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Analysis of Cell Wall and Membrane Contamination of Ribosomal Preparations from *Streptococcus mutans*

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A ribosomal preparation from a cariogenic strain of *Streptococcus mutans* was examined for cell wall and membrane contamination. A biochemical characterization established that the preparation contained 61.0% RNA and 39.0% protein. Carbohydrate was not detected by phenol-sulfuric acid or methyl pentose assays. Glucosyltransferase and D-succinate dehydrogenase, which are cell wall- and membrane-associated enzymes, respectively, were not found. However, D-lactate dehydrogenase, another membrane-associated enzyme, was present in the preparation. A comparison of two-dimensional gel electropherograms of a mixture of cell walls and membranes and the *S. mutans* ribosomal preparation revealed contamination of the latter sample with at least six cell wall- or membrane-associated proteins. Adsorption of a rabbit antiserum raised against the ribosomal preparation with whole *S. mutans* cells abrogated antibodies directed against at least two proteins from the ribosomal preparation. Immunodiffusion plates showed reactivity of this antiserum against preparations of purified lipoteichoic acid from *Streptococcus pyogenes* and *S. mutans*. Adsorption of rat and rabbit antisera against the ribosomal preparation with the cell wall-derived materials glucosyltransferase, lipoteichoic acid, glucan, and a Rantz-Randall extract reduced the concentration of antibodies against the ribosomes by as much as 10-fold. These data indicated that the preparation was contaminated with at least six cell wall proteins, one cell membrane-associated enzyme, and lipoteichoic acid.

Streptococcus mutans has been implicated as one of the major causative agents of dental caries (for a review, see reference 32). The species has been subdivided into seven serotypes (*a* through *g*), all of which are capable of causing carious lesions. Animals immunized with either whole *S. mutans* cells or purified components of this organism have been protected against challenge with virulent organisms, although the efficacy of such preparations has come under scrutiny recently. Rabbit antisera raised against whole *S. mutans* cells have been shown to cross-react with human heart and muscle tissues (18, 25, 42, 43, 49). Glucosyltransferase preparations (GTF) protected animals in some studies (41, 45) but not in others (7, 10, 20). Obviously, the need to develop a more suitable anticaries vaccine exists.

Ribosomal preparations have been used as vaccines in animals to protect against diseases other than dental caries. Protection against the following agents has been established: *Mycobacterium tuberculosis* (52), *Neisseria meningitidis* (46), *Neisseria gonorrhoeae* (12), *Salmonella typhimurium* (1, 29, 50), *Histoplasma capsulatum* (17), *Streptococcus pyogenes* (22, 40), *Streptococcus pneumoniae* (4, 44), *Hemophilus influenzae* (30), *Vibrio cholerae* (27), and *Klebsiella pneumoniae* (37–39).

Previous studies in our laboratory have demonstrated that immunization of rabbits and rats with *S. mutans* ribosomal preparations stimulates salivary and serum antibody and cell-mediated responses against both ribosomes and whole *S. mutans* cells (23; R. L. Gregory, Ph.D. thesis, Southern Illinois University, Carbondale, 1982; R. L. Gregory and I. L. Shechmeister, Ann. N.Y. Acad. Sci., in press), and, furthermore, studies have indicated that these preparations protect gnotobiotic rats from challenge with a virulent organism (manuscript in preparation). These results also established that an antiserum raised against such a preparation cross-reacted with representative strains from all seven serotypes of this organism, but not against human heart tissue. This is an important consideration in the development of a vaccine for possible use in humans against dental caries.

Evidence that protective ribosomal preparations from other bacteria are contaminated with various cell wall or membrane material has been

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reported by a number of laboratories (for reviews, see references 13 and 16). RNA has been suggested to be the active component of a ribosomal preparation from *M. tuberculosis* (52, 53), whereas protein has been reported to be the protective moiety of *H. influenzae* (30) and *S. pyogenes* (40) ribosomes. Various cell wall components, such as protein, lipid, and carbohydrate, have been proposed to be the major component in the *V. cholerae* (27), *S. pneumoniae* (47), *S. typhimurium* (15), and *K. pneumoniae* (37–39) preparations. In this regard, we examined an *S. mutans* ribosomal preparation for the presence of contaminating cell wall or membrane materials. Our results indicated that this preparation was contaminated with a number of such nonribosomal materials.

MATERIALS AND METHODS

Organisms. *S. mutans* 6715, serotype *g*, (kindly provided by R. J. Gibbons, Forsyth Dental Center, Boston, Mass.) was used in these studies. It was originally isolated from a human carious lesion and was subsequently shown to be cariogenic for rats and hamsters. *Escherichia coli* was obtained from our stock culture collection. *S. mutans* 6715 was grown in 3-liter batches of 2% tryptic peptone broth (Difco Laboratories, Detroit, Mich.) containing 1% glucose, 0.8% NaCl, 0.5% yeast extract (Difco), 0.1% K_2CO_3, 0.05% KCl, and 0.05% Na_2HPO_4 in 6-liter Erlenmeyer flasks. *E. coli* was grown in 3-liter batches of 3% tryptic soy broth (Difco) containing 1% glucose in 6-liter Erlenmeyer flasks. Flasks containing either *S. mutans* 6715 or *E. coli* were inoculated with 150 ml of an 18- to 24-h culture of the appropriate organism and incubated without shaking for 5 h at 37°C in 5% CO_2–95% air. After incubation, cells were harvested by centrifugation (10,000 × *g* for 10 min), washed three times with 0.01 M phosphate buffer (pH 7.0) containing 0.01 M $MgCl_2$ (PMB), and frozen at −75°C.

