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Protoplast Formation and Localization of Enzymes in *Streptococcus mitis*

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Cells of *Streptococcus mitis* ATCC 903 were converted to stable protoplasts by the cell wall-degrading M-1 enzyme of the mutanolysin complex isolated from *Streptomyces globisporus*. Over 90% of total glucokinase (EC 2.7.1.2), aminopeptidase (EC 3.4.11.1), and dextranglucosidase (EC 3.2.1.70) was recovered in the cytoplasmic fraction, whereas over 20% of total invertase (β-fructofuranosidase; EC 3.2.1.26) was released during protoplast formation. ATPase (EC 3.6.1.3), chymotrypsin-like protease (EC 3.4.21.1), arginine aminopeptidase (EC 3.4.11.6), and lactate dehydrogenase (EC 1.1.1.27) were detected in Triton X-100 extracts of the cytoplasmic membrane fraction by crossed immunoelectrophoresis in combination with enzyme-staining procedures. By these methods, NADH dehydrogenase (EC 1.6.99.3), aminopeptidase, and lactate dehydrogenase were detected in the cytoplasmic fraction. Aminopeptidases in the cytoplasmic fraction differed from this activity in the membrane fractions in electrophoretic mobility and substrate specificity.

Streptococcus mitis is one of the most frequent organisms in the oral cavity (7) and is most likely a significant factor in human dental caries due to its acidogenicity (22) and high degree of adhesion to the tooth surface (7). *S. mitis* is also one of the most important pathogens in bacterial endocarditis (38).

Many oral bacteria, including *S. mitis*, have enzyme activities that are presumed to interact with substrates in the oral environment. Although some of these activities are extracellular, secreted products, others are cellbound. Thus, the cellular localization and, therefore, the accessibility of such activities to their substrates in intact bacteria are of interest. The determination of the subcellular localization of enzymes by the physical disruption of cells has several disadvantages and sometimes leads to ambiguous results. Thus, many investigators have resorted to the preparation of osmotically fragile forms, such as protoplasts or spheroplasts, that can be rapidly lysed (see reference 25 for a review; 36).

Hen egg white lysozyme has been used to prepare osmotically fragile, spherical, wall-free protoplasts from a number of gram-positive bacteria (25), including a few strains of streptococci, such as *Streptococcus faecium* ATCC 9790 (35, 37), that are highly sensitive to the action of this enzyme. However, many other streptococcal strains, including a broad variety of oral streptococci, are not lysed by the action of hen egg white lysozyme (6). This resistance to cell dissolution may occur despite the hydrolysis of susceptible bonds in the cell wall peptidoglycan, for example, as demonstrated for *Streptococcus mutans* BHT (8). Several examples of successful bacterial dissolution accomplished by the addition of detergents or specific salts after treatment with hen egg white lysozyme have been reported previously (3–5, 8, 9, 32). However, the recent availability of an *N*-acetylmuramoylhydrolase (the M-1 enzyme of the mutanolysin complex; 51, 52) that can hydrolyze bonds in the peptidoglycan and lyse the cells of a broad variety of bacteria (52) has permitted the lysis of a variety of bacterial species (2, 27) and the successful formation of osmotically stable protoplasts of *S. mutans* BHT (30, 39).

The present study describes a procedure for the preparation of stable protoplasts of *S. mitis* ATCC 903, utilizing the M-1 enzyme. The successful preparation of protoplasts of this bacterium made it possible to isolate subcellular fractions (e.g., the cytoplasmic membrane fraction, the cytoplasmic fraction, and the solubilized cell wall fraction) and to analyze these fractions for enzyme activities.

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MATERIALS AND METHODS

Growth conditions. *S. mitis* ATCC 903 was grown at 37°C in a stirred fermentor (FL 101; Biotec, Stock-

holm, Sweden) at pH 6.5, under anaerobic conditions as described earlier (19). The chemically defined growth medium used the FMC medium (47) with the addition of L-cysteine (1 mM) and sucrose (30 mM).

The precultures were inoculated with lyophilized cells and grown overnight in proteose-peptone-glucose medium (19).

Preparation of protoplasts. Cells in the late exponential growth phase were harvested by centrifugation (20,000 × g, 15 min) and washed with 50 mM sodium acetate (pH 8.0) containing 0.2 mM magnesium acetate. Washed cells (7 to 9 mg [dry weight] per ml) were suspended by homogenization with a Teflon tissue homogenizer in a buffer (protoplasting buffer) containing 0.75 M melezitose (unless stated otherwise), 50 mM sodium acetate (pH 8.0), 0.2 mM magnesium acetate, 10 mM sodium fluoride, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was prewarmed to 37°C before protoplast formation was started by the addition of M-1 enzyme to a final concentration of 0.25 mg/ml. After incubation at 37°C, the protoplasts were sedimented (13,000 × *g*, 15 min). This supernatant was dialyzed against 10 mM sodium phosphate (pH 7.0) and called the solubilized cell wall to indicate cell wall or periplasmic material or both that was not sedimented by the centrifugation of the protoplasts. Sedimented protoplasts were washed once in protoplasting buffer and then lysed by suspension in 10 mM sodium phosphate (pH 7.0)–0.2 mM magnesium acetate–DNase (7 U/ml)–RNase (0.3 U/ml). The lysate was centrifuged (3,000 × *g*, 20 min) to remove the remaining whole cells.

