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Effect of Growth Conditions on the Formation of Extracellular Lipoteichoic Acid by *Streptococcus mutans* BHT

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Streptococcus mutans BHT was grown in a chemostat with glucose limitation and at defined dilution rates and pH values. Lipoteichoic acid was estimated by determining the ability of dilutions of culture fluid to sensitize erythrocytes. The greatest amounts of extracellular lipoteichoic acid were produced by organisms growing at a low dilution rate and at pH 6.0 or 6.5. To enable a more accurate estimation of the total amount of extracellular material, rocket immunoelectrophoresis was employed. These results confirmed that the greatest amounts of reactive material were produced by slow-growing organisms, although there were discrepancies between these results and those obtained by hemagglutination. The extracellular material was fractionated by column chromatography and membrane ultrafiltration to yield a lipoteichoic acid-containing fraction and a presumptive deacylated lipoteichoic acid fraction. The relative proportions detected by rocket immunoelectrophoresis differed with the growth conditions, particularly the dilution rate. Analysis of the phenol-extracted cellular material also indicated the presence of deacylated lipoteichoic acid, although less than in the culture fluid.

Lipoteichoic acids (LTAs) occur as membrane components of a number of gram-positive bacteria and characteristically contain a glycolipid in covalent linkage with a glycerol teichoic acid, to which may be attached carbohydrate and D-alanine substituents (32). LTA possesses a number of important biological properties, most of which are dependent on its amphipathic nature (32, 33). These include immunogenicity (32, 33), binding to mammalian cell membranes (2, 3, 33), stimulation of bone resorption in organ culture (13), and activation of the alternate complement pathway (10). Properties that depend on the teichoic acid component are serological specificity (32) and binding to hydroxyapatite (22, 25); the latter is believed to play a role in the adherence of plaque bacteria to the tooth surface (25). The physiological role of LTA has been studied in *Streptococcus faecium*, where it has been shown that LTA but not deacylated LTA inhibits the autolysin (4-6).

By the use of appropriate antisera, LTA can be detected as a surface antigen (27) and in certain instances as an extracellular component (16, 22, 32). A serological survey of a number of strains of lactobacilli and viridans streptococci showed that relatively high concentrations of extracellular LTA were produced by *Streptococcus mutans* strains, particularly BHT (22). Further studies on this strain showed that the extracellular fraction contained micellar LTA in association with polysaccharide and protein, and also deacylated LTA (22). The purified LTA was essentially devoid of carbohydrate substituents (26) and antibody specificity was directed against the glycerol phosphate backbone (19). *S. mutans* BHT also contains a wall glycerol teichoic acid with a relatively high degree of galactosyl substitution (29). This component does not give a detectable reaction with antiserum specific for the glycerol phosphate backbone (unpublished data; A. S. Bleiweis, personal communication) and therefore does not interfere with the serological detection of LTA and deacylated LTA.

The formation of LTA and deacylated LTA by cultures of streptococci has been investigated in detail by Shockman and co-workers. Culture fluid of *S. mutans* FA-1 contained both LTA and deacylated LTA, whereas only the latter component was detected in *S. faecium* culture fluid (16). In each case the components were produced by exponentially growing organisms, although there was a considerable increase in the amounts present during the stationary phase (16). A subsequent study (17) provided definitive evidence that the extracellular material formed by *S. faecium* was derived from cellular LTA by deacylation.

To study the effect of growth conditions on

LTA production, *S. mutans* BHT has been grown in a chemostat with glucose limitation at defined pH values and at different generation times. In many biological systems, such as the mouth, it is considered that organisms grow at an average rate of approximately two generations per day (8, 9, 11), and a chemostat enables such slow growth rates to be achieved.

MATERIALS AND METHODS

Organism. *S. mutans* strain BHT was available from previous studies (19, 22) and had been kindly supplied by R. J. Fitzgerald, Veterans Administration Hospital, Miami, Fla.

