureus* cells incubated in a medium containing glucose and the amino acids found in *S. aureus* murein will incorporate these amino acids almost exclusively into murein (10). Walls may be doubly labeled by incubating cells in such a medium (4AA), first with ^{14}C-glycine, and then with ^{3}H-glycine. When such cells are crushed and incubated, little solubilization of ^{14}C- or ^{3}H-labeled murein takes place (Fig. 1A). When, however, penicillin is present during the second incubation (Fig. 1B), the majority of this newly synthesized wall is dissolved by the murolysin. The wall of the same cell formed during the initial incubation, before the addition of penicillin, is only slightly affected. The small amount of solubilization of ^{14}C (Fig. 1B) may, in part, represent murein synthesized in the presence of penicillin from ^{14}C-labeled wall precursors. The lower content of ^{3}H in the penicillin cell homogenate at zero time represents the sum of a lowered synthesis of murein caused by penicillin action plus a loss of some murein resulting from

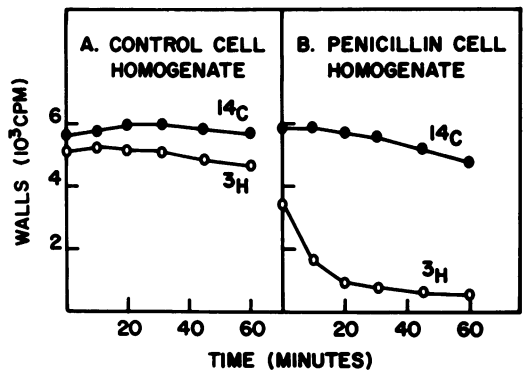

FIG. 1. *Autolysis of walls from glycine-labeled S. aureus H cells grown with and without penicillin. Cells were grown in 5-5-3 medium to late exponential phase ($OD_{582\ nm}$ = 1.8). They were then chilled, washed in 80 mM potassium phosphate buffer (pH 7.3), and concentrated to 8.1 mg (dry weight) per ml in the buffer. (A) Ten milliliters of cell suspension was added to 40 ml of 4AA medium with 2 μc of ^{14}C-2-glycine, and this mixture was incubated with shaking for 10 min at 37 C. The incubation was stopped with ice, and the cells were washed twice in cold buffer and resuspended in 10 ml of the buffer. This 10 ml of cell suspension was then added to 40 ml of 4AA medium with 10 μc ^{3}H-2-glycine and incubated for 10 min at 37 C with shaking. The incubation was stopped with ice, and the cells were washed twice in buffer and taken up in 2 ml of buffer. The 2 ml of suspension of double-labeled cells was then disrupted in a Braun disintegrator with 1.3 ml of Ballotini no. 12 glass beads for 2 min. A 0.7-ml amount of the homogenate was then incubated at 37 C in duplicate, and 0.1-ml samples were taken at various times. The reaction was stopped by boiling. The tube was centrifuged at 30,000 × g for 10 min, washed in buffer, recentrifuged at the same speed, and the pellet was counted in dioxane counting fluid. (B) The procedure was the same as A except that 10 μg per ml of penicillin G was present in the incubation with ^{3}H-glycine.*

autolysis at 0 C during preparation of the homogenate.

In the double-label experiments (Fig. 2) both lysine and alanine are incorporated into material synthesized in the presence of penicillin, which is readily solubilized by autolysis. Only a small amount of solubilization takes place in the homogenate of the control cells grown without penicillin. The ratio of lysine to glycine or of alanine to glycine stays quite constant during autolysis. Thus, the solubilization of any of these amino acids is a measure of release of murein peptide and, hence, of murein autolysis.