Preparation of cytoplasmic and membrane fractions. Protoplast lysates were centrifuged (165,000 × *g*, 1 h) in a type 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The supernatant, designated as the cytoplasmic fraction, was stored at −70°C. The membrane pellet was suspended in and washed three times in 20 mM potassium phosphate (pH 7.0) containing 0.2 mM magnesium acetate at 4°C and centrifuged (165,000 × *g*, 1 h). The washed membranes were suspended in a minimal volume of 50 mM Tris-hydrochloride (pH 8.6) and stored at −70°C until used for detergent extraction and for the determination of *N*-acetylglucosamine and protein content.

Preparation of detergent extracts of the membrane fraction. Membranes (final concentration, 12 to 139 mg/ml) were extracted in 50 mM Tris-hydrochloride (pH 8.6) with 4% (vol/vol) Triton X-100 at 22°C for 2 h. The supernatant fraction obtained after centrifugation at 40,000 × *g* for 30 min at 4°C was divided in 50- and 100-μl portions and stored at −70°C until used for protein, crossed immunoelectrophoresis, and isoelectric focusing analyses. The pelleted material was extracted, centrifuged, and stored again as described above. Residual pellets were washed once by suspension in 50 mM Tris-hydrochloride buffer (pH 8.6), sedimented (40,000 × *g*, 30 min, 4°C), and extracted with 0.1% (wt/vol) sodium dodecyl sulfate (SDS) in 50 mM Tris-hydrochloride (pH 8.6) at 100°C for 2 min. After centrifugation (40,000 × *g*, 30 min, 4°C), the supernatants were removed and stored as described above.

Disruption of cells and preparation of antiserum. Late-exponential-phase cells were suspended by homogenization in 10 ml of lysis buffer to the same cell density as in the protoplasting experiments (7 to 9 mg/ml) and disrupted in the X-press (18). After thawing and addition of DNase (7 U/ml) and RNase (0.3 U/ml), the disrupted cells were shaken at 37°C for 10 min, homogenized in a tissue homogenizer and centrifuged (3,000 × *g*, 20 min) to remove the remaining whole cells. The resulting supernatant in Freund incomplete adjuvant was used to immunize New Zealand white rabbits as described by Harboe and Ingild (11).

Electrophoretic procedures. Crossed immunoelectrophoresis was performed by a modification of the Laurell technique (17) described by Weeke (50). The electrophoresis apparatus (Analysteknik AB, Vallentuna, Sweden) had a circulating buffer system. Barbital-hydrochloride buffer was used. Agarose gels (1% [wt/vol]) containing Triton X-100 (1% [vol/vol]) were prepared from Seakem HGT agarose (Marine Colloids, Inc., Rockland, Maine) and cast on glass plates (50 by 50 by 0.6 mm). Electrophoresis was performed at 24 V/cm for 25 min in the first dimension and at 24 V/cm for 2 h in the second dimension. After completion of the electrophoresis, the gels were pressed and used to prepare zymograms or washed in 0.1 M NaCl for 24 h and in distilled water for 24 h and air dried in preparation for staining with Coomassie brilliant blue R-250 (Inolex Corp., Glenwood, Ill.) by the method of Weeke (50). Isoelectric focusing in polyacrylamide gels was performed as described recently (46).

Visualization of enzyme activities in gels. Specific enzymes were visualized in the gels by incubation in enzyme-staining mixtures. The staining procedures for NADH dehydrogenase (EC 1.6.99.3) and ATPase (EC 3.6.1.3) have been described by Owen and Salton (29), and those for β-fructofuranosidase (invertase) (EC 3.2.1.26) have been described by Sund et al. (46). Immunoprecipitates containing D-lactate dehydrogenase (EC 1.1.1.27) were visualized by the method of Smyth et al. (40), and those containing chymotrypsin-like protease (EC 3.4.21.1) and trypsin-like protease (EC 3.4.21.4) were visualized as described by Uriel (49). Aminopeptidase was detected by the incubation of the immunoplate for 20 min in a solution containing L-arginine-β-naphthylamide or L-leucine-β-naphthylamide (1.37 mM; Sigma Chemical Co., St. Louis, Mo.), $CoCl_2$ (7.5 mM), and Fast Garnet GBC salt (0.055%; Sigma). Polyacrylamide gels were stained for invertase activity after isoelectric focusing as described previously (46).