Growth conditions. The medium consisted of the dialyzable components of a complex medium (28). The complex medium contains Trypticase peptone (Baltimore Biological Laboratory, Cockeysville, Md.), which is essentially free of sucrose (12). The biologically based constituents of 10 liters of medium were dissolved in 1 liter of deionized water and dialyzed at 4°C against three changes of 3-liter aliquots of deionized water with stirring over a period of 72 h. After dissolving the remaining components in the pooled diffusate, glucose was added to a final concentration of 0.5%, and the volume made up to 10 liters. The resulting medium (pH 7.0) was sterilized by filtration (Sartorius filter, 0.2 μm, 100-mm diameter; Sartorius Membranfilter, Göttingen, Germany). Preliminary studies showed that growth was limited at a glucose concentration of 0.5%.

Batch cultures were grown at 37°C in a Multigen fermentor containing 500 ml of culture through which a gas mixture of 95% N_2–5% CO_2 was sparged at a rate of 200 ml/min; the pH was maintained at 6.0 ± 0.05 by the automatic addition of 2 M NaOH. The fermentor and pH controller were supplied by New Brunswick Scientific Co., New Brunswick, N.J.

Continuous culture employed the same medium and gassing procedure and was carried out at constant pH in a BioFlo chemostat of 325-ml capacity (model C30, New Brunswick Scientific Co.). Constant pH was maintained by the automatic addition of 2 M NaOH. The culture vessel was inoculated with an exponentially growing culture in dialyzed medium containing 0.2% glucose. Dilution rates were maintained within a standard deviation of ±1.5% with a maximum tolerance of ±3% of the required value. A dilution rate expressed as $D = 0.1\ h^{-1}$ indicates that 1/10 of the medium was replaced per hour and corresponds to a generation time of approximately 7 h according to the formula: generation time = $\ln 2/D$.

The initial pH of the medium was 7.0, and for cultures grown at pH 7.0 and 8.0 the pH of the inflowing medium was first adjusted to 8.0 with 2 M NaOH. For cultures grown at pH values below 7.0, the pH of the medium was allowed to fall by the natural production of acid by the bacteria; during adjustment of growing cultures to a higher pH, the pH was changed at intervals not exceeding 0.5 pH unit over a period of 3 h. A correction factor was applied to the dilution rate to account for the volume of base required to maintain the correct pH. After each change of growth conditions (dilution rate or pH), cultures were allowed to equilibrate for seven mean generation times (five volume changes) before samples were collected for analysis.

Both outflowing and collected culture fluids were checked daily for their temperature, pH, and density (absorbance at 600 nm). An examination of cultures by the Gram stain showed that the majority of chains (>99%) were composed of 4 to 12 cells irrespective of the growth conditions. The possibility of contamination of cells in the culture vessel was screened for daily by plating cells on both sheep blood and mitis salivarius agar containing 1% potassium tellurite (Baltimore Biological Laboratory, Cockeysville, Md.). To further verify strain specificity, cultures were checked at weekly intervals on API 50 lactobacillus plates (Analytab Products Inc., Plainview, N.Y.). Glucose utilization was measured by a modification (7) of the glucose oxidase method (15) for each condition employed and was found to be in excess of 99.7%.

Dry weights were determined on duplicate known volumes of freshly collected and water-washed cells that were dried to constant weight at 90°C. The cell mass for organisms grown at pH 6.0 varied from 0.79 mg/ml at the lowest dilution rate to 1.21 mg/ml at the highest. In the experiments at dilution rates of 0.1 and 0.2 h^{-1} and different pH values, the dry weight varied between 0.73 and 1.06 mg/ml and 0.55 and 1.34 mg/ml, respectively; in each case the lowest value was obtained at pH 5.5 and the highest was obtained at pH 6.5.

Fractionation of cultures. The culture fluid flowing from the chemostat was collected at 4°C with the aid of a cooling water bath. Cells were routinely harvested at 24-h intervals by centrifugation and washed twice in cold 0.85% NaCl solution (10,000 × *g* at 4°C for 15 min). Washed cells and the bulk of the culture fluid were stored frozen at −20°C. A portion of the culture fluid (30 ml) was passed through a filter (0.22 μm; Millipore Corp.) to remove residual bacteria, dialyzed against 0.85% NaCl, and either examined immediately for serological properties or frozen until required.

Fractionation of culture fluid by ultrafiltration was performed with an Amicon Stirred Ultrafiltration Cell model 12 (Amicon Corp., Lexington, Mass.) containing either a Diaflo XM300 filter or an XM50 filter. Dialyzed culture fluid (20 ml) was concentrated to a volume of 1 ml. The retentate was removed, and the filter was washed twice with a 0.85% NaCl solution (approximately 0.5 ml), the retentate and washings being pooled and made up to 2.0 ml with saline to give a 10-fold concentrate of the original culture fluid; the diffusate was also retained.

