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NOTES

Lysis and Protoplast Formation of Group B Streptococci by Mutanolysin

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Group B streptococci, refractory to previously tested muralysins under physiological conditions, were successfully converted to protoplasts by use of a recently described *N*-acetyl muramidase, mutanolysin, derived from a streptomycete. Purified enzyme was effective, but crude preparations, although degrading cell walls, simultaneously produced peculiar effects of cytoplasmic coagulation, retention of cell shape, loss of some intracellular enzymes, and a rise in optical density. Addition of purified mutanolysin to the array of muralysins (group C streptococcal phage-associated lysin, lysozyme), previously successful in preparing protoplasts of different streptococci, now makes possible enzymatic preparation of protoplasts of streptococci of groups A, B, C, D, G, and H.

Successful enzymatic preparation of protoplasts of group B streptococci by microbial muralysins or lysozyme has not been reported. Because of the need for protoplasts in our studies of toxins such as hemolysin S (2), and in other studies of antigen localization and physiological properties, we investigated the use of a new muralysin (mutanolysin) reported to lyse *Streptococcus mutans* (18), a number of gram-positive bacteria (except staphylococci), and some other streptococci including one of group B (13). We here report the preparation of protoplasts of group B streptococci by use of purified mutanolysin in hypertonic buffer. In addition, an unusual effect of crude mutanolysin was observed: a high enzyme concentration in hypotonic buffer caused an increase in the optical density of cell suspensions, and cell shape was retained despite loss of cell wall and membrane.

Group B streptococcal strains Denver 536-F (type Ic), Denver 148-F (type II), and Denver 429-G (type III) were obtained from G. H. McCracken (University of Texas, Southwestern Medical School, Dallas, Tex.) and SS-615 (type Ia) from R. Facklam (Center for Disease Control, Atlanta, Ga.). Strains were grown in Todd-Hewitt broth (BBL Microbiology) at 37°C for the appropriate time, harvested by centrifugation, washed once with phosphate-buffered saline (pH 7.4), and suspended in appropriate buffers. Hypotonic buffers consisted of either 0.03 M KPO_4 (pH 7.0) or 0.01 M tris(hydroxymethyl)aminomethane (Tris) maleate (pH 7.0), whereas hypertonic buffers were the same with the addition of 20, 30, or 40% sucrose (wt/vol). Mutanolysin preparations were obtained from K. Yokogawa (Dianippon Pharmaceuticals, Ltd., Osaka, Japan). Crude enzyme was known to contain protease, endopeptidase, and other unspecified enzymatic activities (K. Yokogawa, personal communication), and the amount used per 3 ml of cell suspension (optical density at 600 nm [OD_{600}] of 0.400 to 0.750 was 2.75 mg (high concentration) or 0.275 mg (low concentration). Purified enzyme (M-1) was rather insoluble in water and was suspended at a concentration of 1 mg/ml in distilled water, sonicated for 20 s with a Bronwill Biosonik IV (Bronwill, Rochester, N.Y.) at a setting of 30, and diluted 10-fold into distilled water, as per directions of K. Yokogawa (personal communication).

Samples for electron microscopy were fixed in glutaraldehyde (5%, added directly to an equal volume of the sample in buffer), followed by 1% osmium tetroxide in Veronal buffer. Procedures were as previously described (5) with the following exceptions: (i) samples before osmication were held at 4°C in 2.5% glutaraldehyde for 1 to 3 days, instead of in buffered sucrose; (ii) embedment was in Spurr medium (17) with appropriate prior dehydration steps, instead of in Epon; (iii) one set of samples incubated in hypertonic buffer with purified (M-1) enzyme was fixed with glutaraldehyde made up in 0.2 M cacodylate buffer; (iv) another set was fixed with glutaraldehyde made up in the 40% sucrose-phosphate hypertonic buffer instead of in cacodylate buffer.

Results were the same for group B streptococci of serotypes I, II, and III. As judged by a decrease in OD_{600}, they were lysed by either low concentrations of crude mutanolysin or by purified (M-1) mutanolysin, in hypotonic Tris or phosphate buffers. The rate of lysis was influenced by age of the streptococcal culture, the addition of cations, and concentration of crude enzyme (only dilute purified enzyme could be used, as noted above). Stationary-phase cells were more resistant to lysis than log-phase cells. Addition of Fe^{2+} (1 mM) inhibited lysis, and Mg^{2+} and Ca^{2+} (both 1 mM) slowed the rate of lysis.