Measurement of activity. Assay methods for a murein hydrolase could be based on radioactive substrate solubilization, on turbidity lowering, or on colorimetric determination of reactive groupings released. The radioactive method does not require a chemically pure substrate. It does require that only the substrate be labeled. However, all of the substrate need not be labeled. For instance, the radioactive cell walls from cells labeled for short periods, as used for the crushed cell preparations of Fig. 1 and 2, are presumably only radioactive in a small region to either side of the equatorial growth zone of this gram-positive cell, whereas the majority of the wall is nonradioactive (4). On the other hand, accurate measurement of turbidity lowering would demand that a much greater fraction of the wall be suitable substrate. The most suitable substrate seemed to be wall synthesized in the presence of penicillin. To prepare such substrate containing a high proportion of murolysin sensitive wall (i.e., "penicillin wall") cells were incubated in triple-strength 4AA for as long as 6 hr, in the presence and in the absence of penicillin. The upper two curves in Fig. 3A show that, under these conditions, where protein was not being synthesized, the OD of whole cells, with or without penicillin, continued to rise for at least 6 hr. There was little or no cell lysis, although the penicillin concentration used here is more than 150 times the minimal inhibitory concentration for *S. aureus* H.

Although the rate of OD increase in whole cells is similar with or without penicillin, the yield of walls purified from the same amount of penicillin-treated cells (Fig. 3A, lower curves) was markedly less. In part, this is caused by an actual inhibition of the rate of wall synthesis by penicillin. In addition, there is a small but variable preferential loss of penicillin murein compared with control murein in the Park and Hancock procedure (*data not shown*). It is interesting to note that the turbidity of the wall fraction of the control cells is 50% of the tur-

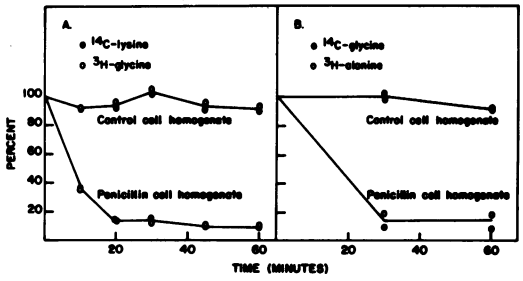

FIG. 2. *Autolysis of doubly labeled cells grown with and without penicillin. The conditions for these experiments are similar to those of Fig. 1, except that no ^{14}C-glycine preincubation was performed and two radioactive amino acids were incubated with the cells at the same time, with and without penicillin. The cells were incubated in the radioactive medium for 20 min, after which they were crushed and autolysis was allowed to take place as in Fig. 1. Results are expressed as per cent of zero time value. (A) Fifteen μc of ^{14}C-U-L-lysine and 6 μc of 2-^{3}H-glycine were used. (B) Thirty μc of ^{3}H-U-DL-alanine and 3 μc of 1-^{14}C-glycine were used.*

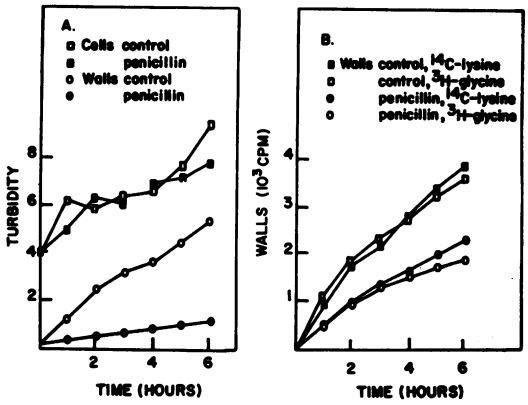

FIG. 3. *Six-hour incubation in 4AA. The preliminary preparation and incubation of cells in 4AA is similar to Fig. 1 with some exceptions: each of the two flasks contained 20 ml of triple-strength 4AA (see text); 26 mg (dry weight) of washed cells in 5 ml of buffer; 0.25 μc of ^{14}C-U-L-lysine and 2.1 μc of ^{3}H-2-glycine; 10 μg per ml of penicillin was present in one flask. At intervals during the incubation the OD was taken; 2-ml samples were removed and treated essentially by the method of Park and Hancock (10), and the resulting wall preparations were counted and the OD measured. (A) The OD of whole cells taken directly from the incubation in triple-strength 4AA is shown in the two curves at the top of the graph. The bottom two curves are the OD of the walls isolated from an amount of cells identical to that shown in the top of the graph. (B) The counts per min of the same wall fractions shown in the lower curves of Fig. 3A. The points represent the radioactivity of walls from a sample of cells that weighed 0.52 mg (dry weight) at zero time.*

