Protoplast and Cytoplasmic Membrane Preparations from 
Streptococcus sanguis and Streptococcus mutans

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Protoplasts were prepared from Streptococcus sanguis and some S. mutans serotypes by use of lysozyme (EC 3.2.1.17) under particular conditions: cells had to be grown in DL-threonine (20 mM) and harvested in early exponential phase. The efficiency of protoplast formation was enhanced by two additional steps: plasmolysis (in 12% PEG), prior to addition of lysozyme, and a swirling phase, after the enzymic action. This procedure allowed us to obtain clean protoplasts, with only 0.5% contamination by bacterial cell walls. Up to 90% protoplast lysis was obtained in 0.5 M-NaCl. Cytoplasmic membrane purification was achieved by centrifugation on a glycerol cushion.

INTRODUCTION

Streptococcus sanguis and Streptococcus mutans are commonly associated with dental caries (Guggenheim, 1968). The latter species appears to be very heterogeneous, and has been divided into five biotypes (Shklair & Keene, 1976), four genetic groups (Coykendall, 1974) and eight (a to h) serological groups (Bratthall, 1970; Perch et al., 1974; Beighton et al., 1981). Since purified serotype antigens were proposed as possible vaccines against dental caries (Taubman, 1973) much attention has been given to those characterized as wall-associated polysaccharides containing, in some cases, lipoteichoic acid (Silvestri et al., 1978; Vaught & Bleiweis, 1974). Because of the poor cross-reactivity between the different serotypes, more recent studies have dealt with the characterization of extracellular or wall-associated proteins (Russell & Lehner, 1978; Russell, 1979; Scholler et al., 1981). The difficulty in obtaining pure plasma membranes, free of cell wall components, from S. mutans and S. sanguis stems from the lack of efficient methods of protoplast preparation. Hence their cytoplasmic membranes are poorly characterized. Spheroplast induction with phage-associated lysins has been described for streptococci of Lancefield groups A, C, D and H (Krause, 1972; Bleiweis & Zimmermann, 1961; Calandra et al., 1975), but not for oral streptococci. Except for those belonging to Lancefield group D, most streptococci are very resistant to lysozyme lysis (Smith, 1973).

Lysozyme (N-acetylmuramidase, EC 3.2.1.17) exerts its lytic effect through hydrolysis of specific glycosidic linkages in its substrate, the peptidoglycan of the bacterial cell wall. Streptococci, and especially strains of S. mutans and S. sanguis, are considered refractory to lysozyme action (Gibbons et al., 1966) and few studies have shown lysis of S. mutans cell walls with the enzyme alone (Coleman et al., 1970). Bacterial resistance to lysozyme may stem from several factors including the three-dimensional structure of peptidoglycan (Chiu et al., 1974; Formaneck et al., 1974), the presence of teichoic acids covalently bound to peptidoglycan (Joseph & Shockman, 1975; Markham et al., 1975), the presence of unacetylated glucosamine residues in peptidoglycan (Hayashi et al., 1973) and surface adsorption of macromolecules from growth media (Chassy, 1976). Procedures used to enhance sensitivity to lysozyme include use of

Abbreviations: DAP, diaminopimelic acid; PEG, polyethylene glycol.

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appropriate culture media and age of culture, and the addition of high concentrations of sucrose, detergents and salts (Coleman et al., 1970; Eisenberg & Lillmars, 1975; Chassy, 1976; Pollock et al., 1976; Shockman et al., 1979; Goodman et al., 1981). Thus, lysozyme can reach and degrade the murein layer after acetylation of cell wall muramic acid residues in exponential phase cultures of streptococci (Eisenberg & Lillmars, 1975), or by inclusion of DL-threoneine in culture media, which interferes with cell wall cross-linking in S. mutans by a penicillin-like inhibition of lysine incorporation (Chassy, 1976). Destabilization of cell wall can also be achieved by resuspending cells in dilute Tris/HCl buffer supplemented with high concentrations of sucrose or polyethylene glycol (PEG), and by adding lysozyme in considerable excess relative to total cell peptidoglycan (Witholt & Boekhout, 1978; Owen & Freer, 1972; Johnston & Gotschlich, 1974). Although all these procedures were effective in the lysis of S. sanguis and S. mutans cells, no satisfactory method for conversion of these bacteria into stable protoplasts with lysozyme has yet been described.

