Preparation of Protoplasts of Group H Streptococci

(Streptococcus sanguis)

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Stable protoplasts of several strains of group H streptococci (Streptococcus sanguis) can be prepared by use of group C streptococcal phage-associated lysin in the presence of 30% raffinose. Sucrose cannot be substituted for raffinose. Protoplast formation did not require the addition of Mg²⁺; however, this cation enhanced their stability. Some other strains, also presumptive group H streptococci, were not sensitive to phage-associated lysin.

Several strains of group H streptococci can be lysed enzymatically by Streptomyces albus enzymes (1, 11, 12, 14), competent cell autolysins (16), lysozyme at high pH plus sodium dodecyl sulfate (1, 3), or group C phage-associated lysin (PAL) (8, 10, 17). However, preparation of stable group H streptococcal protoplasts has apparently proved difficult and has not been previously reported. Perry (13) recently reported the preparation of spheroplast membranes from strain Wicky, utilizing PAL and ethylenediaminetetraacetic acid (EDTA) in 20% sucrose; however, he did not report on the stability of the spheroplasts. Although the most frequently used procedures for preparation of streptococcal protoplasts have utilized various concentrations of sucrose or NaCl (7), earlier attempts to prepare protoplasts of group H strains with sucrose and NaCl as osmotic stabilizers were unsuccessful in our laboratory. Because of an interest in developing a method to determine the subcellular location of group H streptococcal cellular constituents such as the competence-associated proteins (15, 17), we report here a procedure for preparing stable protoplasts of some strains of group H streptococci utilizing PAL and raffinose.

MATERIALS AND METHODS

Strains and growth conditions. The following strains of streptococci (all originally considered to be group H and Streptococcus sanguis) from the Laboratory of Streptococcal Diseases (LSD) culture collection were used: Wicky 4 Ery⁺ (LSD 72 × 7), Wicky 4 Ery⁺Rif⁺ (LSD 73 × 40), Blackburn NCTC 10231 (LSD 71 × 26), SBE II/II NCTC 7865, ATCC 10558 (LSD 72 × 33), Channon NCTC 7869 (LSD 71 × 55), K208 ATCC 8144 (LSD 71 × 23), FW213 (LSD 72 × 42), FW225 (LSD 71 × 43), and Sanguis II NCTC 7864 (LSD 72 × 35). Each strain was grown in 100 ml of P broth (6) to 100 Klett-Summerson units (no. 520 filter), harvested by centrifugation at 10,000 × g for 10 min, washed once with distilled water, and suspended in 5 ml of buffer consisting of 0.05 M sodium phosphate (pH 6.5), 0.85% NaCl, and 10⁻⁴ M 2-mercaptoethanol (Eastman-Kodak). This buffer, also termed “hypotonic” buffer, was used as stock buffer for preparation of other buffers containing sucrose or raffinose and termed “hypertonic” buffers. Addition of the appropriate concentration of osmotic stabilizer (sucrose or raffinose), MgCl₂, and EDTA are described below.

PAL preparation. PAL was obtained from a C, phage lysate of 500 ml of late-log group C streptococcal strain 26RP66 by the method of Fox (5). Phage and debris were removed from the lysate by centrifugation at 100,000 × g for 2 h. The PAL titer was determined by a modified method of Markowitz and Dorfman (9). One unit of PAL is defined as the amount required to reduce the turbidity of a 5-ml suspension of group A streptococcus C203S by 1 turbidity unit at 1 h at 37 C when read with a no. 520 filter in a Klett-Summerson photometric colorimeter. The cells were initially suspended in hypotonic buffer plus 10⁻⁴ M EDTA to give a turbidity of about 100. Since the titer of PAL varied with each preparation (from 1 × 10⁴ to 6 × 10⁴ units/ml of PAL preparation), only one preparation was used for each set of experiments.

Protoplast preparation. The amount of PAL required to prepare protoplasts of the various strains was determined prior to use and was such that, when added to 3 ml of appropriate cell suspension in hypotonic buffer, there was a 70 to 90% decrease in the turbidity in 5 min at 37 C. Strains resistant to lysis by PAL were so designated when no decrease in turbidity occurred after addition of PAL in an amount 10-fold greater than that required to lyse sensitive strain Wicky 4 Ery⁺ (WE4). Protoplasts or PAL-treated streptococci were prepared by incubation of streptococci in hypotonic buffer with PAL (about 5 × 10⁴ to 10 × 10⁴ units/ml of PAL) at 37 C for 0.5 to 1 h. When appropriate, whole cells could be removed...
by centrifugation at 2,000 × g for 10 min. The turbidity of samples was determined at 5 min intervals with a Beckman DU recording spectrophotometer at 520 nm after dilution of the protoplast mixture into either distilled water or raffinose- or sucrose-containing buffer. The initial turbidity of the dilutions was adjusted to an optical density of approximately 0.400.