Fractionation of culture fluid by column chromatography was performed in a few instances. Dialyzed culture fluid was freeze-dried, and an appropriate amount was dissolved in 1 ml of ammonium acetate solution (0.2 M, pH 6.9) to which was added approximately 100 μg of deoxyribonuclease and ribonuclease (Sigma Chemical Co., St. Louis, Mo.). After incubation for 4 h at 37°C, the suspension was centrifuged (12,000 × *g* for 2 min), and the supernatant was applied to a column (40 by 2.6 cm) of Ultrogel AcA22 (LKB Pro-

dukter AB, Bromma, Sweden). The column was eluted with ammonium acetate buffer (0.2 M, pH 6.9), and the eluate was monitored at 206 and 280 nm and by analysis for phosphorus. The two major phosphate-containing peaks had the chromatographic properties of LTA and deacylated LTA, respectively; in confirmation, only the higher-molecular-weight fraction sensitized sheep erythrocytes by the procedure described below.

LTA preparations. Preparations of LTA from *Lactobacillus casei* NCTC 6375, *Lactobacillus fermentum* NCTC 6991, and batch-grown *S. mutans* BHT were available from a previous study (22). The teichoic acid components of LTA from *S. mutans* BHT and *L. fermentum* were prepared by deacylation with methanolic KOH (34).

For comparing the relative amounts of LTA in organisms grown under different conditions, cell suspensions (approximately 10 mg/ml) were extracted with hot aqueous phenol (30), and the aqueous phase was dialyzed against 0.85% NaCl. By knowing the volume of the aqueous extract, the corresponding concentration of cells that yielded the LTA extract could be calculated.

Serological methods. The previously described procedure was followed for obtaining antisera to *L. casei* LTA (31); all the reported tests were carried out on an antiserum from one rabbit (409), where the specificity against the glycerol phosphate backbone of LTA had been verified (31). Procedures have also been described previously for immunoelectrophoresis (22), hemagglutination (14), and culture fluid titer (22). The last method relies on the assessment of the maximum dilution of LTA needed for maximum sensitization of sheep erythrocytes; the only modification to the previously employed method was the use of serial 1.5-fold dilutions rather than 2-fold dilutions.

In preliminary studies the total amount of serologically reactive material in culture fluid was determined by the quantitative precipitin method (18), and the results were then expressed as the maximum amount of antibody calculated to be precipitable by 1 ml of culture fluid (22). The results obtained for different culture fluids by this method were directly related to those obtained by rocket immunoelectrophoresis (described below), and the latter procedure was therefore employed in all the reported studies.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis (1) was performed with equipment supplied by LKB Produkter AB, Bromma, Sweden. Glass plates (8.4 by 9.4 cm) were coated with 1% agarose in 0.01 M phosphate buffer, pH 7.15, containing 0.01% sodium azide and 200 μl of antiserum 409 (12 ml of gel per plate). Antigen samples (5 μl) were pipetted into 2.5-mm-diameter wells cut into the agarose, and the electrophoresis was run in phosphate buffer (0.01 M, pH 7.15) at 200 V (35 mA/plate) for 2 h.

The temperature was controlled at 10 to 12°C by circulating cold water. When the electrophoresis was completed, the plates were covered with filter paper presoaked in 0.85% NaCl and dried at 37°C for 24 h. The plates were then washed over a period of 24 h with three changes of 0.85% NaCl, covered with filter paper presoaked in deionized water as described above, and dried at 37°C. The precipitin lines in the agarose were stained by a 10-min immersion into a 0.5% solution of Coomassie brilliant blue R (Coomassie brilliant blue R, 5 g; 96% ethanol, 450 ml; glacial acetic acid, 100 ml; distilled water, 450 ml). The agarose was then destained with the same solvent mixture. After staining, the heights of the peaks were measured from the top of the well to the top of the peak. The rocket heights in centimeters per 10 μg of cell equivalent were then calculated. In later studies, a photographic record was made of the gel immediately after electrophoresis (Polaroid Corp., Cambridge, Mass.).