Use of the purified (M-1) enzyme in hypotonic buffer (either Tris or phosphate) resulted in a 96% decrease in OD_{600} in 30 min. The streptococci (Fig. 1) became lysed for the most part, and only an occasional cell or cell wall fragment was seen among the residual membranes and cytoplasmic debris (Fig. 2).

When this enzyme was used in hypertonic buffer (40% sucrose with either Tris or phosphate), OD_{600} fell only 34% in 30 min. Morphologically characteristic protoplasts and expelled mesosomal vesicles resulted, and these differed in electron density according to whether initial fixation was in cacodylate-buffered glutaraldehyde (Fig. 3) or in sucrose-phosphate-buffered glutaraldehyde (Fig. 4). Dilution of such preparations into distilled water resulted in a subsequent 97 to 98% decrease in OD_{600}, whereas only a 28 to 32% fall occurred on dilution into 40% sucrose. These percentages remained approximately the same for at least 60 min of enzyme exposure. Detection of aldolase in the original supernatant fluids as an intracellular marker (3) showed a loss of only 1 to 4% of total intracellular aldolase from the cells during this period of protoplasting. The use of crude mutanolysin in low concentration, in either hypotonic or hypertonic buffer, resulted in OD_{600} decreases that ranged from 85 to 98% in 30 min. (See Table 1 for example in hypotonic buffer.) Since the decreases indicated no protoplast formation, these samples were not examined further.

Crude mutanolysin in high concentration in hypotonic buffer caused a curious increase in OD_{600}, ranging from 50 to 82% in 30 min. (See Table 1 for example.) Neither high-speed mixing nor addition of deoxyribonuclease lowered the OD_{600}. Electron microscopy showed a progressive layered fragmentation, thinning, and ultimate loss of cell wall; a progressive increase in number and size of electron-lucent particle-containing areas in the cytoplasm; progressive patchy loss of cytoplasmic membrane; and increasing coagulation and electron density of residual cytoplasm (Figs. 5–7). These resultant bodies, unlike intact protoplasts produced by purified enzyme in hypertonic buffer, lost some cytoplasmic content, as indicated by the detection of 22% of the intracellular aldolase in the supernatant fluid after centrifugation. Furthermore, suspension of these bodies in hypotonic buffer did not result (as it did for protoplasts) in a decrease in the OD or in release of deoxyribonucleic acid as judged by the absence of any change in viscosity. Thus, although the cytoplasmic membrane was disrupted or absent, these bodies were not osmotically labile and, in fact, retained their general form and coagulated appearance even after exposure to sodium dodecyl sulfate (Fig. 8).

The reason for the peculiar coagulating action of crude mutanolysin on group B streptococci, in addition to its degradation of cell wall, is unknown, but undoubtedly results from the presence of proteases, peptidases, and other unspecified activities in the crude mixture (Yokogawa, personal communication). This effect probably accounts in part for the earlier observations of Hamada et al. (11) that high levels of partially purified mutanolysin produced less of a decrease in OD during the same time of exposure than did lower levels.

Successful protoplast preparation requires rupture or removal of bacterial cell wall under conditions that are osmotically supportive and otherwise physiological. Our experience with mutanolysin indicates that crude preparations, although active in removing and degrading cell

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FIG. 1. *Group B streptococcus, type III, Denver 429 G. Control, standard fixation (see text). All magnifications, ×35,000; bars = 1.0 μm. Abbreviations: cw = cell wall; f = fragmenting cell wall; cm = cytoplasmic membrane; mv = mesosomal vesicles; el = electron-lucent areas containing particles (p).*