Preparation of ribosomes. Ribosomes were prepared by a modification of the procedure of Youmans and Youmans (52). Briefly, the pelleted, frozen *S. mutans* 6715 and *E. coli* cells were thawed rapidly at 37°C, and 1 g was suspended with 1 g of microglass beads (0.17 to 0.18 mm) in 1 ml of a solution containing 10^{-4} M phosphate buffer (pH 7.0), 0.44 M sucrose, 0.25% sodium dodecyl sulfate, 3×10^{-2} M $MgCl_2$, and 3 μg of DNase (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were then broken by shaking for three 2-min cycles in a Braun homogenizer (B. Braun Co., Melsungen, Germany), and the unbroken cells and cellular debris were removed by two low-speed centrifugations (27,000 × *g* and 47,000 × *g* for 10 min each). The ribosomes in the supernatant were harvested by centrifugation at 250,000 × *g* for 2.5 h, and the ribosomal pellet was resuspended in PMB at a concentration of 100 mg (wet weight) per ml. An equal volume of PMB containing 0.5% sodium dodecyl sulfate was added, gently rotated by hand for 20 min at room temperature, and placed in an ice bath overnight; precipitated sodium dodecyl sulfate was then removed by two 15-min low-speed centrifugations at 37,000 × *g*, and the ribosomes in the supernatant were harvested as described above. The ribosomal preparations were then subjected to five successive washes in PMB at 250,000 × *g* for 2.5 h each, two 20-min low-speed centrifugations at 47,000 × *g*, and filtration through sterile 0.45-μg Millipore filters (Millipore Corp., Bedford, Mass.). These preparations were then standardized on the basis of protein content. Inoculation of the preparations on appropriate media did not reveal the presence of viable organisms.

Immunization of animals. Rabbits and rats were immunized as reported previously (Gregory, Ph.D. thesis), and sera were obtained from these animals.

Chemical and physical analyses of ribosomal preparations. The amounts of RNA and DNA in the ribosomes were measured by the orcinol (51) and diphenylamine (14) methods, respectively, using D-ribose and 2-deoxy-D-ribose as standards. The amounts of protein, carbohydrate, and methyl pentose were determined by the Lowry (8), phenol-sulfuric acid (3), and Ashwell (2) procedures, respectively, using bovine serum albumin, glucose, and rhamnose as standards.

For sucrose gradient analyses, 0.5 ml of the ribosomal preparation containing 0.8 mg of RNA was layered over 5 ml of a 5, 10, 15, and 20% (wt/vol) discontinuous, stepwise sucrose gradient prepared in 0.02 M Tris-hydrochloride buffer (pH 7.8) containing either 10^{-2} or 10^{-4} M $MgCl_2$. The gradient was centrifuged for 70 min at 243,000 × *g*, and fractions were collected by upward displacement with mineral oil. The absorbance of each fraction at 260 nm was measured in a Gilford spectrophotometer. The sedimentation coefficients of *S. mutans* 6715 ribosomes and their subunits were determined by the method of Martin and Ames (31) with ribosomes and subunits from *E. coli* as 70S, 50S, and 30S markers.

The ratios of the optical density at 260 nm to those at 280 and 235 nm were determined for the ribosomal preparation to detect cell wall or membrane contamination (5, 47).

Preparation of *S. mutans* subcellular materials. *S. mutans* subcellular materials were obtained by using modifications of the techniques described by Cooper et al. (11) and Peterman (35). Briefly, 1 g of cells was mixed with 1 ml of PMB and 1 g of microglass beads. The cells were disrupted in a Braun homogenizer, and the suspension was centrifuged at 10,000 × *g* for 10 min. The pellet was resuspended in 15 ml of PMB, and the glass beads were removed by decanting the lighter cells, cell walls, membranes, and cell debris. RNase A type I (Sigma) was added to effect a concentration of 3 μg/ml. The mixture was kept in an ice bath for 15 min and then centrifuged for 20 min at 10,000 × *g*, and the pellet was washed twice with 1 M NaCl at 10,000 × *g* and once with double-distilled water (dH_2O) at 9,000 × *g*. Most of the cell membranes were left in the 9,000 × *g* supernatant, which was gently removed and saved, and the cell walls were gently washed off the remaining heavier cell pellet with 5 ml of dH_2O. The cell wall suspension was layered over a stepwise, discontinuous, 40, 50, 60, and 70% (wt/vol) sucrose gradient and centrifuged for 15 min at 10,000 × *g*. The cell wall band (top band) was harvested and washed twice in dH_2O and the alternating sucrose gradient, and the washing procedures were repeated until a single band was obtained. This material was then washed three times and suspended in 15 ml of dH_2O.