Owen & Freer (1972) showed the efficiency of whole-cell plasmolysis, before removal of cell wall with lysozyme, for the preparation of mesosomal and cytoplasmic membranes from Micrococcus lysodeikticus. We tested the effect of such treatment for the preparation of stable protoplasts from S. sanguis and S. mutans strains representative of each of Bratthall's serological groups. The present paper describes a method for preparation of cytoplasmic membranes from protoplasts of S. sanguis OMZ9 and different S. mutans serotypes.

**METHODS**

**Bacteria and growth conditions.** Streptococcus sanguis OMZ9 was used as a reference strain throughout this study. The following S. mutans serotypes were used: OMZ51 (b), OMZ70 (c), OMZ176 (d), B-2 (e), OMZ175 (f). The origin and maintenance of all strains were described by Scholler et al. (1981).

Cells of S. sanguis used for conversion to protoplasts were grown in Brain Heart Infusion Broth (BHI, Difco) supplemented with 20 mM-DL-threonine at 37°C (Chassy, 1976). The bacteria were harvested by centrifugation (5000 g, 20 min, 4°C) in mid-exponential phase (OD$_{660}$ = 1-0) and washed twice in 10 mM-Tris/HCl buffer pH 7.2 (referred to as ‘Tris-buffer’ throughout this paper) before as described below.

The efficiency of protoplast formation was monitored by measuring the cell-associated peptidoglycan and DNA at each step of the preparation. meso-Diamino[3H]pimelic acid ([3H]DAP) was employed to label peptidoglycan in vivo. Cells of S. sanguis OMZ9, grown as previously described, were transferred into 100 ml BHI containing [3H]DAP (25 mm; 4 Ci mmol$^{-1}$; 148 GBq mmol$^{-1}$), and allowed to grow for three mean generation times. DNA was labelled with [3H]thymidine by growing bacteria as above, in 100 ml BHI supplemented with DL-threonine (20 mm), deoxyadenosine (20 µM) and [methyl-3H]thymidine (0.5 µM; 2 Ci mmol$^{-1}$; 74 GBq mmol$^{-1}$). Labeled cells were harvested in mid-exponential phase and used for protoplast formation.

**Protoplast formation.** Washed cells were resuspended to one-tenth of the culture volume in Tris-buffer containing 6% (w/v) PEG 4000. Lysozyme was added to final concentrations between 125 and 1000 µg per ml of initial culture volume, and the suspensions were incubated for 1 h at 30°C.

Optimal PEG concentration for protoplast stability was determined by suspending protoplasts for 24 h in Tris-buffer containing various PEG concentrations.

In the procedure involving plasmolysis and swirling, washed cells were allowed to equilibrate for 2 h at 30°C in Tris-buffer containing 12% PEG. After lysozyme treatment, the suspension was made 6% with respect to PEG, and subjected to swirling (100 r.p.m.) at 30°C for 1 h.

**Preparation of membranes.** Protoplasts were sedimented from the suspending medium by centrifugation (8000 g, 20 min, 4°C), washed twice with Tris-buffer containing 6% PEG and resuspended (2 h, 30°C) in 10 ml Tris-buffer with or without 15% PEG. In both cases lysis was performed in one of four ways: (i) sonication, (ii) freeze-thawing procedures, (iii) osmotic shock (addition of 0.5 M-NaCl to Tris-buffer or resuspension of protoplasts in buffer with or without 15% PEG), and (iv) treatment with SDS (2%, w/v).

Lysates were centrifuged three times at 6000 g for 20 min at 4°C to remove any residual protoplasts or bacteria. The 6000 g supernatant, after 15 min treatment with DNAase (10 µg ml$^{-1}$) and RNAase (10 µg ml$^{-1}$), was centrifuged at 200000 g for 30 min at 4°C. The membrane pellet was washed twice, resuspended in Tris-buffer and layered on a glycerol cushion. Centrifugation was carried out in an SW-25 rotor at 40000 g for 60 min at 4°C. The upper aqueous phase was carefully removed and centrifuged (200000 g, 30 min, 4°C), and the sedimented membranes were washed three times with Tris-buffer and stored as a thick suspension at ~20°C.

**Monitoring procedures.** The efficiency of protoplast formation was assessed by monitoring the extent of [3H]DAP labelling of cells at each stage of the procedure. Samples (1 ml) were collected on membrane filters (pore size 0.22 µm, Millipore) and immediately precipitated with 10% (w/v) cold TCA. The dried filters were
transferred to glass vials containing toluene supplemented with PPO (0-5%, w/v) and POPOP (0-01%, w/v) before counting in an SL-32 liquid scintillation counter (Intertechnique).