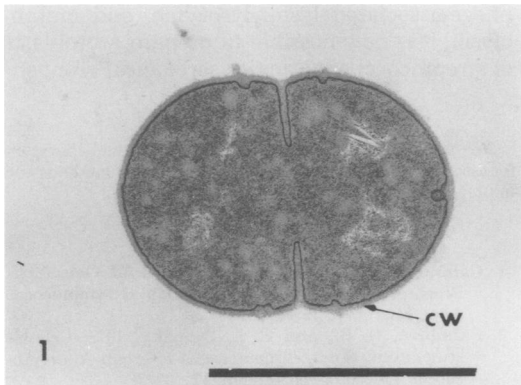
FIG. 2. *Same after exposure (30 min, 37°C) in hypotonic buffer to purified (M-1) mutanolysin. Standard fixation.*

FIG. 3. *Protoplasts resulting from exposure (30 min, 37°C) in hypertonic buffer to purified (M-1) mutanolysin. Standard fixation.*

FIG. 4. *Same as Fig. 3, but fixed initially in 2.5% glutaraldehyde made up in 40% sucrose-phosphate hypertonic buffer instead of cacodylate buffer (see text).*

FIG. 5–7. *Representative examples of progressive stages of effects of crude mutanolysin in hypotonic buffer (10, 20, and 30 min, 37°C). Standard fixation.*

FIG. 8. *Result of exposing 30-min sample (Fig. 7) to sodium dodecyl sulfate, 0.05%, for 30 min at 25°C, followed by standard fixation.*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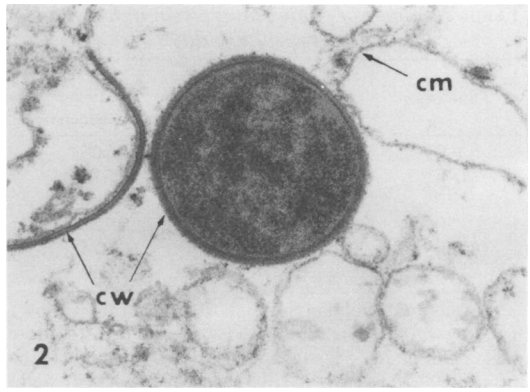
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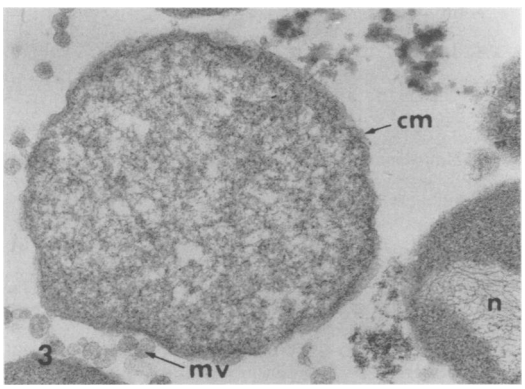
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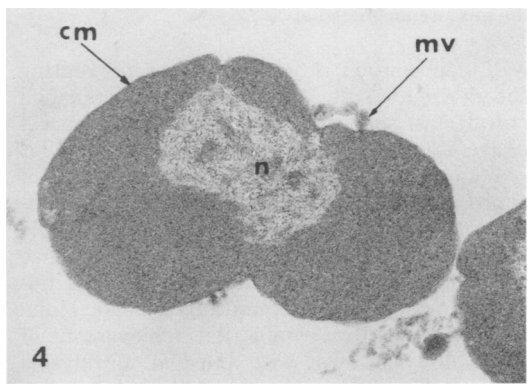
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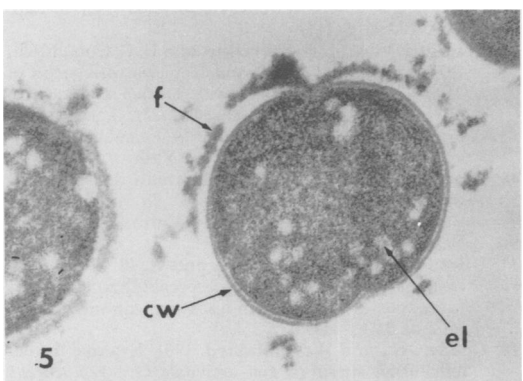
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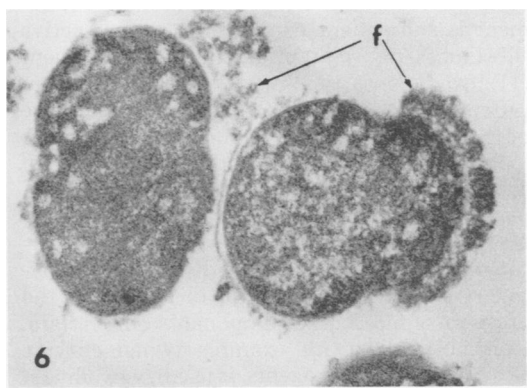
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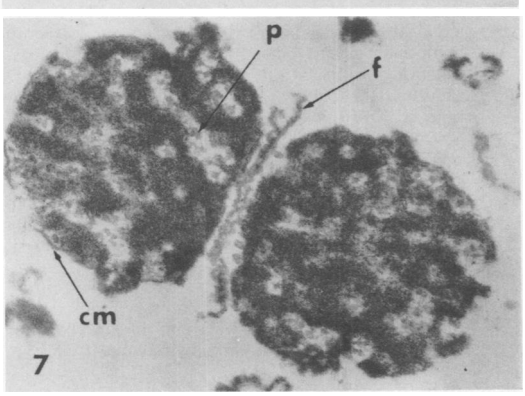
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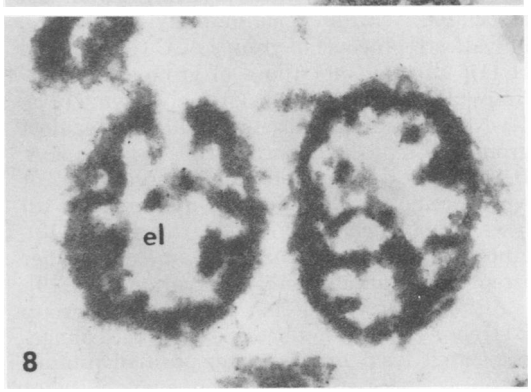
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TABLE 1. *Effect of crude mutanolysin in hypotonic buffer on cell turbidity*[a]