DNA analysis. The release of intracellular deoxyribo nucleic acid (DNA) was measured colorimetrically by the diphenylamine reaction (2).

RESULTS AND DISCUSSION

Since raffinose solutions are effective stabilizers of protoplasts (4) and have been successfully used in this laboratory to produce protoplasts of group A streptococci (A. L. Schade, unpublished observations), a similar method was applied to WE4 cells. The stability of the PAL-treated streptococci was initially assessed by measuring the change in turbidity of cell suspension after dilution into either water or hypertonic raffinose buffer (Table 1). Little or no decrease in the turbidity of protoplast suspensions in raffinose solutions indicates that the protoplasts are stabilized. There was only a 6 to 22% decrease in the turbidity of the PAL-treated organisms in 30% raffinose. However, when diluted into water, there was a marked decrease of 90 to 95% in the turbidity. A decrease of 29% in the turbidity of dilutions into raffinose buffer occurred when 20% rather than 30% raffinose was used. The decrease in turbidity of cell suspensions in the absence of PAL was negligible during the period of incubation.

Added Mg²⁺ was not required to prepare WE4 protoplasts in raffinose. In marked contrasts, group A streptococcal protoplasts prepared from strain C203S required 10⁻¹ M Mg²⁺ for stability (unpublished data). The only Mg²⁺ present in WE4 protoplast preparations was that contributed by PAL, which was prepared in the presence of Mg²⁺. The final concentration of this cation in the protoplast mixture was approximately 10⁻⁴ to 10⁻² M as calculated from dilution data. WE4 protoplasts prepared either in this concentration of Mg²⁺ (that in the PAL preparation) or in 10⁻³ M Mg²⁺ were stable (i.e., there was little to no decline in turbidity of the protoplast suspension in hypertonic buffer over the period of 5 to 40 min after addition of PAL—additional measurements at 5 to 35 min not shown) in hypertonic buffer (Table 1), but were fragile on centrifugation at 10,000 × g for 20 min, as shown by increased gross viscosity. This increase in viscosity on centrifugation was not observed when the protoplasts were prepared in raffinose buffer with 10⁻¹ M Mg²⁺ and, in fact, less than 10% of the DNA was released from protoplasts under these conditions. It appears that a Mg²⁺ supplement is not essential for protoplast preparation of group H streptococci, although Mg²⁺ does appear to decrease the protoplast fragility noted after they were centrifuged. When Mg²⁺ in high concentration was used, much larger amounts of PAL are required because of the inhibitory effect of Mg²⁺. For example, 10 times more enzyme was required when 10⁻¹ M Mg²⁺ was added than when Mg²⁺ was omitted. Whether the major inhibitory effect of Mg²⁺ is its action on the PAL enzyme or on its stabilization of the cell was not examined.

Attempts to prepare stable protoplasts of WE4 in various concentrations of sucrose were unsuccessful (Table 1). The addition of Mg²⁺ or EDTA did not enhance stabilization of protoplasts as evidenced by the marked decrease in turbidity of the cell suspensions when diluted into hypertonic buffer (58 to 75% decrease). Concomitant with this decrease in turbidity there was a significant release of DNA. Approximately 50% of the total DNA of the “sucrose stabilized protoplasts” was in the supernatant after centrifugation of the protoplasts at 10,000 × g for 20 min. These findings differ from those of Perry (13) who used PAL plus a phosphate buffer containing sucrose and EDTA to obtain spheroplasts from Wicky cells. Whether differences in strains accounts for this result is

| Osmotic stabilizer           | Additions     | Decrease (%) in turbidity after 40 min of incubation with PAL and dilution into: |
|-----------------------------|---------------|---------------------------------------------------------------------------------|
|                             |               | Water⁺                        | Hyper-tonic buffer⁺                                      |
| Raffinose (30%)             | None⁺         | 94                           | 6                                                    |
|                             | 10⁻¹ M Mg⁺    | 89                           | 15                                                   |
|                             | 10⁻² Mg⁺      | 97                           | 22                                                   |
|                             | 10⁻³ Mg⁺      | 95                           | 20                                                   |
|                             | 10⁻² M EDTA   | 90                           | 22                                                   |
|                             | 10⁻¹ M Mg⁺    | 88                           | 29                                                   |
| Raffinose (20%)             | None          | 92                           | 64                                                   |
| Sucrose (30%)               | None          | 83                           | 64                                                   |
| Sucrose (20%)               | None          | 95                           | 72                                                   |
|                             | 10⁻¹ M Mg⁺    | 68                           | 58                                                   |
|                             | 10⁻² M EDTA   | 91                           | 75                                                   |

⁺ Buffer was the stock buffer, described in Materials and Methods, plus appropriate concentrations of raffinose or sucrose.

⁺ Either water or stock buffer with additions but without added sucrose or raffinose.