Electron microscopy. Protoplasts and whole cells were initially fixed in protoplasting buffer with 2% (vol/vol) glutaraldehyde for 45 min at 4°C and then in 2% glutaraldehyde in 0.1 M cacodylate (pH 7.3) containing 0.1 M sucrose for 24 h at 4°C. After being washed at 4°C for 24 h in 0.1 M cacodylate (pH 7.3) containing 0.1 M sucrose, the protoplasts were post-fixed with 2% (vol/vol) OsO_4 in *S*-collidine (pH 7.3) for 90 min at 4°C and then dehydrated in ethanol and embedded in Epikote. Thin sections prepared with an LKB-Ultratome V were examined in a Philips 400 electron microscope.

Enzyme assays. Glucokinase (EC 2.7.1.2) activity was determined after the incubation of samples in 130 mM potassium phosphate (pH 7.5), 50 mM glucose, 10 mM ATP, and 10 mM $MgCl_2$ in a total volume of 2.0 ml for 20 min at 37°C. The reaction was terminated by boiling for 5 min. NADP (3 mM) and glucose-6-phosphate dehydrogenase (1 to 3 U) were then added, and the amount of NADPH formed during 15 min at

37°C was determined spectrophotometrically at 340 nm. The amount of NADPH formed is directly proportional to the amount of glucose-6-phosphate. Corrections for the amount of glucose-6-phosphate present in the enzyme sample were made. Dextranglucosidase (EC 3.2.1.70) and invertase (EC 3.2.1.26) were determined as described earlier (44). The method of Linder et al. for the determination of aminopeptidase (20) was modified by the addition of 0.5 mM $CoCl_2$ to the reaction mixture. Protease activity was determined by the method of Kunitz (16). All enzyme activities are expressed in katals. One katal (kat) is the amount of enzyme catalyzing the formation of 1 mol of product per s under the conditions described.

Miscellaneous procedures. *N*-acetylglucosamine was determined by the method of Morgan and Elson (28). Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as the standard. When membrane detergent extracts were analyzed by isoelectric focusing in polyacrylamide gel, Triton X-100 was removed from the sample before focusing by the method of Holloway (14), using Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, Calif.).

Chemicals. Melezitose was obtained from Fanstiehl Laboratories, Waukegan, Ill. Mutanolysin (M-1 lytic enzyme) was a generous gift from K. Yokogawa, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. PMSF was purchased from Sigma.

RESULTS

The procedure of Parks et al. (30) required some modifications when applied to the studies of *S. mitis* ATCC 903 described here. PMSF (0.5 mM) was added to the protoplasting buffer to inhibit serine protease activity. This concentration of PMSF inhibited the proteolytic activity on casein of the M-1 preparation used by over 90%. Sodium fluoride (10 mM) was added to the protoplasting buffer to prevent a decrease in the pH of the incubation mixture. In the absence of sodium fluoride, the pH decreased to below 5.5 after 30 min at 37°C, probably due to the metabolism of intracellularly stored polyglucose (22). The optimum osmotic stabilization, as determined by the release of the intracellular enzyme glucokinase (Fig. 1), was at a melezitose concentration of 0.75 M (4% of total glucokinase).

Formation of protoplasts. The incubation of exponential-phase cells of *S. mitis* ATCC 903 with M-1 enzyme in protoplasting buffer as described above resulted in maximal osmotic fragility in about 30 min (Fig. 2). After that time, about 10% of the initial cell turbidity remained after the dilution of the suspension in 0.01 M sodium phosphate, pH 7.0. Electron micrographs of thin sections of untreated cells of *S. mitis* ATCC 903 showed cells surrounded by a typical cell wall structure and fimbriae-like appendages (Fig. 3A), whereas cells treated with M-1 enzyme in protoplasting buffer for 20 min appeared as round bodies devoid of a cell wall and fimbriae (Fig. 3B). Some of these bodies

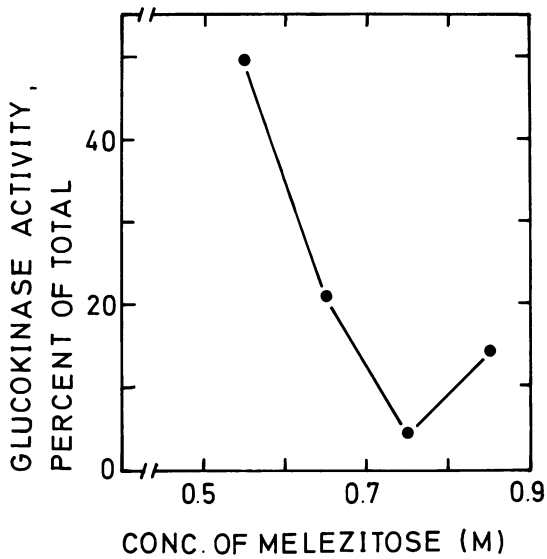

FIG. 1. Osmotic stabilization of protoplasts of *S. mitis* ATCC 903. The cells were suspended in protoplasting buffer containing M-1 enzyme and various concentrations of melezitose. After 30 min at 37°C, the protoplasts were sedimented (13,000 × *g*, 15 min), and the glucokinase activity present in the supernatants and in the pelleted protoplasts was determined. The percentage of the sum of the activities in the two fractions present in the supernatants is shown.

