L-Form-Like Colonies of *Staphylococcus aureus* Induced by an Extracellular Lytic Enzyme from *Pseudomonas aeruginosa*

M. A. FALCON,* T. B. MANSITO, A. CARNICERO, AND A. M. GUTIERREZ-NAVARRO

Departamento de Microbiología y Biología Celular, Facultad de Biología, Universidad de La Laguna, Tenerife, Canary Islands, Spain

An extracellular enzyme produced by *Pseudomonas aeruginosa* had a lytic effect on lyophilized *Staphylococcus aureus* cells. It was purified from the culture supernatant by ammonium sulfate fractionation followed by column chromatography with P cellulose and Sephadex G-50. The molecular weight of the enzyme was estimated to be 19,000 ± 1,750 with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The pI of the enzyme was estimated to be 8.5 with isoelectric focusing. The enzyme was inactive in 4% NaCl–40 mM sodium phosphate buffer or at pH values lower than 6.0 or higher than 11.0; however, it was not affected by 1 M sucrose or 0.25% heat-denatured horse serum. The action of the enzyme on cultures of *S. aureus* resulted in the presence of many cells lacking cell walls. In addition, when cultivation was carried out on osmotically stabilized solid media, these cell wall-deficient cells developed in L-form colonies.

Substances excreted in the culture media by *Pseudomonas aeruginosa* have been largely involved in several aspects of the biology of this bacterium. Some of them, proteases and elastases, have been reported as virulence factors (9, 13, 14). Some proteases, collectively called staphylolytic substances, have been purified by several authors who reported them as enzymes exhibiting in vitro lytic action on lyophilized cells of *Staphylococcus aureus* (6, 8, 18, 19).

In our laboratory, an interaction between *P. aeruginosa* and *S. aureus* was studied and reported as an amensalistic relation resulting in inhibition of the growth of *S. aureus* (2) caused by extracellular substances from *P. aeruginosa*, active not only on lyophilized cells but on growing cells of *S. aureus* as well (15).

The possibility that staphylolytic substances would act on the cell walls of members of the family *Micrococcaceae* was considered by Lache et al. (8), and we have reported that the action of the staphylolytic substances from *P. aeruginosa* results in structural damages in the cell wall of *S. aureus* (11).

Taking these facts into account, it was considered necessary to explore the possibility that some of those substances induce forms of *S. aureus* which lack cell walls and are able to grow as L-form colonies. If this is so, the results could explain therapeutic failures in patients simultaneously infected with *P. aeruginosa* and *S. aureus*.

The aim of this paper is to report the formation of L-form colonies by *S. aureus* cells when cultured in the presence of a purified extracellular enzyme from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* PAKS I, kindly supplied by B. Wretlind, was used as a source of the staphylolytic enzyme. This strain was originally isolated in 1970 from a urine specimen at the Karolinska Hospital, Stockholm, Sweden (18), and it was selected for this work on the basis of its ability to produce extracellular proteases (18).

S. aureus ATCC 6538 was used as the target microorgan-

ism for the staphylolytic enzyme. Both bacteria were maintained on Nutrient Agar (Oxoid Ltd., London, England).

In order to produce the lytic enzyme, *P. aeruginosa* was grown in a yeast extract-salts-glucose medium (MYG medium) with the following composition (per liter): glucose, 1.25 g; $(NH_4)_2SO_4$, 1 g; K_2HPO_4, 7 g; KH_2PO_4, 3 g; sodium citrate · $7H_2O$, 0.5 g; $MgSO_4$ · $7H_2O$, 0.1 g; yeast extract (Oxoid), 7.5 g (pH 7.2).

Identification of *S. aureus* was carried out by using the API Staph test (Analytab Products, Plainview, N.Y.) and by performing a coagulase test by the method of Fisk (1).