The membranes were washed thrice in dH_2O for 15

TABLE 1. Biochemical analyses of various compounds in different preparations of *S. mutans* 6715

Biochemical compound	Amt (%) in the following prep:					
	Ribosomal	Rantz-Randall	LTA	Membrane	Glucan	GTF
Pentose (RNA)	61.0	61.6	0.0	57.4	61.0	58.5
Protein	39.0	18.3	0.0	27.6	1.9	31.1
Total neutral carbohydrate	0.0	18.3	0.0	15.0	37.1	10.4
Methyl pentose	0.0	0.9	0.0	0.0	0.0	0.0
DNA	0.0	1.3	0.0	0.0	0.0	0.0
LTA	−	+	+	+	−	−

min each at 10,000 × *g*, resuspended in 1 ml of dH_2O, and then layered over another discontinuous sucrose density gradient consisting of 1.0 and 2.0 M sucrose. The gradient was centrifuged for 20 h at 100,000 × *g*, and the rotor was stopped without use of a brake. The membranes, which banded at the interface of the two concentrations of sucrose, were harvested and washed three times in dH_2O, and the gradient step was repeated once. The membranes were then washed thrice and resuspended in 5 ml of dH_2O.

Glucan was obtained by the procedure described by Gibbons and Banghart (21). Briefly, *S. mutans* 6715 was grown in 2 liters of 3% Todd-Hewitt broth (Difco) containing 4% sucrose for 48 h without shaking, the cells were harvested, and the supernatant was saved. The cells were washed once with 100 ml of 0.01 N NaOH, and the two cell-free supernatants were combined. Glucan was precipitated with 4 liters of cold 95% ethanol and centrifuged for 20 min at 20,000 × *g*. The pellet was dissolved in 500 ml of dH_2O, and the glucan was reprecipitated with 750 ml of cold 95% ethanol. The mixture was centrifuged for 20 min at 10,000 × *g*; the pellet was resuspended in 250 ml of 10% (vol/vol) trichloroacetic acid and centrifuged for 20 min at 5,000 × *g* to remove the precipitated protein; and the supernatant was dialyzed against dH_2O for 48 h to remove the remaining trichloroacetic acid.

GTF was isolated by the procedure described by Johnson et al. (28). Briefly, *S. mutans* 6715 was grown in a dialyzed medium (28) for 18 h. During this time, 5 N NaOH was added intermittently until acid production stopped. The cells were pelleted, and 2 liters of dH_2O was added to the cell-free supernatant to lower the ionic strength. A 250-ml portion of washed Bio-Gel HA (Bio-Rad Laboratories, Richmond, Calif.) was added, stirred constantly for 3 h, and allowed to sediment by gravity overnight. The supernatant was decanted, and the HA was washed three times each with 2 liters of 0.1 M phosphate buffer (pH 6.8), resuspended in 1 liter of the same buffer, and packed into a column (2.5 by 70 cm). The buffer was passed through the column until the eluate had an optical density at 280 nm of near zero; 0.2 and 0.4 M phosphate buffers (pH 6.8) were then passed through the column, and 5-ml fractions were collected. The fractions which had an optical density peak at 280 nm were saved. The presence of GTF activity was tested qualitatively by incubating 0.5 ml of each saved fraction with 0.5 ml of 5% sucrose and 2 drops of toluene for 24 h. A 1-ml portion of 100% ethanol was added, and the tube was shaken. GTF was considered to be present if a turbidity persisted after shaking. The fractions containing GTF were then pooled.

Lipoteichoic acid (LTA) was prepared (19) by using cell walls and membranes obtained as described above. Equal volumes of cell walls and membranes were mixed, lyophilized, and then resuspended in 20 ml of dH_2O. A 20-ml portion of 90% phenol was added, and the preparation was vigorously mixed for 15 min at 67°C. It was then centrifuged for 10 min at 16,000 × *g*, and the upper aqueous phase was collected. The lower phenol phase was washed once with dH_2O, and the two aqueous phases were pooled and dialyzed for 48 h against frequent changes of dH_2O. The dialyzed solution was incubated with 1 drop of toluene per 10 ml and 4 mg each of RNase, DNase, and trypsin (Sigma) per ml for 48 h. The solution was then dialyzed overnight against dH_2O, trichloroacetic acid was added to make a 10% (vol/vol) concentration, and the mixture was stirred for 2 h at 4°C. Five volumes of cold 95% ethanol was added and stirred slowly for 24 h at 4°C. The precipitated LTA was removed by centrifugation for 20 min at 10,000 × *g* and dissolved in 0.01 M phosphate-buffered saline (pH 7.2).

A Rantz-Randall extract was prepared (36) by centrifuging *S. mutans* 6715 grown in 40 ml of 3% Todd-Hewitt broth for 24 h, suspending the pellet in 40 ml of 0.5 M NaCl, autoclaving at 121°C for 20 min, and recentrifuging. The supernatant was then removed and saved.

Concentrations of RNA, protein, total neutral carbohydrate, methyl pentose, and DNA in these preparations were determined as described above.