Protoplast integrity was assessed by measuring non-sedimentable TCA-precipitable $^{3}$Hthymidine radioactivity of incubation mixtures. Samples (10 to 30 μl) were spotted on 3MM Whatman filters, precipitated with 10% TCA and counted as previously described. Protoplast leakage was determined by measuring TCA-precipitable $^{3}$Hthymidine radioactivity released into the supernatant fraction of sedimented incubation mixtures. Purity of membrane preparations was evaluated by counting residual TCA-precipitable $^{3}$H]DAP and $^{3}$H]thymidine label present in membrane suspensions.

The different steps in protoplast and membrane preparations were also followed by electron microscopy. Protoplast pellets were fixed for 2 h in a mixture of 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0-1 m-cacodylate buffer (pH 7-4), washed with cacodylate buffer and postfixed in 1% (w/v) osmium tetroxide for 1 h, dehydrated with ethanol and embedded in Epon. Membrane preparations after osmium tetroxide postfixation were first embedded in 1% (w/v) agar, before dehydration and embedding in Epon.

Analytical procedures. Protein was determined by a modified Lowry method (Dulley & Grieve, 1975), with bovine serum albumin as standard, in the presence of 2% (w/v) SDS. Total hexose was estimated with the anthrone reagent, with glucose as standard (Morris, 1948). DNA was determined by the fluorimetric method of Thomas & Farquhar (1978), with calf thymus DNA as standard. Total lipid was extracted according to Osborn et al. (1972) and the phosphorous content of the membranes, determined either by thymidine-incorporated radioactivity or by fluorimetry, represented 1% and 0-17% of total cell DNA respectively. The total membrane

RESULTS

Protoplast formation. The susceptibility of $S$. sanguis OMZ9 to different lysozyme concentrations, under various conditions, was assayed by measuring the residual $^{3}$HDAP contained in protoplasts. Incubation of $S$. sanguis suspensions with 500 to 1000 μg lysozyme ml$^{-1}$ for 1 h resulted in a loss of 85% of the $^{3}$HDAP label (Fig. 1). Plasmolysis for 2 h in Tris-buffer containing 12% PEG before lysozyme treatment allowed an additional loss of $^{3}$HDAP of 10% (Fig. 1). When a swirling step (1 h) in Tris-buffer containing 6% PEG followed plasmolysis and lysozyme treatment, only 1-45% of the $^{3}$HDAP remained sedimentable (Fig. 1, Table 1).

PEG concentration was chosen after assessing the effect upon protoplast stability of various concentrations (4 to 12%) in Tris-buffer, after 24 h incubation of cell suspensions obtained by a 1 h treatment with lysozyme (500 μg ml$^{-1}$). A 6% PEG concentration gave minimal lysis and was employed during enzymic digestion and swirling, resulting in negligible lysis (0-48 to 1-4%) throughout protoplast preparation (Table 1).

Protoplast lysis and membrane preparation. The efficiency of protoplast disruption was investigated by four different lysis procedures (osmotic and detergent shocks, sonic oscillation, freeze-thawing) in either hypotonic (without PEG) or hypertonic (15% PEG) Tris-buffer (Table 2). The lysates were centrifuged at 200000 g for 30 min at 4 °C and the percentage of lysis was calculated from the amount of $^{3}$Hthymidine radioactivity found in the supernatant fraction compared to that found in untreated protoplasts. Addition of 2% SDS or 0-5 m-NaCl to hypotonic Tris-buffer resulted in maximal lysis and a 90% release of $^{3}$Hthymidine from the cells (Table 2). However, SDS is known to solubilize most membranes (Moldow et al., 1972; Helenius & Simons, 1975) and hence NaCl (0-5 m) in hypotonic buffer was the method of choice.

After DNAase and RNAase treatment of the lysates, membranes were collected by centrifugation at 200000 g (30 min at 4 °C), and purified on a glycerol cushion.