Preparation of the staphylolytic enzyme. In order to obtain the staphylolytic enzyme, *P. aeruginosa* was grown for 18 h (until about 10^9 CFU/ml) in 5-liter conical flasks containing 2 liters of MYG medium on a rotatory shaker at 37°C. After that, cultures were centrifuged at 5,000 × *g* for 15 min and the cell pellet was discarded. The supernatant demonstrated lytic activity on lyophilized cells of *S. aureus*. A 560-g portion of ammonium sulfate was added to each liter of supernatant under continuous stirring, and the mixture was allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 17,000 × *g* for 30 min and dissolved in 10 mM sodium phosphate buffer (pH 8.5).

Desalting of the sample was carried out by chromatography through a column (21 by 3 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 10 mM sodium phosphate buffer (pH 8.5), which was also used as eluent. A 25-ml sample was applied to the column. The flow rate was 30 ml/h, and 5-ml fractions were collected. The contents of tubes 12 to 20 were combined, and they were found to contain most of the staphylolytic activity.

A sample (19 ml) of the volume of the lytic solution recovered from the Bio-Gel column was passed through a P-cellulose (Cellex-P, Bio-Rad) column (18 by 4 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). Elution was carried out with a continuous gradient of ionic strength and pH obtained with mixtures of 20 mM sodium phosphate buffer (pH 8.5) and 100 mM sodium phosphate buffer (pH 10.0). The flow rate was 82.2 ml/h, and 8-ml fractions were collected.

Fractions 42 to 52 from P-cellulose chromatography were combined, concentrated by reverse dialysis against polyeth-

---

* Corresponding author.

ylene glycol, and dialyzed against 30 mM sodium phosphate buffer (pH 7.5). The volume recovered from the dialysis was concentrated to a 4-ml volume by reverse dialysis against polyethylene glycol and was passed through a Sephadex G-50 column (61 by 3 cm) equilibrated with 30 mM sodium phosphate buffer (pH 7.5), which was also used as eluent. The flow rate was 5.3 ml/h, and 4-ml samples were collected.

All operations were conducted at 4°C, and this procedure led to a 122-fold increase in specific activity over the original culture liquor.

Protein contents were determined by the method of Bradford (3).

Electrophoresis. Samples of purified staphylolytic enzyme (17 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method described by Hames (7) for 4 h at 80 mA. The proteins were detected by staining the gels with Coomassie blue.

Isoelectric focusing. A sample of purified staphylolytic enzyme (200 ng) was focused in a Phast System (Pharmacia Inc., Piscataway, N.J.) with Phast Gel IEF (Pharmacia) (pH 3 to 9). As pI markers, the following were used: trypsinogen (pI 9.3), lentil lectin basic band (pI 8.65), lentil lectin medium band (pI 8.45), lentil lectin acidic band (pI 8.15), myoglobin basic band (pI 7.35), myoglobin acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase (pI 5.85), α-lactalbumin (pI 5.20), soy trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.50). Proteins in the gel were detected by staining with Coomassie blue.

Assay of the lytic activity. Lytic activity was normally assayed at 37°C, and the reduction of turbidity at 535 nm of a suspension of lyophilized S. aureus cells (having an initial turbidity of 0.5 at that wavelength) in 10 mM sodium phosphate buffer (pH 8.5) was determined photometrically. These suspensions contained about 10^8 CFU/ml. One lytic enzyme unit (LU) was defined as the amount of enzyme necessary to cause a turbidity decrease of 0.01 absorbance units per minute in such conditions.

The activity of the lytic enzyme was also assayed on suspensions of lyophilized P. aeruginosa cells in 10 mM sodium phosphate buffer. As for S. aureus, the suspensions had an initial turbidity of 0.5 A_{535} units.

Study of conditions for lytic activity. To study the effects of pH on the activity of the lytic enzyme, suspensions of lyophilized cells of S. aureus were prepared in 10 mM sodium phosphate buffer followed by 10 mM ammonium acetate buffer, which had pH values that ranged from 4.0 to 11.0. The lytic enzyme (40 LU/ml) was added to these suspensions, and the decrease in turbidity at 535 nm over a 5-min period was determined.