(less than 10%) were partly deformed and without a continuous outer delineation.

Cytoplasmic membranes isolated from protoplast lysates contained less than 0.5% glucosamine and, therefore, seemed essentially free of cell wall material. In an attempt to increase cell wall hydrolysis, 100 μg of hen egg white lysozyme per ml was added simultaneously with M-1 to the suspension of cells in protoplasting buffer at zero time. Surprisingly, hen egg white lysozyme was found to inhibit the attainment of osmotic fragility (Fig. 2). When added after 20 min of incubation with the M-1 enzyme, hen egg white lysozyme had no effect on protoplast formation (data not shown).

Cellular localization of enzyme activities. During protoplast formation, protein was released to the supernatant buffer, resulting in a release of over 28% of the total protein after 45 min (Fig. 4). In contrast, nearly all of the glucokinase (96%), aminopeptidase (93%), and dextranglucosidase (95%) remained with the protoplasts—an outcome consistent with a cytoplasmic or membrane location of these three activities. Substantial amounts of fructofuranosidase activity were released during the incubation; nearly 20% of this activity was released after 45 min. However, the kinetics of release were unusual in that nearly half of the activity that was found in the

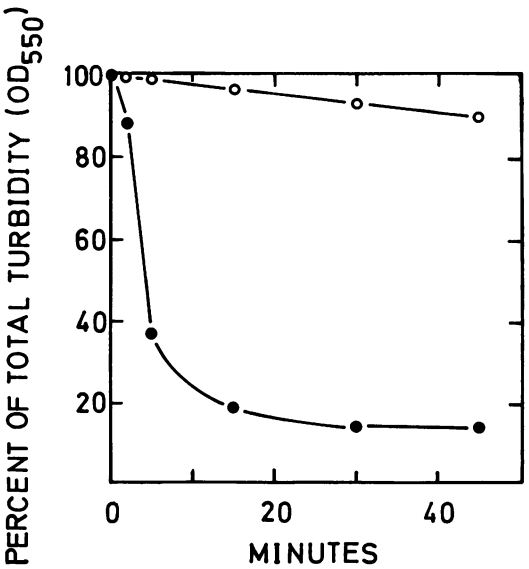

FIG. 2. Attainment of osmotic fragility during incubation of *S. mitis* ATCC 903 with 250 μg of M-1 enzyme per ml (●) or with 250 μg of M-1 and 100 μg of lysozyme per ml (○) in the protoplasting buffer. The percentage of the initial turbidity (OD_{550}) remaining after a 40-fold dilution of the cell suspension in 10 mM sodium phosphate (pH 7.0) is plotted against the incubation time.

supernatant was released between 20 and 45 min, well after the cells had attained maximum osmotic fragility.

Triton X-100 extraction of the membrane fraction. The extraction of bacterial cytoplasmic membranes by the nonionic detergent Triton X-100 has proved to be a convenient method for the subsequent analysis of membrane antigens by crossed immunoelectrophoresis provided that membrane fractions with high initial protein concentrations can be obtained (40). The extraction of membrane fractions, containing 12 to 139 mg of protein per ml, was performed twice with 4% Triton X-100 and solubilized about 90% of the membrane protein (Table 1). The initial concentration of protein in the membrane fraction did not seem to affect the distribution of protein in the extracts. No visible particulate residue remained after treatment with 0.1% SDS.

Examination of membrane and cytoplasmic contents by crossed immunoelectrophoresis. A maximum of 33 immunoprecipitates were detected after crossed immunoelectrophoresis of Triton X-100 extracts of membrane fractions, whereas the maximum number for the cytoplasmic fraction was 73. As expected, these numbers varied with the concentration of both antigen and antibody. The detergent extracts of the membrane fraction contained many more slowly migrating antigens than did the cytoplasmic fraction (see Fig. 6A and 7A). Significant differences were not observed in the profile of bands present in the successive membrane extracts (Fig. 5), suggesting a lack of selectivity of the extractants.

Characterization of immunoprecipitates of the cytoplasmic membrane extract by zymogram procedures. Many bacterial enzymes associated with metabolic processes have been shown to be