Analysis of membranes for purity and biological activity. Each membrane preparation from $S$. sanguis was analysed for contamination by either cell wall or cytoplasmic constituents. Radioactive DAP represented only 0-5% of the original whole-cell radioactivity (Table 1). The nucleic acid content of the membranes, determined either by thymidine-incorporated radioactivity or by fluorimetry, represented 1% and 0-17% of total cell DNA respectively. The total membrane
Fig. 1. Effect of various amounts of lysozyme, plasmolysis and swirling on lysozyme-induced protoplast formation. Remaining cell wall associated [$^3$H]DAP radioactivity was measured in the sedimented cells after incubation of bacteria for 1 h at 30 °C with various amounts of lysozyme in Tris-buffer supplemented with 6% PEG, without plasmolysis (○), with plasmolysis (■) or with plasmolysis and swirling (▲).

Table 1. Degree of lysis and cell wall contamination during preparation of protoplasts and membranes from S. sanguis

<table>
<thead>
<tr>
<th>Successive treatments</th>
<th>Percentage (w/v) of PEG in Tris-buffer</th>
<th>Lysis* (%)</th>
<th>Residual cell wall DAP† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmolysis (2 h)</td>
<td>12</td>
<td>0.48</td>
<td>100</td>
</tr>
<tr>
<td>Incubation with lysozyme (1 h)</td>
<td>6</td>
<td>1.40</td>
<td>5.1</td>
</tr>
<tr>
<td>Swirling (1 h)</td>
<td>6</td>
<td>1.37</td>
<td>1.45</td>
</tr>
<tr>
<td>Lysis (0.5 M-NaCl)</td>
<td>0</td>
<td>92</td>
<td>—</td>
</tr>
<tr>
<td>Cytoplasmic membrane preparations</td>
<td>0</td>
<td>—</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* The percentage of lysis was calculated as the fraction of total [$^3$H]thymidine radioactivity found in the supernatant of samples at the end of each treatment per OD~540~ unit.
† The percentage of cell wall contamination was measured as the fraction of total [$^3$H]DAP radioactivity found in the pellet of samples at the end of each treatment per OD~540~ unit.

Table 2. Efficiency of different methods of protoplast lysis in either hypotonic or hypertonic medium

<table>
<thead>
<tr>
<th>Method of lysis</th>
<th>Lysis* of protoplasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In hypotonic medium (Tris-buffer)</td>
</tr>
<tr>
<td>SDS (2%, w/v)</td>
<td>91</td>
</tr>
<tr>
<td>NaCl (0.5 M)</td>
<td>92</td>
</tr>
<tr>
<td>Freeze-thawing</td>
<td>6</td>
</tr>
<tr>
<td>Sonic oscillation</td>
<td>6</td>
</tr>
<tr>
<td>Resuspension in 1 M-phosphate buffer pH 7.2</td>
<td>33</td>
</tr>
</tbody>
</table>

* The percentage of lysis is calculated as the fraction of total [$^3$H]thymidine radioactivity found in the supernatants.

fraction also contained less than 1% of the glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme (Osborn et al., 1972) present in intact protoplasts.

The activity of ATPase, a membrane enzyme (Salton, 1974), was measured to determine whether the procedure used in membrane purification released membrane-associated enzymes. No ATPase activity was found in the different supernatants during protoplast formation. The S. sanguis membrane fractions contained 1.7 units of ATPase activity [66.6% of total activity,
Cytoplasmic membranes of oral streptococci

Fig. 2. Electron micrographs of thin sections of S. sanguis OMZ9 after different treatments. (a) Control bacteria; (b) cells after treatment for 1 h with lysozyme (500 μg ml⁻¹); (c) cells prior to lysozyme treatment but after plasmolysis (2 h at 30 °C in presence of 12% PEG); (d) cells after plasmolysis, lysozyme treatment and swirling (1 h at 30 °C in presence of 6% PEG). Progressive solubilization of the cell wall occurred during the above mentioned treatments and a typical protoplast apparently devoid of cell wall material can be seen in (d). The bar markers represent 0.1 μm.

Progressive solubilization of the cell wall occurred during the above mentioned treatments and a typical protoplast apparently devoid of cell wall material can be seen in (d). The bar markers represent 0.1 μm.

specific activity 0.2 units (mg protein)⁻¹] whereas 0.6 units (23.8%, 0.0012 units mg⁻¹) was found in the cytoplasmic fraction, and 0.24 units (9.5%) in the different membrane washes, indicating that rather low release of enzyme occurred during this procedure.

Chemical analysis of the membranes. The results of the gross chemical analysis of S. sanguis cytoplasmic membranes are expressed as percentages of the total dry weight of membranes. Total protein accounted for 62% of the membrane preparation. Total lipids comprised 20% of the dry weight, with a phosphorus/lipid ratio of 0.045. Total hexose content of the membrane was relatively low, representing only 4.2%, which agrees with the work of others (Owen & Freer, 1972; Van de Rijn & Kessler, 1979).