To study the effect of the temperature, suspensions of lyophilized S. aureus cells in 10 mM sodium phosphate buffer (pH 8.5) were incubated for 15 min at 12, 20, 30, 37, 45, 50, and 56°C, and the lytic enzyme (40 LU/ml) was then added to each of these suspensions. The decrease in turbidity at 535 nm over a 5-min period was determined.

The effect of the molarity of the buffer was determined by using 5, 10, 20, 30, 40, and 50 mM (all at pH 8.5) sodium phosphate buffer to prepare the suspensions of lyophilized S. aureus cells. The lytic enzyme (40 LU/ml) was added to each of these suspensions, and the decrease in turbidity at 535 nm over a 5-min period at 37°C was monitored.

The activity of the lytic enzyme was also assayed under various conditions which, according to the literature, are required for growth of L-form colonies. Firstly, Brorsons' medium (4) was used with modifications (1 M sucrose was substituted for NaCl). This medium contained (per liter)

the following: brain heart infusion broth (Oxoid), 37 g; $MgSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 0.006 g; thyamine pyrophosphate chloride (Sigma Chemical Co., St. Louis, Mo.), 0.002 g; nicotinic acid (Sigma), 0.004 g. This medium was normally used at pH 7.5, but when necessary, its pH was raised to 8.5 by adding 10 mM sodium phosphate buffer instead of distilled water.

Secondly, the activity of the lytic enzyme was assayed in 10 mM sodium phosphate buffer (pH 8.5) containing 4% (wt/vol) NaCl, 1 M sucrose, or 0.25% (vol/vol) horse serum (heat denatured according to the method recommended by Makino [10]).

Thirdly, the activity was also assayed in MYG medium with or without 1 M sucrose, as well as in MYG medium modified by raising its pH to 8.5 and decreasing the phosphate content to 10 mM. In all cases, the activity was measured by monitoring not only the drop in turbidity of the lyophilized-cell suspensions but also by viable count on agar (2%, wt/vol) containing MYG medium.

Effects of the lytic enzyme on cultures of S. aureus. In order to study the effects of the lytic enzyme on the growth of S. aureus, this bacterium was grown in horse serum in MYG medium with or without 1 M sucrose. Cultures were incubated at 37°C on a rotatory shaker until they numbered about 10^9 CFU/ml. The lytic enzyme (40 LU/ml) was then added, and the incubation was prolonged for one more hour. Samples were collected at 15-min intervals. For each sample, turbidity at 535 nm and the number of viable cells were determined. The viable counts were performed on plates containing agar-solidified (2%, wt/vol) MYG medium with or without 1 M sucrose or 0.25% horse serum.

Detection of L-forms. L-like forms were detected by the following criteria: ability to form fried-egg colonies, incrustation into the agar, negative reaction against Gram staining, reversibility to normal colonies, and absence in MYG medium without sucrose and horse serum.

Electron microscopy. For examination, cells were fixed at 4°C for 4 h in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% $MgSO_4$ and 1 M sucrose. The fixed cells were harvested by centrifugation, and the pellet was embedded in 2% Noble agar (Difco Laboratories, Detroit, Mich.) in cacodylate buffer and washed three times in the same buffer. Specimens were then postfixed for 5 h at 4°C in 1% OsO_4 in the same buffer. At each step, 0.1% $MgSO_4$ and 1 M sucrose were added.

After being washed several times in cacodylate buffer, specimens were dehydrated in a graded acetone series and embedded in Spurr resin. Thin sections were cut on an LKB Ultratome III and poststained with uranyl acetate for 30 min and then with lead nitrate for 5 min. Sections were examined on a Hitachi H300 transmission electron microscope at 75 kV. When samples were obtained from hypertonic cultures, all the fixation and washing buffers contained 1 M sucrose.

RESULTS AND DISCUSSION

Purification of the lytic enzyme. In supernatant fluids of 18-h-old cultures of P. aeruginosa PAKS I in MYG medium, staphylolytic activity (63.3 LU/ml) was detected for a protein content of 1.13 mg/ml. After purification, the specific activity was increased 122.23-fold. Table 1 shows a summary of the purification procedure.