bidity of the whole control cells, suggesting a very high ratio of wall to protoplasm in these latter cells. In Fig. 3B are shown the radioactivity of the same wall fractions whose turbidity is shown in the lower curves of Fig. 3A. These curves show good incorporation rates even at 6 hr. The glycine to lysine ratios stayed approximately constant over the 6 hr. Calculations based on the specific radioactivity of precursor amino acid and product wall show that, at 6 hr in the penicillin wall, over 75% of the total wall has been newly synthesized in the 4AA medium, the balance being old wall.

An assay for lysis by the 100,000 × *g* supernatant fraction for OD gives results similar to the radioactive assay (Fig. 4). At 40 min, the OD is 50% of the initial value, and the radioactivity is 50% of the final value. In subsequent work, OD and radioactivity assays were used interchangeably.

Attempts were made to assay the enzyme in various cell fractions by adding exogenous radioactive substrate. Activity is present mainly in the 100,000 × *g* supernatant fraction, and the cell wall fraction with much lesser amounts in the washed membrane fraction. The true amount of activity present in the supernatant fraction, relative to the wall fraction (3:1 as measured), is difficult to judge accurately, since adding the murein of the wall fraction to the radioactive wall substrate lowers the substrate specific activity. In addition, enzyme does not easily come off the wall as seen in other autolytic systems (13). Both of these factors act to give an underestimate of wall activity. Some of the wall enzyme may result from poorly reversible binding of cytoplasmic enzyme to wall after cell breakage. Even if the relative amounts of enzyme were accurately assayable, little might be contributed toward an understanding of the physiology of the action of this enzyme(s). No murolysin is found in the culture medium. With all these points in mind, we chose to study the cytoplasmic fraction for its ease of extraction and assay.

Activity as a function of pH. In Fig. 5, curve A, which was obtained with citrate and tris(hydroxymethyl)aminomethane (Tris) buffers, is markedly different from curve B, which was obtained with phosphate buffer. In curve A, the ionic strength was held near optimal level with KCl. When 0.1 M phosphate buffer alone (curve B) was used, a markedly different activity was seen. In this latter curve, the ionic strength was slightly supraoptimal and, of course, changed with the *p*H; this minor change of ionic strength is, however, distinctly a second-order variable as judged from ionic strength effects (Fig. 6).