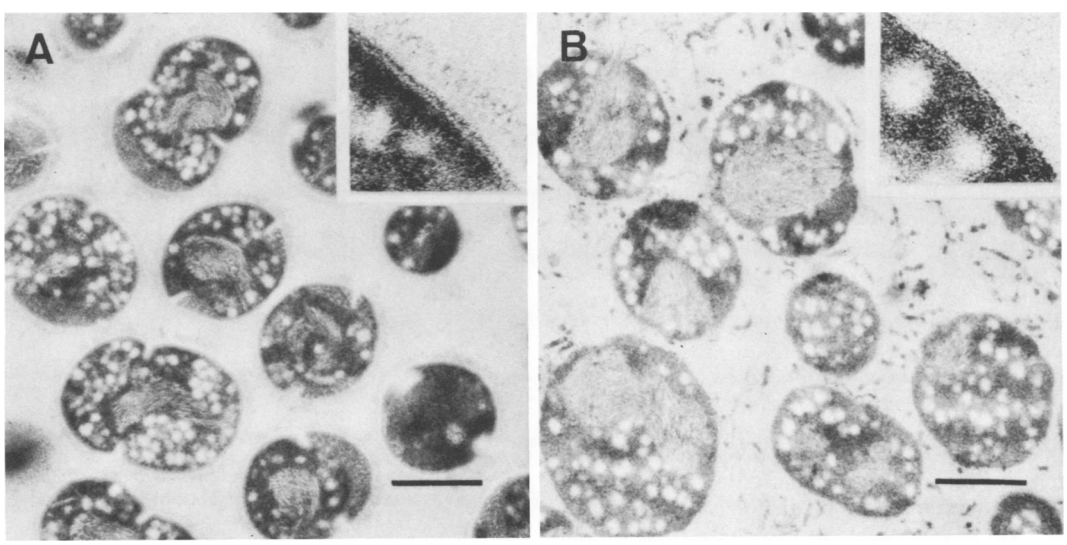

FIG. 3. Electron micrographs of thin sections of *S. mitis* ATCC 903 before treatment with M-1 enzyme (A) and after 20 min of treatment in protoplasting buffer (B). The bar equals 0.5 μm. Magnification of insets, ×130,000.

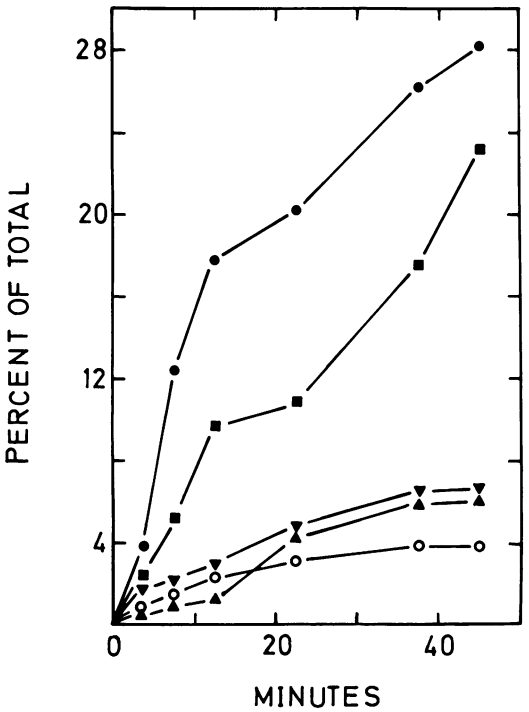

FIG. 4. Release of specific enzymes and protein from *S. mitis* ATCC 903 during protoplast formation. The percentage of total protein and enzyme activities released during incubation with the M-1 enzyme is plotted against the time of incubation. The total protein and enzyme activities were calculated from the sum of the activities recovered in the protoplasting buffer supernatants and in the pelleted protoplasts (13,000 × *g*, 15 min) after 30 min of protoplast formation. The total protein and enzyme activities recovered with 9.6 mg (dry weight) of cells is given below in parentheses. Symbols: ●, protein (3.8 mg); ○, glucokinase (115 nkat); ▲, aminopeptidase (10.8 nkat); ▼, dextranglucosidase (2.5 nkat); ■, invertase (β-fructofuranosidase; 18.6 nkat).

TABLE 1. Detergent extraction of the membrane fraction of *S. mitis* ATCC 903[a]

Membrane fraction	Amt of protein		% Protein extracted
	mg	mg/ml	
Isolated membranes	3.5	11.9	
First Triton X-100 extract	2.1	7.0	60
Second Triton X-100 extract	1.1	3.8	31
Buffer wash	0.1	0.2	3
SDS extract	0.1	0.2	3
Recovery (%)			97

[a] A total of 424 mg (dry weight) of cells was converted to protoplasts. The protoplasts were lysed in hypotonic buffer, and cytoplasmic membranes were isolated by centrifugation of the lysate at 165,000 × *g* for 1 h.

associated with the cytoplasmic membrane (34). Immunoprecipitates containing ATPase, lactate dehydrogenase, aminopeptidase, NADH dehydrogenase, and chymotrypsin-like protease activities present in the first Triton X-100 extract by the membrane fraction of *S. mitis* ATCC 903 were visualized by zymogram procedures after crossed immunoelectrophoresis (Fig. 6). Four enzyme-active immunoprecipitates were detected for ATPase (Fig. 6B) of which three stained intensely. For lactate dehydrogenase and chymotrypsin-like protease activities, two and four immunoprecipitates, respectively, were detected. These precipitates were sharp (Fig. 6C and A). Three intensely stained precipitates were detected in gels stained for aminopeptidase with arginine-β-naphthylamide as the substrate (Fig. 6D). None of these precipitates stained when leucine-β-naphthylamide was used as the substrate. NADH dehydrogenase activity was found associated with only one very small precipitate (Fig. 6E). Immunoprecipitates with invertase or trypsin-like protease activity were not detected by appropriate zymogram procedures. All enzyme-stained gels could be subsequently stained with Coomassie brilliant blue. By this method, the identification of the immunoprecipitates in the Coomassie brilliant blue-stained pattern could be achieved, as shown for the