Time (min)	OD_{600}	
	High concn	Low concn
0	0.499	0.508
5	0.561	0.478
10	0.792	0.293
20	0.779	0.126
30	0.749	0.074
60	0.678	0.052

[a] Crude mutanolysin in high (0.5 mg/ml) or low (0.05 mg/ml) concentration was added to late log-phase cells of 75 × 9 suspended in 3 ml of hypotonic buffer (0.03 M KPO_4, pH 6.5, plus 0.85% NaCl) and the mixture incubated at 37°C.

wall, can contain other substances preventing the desired result. Although some unpurified muralysins, such as the group C streptococcal phage-associated lysin or the staphylococcal LS enzyme, can be used to prepare protoplasts of appropriate bacteria (4, 16), untested detrimental effects of extraneous components are possible though not necessarily obvious as in the instance of crude mutanolysin. The desirability of homogeneous enzyme preparations is clear. However, it is equally obvious that achievement of cell wall lysis, even with purified muralysins such as lysozyme when an additional requirement is subsequent exposure to surface-active substances (6, 7) or prior chemical treatment (11), can be unsatisfactory for protoplast preparation because of the non-physiological nature of the additives or treatments.

In addition, knowledge of the site of action of a muralysin on some peptidoglycans does not permit prediction of its activity against a previously untested bacterial cell wall or peptidoglycan—particularly one of which the peptidoglycan structure is not known, or on which the presence of interfering components is uncertain. It is not known, for example (when enzyme alone, without other agents, is used), why phage-associated lysin (an L-ala-muramyl-amidase; 8, 9) lyses streptococci of groups A, C (14, 15), and H (1), although not those of groups B and G (unpublished observations); why lysozyme (a B-1,4-*N*-acetyl-muramidase: 10) is effective against group D streptococci but not those of groups A, B, C, G, or H (unpublished observations); or why mutanolysin (an *N*-acetyl-muramidase; 19) lyses streptococci of groups B (this report; 13), groups A, C, G, and H, and *S. mutans* and other streptococci (unpublished observations) (13, 19).

Although preparation of protoplasts of group B streptococci had not been possible with phage-associated lysin or lysozyme, purified mutanolysin is successful. With the availability of phage-associated lysin, lysozyme, and mutanolysin, it is now possible to prepare protoplasts of streptococci of all major serological groups.