Analysis of possible cell wall or membrane contamination of *S. mutans* 6715 ribosomal preparations. (i) Radiolabeling experiments. *S. mutans* 6715 was grown for two or three passages in 3% Todd-Hewitt broth supplemented with 1% unlabeled isotope and passed three times in 3% Todd-Hewitt broth containing D-[^{14}C]alanine (0.05 μCi/ml, 56.2 mCi/mmol), D-[^{3}H]glucose (0.5 μCi/ml, 7 Ci/mmol), D-[^{3}H]glycerol (0.05 μCi/ml, 200 mCi/mmol), or [^{3}H]uracil (1.0 μCi/ml, 40 Ci/mmol) (New England Nuclear Corp., Boston, Mass.). The cells were harvested, and a sample of each was saved for later use.

Ribosomes, cell walls, and membranes were prepared from the rest of the cells as described above. Portions of the four whole cell samples and their corresponding ribosome, cell wall, and membrane suspensions were placed on filter paper squares and dried overnight, and the radioactivity in each was counted in a liquid scintillation counter. Portions of all of the preparations were also evaporated to dryness to determine the dry weight of each sample. The counts per minute per milligram of each sample were calculated by dividing the counts per minute of each by the

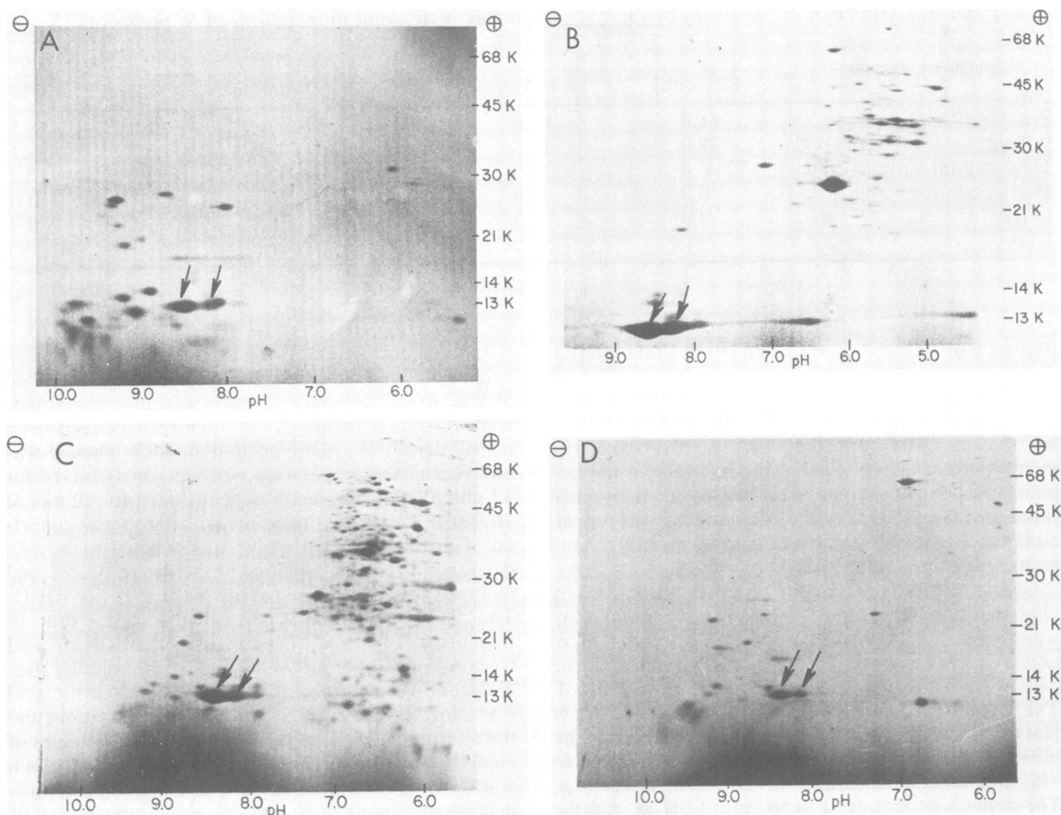

FIG. 1. Representative two-dimensional electropherograms of *S. mutans* 6715 and *E. coli* proteins. The molecular weights ($\times 10^3$) are listed to the right of the gel, and the pHs are listed on the bottom. The basic end (−) of the gel is to the left, and the acidic end (+) is to the right. The arrows represent RNase. (A) *S. mutans* 6715 ribosomes. (B) *S. mutans* 6715 cell walls and membranes. (C) *S. mutans* 6715 whole cells. (D) *E. coli* ribosomes.

respective dry weight in milligrams.

(ii) One- and two-dimensional gel electrophoresis. (a) Separation of various extracts of *S. mutans* 6715 and *E. coli*. Two-dimensional gel electrophoresis of *S. mutans* 6715 ribosomes, cell walls and membranes, and whole cells and *E. coli* ribosomes was done by the nonequilibrium pH gradient electrophoresis procedure of O'Farrell et al. (34).