The height of the rocket is influenced by the amount of antibody in the serum, and all reported studies employed the same collection of antiserum, which contained 2.2 mg of antibody against LTA as determined by the quantitative precipitin method. The mean rocket height for the *L. casei* LTA standard (1.25 μg) included in each of the 13 different experiments was 1.97 cm, with a standard deviation of 0.15 cm and a coefficient of variation of 7.7%.

RESULTS

Estimation of extracellular LTA by hemagglutination. The application of the hemagglutination method is shown in Table 1, which compares culture fluids from organisms grown at $D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$ and at pH 6.0. The maximum hemagglutination titers of 1,600 in each case show that the LTA component in each culture fluid reacts equally well with the antiserum. However, the culture fluids differ in the extent to which they retain full sensitizing capacity on dilution, so that the culture fluid titers for cells grown at $D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$ are 60 and 18, respectively. The maximum hemagglutination titer of 1,600 was also obtained for the other culture fluids that are described below. Treatment of culture fluid with hot aqueous phenol had no detectable effect on these properties.

Effect of growth conditions on the culture fluid titer. Figure 1 provides a comparison

TABLE 1. *Estimation of culture fluid titers by determining the maximum dilution of culture fluids capable of fully sensitizing erythrocytes as shown by hemagglutination titer*

Culture fluid dilution	Hemagglutination titer	
	$D = 0.05\ h^{-1}$	$D = 0.5\ h^{-1}$
5	1,600	1,600
7.5	1,600	1,600
12	1,600	1,600
18	1,600	1,600
27	1,600	800
40	1,600	800
60	1,600	400
90	800	200

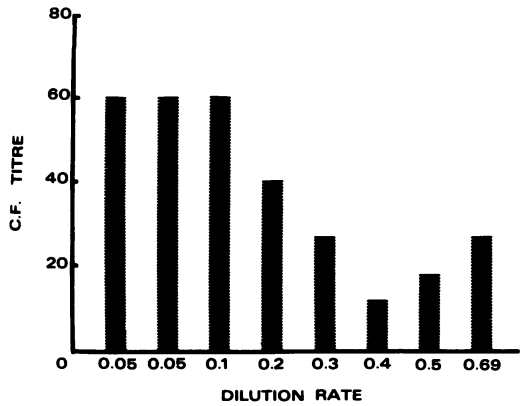

FIG. 1. *Estimation of the relative amounts of extracellular LTA produced at pH 6.0 and different dilution rates by determining the culture fluid (C.F.) titer. The C.F. titer is defined as the maximum dilution of culture fluid that fully sensitizes erythrocytes for hemagglutination.*

of the culture fluid titers for organisms grown at pH 6.0 and dilution rates between 0.05 and 0.69 h^{-1}. Two values for $D = 0.05$ h^{-1} are given because organisms were grown under conditions of increasing dilution rate to 0.69 h^{-1} and then returned to $D = 0.05$ h^{-1} to ensure that the differences between slow- and fast-growing cells were reproducible. The results, which are typical of those obtained in three experiments carried out at pH 6.0, indicate a trend towards a decreasing amount of LTA for faster-growing cells. The culture fluid titers in the different experiments at a particular dilution rate always agreed to within one dilution.

The culture fluid titer has been shown to depend on both the pH of growth and the dilution rate (Table 2). Organisms were grown at pH values between 5.5 and 8.0 at $D = 0.1$ h^{-1} and between 5.5 and 7.5 at $D = 0.2$ h^{-1}; at this faster dilution rate, stable conditions of growth could not be achieved at pH 8.0 and "wash-out" occurred. A comparison of the results shows that more LTA was detectable in the culture fluid of the slower-growing organisms ($D = 0.1$ h^{-1}) at pH 6.5 and below, but at pH 7.0 and 7.5 there was no difference.

Examination of culture fluids by rocket immunoelectrophoresis. Rocket immunoelectrophoresis of dialyzed culture fluid from chemostat-grown organisms and of the LTA fraction from batch-grown organisms showed that there was a linear relation between rocket height and the amount of material over the ranges shown in Fig. 2. The teichoic acid components isolated from the LTA fraction of *L. fermentum* and *S. mutans* BHT gave rocket heights that were 3.7 and 4.0 times those given by a comparable weight of LTA, respectively.