The purified enzyme migrated as a single protein band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis; its molecular weight was estimated to be 19,000 (Fig. 1).

Isoelectric focusing of the purified enzyme also revealed one band at pH 8.5.

TABLE 1. Purification of the staphylolytic enzyme produced by *P. aeruginosa* PAKS 1

Source of fraction	Lytic activity (LU/ml)	Protein (mg/ml)	Sp act (LU/mg of protein)	Purification (fold)
Supernatant fluid	63.33	1.13	56.08	1
Dialysis	2,833.00	3.90	845.00	14.8
Bio-Gel P-2 chromatography	1,189.50	1.46	811.00	14.5
P-cellulose chromatography	173.30	0.05	3,466.60	61.9
Sephadex G-50	203.00	0.03	6,855.00	122.23

Conditions for lytic activity. Lytic activity was maximal at pH 8.5, at 37°C, and in 10 mM sodium phosphate buffer. The enzyme was inactive when pH values were lower than 6.0 or higher than 10.5 and when the molarity of the buffer was equal to or greater than 40 mM. These results are similar to those reported by Burke and Pattee (6) from a study of a staphylolytic enzyme from *P. aeruginosa* X.

In view of the results mentioned above, lytic enzyme was assayed in 10 mM sodium phosphate buffer, pH 8.5, at 37°C, unless otherwise stated. Under such conditions, the addition of the lytic enzyme (40 LU/ml) to the suspensions of lyophilized *S. aureus* cells resulted in a decrease in their absorbance (0.41 A_{535} units) concomitant to a drop in the number of viable units, reflected by the facts that after 1 min of incubation the viable number was three times lower than the initial one and that after 5 min of incubation the viable number was 50 times lower.

When the incubation buffer contained 4% NaCl, the lytic enzyme was not active.

In 1 M sucrose-containing buffer, the absorbance of the suspensions decreased slightly (0.15 A_{535} units), after 5 min of incubation in the presence of the lytic enzyme (40 LU/ml). With respect to the viable number after 5 min of incubation, when counts were carried out in MYG medium without sucrose, the number of viable cells observed was 50 times
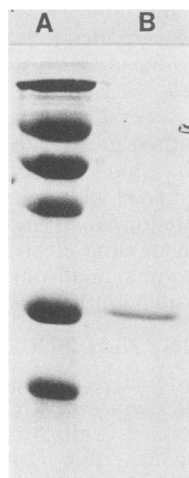

FIG. 1. Electrophoretic pattern on sodium dodecyl sulfate-polyacrylamide gel (12% reticulation) of the staphylolytic enzyme produced by *P. aeruginosa* PAKS 1. Lane A, Molecular weight markers (10 μg of each): bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100). Lane B, Staphylolytic enzyme (17 μg).
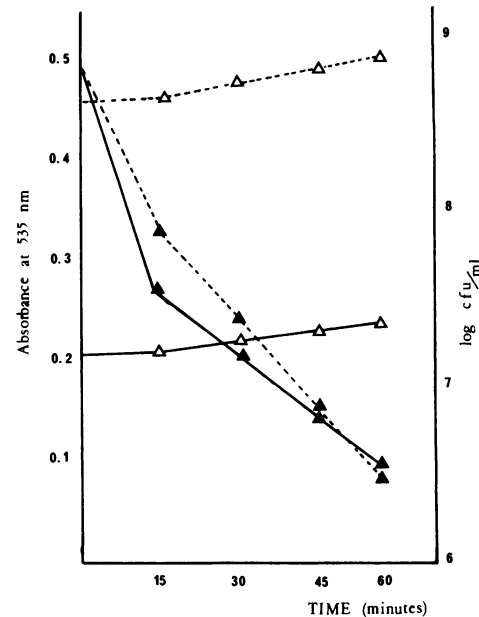