(b) Immunoprecipitation of electrophoretically separated antigens. The first-dimension O'Farrell gels were prepared, electrophoresed, and extruded into 1 ml of appropriately diluted rabbit and rat sera raised against the following antigens: *S. mutans* 6715 ribosomes (including a sample adsorbed five times for 10 min each with strain 6715 cells), strain 6715 cells, and *E. coli* ribosomes. The gels were incubated for 36 h at room temperature, washed exhaustively for 72 h with phosphate-buffered saline to remove nonprecipitated antigens and antibodies, and then stained.

(iii) Electron microscopy of *S. mutans* 6715 ribosomal preparations. The ribosomal preparations were examined under a transmission electron microscope for size and membrane contamination. The preparations were stained with 2% phosphotungstic acid at a pH of 5.5 for 1 min (26). The percentage of membrane contamination was determined by counting the number of ribosomal and membrane particles in an average field, dividing the number of ribosomal particles by the total number of particles, and multiplying by 100.

(iv) Enzyme assays. GTF activity was measured by determining, in a Serometer (Malinkrodt, Inc., St. Louis, Mo.), the amount of reducing sugar present after incubation of 1 ml each of the ribosomal preparation (1 mg of ribosomal protein per ml) and 5% (wt/vol) sucrose for 10 min at 37°C. Purified GTF and glucan were used as positive and negative controls, respectively.

D-Succinate and D-lactate dehydrogenases were measured by coupling the enzymes via phenazine methosulfate to the reduction of thiazolyl blue described by Miller and Morse (33). A preparation of purified cell membranes was used as a positive control.

(v) Immunodiffusion. Briefly, wells were punched out in plates containing 0.9% NaCl, 0.5% agarose, 0.14% sodium barbital, 0.1% NaN_3, and 0.00001% trypan blue dye (pH 8.4), and various antisera and antigens were placed in the wells. The plates were incubated for 2 to 3 days in a humid atmosphere at room temperature and examined for precipitin lines. A rabbit antiserum raised against *S. pyogenes* LTA (kindly prepared [19] and supplied by R. W. Jackson,

Southern Illinois University, Carbondale) was used to determine the presence or absence of LTA in various preparations.

(vi) Passive hemagglutination. The technique described by Campbell et al. (9) was used to measure the concentration of rabbit and rat antibodies against tannic acid-treated, ribosome-coated erythrocytes. Samples of the antisera were adsorbed five times for 10 min each at room temperature with the various extracts of *S. mutans* 6715, and the concentrations of antibodies against the coated cells were measured.

TABLE 2. GTF activity of ribosomal, GTF, and glucan preparations from *S. mutans* 6715

Prep	GTF activity[a]
GTF	9.50
Glucan	0.00
Ribosomal	0.00

[a] Expressed as units of dextran sucrase per milligram of protein.

RESULTS

Various preparations, such as ribosomes, cell walls, membranes, GTF, and glucan, were assayed by using a variety of techniques to determine whether *S. mutans* 6715 ribosomes were contaminated with nonribosomal materials. These techniques included: (i) biochemical assays, such as those for RNA, protein, total neutral carbohydrate, methyl pentose, and DNA; (ii) biophysical tests, such as optical density ratios, sucrose density gradients, electron microscopy, and two-dimensional electrophoresis; (iii) biological assays, in which various cell wall- and membrane-associated enzyme activities were measured; (iv) radiological tests, involving the labeling of portions of the cell wall or membrane (or both) of strain 6715 with different radioisotopes; and (v) immunological tests, including immunodiffusion, immunoadsorption, and immunoprecipitation. With the results from these experiments, it was possible to ascertain the relative amounts and numbers of the specific entities that may have contaminated the ribosomal preparations.

Biochemical and biophysical analyses of various preparations. The biochemical compositions of the various preparations are shown in Table 1. The ribosomal preparation contained 61.0% RNA and 39.0% protein, and carbohydrate was not detected by either the phenol-sulfuric acid or the methyl pentose assay. In addition, the preparation had an optical density at 260 nm/optical density at 235 nm ratio of 1.62 and an optical density at 260 nm/optical density at 280 nm ratio of 1.93.

The results of the electron microscopy study revealed that the ribosomal particles measured 15.0 to 18.0 by 19.0 to 22.0 nm in size, which is well within the size range of procaryotic ribosomes (26). The percentage of ribosomal particles was greater than 99% in all of the ribosomal preparations examined. The sedimentation coefficient of undissociated *S. mutans* 6715 ribosomes was 70S, and they dissociated into 54S and 32S subunits.

Two-dimensional electrophoretic separations of various extracts of *S. mutans* 6715 and *E. coli* were done to analyze the possible contamination of strain 6715 ribosomes by cell wall or membrane material. *E. coli* ribosomes were used as a control for the technique. The results of the electrophoretic separation of *S. mutans* 6715 ribosomes, cell walls and membranes, and whole cells and *E. coli* ribosomes are presented in Fig. 1.