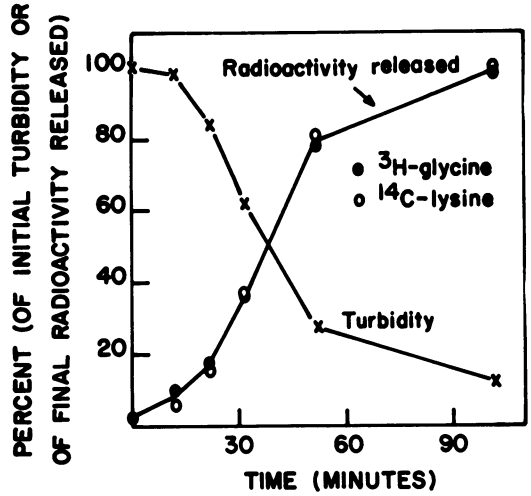

FIG. 4. *Comparison of turbidity lowering and radioactivity release. The substrate was purified murein from cells incubated for 3.5 hr with penicillin and* ^{14}*C-lysine essentially as in the procedure described in the legend to Fig. 3. The specific radioactivity of the murein was 9,000 counts/min per mg. The enzyme was a 100,000 × g supernatant fraction from cells crushed with glass beads in a Braun disintegrator. The enzyme was in 0.09 M potassium phosphate buffer (pH 7.3). Radioactive penicillin murein (0.4 mg) and enzyme from 49 mg (dry weight) of cells were in a total volume of 3 ml. The cells were incubated at 37 C for 100 min. Turbidity at 582 nm was followed, samples were boiled for 3 min, and radioactivity was assayed in the 40,000 × g, 10-min supernatant. Results for turbidity are expressed as per cent of the zero time value, and results for radioactivity are expressed as per cent of radioactivity released at 100 min; essentially all radioactive wall was solubilized by this time.*

Curves A and B of Fig. 5 were made with the same enzyme preparation.

Ionic strength and other effects. Effect of ionic strength on activity at optimal *p*H is shown in Fig. 6. In the curve shown, sodium phosphate was both the buffer and the "salt." Roughly similar curves were obtained when the major salt was KCl and only minor amounts of buffers were used (*data not shown*). Other properties found (*data not shown*) include the following facts. Ionic strengths in excess of 1 were markedly inhibitory to the supernatant enzyme. Magnesium ion was without effect on activity of the supernatant fraction or of the wall enzyme. One molar sucrose gave 90% inhibition of the supernatant enzyme. The presence or absence of teichoic acid on wall does not alter its atcivity as substrate for added supernatant enzyme. Added supernatant enzyme was without effect on whole cells.

DISCUSSION

Mitchell and Moyle (9) first showed autolysin activity in staphylococci 12 years ago. Here we are defining an autolysin as an enzyme in the cell wall itself or internal to the cell wall (i.e., not an extracellular lytic enzyme) that is capable of digesting the wall of that same cell. Mitchell and Moyle were able to prepare "protoplasts" from *S. aureus* Duncan and from several other *S. aureus* strains, merely by allowing the cells to stand in buffered 1.2 M sucrose, 0.3 ionic strength. The *p*H optimum was a broad one, peaking at *p*H 5.8. Walls remaining after autolysis were hemispheric, the attack having been in a ring shaped zone around the cell perpendicular to an equatorial ring. This enzymatic activity was apparently exclusively in the "wall fraction" of broken cell preparations in strain Duncan, but the mechanism of action was not investigated. The reason for the greater sensitivity of the attacked zone to the thickened, so-called equatorial ring, is complex. If the region attacked is the growth ring of the cell, the enzyme may be localized in this region perhaps, in part, bound to adjacent adherent membrane. In addition, it is likely that newly made murein has a lower strength in this ring because of thinness or lowered cross-linking, or both, and is therefore more susceptible to the autolysin. In any case, the vastly increased autolytic susceptibility of this putative growth ring is comparable to the increased susceptibility of penicillin murein over normal murein as reported in the present paper. Such a differential sensitivity suggests a possible role of the autolysin in cell death by penicillin or at least in the final lysis. The work of Schwarz et al. (14) strongly suggests this for *Escherichia coli*. However, the irreversible damage caused by penicillin may come at an earlier point, and the lysis of the cell itself may be a more passive, and in most cases, obligate stage after an earlier irreversible action of penicillin on murein synthesis.

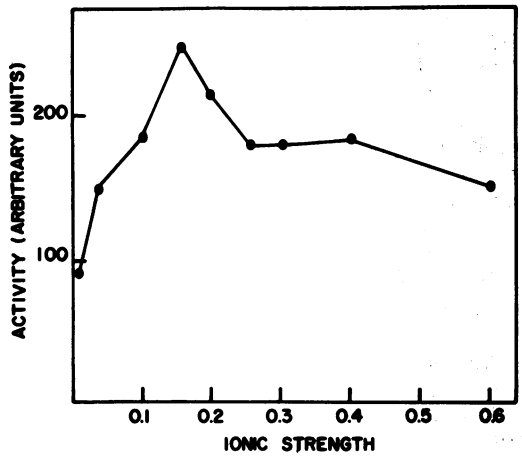

FIG. 6. *Effect of ionic strength on activity at optimal pH. Sodium phosphate was both the buffer and the "salt."*