It was necessary to confirm that the collection of cultures for up to 24 h in a cold bath would not influence these serological results, and a comparison was therefore made of the amounts of material detectable by rocket immunoelectrophoresis for cultures (pH 6.0, $D = 0.05$ and 0.2

TABLE 2. *Culture fluid titer for S. mutans BHT grown at different pH values and dilution rates of 0.1 and 0.2 h^{-1}*

pH	Titer at D:	
	0.1 h^{-1}	0.2 h^{-1}
5.5	60	18
6.0	90	27
6.5	90	40
7.0	40	40
7.5	40	40
8.0	40	

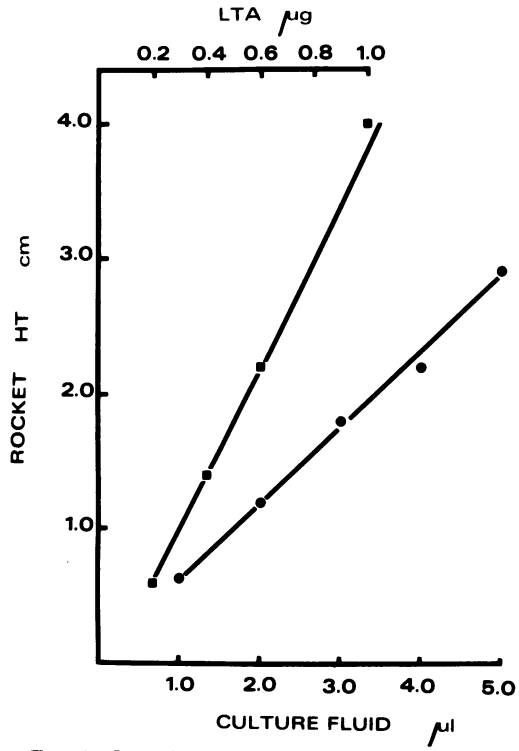

FIG. 2. *Quantitation of LTA fractions by rocket immunoelectrophoresis against antiserum to L. casei LTA. A straight-line relation is shown between the rocket height for S. mutans cellular LTA (■) and the amount applied (upper axis), and similarly between the rocket height for dialyzed culture fluid from organisms grown at pH 6.0 and $D = 0.05$ h^{-1} (●) and the volume of culture fluid applied (lower axis).*

h^{-1}) collected immediately on exit from the chemostat and after storing at 0 to 4°C for 24 h. The results showed that storage had no detectable effect on the rocket height (Fig. 3) or appearance. In contrast storage of batch-grown organisms at 0 to 4°C for 24 h resulted in a doubling of the height of the rocket obtained for the culture fluid (Fig. 3). It was also shown that prolonged storage and repeated thawing of culture fluid (up to 6 months) did not influence the result obtained by rocket immunoelectrophoresis, nor was the rocket changed in its height or appearance by phenol extraction of culture fluid.

Effect of growth conditions on the cellular and extracellular products detected by rocket immunoelectrophoresis. The effect of growth conditions on the production of serologically active material was examined by comparing the rocket heights for the cellular and extracellular fractions; rocket heights for cell extracts were standardized on 10 μg (dry weight) of cells and for culture fluid on the volume corresponding to 10 μg of cells.

The results obtained for organisms grown at pH 6.0 and different dilution rates are given in Fig. 4. The values for the culture fluid follow the same trend as was observed for the culture fluid titer, namely, a greater amount of reactive material at the lower dilution rates. The ratio of rocket height for extracellular to cellular material varied from 8.3:1.0 at $D = 0.05\ h^{-1}$ to 1.5:1.0 at $D = 0.69\ h^{-1}$. By comparison the ratio for cells grown overnight at pH 6.0 in batch culture containing 0.5% was 2.0:1.0.

For the three different series of studies that were performed, rocket heights at a particular dilution were very similar to the values shown in the figure. The coefficient of variation was 10%, which is only marginally greater than the coefficient of variation (7.7%) with standard LTA on rocket immunoelectrophoresis.

The pH of growth was also shown to influence the amounts of reactive material (Fig. 5), particularly for organisms grown at $D = 0.1\ h^{-1}$, where there were greater variations in the amounts of both cellular and extracellular material than for organisms grown at $D = 0.2\ h^{-1}$.