FIG. 2. Evolution of the optical density (——) and viable cells (– – – –) of cultures of *S. aureus* in MYG medium at pH 8.5 containing 10 mM sodium phosphate buffer and 0.25% heat-inactivated horse serum (▲) and in the same medium to which 1 M sucrose was added (△). In both experiments, purified lytic enzyme (40 LU/ml) was added at time zero.

lower than the initial one, whereas in the presence of 1 M sucrose in the medium the number of viable cells was only 20 times lower than the initial one. This finding indicated that, in the presence of sucrose, the lytic enzyme remained active, as reflected by the decrease in the number of viable cells. The absorbance of the suspensions did not decrease. Therefore, sucrose, an osmotic stabilizer, prevented cellular lysis.

Heat-inactivated horse serum did not seem to affect the activity of the lytic enzyme, since both optical density and viable cell numbers were not significantly different from those observed in sodium phosphate buffer without horse serum.

The results obtained indicated that the activity of lytic enzyme on growing *S. aureus* cells had to be assayed in media without NaCl as a stabilizer. Sucrose as an osmotic stabilizer and horse serum were considered appropriate to assay that activity.

In Brorsons' medium (in which 1 M sucrose replaced NaCl), the activity was slight at pH values of 7.5 and 8.5 (the decreases in optical density during 5 min of incubation were only 0.03 and 0.09, respectively). Similar values of activity were detected in MYG medium at pH 7.5. However, the lytic activity measured in MYG medium with a pH which was raised to 8.5 (by using 10 mM sodium phosphate buffer instead of water) was similar to that observed in sodium phosphate buffer with a pH of 8.5 (the decrease in optical density after 5 min of incubation was 0.40 A_{535} units).

Induction of L-like forms of *S. aureus*. Figure 2 shows the evolution of cultures of *S. aureus* during 1 h after the addition of the lytic enzyme (40 LU/ml). The cultures were incubated in modified MYG medium (pH 8.5; 10 mM sodium phosphate buffer was used instead of water) at 37°C until the cell density reached about 5 × 10⁸ CFU/ml, at which point the lytic enzyme was added (time zero in Fig. 2). In the absence of sucrose, both the optical density and the number
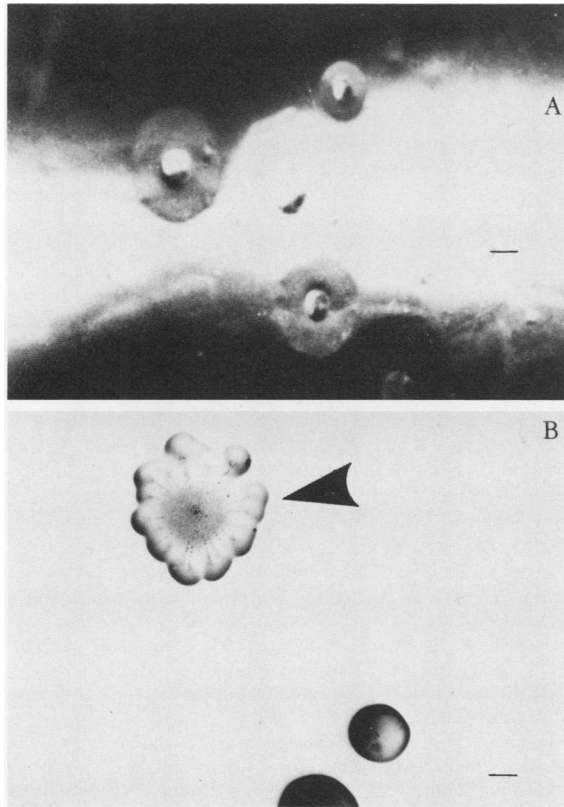
FIG. 3. (A) Fried-egg colonies, obtained by subculturing on solid MYG medium containing 1 M sucrose, from a culture of *S. aureus* after treatment for 1 h with the purified lytic enzyme (40 LU/ml) in liquid MYG medium. (B) Micrograph showing the aspect of one of these colonies after incubation for 24 h (arrow). Bars, 0.5 mm.