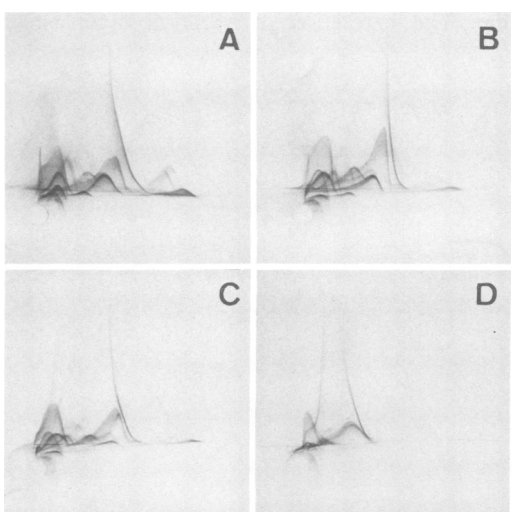

FIG. 5. Crossed immunoelectrophoresis of different consecutive detergent extracts of the cytoplasmic membrane fraction of *S. mitis* ATCC 903. (A) First Triton X-100 extract; (B) second Triton X-100 extract; (C) supernatant after buffer wash; and (D) final SDS extract. The antigen preparations contained 70 μg of protein.

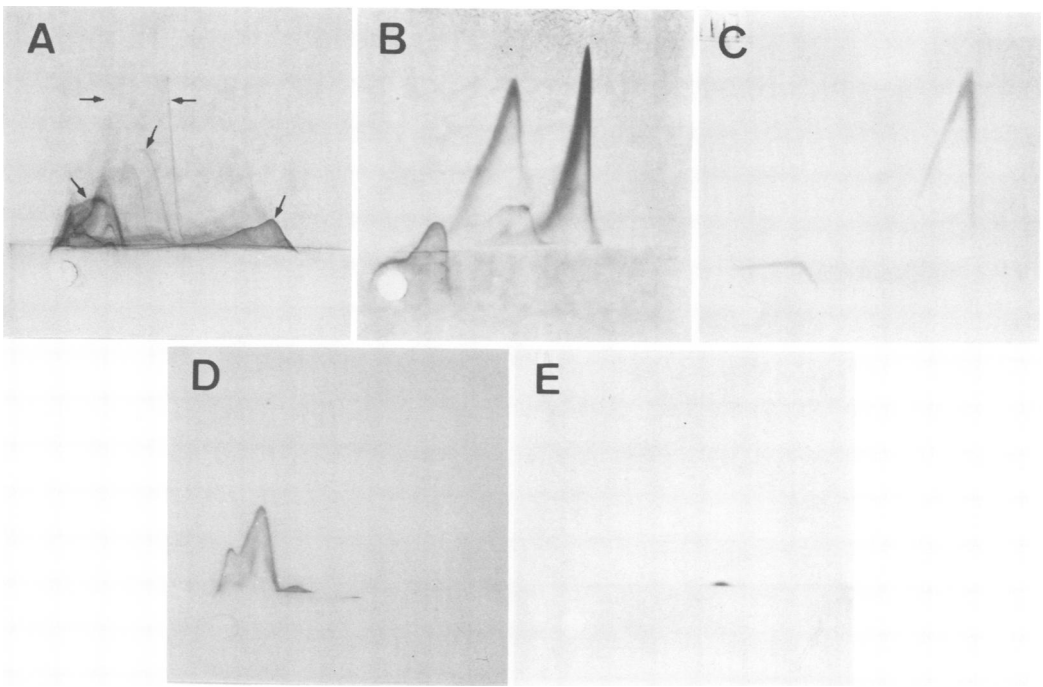

FIG. 6. Crossed immunoelectrophoresis of a detergent extract of the cytoplasmic membrane fraction of *S. mitis* ATCC 903. Cytoplasmic membranes were extracted with 4% (vol/vol) Triton X-100. A 10-μl amount of the first Triton X-100 extract (70 μg of protein) was used. In (B to E), the gels were stained to reveal the various enzyme activities present as follows: (B) ATPase; (C) lactate dehydrogenase; (D) aminopeptidase; and (E) NADH dehydrogenase. In (A), a gel previously stained for enzyme activity was subsequently stained with Coomassie brilliant blue. The arrows in (A) show the precipitates that stained for chymotrypsin-like protease activity.

chymotrypsin-like protease (Fig. 6A). Brogren and Bøg-Hansen (1) reported that the enzyme-staining reactions may be due to nonspecific reactions, such as the entrapment of enzyme or the affinity of the precipitate for the dye. However, we found that no precipitates stained for more than one enzyme activity. Furthermore, stained precipitates were not detected when the enzyme substrate was omitted from the enzyme-staining mixture (ATPase and NADH dehydrogenase).