Molecular weights were determined in the second dimension with Bio-Rad low-molecular-weight markers. pHs were determined by cutting a first-dimension gel which had been electrophoresed without a sample into 0.5-cm-length pieces, placing these into 2 ml of dH_2O, and measuring the pH. There were 55 and 56 isolated protein spots in *S. mutans* 6715 and *E. coli* ribosomes, respectively (black spots in Fig. 1A and D). Most ribosomal proteins are very basic and appear in the left-hand side of the electropherogram, whereas many nonribosomal proteins are acidic and therefore appear in the right-hand side (34). Only 9 *S. mutans* 6715 ribosomal proteins were found at an acidic pH (Fig. 1A), whereas approximately 29 proteins from the *E. coli* ribosomal preparation were found at an acidic pH (Fig. 1D). This indicated that the *E. coli* ribosomal preparation contained a higher proportion of contaminating proteins.

A comparison of the *S. mutans* 6715 ribosomal, cell wall and membrane, and whole cell electropherograms revealed that at least six cell wall or membrane proteins contaminated the ribosomal preparation. However, some, but not all, of the ribosomal spots could be identified in separations of whole cells or cell walls and membranes or both, although many could not. For example, 13 *S. mutans* 6715 ribosomal proteins could absolutely be correlated with 13 cellular proteins, and 6 *S. mutans* 6715 ribosomal proteins could be correlated with 6 cell wall and membrane protiens. It is probable that some strain 6715 ribosomal proteins contaminated the cell wall and membrane preparation, and, of course, the gel containing the total cellular proteins (Fig. 1C) would be expected to contain most of the ribosomal proteins. However, 42 *S. mutans* 6715 ribosomal proteins could not be correlated with certainty with spots on the *S. mutans* 6715 total cellular protein gel. The reason for this is not known, but it has been observed by other investigators (J. Parker,

TABLE 3. Dehydrogenase activities of ribosomal and cell membrane preparations from *S. mutans* 6715

Prepn	D-Succinate dehydrogenase activity[a]	D-Lactate dehydrogenase activity[a]
Cell membrane	2.83	4.33
Ribosomal	0.00	0.20

[a] Expressed as units of enzyme per milligram of protein.

Southern Illinois University, Carbondale, personal communication). Conversely, at least 200 cell wall or membrane proteins were not present in the *S. mutans* 6715 ribosomal preparations (Fig. 1A and B), indicating that most of these proteins were removed during the purification of the ribosomes.

Biological and radiological analyses. Assays for GTF (Table 2), and D-succinate and D-lactate dehydrogenase (Table 3) activities revealed a small amount of cell membrane contamination in the ribosomal preparation.

It has been previously established that D-glucose is incorporated into the peptidoglycan of *S. mutans*, that D-glycerol is incorporated into LTA, and that D-alanine is incorporated into both peptidoglycan and LTA (6). Four different cultures of *S. mutans* 6715 were labeled for 5 h with, respectively, D-[^{3}H]glucose, D-[^{3}H]glycerol, D-[^{14}C]alanine, and [^{3}H]uracil (used as a positive control to ensure that the ribosomes could be labeled). These cells were broken; ribosomal, cell wall, and membrane preparations were obtained; and the radioactivity in each, as well as in the whole cells, was counted. In addition, the dry weight of each sample was determined, and the percentage of the total cellular radioactivity in each subcellular fraction was calculated by dividing the counts per minute per milligram of each fraction by the counts per minute per milligram of the whole cell samples. The results are presented in Table 4.

Ribosomes from cells radiolabeled with glucose, glycerol, and alanine had 0.44, 0.08, and 0.20%, respectively, of the radioactivity of the corresponding whole cell samples. The cell wall or membrane preparations, which were used as positive controls to establish that the labels were incorporated into these materials, had 51.65, 57.70, and 80.98% of the radioactivity, respectively.

The ribosomes from the control uracil-labeled cells had 2.44% of the total cellular radioactivity, indicating that ribosomal RNA was specifically labeled. The cell wall preparation isolated from these cells contained 7.43% of this isotope, implying that the sample was contaminated with RNA. The reason for the fact that only 9.87% of the radioactivity was recovered in the ribosomal and cell wall samples was probably that the recovery of ribosomes was very low. These results suggest that the cell walls, and possibly the membranes from the glucose-, glycerol-, and alanine-labeled cells, were also contaminated with a small amount of RNA.

Although the radioactivities in the ribosomal samples from cells labeled with glucose and alanine (both of which are incorporated into peptidoglycan) were only 0.44 and 0.20%, respectively, of the activities in the corresponding whole cells, this may indicate some small but unknown amount of cell wall contamination of the ribosomes. Even though the percentage of radioactivity of the ribosomal sample from the glycerol LTA experiment was only 0.08%, other results, such as those of the alanine radioisotope experiment (0.20% of the activity) and the immunoadsorption and immunodiffusion experiments (see below), suggest that a small but undefined amount of LTA was present in the ribosomal preparations.

Immunological analyses. Figure 2A shows the results of an immunodiffusion plate with an antiserum raised in rabbits against acylated *S. pyogenes* LTA reacting with deacylated and acylated *S. pyogenes* LTA (kindly supplied by R. W. Jackson), *S. mutans* 6715 ribosomes, acylated LTA, and a Rantz-Randall extract. This serum produced a line of identity between all of the latter four antigens. These results indicated that: (i) the ribosomal preparation contained at least partially acylated LTA; (ii) *S. mutans* 6715 LTA was similar to *S. pyogenes* LTA; and (iii) the Rantz-Randall extract contained LTA.