FIG. 4. (A) Electron micrograph of specimen obtained from sections of *S. aureus* grown in MYG medium. Cell wall is apparent. (B) Electron micrograph of specimen obtained from sections of *S. aureus* treated for 1 h with purified lytic enzyme (40 LU/ml). Bars, 0.5 μm.

of viable cells decreased abruptly, but in the presence of 1 M sucrose, the optical density of the cell suspensions was maintained during the time of incubation. When viable counts were performed on solid MYG medium with 1 M sucrose, the viable cell numbers remained constant, but when determined on MYG medium without sucrose, low viability was detected. These results indicate that the lytic enzyme was active not only on lyophilized cells but also on cultures of *S. aureus*.

In the presence of sucrose, optical density and number of viable cells remained unchanged, and since sucrose did not inhibit the lytic enzyme, it must be assumed that it protected the cells against lysis. This interpretation was consistent with the idea that the lytic enzyme degraded cell wall components of *S. aureus*. MYG medium was hypotonic with respect to the cytoplasm, and thus the cells underwent lysis, caused by a deficiency in the cell walls; contrarily, MYG medium containing 1 M sucrose was hypertonic or isotonic and, consequently, the cells were protected against cellular lysis.

This conclusion was supported by the fact that during the counting of viable cells on solid MYG medium containing 1 M sucrose and 0.25% horse serum, L-like colonies were detected (Fig. 3A). These colonies were identified as L-form colonies according to the terminology proposed by McGee et al. (12) because of their fried-egg appearance and their ability to incrust into the agar and because they were whitish and formed gram-negative cocci. After 48 h of incubation at 37°C, they reverted to normal colonies (Fig. 3B); they

exhibited a golden color, were convex, did not incrust into the agar, and formed gram-positive cocci. These cocci were coagulase positive, and their profiles in the API Staph system were compatible with *S. aureus*.

In addition, an examination by electron microscopy of specimens from cultures of *S. aureus* in liquid MYG medium containing both 1 M sucrose and the lytic enzyme (40 LU/ml) revealed the presence of many cells lacking cell walls (Fig. 4), which, according to our interpretation, could be responsible for the formation of L-form colonies.

Several strains of *S. aureus* have been described as able to produce spontaneous L-forms (5), but repeated incubations in both solid and liquid media with or without 1 M sucrose failed to produce spontaneous L-forms of the staphylococcal strain used in this work.

The results presented here indicated that the lytic enzyme produced by *P. aeruginosa* PAKS I can act on *S. aureus*, inducing the formation of cell wall-deficient forms which, when cultivated on solid media with 1 M sucrose, produced L-form colonies. This conclusion is similar to that reported by Makino (10) on induction of L-forms of *S. aureus* by treatment with lysostaphin, a lytic enzyme produced by *Staphylococcus staphylolyticus* acting on the cell wall components of the staphylococci.

Burke and Pattee (6) reported the induction of cell wall-deficient forms of *S. aureus* by treatment with a staphylolytic enzyme from *P. aeruginosa*. However, the detection of those forms was made only on the basis of the

osmotic sensitivity of the cells, and L-form colonies were not found by these authors, who also described lytic activity of the staphylolytic enzyme on *P. aeruginosa* under certain conditions; in our case, activity of the lytic enzyme on *P. aeruginosa* PAKS I was not detected after repeated attempts. In addition, homogeneity of the lytic enzyme was not accomplished by these authors and, consequently, data about molecular weight and pI were not reported. Lache et al. (8) reported an alternative method for purification of the same enzyme, but no characteristics were given.

Finally, Stepnaya et al. (16, 17) have recently published the isolation of two bacteriolytic enzymes from members of the family *Pseudomonadaceae*. One of them is lytic on *S. aureus*, but its molecular weight (15,000) and pI (5.3) are quite different from those of the enzyme we isolated. In addition, the enzyme purified by these authors also exhibited caseinolytic activity. The staphylolytic enzyme we purified was found to be inactive on casein.