Partial fractionation of extracellular LTA and deacylated LTA. Culture fluids and representative cell extracts were fractionated on an XM300 membrane. Appropriate concentrations of the retentate and diffusate were examined by hemagglutination, rocket immunoelectrophoresis, and in some instances immunoelectrophoresis.

By the hemagglutination procedure, it was shown that in each case the retentate had the original culture fluid titer and the amount of LTA in the diffusate was insufficient to fully sensitize erythrocytes; the maximum titer for the first dilution of 1:5 was 200 or less for 12 of the 22 fractions tested and 400 to 800 for the remainder, compared with 1,600 for fully sensitized erythrocytes.

Examination by immunoelectrophoresis of the XM300 retentate from a typical experiment showed the presence of two components, the faster having the same mobility as the single

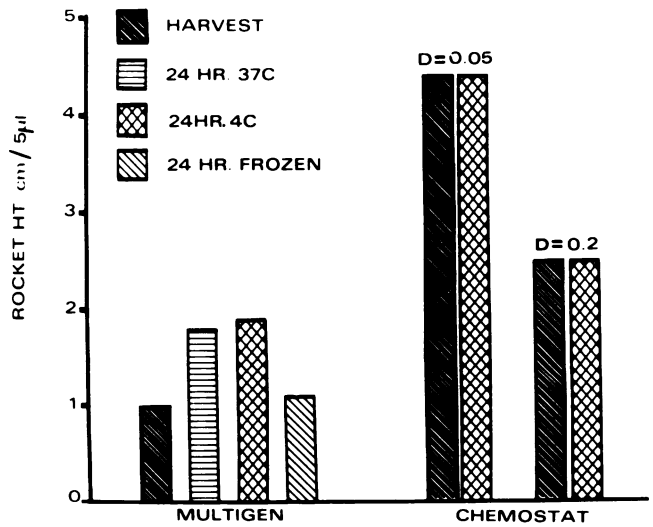

FIG. 3. *Effect of storage of cultures for 24-h periods at different temperatures on the resultant serological activity of the culture fluid. The results compare batch-grown organisms (Multigen) and organisms grown in the chemostat at pH 6.0 and two dilution rates (D = 0.05 and 0.2 h^{-1}).*

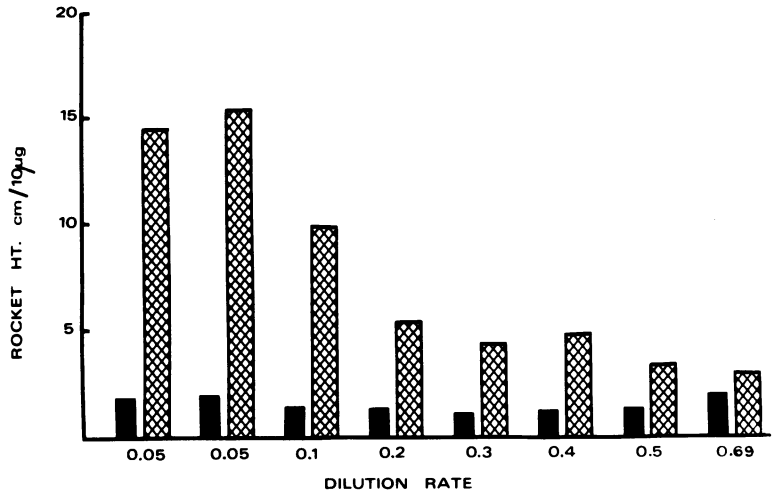

FIG. 4. *Estimation of the serological reactivities of cultures grown at pH 6.0 and different dilution rates. The results compare the values obtained by rocket immunoelectrophoresis of cell extracts (solid bars) and culture fluid (hatched bars).*