Figure 2B shows the results of an immunodiffusion plate with rabbit anti-6715 ribosome serum reacting with *S. mutans* 6715 ribosomes, acylated LTA, GTF, and glucan. This serum produced a line of identity between the glucan, LTA, and strain 6715 ribosomes. These results show that this serum recognized two components of the glucan sample and one from the GTF preparation, suggesting contamination of

TABLE 4. Analysis of ribosomal preparations with radioisotopes

Radioisotope/substance incorporated into	Amt of isotope (% of whole cell content) in the following prepn:		
	Ribosome	Cell wall	Cell membrane
D-[^{3}H]glucose/peptidoglycan	0.44	51.65	NA[a]
D-[^{3}H]glycerol/LTA	0.08	NA	57.70
D-[^{14}C]alanine/peptidoglycan and LTA	0.20	80.98	NA
[^{3}H]uracil/RNA	2.44	7.43	NA

[a] NA, Not applicable.

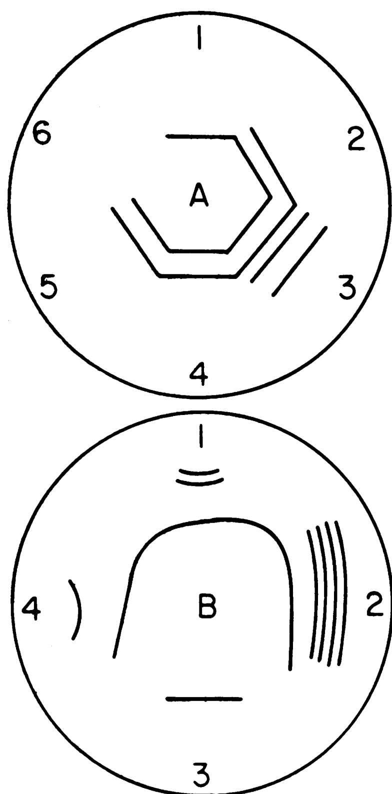

FIG. 2. Immunodiffusion in plates. (A) center, rabbit anti-*S. pyogenes* LTA serum; 1, deacylated *S. pyogenes* LTA; 2, *S. mutans* 6715 ribosomes; 3, *S. pyogenes* LTA; 4, *S. mutans* 6715 LTA; 5, Rantz-Randall extract of *S. mutans* 6715; 6, saline. (B) center, rabbit anti-6715 ribosome serum; 1, *S. mutans* 6715 LTA; 2, *S. mutans* 6715 ribosomes; 3, *S. mutans* 6715 glucosyltransferase; 4, *S. mutans* 6715 glucan.

the ribosomes with these materials.

Table 5 shows the results of passive hemagglutination assays against *S. mutans* 6715 ribosomes with rabbit and rat antisera raised against ribosomes which had been adsorbed with the following materials: a Rantz-Randall extract, GTF, glucan, LTA, *S. mutans* 6715 cells and ribosomes, and *E. coli* ribosomes.

Untreated rabbit and rat sera from animals immunized with *S. mutans* 6715 ribosomes had passive hemagglutination titers against strain 6715 ribosomes of 10,240 and 2,048, respectively. When the sera were adsorbed with strain 6715 ribosomes, the titers decreased to 10 and 2, respectively. However, when the antisera were adsorbed with *E. coli* ribosomes, the titers did not decrease. The adsorption of these sera with either *S. mutans* 6715 cells, GTF, glucan, LTA, or a Rantz-Randall extract decreased the titer against strain 6715 ribosomes by various degrees up to a 10-fold decrease.

All of the preparations except LTA were contaminated with pentose (Table 1). LTA was detected in the Rantz-Randall extract, but was not found in either the glucan or the GTF samples. These results indicated that the adsorption removed antibodies against an antigenic determinant(s) common to all of these preparations. Since the Rantz-Randall extract, GTF, glucan, and LTA are components of the *S. mutans* 6715 cell wall, we suggest that the adsorption removed antibodies against cell wall antigens, and therefore the ribosomal preparation was contaminated with a cell wall determinant(s).

Various antisera were used to precipitate electrophoretically separated proteins obtained from *S. mutans* 6715 ribosomes, cell walls and membranes, and whole cells to identify the cell wall or membrane material present in *S. mutans* 6715 ribosomal preparations and to compare the proteins from the preparations listed above with each other. Figure 3 shows the results of precipitating *S. mutans* 6715 ribosomal proteins with rabbit anti-6715 ribosome serum (lane A), anti-6715 ribosome serum which had been adsorbed with *S. mutans* 6715 cells (lane B), and normal rabbit serum (lane C). Lanes A and B contained eight and six precipitated proteins, respectively. Similar results have been reported with anti-*E. coli* ribosome serum and *E. coli* ribosomal proteins (48). Two of the precipitated proteins seen in lane A were not seen or were greatly reduced in lane B (arrows). (Due to loss of photographic resolution, the bands in lane B are difficult to establish, but the differences between the proteins in lanes A and B at the arrows are apparent on the original gels.) This indicated that some of the antibodies produced against *S. mutans* 6715 ribosomes were removed by adsorption with whole cells and, therefore, that there was some contamination of the ribosomes with cell wall determinants. In the serum from normal, unim-