ACKNOWLEDGMENT

LITERATURE CITED

1. **Baird-Parker, A. C.** 1963. A classification of Micrococci and Staphylococci based on physiological and biochemical tests. J. Gen. Microbiol. **30**:409–427.
2. **Blanco, M. T., M. A. Falcón, and A. Chordi.** 1982. Estudio de la interacción establecida entre *Staphylococcus aureus* y *Pseudomonas aeruginosa* cuando se cultivan en un medio semisintético. Microbiol. Esp. **35**:33–41.
3. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brorsons, J. E., M. E. Lundholm, and S. Seeberg.** 1973. L-colony production by different strains of *Staphylococcus aureus* in the absence of antibiotic. J. Infect. Dis. **128**:335–339.
5. **Brorsons, J. E., M. E. Lundholm, and S. Seeberg.** 1975. Properties of some *Staphylococcus aureus* strains associated with spontaneous L-phase transformation. Scand. J. Infect. Dis. **7**:51–54.
6. **Burke, M. E., and P. A. Pattee.** 1967. Purification and charac-
terization of a staphylolytic enzyme from *Pseudomonas aeruginosa*. J. Bacteriol. **93**:860–865.
7. **Hames, B. D.** 1981. An introduction to polyacrylamide gel electrophoresis. *In* B. D. Hames and D. Rickwood. (ed.), Gel electrophoresis of proteins: a practical approach. IRL Press, London.
8. **Lache, M., W. R. Hearn, J. W. Zyskind, D. J. Tipper, and J. L. Strominger.** 1969. Specificity of bacteriolytic substances isolated from *Pseudomonas aeruginosa*. Fed. Proc. **25**:752–757.
9. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):S94–S99.
10. **Makino, T.** 1983. Induction of L-phase variant from protoplast of *Staphylococcus aureus*. Microbiol. Immunol. **27**:749–755.
11. **Mansito, T. B., M. A. Falcón, J. Moreno, A. Carnicero, and A. M. Gutiérrez-Navarro.** 1987. Effects of staphylolytic enzymes from *Pseudomonas aeruginosa* on the growth and ultrastructure of *Staphylococcus aureus*. Microbios **49**:55–64.
12. **McGee, Z. A., R. G. Wittler, H. H. Gooder, and P. Charache.** 1971. Wall-defective microbial variants: terminology and experimental design. J. Infect. Dis. **123**:433–438.
13. **Morihara, K.** 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. J. Bacteriol. **88**:745–757.
14. **Morihara, K., and J. Y. Homa.** 1985. *Pseudomonas* proteases, p. 41–79. *In* I. A. Holder (ed.), Bacterial enzymes and virulence. CRC Press, Inc., Boca Raton, Fla.
15. **Perestelo, F. R., M. T. Blanco, A. M. Gutiérrez-Navarro, and M. A. Falcón.** 1985. Growth inhibition of *Staphylococcus aureus* by a staphylolytic enzyme from *Pseudomonas aeruginosa*. Microbios Lett. **30**:85–94.
16. **Stepnaya, O. A., A. I. Severin, and I. S. Kulaev.** 1986. Some physico-chemical properties of lytic proteinase L_2 of the enzymatic preparation of bacterial lysoamidase. Biokhimiya **51**:909–915.
17. **Stepnaya, O. A., A. I. Severin, and I. S. Kulaev.** 1986. Bacteriolytic enzymes from the preparation of lysoamidase isolated from the bacterium of the Pseudomonadaceae family. Biokhimiya **51**:1117–1123.
18. **Wretlind, B., L. Sjöberg, and T. Wadström.** 1977. Protease deficient mutants of *Pseudomonas aeruginosa*: pleiotropic changes in activity of other extracellular enzymes. J. Gen. Microbiol. **103**:329–336.
19. **Zyskind, J. W., P. A. Pattee, and M. Lache.** 1965. Staphylolytic substances from species of *Pseudomonas*. Science **147**:1458–1459.