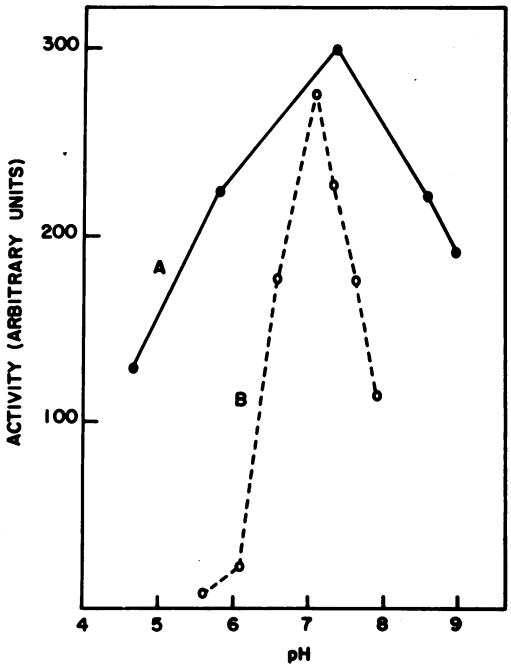

FIG. 5. *Effect of pH on activity using the radioactive assay. The enzyme preparation was 100,000 × g supernatant fraction dialyzed overnight against 0.001 M phosphate buffer (pH 7.3) and it was assayed as described in the methods section. Curve A is obtained with 0.1 M KCl and 0.01 M buffer [sodium citrate (pH 4.6 to 5.8) or Tris-hydrochloride (pH 7.4 to 9.0)]; curve B is obtained with 0.1 M sodium phosphate alone. One arbitrary unit equals 1 pmole of radioactive lysine solubilized per min under the conditions of the assay as described.*

Other lytic enzymes in *S. aureus* have been reported, including an extracellular enzyme that releases reducing groups in strain 524 (12), and, in strain Copenhagen, an autolytic amidase associated with an *N*-acetyl glucosaminidase activity (18). Strain Oeding 8507 has a similar amidase (Huff, Silverman, and Adams, Bact. Proc., p. 47, 1969). As will be shown in a subsequent paper (Singer, Takebe, Wise and Park, *in preparation*), the major autolytic activity of strain H is an amidase, as in strains Copenhagen and Oeding 8507, but glycosidase activity in strain H is probably quite low. Bacteriophage induced virolysins have been reported for staphylococcus (5, 11).

The autolysin(s) described here, as well as autolysins in general, should probably not be so called. Autolysis, when defined as lysis of a cell by its own enzymes, describes a suicidal act if the cell is still capable of reproducing. If the cell is not still capable of reproducing, autolysis might be useful for the common good, in the sense of helping in the recyclization of chemical elements in nature. Neither of these uses of autolysis is of very immediate important survival value for the microorganism possessing the autolysin, and autolysis is therefore probably only a fortuitous function of the enzyme. This makes it seem that our denotation of any enzyme as an autolysin is merely an admission of ignorance of its true physiological significance. Current enzyme nomenclature demands that an enzyme be given a name which denotes its most important role for the particular organism which possesses it. The true function of such a wall-attacking enzyme is unknown but it may act in cell division. Chatterjee and co-workers (2, 3) have found a pleiotropic mutant type of *S. aureus* H which numbers among its numerous defects: (i) an inability to divide properly and (ii) a 10-fold lowering of the autolytic activity found in the soluble fraction of the cell. However, a direct causal nexus between these two properties is lacking at the present time.

ACKNOWLEDGMENTS

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