Characterization of immunoprecipitates of the cytoplasmic fraction by zymogram procedures. The enzyme staining of immunoprecipitates of the cytoplasmic fraction revealed the presence of ATPase (Fig. 7B), lactate dehydrogenase (Fig. 7C), aminopeptidase (Fig. 7D), and NADH dehydrogenase (Fig. 7E). A single, small immunoprecipitate staining for ATPase activity was detected (Fig. 7B). For lactate dehydrogenase, a single sharp, stained precipitate was found (Fig. 7C). Aminopeptidase activity was found associated with three stained precipitates (Fig. 7D), and NADH dehydrogenase was found associated with two stained precipitates (Fig. 7E). In contrast to the aminopeptidase immunoprecipitates of the membrane detergent extract, all three amino peptidase precipitin arcs of the cytoplasmic fraction stained identically with arginine-β-naphthylamide and leucine-β-naphthylamide as the substrates.

Based on differences in the electrophoretic profiles, staining intensities, and substrate specificities, it was concluded that none of the aminopeptidases of the cytoplasmic fraction was identical to any of the aminopeptidases of the membrane detergent extract. Immunoprecipitates possessing invertase and protease activities could not be detected in the cytoplasmic fraction by zymogram techniques. However, invertase could be visualized by the zymogram procedures as an intensely stained band in the first dimension of crossed immunoelectrophoresis of the cytoplasmic fraction. The presence of immunoglobulins specific for invertase in the antiserum preparation was tested by crossed immunoelectrophoresis of invertase of *S. mitis* ATCC 903 purified to homogeneity (43). A sharp and intensely stained immunoprecipitate was visualized by staining with Coomassie brilliant

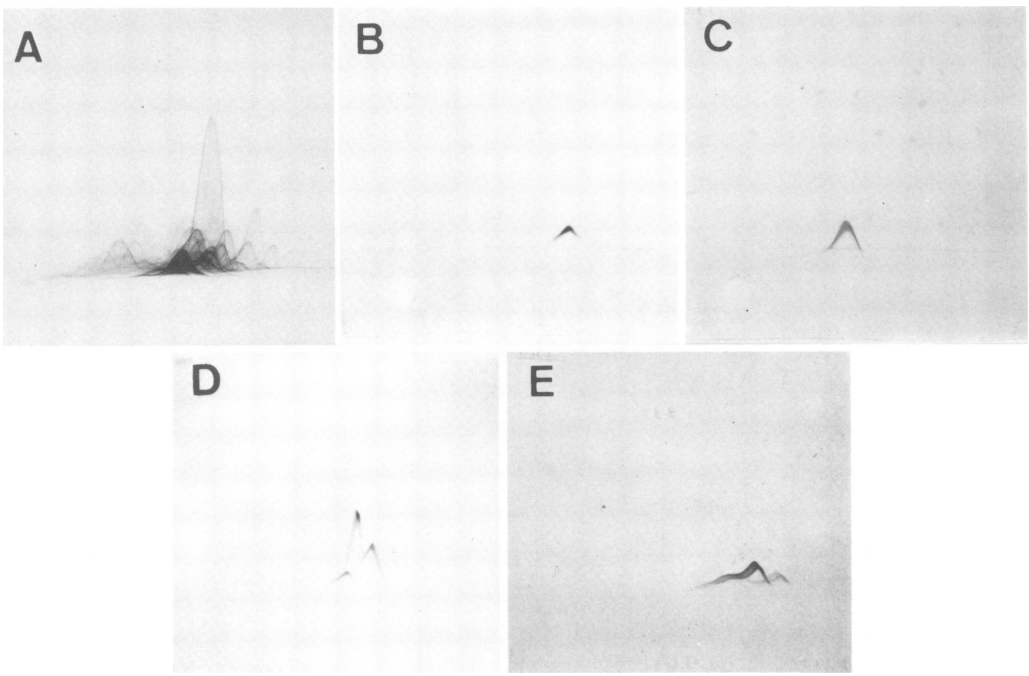

FIG. 7. Crossed immunoelectrophoretic analyses of the cytoplasmic fraction of *S. mitis* ATCC 903. The cytoplasmic fraction was prepared from a protoplast lysate and concentrated five-fold to 7.0 mg of protein per ml. A 10-μl amount (70 μg of protein) was used in the electrophoresis. (A) Immunoprecipitates stained with Coomassie brilliant blue. The characterization of the immunoprecipitates was performed with similar gels by staining for various enzyme activities as follows: (B) ATPase; (C) lactate dehydrogenase; (D) aminopeptidase; and (E) NADH dehydrogenase.

blue (data not shown). This immunoprecipitate did not stain for invertase activity. From these findings, it was concluded that unlike most enzymes, invertase of *S. mitis* ATCC 903 does not retain its activity after precipitation with specific antibodies (1).