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LITERATURE CITED

1. **Calandra, G. B., K. M. Nugent, and R. M. Cole.** 1975. Preparation of protoplasts of Group H streptococci. Appl. Microbiol. **29**:90-93.
2. **Calandra, G. B., and E. L. Oginsky.** 1975. Cellular streptolysin S-related hemolysins of group A streptococcus C203S. Infect. Immun. **12**:13-28.
3. **Calandra, G. B., and T. S. Theodore.** 1975. Cellular location of streptolysin O. Infect. Immun. **12**:750-753.
4. **Cole, R. M.** 1968. The structure of the Group A streptococcal cell and its L-form, p. 5-42, *In* R. Caravano (ed.), Current research on group A *Streptococcus*. Excerpta Medica Foundation, New York.
5. **Cole, R. M., T. J. Popkins, R. J. Boylan, and N. H. Mendelson.** 1970. Ultrastructure of a temperature-sensitive Rod$^-$ mutant of *Bacillus subtilis*. J. Bacteriol. **103**:793-810.
6. **Coleman, S. E., I. van de Rijn, and A. S. Bleiweis.** 1970. Lysis of grouped and ungrouped streptococci by lysozyme. Infect. Immun. **2**:563-569.
7. **Coleman, S. E., I. van de Rijn, and A. S. Bleiweis.** 1971. Lysis of cariogenic and noncariogenic oral streptococci with lysozyme. J. Dent. Res. **50**:939-943.
8. **Fischetti, V. A., E. C. Gotschlich, and A. W. Bernheimer.** 1971. Purification and physical properties of Group C streptococcal phage-associated lysin. J. Exp. Med. **133**:1105-1117.
9. **Fischetti, V. A., J. B. Zabriskie, and E. C. Gotschlich.** 1974. Physical, chemical and biological properties of Type 6 M-protein extracted with purified streptococcal phage-associated lysin, p. 26-36. *In* M. J. Haverkorn (ed.), Streptococcal disease and the community. American Elsevier Publishing Co., New York.
10. **Ghuysen, J.-M., and G. D. Shockman.** 1973. Biosynthesis of peptidoglycan, p. 37-130. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, Inc., New York.
11. **Glick, A. D., J. M. Ranhand, and R. M. Cole.** 1972. Degradation of group A streptococcal cell walls by egg-white lysozyme and human lysosomal enzymes. Infect. Immun. **6**:403-413.
12. **Gooder, H., and W. R. Maxted.** 1961. External factors influencing structure and activities of *Streptococcus pyogenes*, p. 151-173. *In* G. G. Meynell and H. Gooder (ed.), Microbial reaction to environment. Eleventh Symposium of the Society for General Microbiology. Cambridge University Press, Cambridge, England.
13. **Hamada, S., M. Torii, S. Kotani, N. Masuda, T. Ooshima, K. Yokogawa, and S. Kawata.** 1978. Lysis of *Streptococcus mutans* cells with mutanolysin, a lytic enzyme prepared from a culture liquor of *Streptomyces globisporus* 1829. Arch. Oral Biol. **23**:543-549.
14. **Krause, R. M.** 1958. Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage-associated lysin. J. Exp. Med. **108**:803-821.
15. **Krause, R. M., and M. McCarty.** 1962. Studies on the chemical structure of the streptococcal cell wall. II. The composition of group C cell walls and chemical basis for serologic specificity of the carbohydrate moiety. J. Exp. Med. **115**:49-62.
16. **Popkin, T., S. Theodore, and R. M. Cole.** 1971. Electron

microscopy during release and purification of mesosomal vesicles and protoplast membranes from *Staphylococcus aureus*. J. Bacteriol. **107**:907-917.
17. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastr. **26**:21-43.
18. **Yokogawa, K., S. Kawata, S. Nishimura, Y. Ikeda, and Y. Yoshimura.** 1974. Mutanolysin, bacteriolytic agent for cariogenic streptococci: partial purification and properties. Antimicrob. Agents Chemother. **6**:156-165.
19. **Yokogawa, K., S. Kawata, T. Takemura, and Y. Yoshimura.** 1975. Purification and properties of lytic enzymes from *Streptomyces globisporus* 1829. Agr. Biol. Chem. **39**:1533-1543.