⁺ Concentration of Mg⁺⁺ was approximately 10⁻⁴ to 10⁻² M.
uncertain. The addition of EDTA did not impair or enhance production of protoplasts in raffinose buffer. However, some protoplast preparations in EDTA-raffinose were fragile and ruptured during such mild procedures as pipetting. Addition of $10^{-1}$ M Mg$^{2+}$ to the EDTA-raffinose eliminated this fragility. In fact, less than 5% of the DNA was released from protoplasts prepared in $10^{-2}$ M EDTA-$10^{-1}$ M Mg-raffinose.

Another criterion used to assess the preparation and intactness of the PAL-raffinose-prepared protoplasts was examination by electron microscopy (Fig. 1). After 45 min of incubation, random destruction of the cell wall occurred, and only protoplasts, mesosomal vesicles, and cell wall fragments in the surrounding medium remained. As shown in these experiments, raffinose as the hypertonic stabilizer provides an adequate solution to the problem of stabilizing strains of streptococci that lyse in sucrose or NaCl.

This protoplast procedure has been successfully used to prepare protoplasts of streptococcal strains Blackburn, Channon, SBE I/II, and Wicky 4 Ery$^R$Rif$^R$. Other strains, including K208, FW213, FW225, and Sanguis II, were refractory to cell wall lysis by PAL. Preliminary evidence, including serological and phage adsorption studies (unpublished observations) suggest that these latter strains are not group H (Streptococcus sanguis), whereas the former strains are. We are now assessing PAL lysis susceptibility as a possible taxonomic aid. Also, we have used the PAL-raffinose-prepared protoplasts of Wicky 4 Ery$^R$Rif$^R$ to obtain transforming DNA; others in this laboratory are successfully employing this method in investigation of the cellular localization of competence factor in strain Challis.

LITERATURE CITED

1. Biswas, G. D., and A. W. Ravin. 1971. Heterospecific transformation of Pneumococcus and Streptococcus. IV. Variations in hybrid DNA produced by recombination. Mol. Gen. Genet. 116:1-22.
2. Burton, K. 1956. A study of the conditions and the mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochemistry 52:318-323.
3. Coleman, S. E., I. van de Rijn, and A. S. Bleiweis. 1970. Lysis of grouped and ungrouped streptococci by lysozyme. Infect. Immunity 2:563-569.
4. Corner, T. R., and R. E. Marquis. 1969. Why do bacterial protoplasts burst in hypertonic solutions? Biochim. Biophys. Acta 183:544-558.
5. Fox, E. N. 1963. Intracellular M protein of group A streptococci. J. Bacteriol. 85:536-540.
6. Friend, P. L., and H. D. Slade. 1966. Characteristics of
group A streptococcal bacteriophages. J. Bacteriol. 92:148-154.
7. Gooder, H., and W. R. Maxted. 1958. Protoplasts of group A beta-haemolytic streptococci. Nature (London) 182:806-809.
8. Leonard, C. G., J. M. Ranhand, and R. M. Cole. 1970. Competence factor production in chemically defined media by noncompetent cells of group H Streptococcus strain Challis. J. Bacteriol. 104:674-683.
9. Markowitz, A., and A. Dorfman. 1962. Synthesis of capsular polyaccharide (hyaluronic acid) by protoplast membrane preparations of group A streptococcus. J. Biol. Chem. 237:273-279.
10. Maxted, W. R. 1957. The active agent in nascent phage lysis of streptococci. J. Gen. Microbiol. 16:584-595.
11. Pakula, R., M. Piechowska, E. Bankowska, and W. Walczak. 1962. A characteristic of DNA-mediated transformation systems of two streptococcal strains. Acta Microbiol. Pol. 11:205-211.
12. Pakula, R., and M. Tyc. 1956. Use of the lytic factor from Streptomyces strain (actinomycetin) for extraction of deoxyribonucleic acid from bacteria. Med. Dosw. Mikrobiol. 8:523-528.
13. Perry, C. 1974. Binding of streptococcal competence factor by the spheroplast membrane of a group H streptococcus. J. Bacteriol. 117:702-707.
14. Perry, D., and H. D. Slade. 1962. Transformation of streptococci to streptomycin resistance. J. Bacteriol. 83:443-449.
15. Ranhand, J. M. 1974. Inhibition of the development of competence in Streptococcus sanguis (Wicky) by reagents that interact with sulphhydryl groups: discernment of the competence process. J. Bacteriol. 118:1041-1050.
16. Ranhand, J. M., and R. M. Cole. 1971. Demonstration of transforming deoxyribonucleic acid in an autolysate of a group H streptococcus. J. Bacteriol. 106:712-713.
17. Ranhand, J. M., and R. M. Cole. 1972. Lysis of streptococci by an extracellular lysis produced by competent group H streptococcus strain Challis. J. Gen. Microbiol. 71:190-202.