Isoelectric focusing. The presence of invertase activity in all three subcellular fractions was investigated further by isoelectric focusing in thin-layer polyacrylamide gels in combination with zymogram procedures. Both the cytoplasmic fraction and the solubilized cell wall fraction showed a single band staining for invertase activity identically positioned in the gel at pI 4.25 (data not shown). Invertase activity was not detected in the detergent extract of the cytoplasmic membrane. It is possible that this activity is sensitive to inactivation by the detergent used.

DISCUSSION

Protoplast and spheroplast formation in an osmotically stabilizing environment is widely used in studies concerned with the localization of enzymes or other cell components. In *Escherichia coli* and other gram-negative bacteria, a group of enzymes which are selectively released during spheroplast formation have been localized in a region of the cell between the cytoplasmic membrane and the cell wall called the periplasmic space. Although reports on periplasmic enzymes in gram-positive bacteria are scarce, enzymes have been found located at or near the cell surface of gram-positive bacteria (24). Indirect evidence for the location near the cell surface of invertase of *S. mitis* ATCC 903 has been obtained previously by immunological and biochemical methods (45). In the present studies, although the major part of invertase activity was recovered in the cytoplasmic fraction, a fraction (e.g., 25%; Fig. 4) of the total invertase activity was released to the supernatant fraction during protoplast formation. Whether these observations reflect a cell wall or periplasmic space-like location of part of the invertase activity of *S. mitis* or leakage of cytoplasmic invertase through the membrane is uncertain. Isoelectric focusing analyses failed to yield evidence of the presence of chemically different invertases in the solubilized cell wall fraction and in the cytoplasmic fraction. A similar selective release of invertase from *Neurospora crassa* was reported earlier (48).

The inhibition of invertase activity of *S. mitis* by immunoglobulins is interesting and will be

further investigated. Hamada and Mizuno (10) reported that glucosyltransferase of *S. mutans*, a sucrose-active enzyme, was inhibited by an antiserum.

The localization of proteins within the cell has proved to be of great value in furthering our understanding of the distribution of functions in the cell. Our investigation clearly demonstrated the presence of biochemically and immunochemically distinct peptide hydrolases associated with the membrane and in the cytoplasmic fraction.

The data presented here suggest the following hypothesis for the utilization of peptides or proteins by *S. mitis*. Membrane-associated proteases and peptidases would be responsible for both hydrolysis to lower-molecular-weight peptides and their subsequent translocation through the membrane into the cytoplasm. The cytoplasmic peptidases would be responsible for the hydrolysis of these peptides into their constituent amino acids. This close-coupling model of transport and hydrolysis suggested for the membrane-associated proteases of *S. mitis* was previously proposed for peptide transport in eucaryotes (31). However, it is also possible that one or more of the membrane-associated proteases may be important in the processing of secreted proteins.

Reports on membrane-associated proteases and peptidases in bacteria are scarce. Smyth et al. (41) showed the presence of chymotrypsin-like protease in the cytoplasmic membrane of *E. coli* by crossed immunoelectrophoresis. Miller and Becker (26) found evidence for membrane-associated oligopeptidase activity in *Pseudomonas aeruginosa*. The presence of cell-bound proteolytic activity in viridans streptococci has been anticipated by some authors. Sund and Linder (42) claimed that the autolysins of *S. mitis* ATCC 903 had an endopeptidase type of action. Jacques and Wittenberger (15) found that the observed inactivation of fructosyltransferase in *Streptococcus salivarius* was most likely due to cell-bound proteolytic activity.

The cytoplasmic location of dextranase demonstrated by the present investigation suggests that this enzyme may not be involved in the degradation of dental plaque dextrans as suggested earlier (21). However, it must be emphasized that strains of *S. mitis* are prone to autolysis (42). Furthermore, autolysis has been found to be the only mechanism for the release of hyaluronidase from *S. mitis* ATCC 903 (18). This enzyme has been considered a typical extracellular enzyme in bacteria because of its high-molecular-weight substrate.

Bacterial membrane-associated ATPase enzyme complexes have been shown to participate in proton transport (12) and in the exclusion of sodium ions from the interior of the bacterial cell (13). Indirect evidence for a role for ATPase in sugar transport (33) and acid production (22) in *S. mitis* ATCC 903 has been obtained earlier. To our knowledge, the detection of ATPase by crossed immunoelectrophoresis of detergent extracts of cytoplasmic membranes of *S. mitis* ATCC 903 (Fig. 6B) is the first evidence for the presence of this enzyme complex in oral streptococci.

The possibility of isolating and identifying specific cell wall, membrane, and cytoplasmic antigens by the procedures described herein may be of value in streptococcal research, e.g., in the isolation of adhesins and antigens suitable for the production of streptococcal vaccines and for the selection of marker molecules specific for different cell fractions. From our results, it can be concluded that aminopeptidase assayed with leucine-β-naphthylamide as the substrate is a suitable cytoplasmic marker in *S. mitis* and that ATPase and chymotrypsin-like protease may be used as markers for the cytoplasmic membrane. Further studies are needed for the isolation and characterization of specific cell wall markers.

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