TABLE 5. Passive hemagglutination (PHA) assay of rabbit and rat anti-6715 ribosome sera adsorbed with various preparations

Prepn serum adsorbed with	PHA titer against *S. mutans* 6715 ribosomes with serum from:	
	Rabbits	Rats
None	10,240	2,048
S. mutans 6715 ribosomes	10	2
E. coli ribosomes	10,240	2,048
S. mutans 6715 cells	1,280	128
GTF	1,280	512
LTA	1,280	512
Rantz-Randall extract	1,280	256
Glucan	640	256

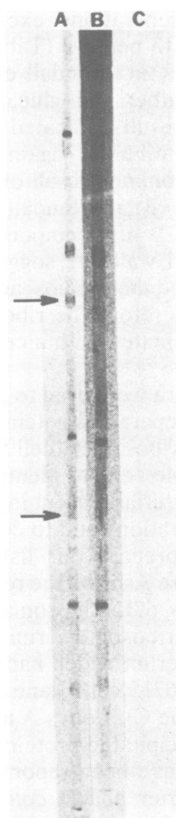

FIG. 3. One-dimensional gels of *S. mutans* 6715 ribosomal proteins precipitated with rabbit anti-6715 ribosome serum (lane A), rabbit anti-6715 ribosome serum adsorbed with *S. mutans* 6715 whole cells (lane B), and normal rabbit serum (lane C). The arrows indicate those proteins seen in lane A that were not seen or were greatly reduced in lane B.

munized rabbits, there were no antibodies that precipitated *S. mutans* 6715 ribosomal proteins (lane C).

DISCUSSION

The results presented in this report describe the cellular contamination of an immunogenic ribosomal vaccine from *S. mutans*. A representative preparation was shown to contain 61.0% RNA and 39.0% protein. Total neutral carbohydrate, methyl pentose, and DNA were not detected. The optical density ratios and the assays for GTF and D-lactate dehydrogenase activities could not detect cell wall or membrane contamination. However, more sensitive methods such as two-dimensional electrophoresis, immunoprecipitation, D-succinate dehydrogenase, radioisotope, immunoadsorption, and immunodiffusion assays disclosed a small, but definite, contamination of the ribosomal preparation with at least six cell wall proteins (possibly derived from peptidoglycan), one cell membrane-associated enzyme (D-succinate dehydrogenase), and LTA. This indicates that even with the rather laborious procedure used to obtain these preparations in a purified state, they were still contaminated with minute amounts of cell wall and membrane material, although this was only observed with very sensitive assays.

The results and conclusions presented here concerning cell wall or membrane contaminants in ribosomal vaccines are in agreement with those of other investigators (13, 16). In light of this evidence, one might raise the question of whether the ribosomes make any contribution to the immunogenicity of the preparation. Since the first report of the isolation of immunogenic ribosomal fractions (52), there have been conflicting reports regarding the exact chemical nature and derivation of the immunogenic moiety. In this regard, the following hypotheses have been postulated to explain the possible mode of action of ribosomal vaccines (16): (i) these preparations contain messenger RNA, which codes for and is translated into microbial cell surface antigens in the immunized individuals; (ii) the ribosomal preparations are contaminated with microbial surface antigens; (iii) same as (ii) but the ribosomes serve as an adjuvant for the contaminating antigens; (iv) antibodies to certain integral determinants of the ribosomes in the preparations cross-react with microbial cell surface antigens; and (v) ribosomes are associated with or are exposed to the cell surface during some phase of microbial replication.

At present, no generalization can be made about the nature of the protective moiety or immunogen in ribosomal preparations. Double-stranded RNA has been claimed to be the active substance in the *M. tuberculosis* (53) vaccine, and lipopolysaccharide has been claimed to be the active substance in the *N. gonorrhoeae* (12) vaccine. Ribosomal protein is regarded as the active component in the *H. capsulatum* (17), *H. influenzae* (30), and *N. meningitidis* (46) vaccines. In this regard, preliminary results indicated that protein serves as the active substance in the *S. mutans* ribosomal preparation. The first three hypotheses presented above have been used to explain the mode of action of several of the ribosomal vaccines, whereas data to support the latter two hypotheses have not been reported.

Although contamination from the cell wall and membrane was identified in the *S. mutans* 6715 ribosomal preparation, it was not possible to establish a role in protective immunity for these contaminants. It may be possible that one or more of these materials are responsible for protection against dental caries. Therefore, hypoth-

eses (ii) and (iii) are the most likely ones in regard to this vaccine. The preparation of more highly purified ribosomes which are devoid of such contamination may help to clarify this question. The protective effect of the *S. typhimurium* preparation can be removed, without altering the metabolic function, by washing the ribosomes in 1 M NH_4Cl (24). The use of such techniques to prepare pure ribosomes from *S. mutans* may be useful. We have not yet achieved such a preparation, although we hope to initiate such studies in the near future.

Experiments are currently in progress to isolate and purify the *S. mutans* ribosomal immunogen and to elucidate the mechanism by which it induces immunity and protection.

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