Electron microscopy. All steps in protoplast and membrane preparations were further followed by electron microscopy to confirm the effectiveness of the method. Figure 2 illustrates the different stages in protoplast formation after 1 h incubation in the reaction mixture with lysozyme alone (Fig. 2b), with preliminary plasmolysis (Fig. 2c), and with additional swirling (Fig. 2d). While initially the streptococci had a typical cell wall (Fig. 2a), progressive elimination of wall material was seen after the different treatments, and wall contamination was
minimal after plasmolysis and swirling (Fig. 2d). Thin sections of cytoplasmic membranes after centrifugation on a glycerol cushion appeared to be completely devoid of cell wall contamination (Fig. 3).

Application of the method to other serotypes. The results described above showed that cytoplasmic membrane preparations could be obtained from S. sanguis OMZ9. The same procedure, outlined in Fig. 4, was successfully used to obtain cytoplasmic membranes from different S. mutans serotypes.

DISCUSSION

With the exception of Siegel et al. (1981), who used mutanolysin to induce spheroplasts, no suitable method has been described for preparation of plasma membranes from S. sanguis or S. mutans. We report a method for producing membranes from these species by a preliminary conversion of bacteria into stable protoplasts with lysozyme. Acetylation of bacterial cells according to Eisenberg & Lillmars (1975) did not provide any advantage in protoplast formation (data not shown). In order to enhance lysozyme sensitivity, bacteria from exponential phase cultures grown in the presence of DL-threonine (Chassy, 1976) were diluted in Tris/HCl buffer (Witholt & Boekhout, 1978) supplemented with PEG (Owen & Freer, 1972). After determination of optimal concentrations of lysozyme (500 µg ml⁻¹) and PEG (6%), we tested the effect of plasmolysis and swirling on the efficiency of protoplast formation. Exposure of cells to 12% PEG increased their sensitivity to lysozyme and increased the yield of protoplasts. This was further improved by a swirling step after PEG dilution. Chemical and ultrastructural studies indicated the almost complete lack of cell wall material in the protoplast preparations. Addition of 0.5 M-NaCl to osmotically sensitive protoplasts suspended in hypotonic Tris-buffer resulted in up to 90% lysis, and membranes prepared in this way contained only a small amount of the peptidoglycan DAP. This method allowed us to prepare cytoplasmic membranes from the different S. mutans serotypes.

The gross chemical composition of S. sanguis cytoplasmic membranes appeared to be in broad agreement with that reported by Kessler & Van de Rijn (1979). Contamination of the membranes by either extracellular or intracellular components was less than 1%, as estimated by the peptidoglycan or DNA content, and the protein/phospholipid ratio was in accord with those generally found for Gram-positive bacteria (Owen & Freer, 1972; Van de Rijn & Kessler, 1979).

It should be pointed out that the above results are drawn from membranes with minor cytoplasmic or wall contamination. The enzyme treatment and washing steps do not appear to affect the integrity of the membranes, which can be compared to membranes obtained by other methods (Siegel et al., 1981).
Washed cells (grown in BHI containing 20 mM-DL-threonine)

Plasmolysis (Tris/HCl, 10 mM, pH 7.2 ['Tris-buffer'] containing 12% (w/v) PEG, 2 h, 30 °C)

Protoplast formation (lysozyme, 500 μg ml⁻¹, 1 h, 30 °C)

Swirling (dilution with 1 vol. Tris-buffer, 1 h, 30 °C)

Centrifugation (8000 g, 20 min, 4 °C)

Washed protoplasts (suspended in Tris-buffer, 2 h, 30 °C)

Lysis (NaCl, 0.5 M; RNAase and DNAase 10 μg ml⁻¹)

Centrifugation × 3 (6000 g, 10 min, 4 °C)

Pellet (discard) Supernatant containing cytoplasmic membranes

Centrifugation (200000 g, 30 min, 4 °C)

Supernatant (discard) Pellet resuspended in Tris-buffer

Centrifugation on glycerol cushion (40000 g, 60 min, 4 °C)

Aqueous phase containing purified cytoplasmic membranes

Fig. 4. Procedure used to obtain cytoplasmic membrane preparations from protoplast suspensions.

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REFERENCES


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