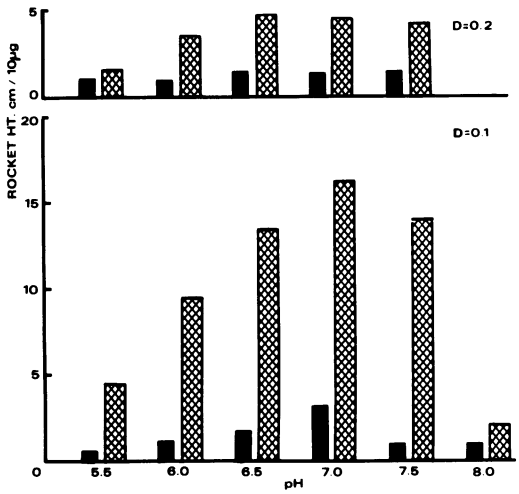

FIG. 5. *Estimation of the serological reactivities of cultures grown at D = 0.2 h^{-1} and pH 5.5 to 7.5 and at D = 0.1 h^{-1} and pH 5.5 to 8.0. The results compare the values obtained by rocket immunoelectrophoresis of cell extracts (solid bars) and culture fluid (hatched bars).*

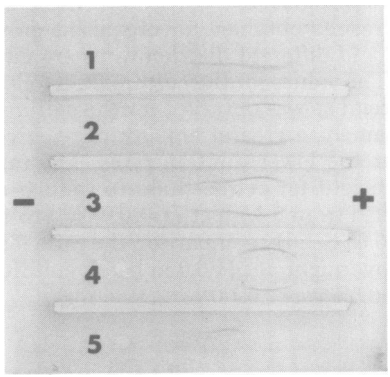

FIG. 6. *Immunoelectrophoresis of LTA fractions from organisms grown at D = 0.1 h^{-1} and pH 6.0. From top to bottom, the figure shows the precipitin patterns obtained against LTA antiserum after electrophoresis at 15 V/cm for 10 min for the following: 1, retentate from an Amicon XM300 membrane (×10); 2, retentate formed after passing the diffusate of 1 through an Amicon XM50 membrane (×10); 3, high-molecular-weight fraction obtained by column chromatography (5 µg); 4, low-molecular-weight fraction obtained by column chromatography (5 µg); 5, standard LTA from L. casei NCTC 6375 (2.5 µg).*

component detectable in the diffusate (Fig. 6). The LTA fraction obtained by column chromatography of culture fluid also contained the two components, whereas the deacylated LTA fraction only contained the faster-moving component (Fig. 6).

Examination of the XM300 retentate and diffusate by rocket immunoelectrophoresis showed that the retentate gave a clearly defined rocket, whereas the diffusate gave a less-distinct rocket (Fig. 7). When the two components were mixed, a single rocket was produced whose height approximated the sum of the individual components (Fig. 7). Further fractionation of the XM300 diffusate on an XM50 membrane showed that part of the material was diffusible (Fig. 7) and also that the mixture of retentate and diffusate again gave a rocket whose height approximated the sum of the individual components.

The results of these experiments indicate that

ultrafiltration of culture fluids and cell extracts through an XM300 membrane yields a retentate containing LTA and also deacylated LTA and a diffusate which is predominantly deacylated LTA and which can be further fractionated on an XM50 membrane.

Effect of growth conditions on the relative amounts of XM300 retentate and diffusate. The retentate and diffusate from three cell extracts from cultures grown at pH 6.0 and the culture fluids from all the dilutions at pH 6.0 were examined by rocket immunoelectrophoresis, and the percentage of material recovered in each fraction was estimated (Fig. 8). The total recovery was 95 to 100% for cellular extracts and averaged 95% for the extracellular material. For the cellular products, a greater proportion of material was retained by the XM300 membrane. A similar comparison was made between culture fluids of organisms grown at $D = 0.1$ and 0.2 h^{-1} and at different pH values.

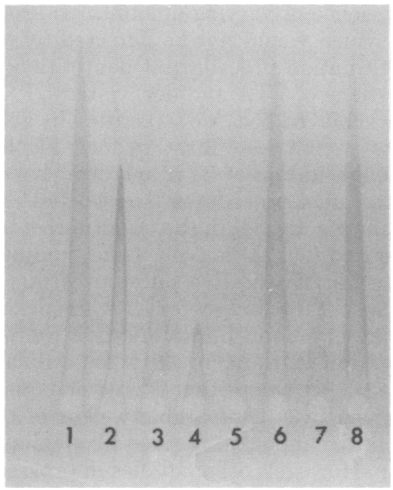

FIG. 7. *Rocket immunoelectrophoresis of fractionated and reconstituted culture fluid from organisms grown at $D = 0.1\ h^{-1}$ and pH 6.0. From left to right the figure shows the rockets formed by 5 μl of the following: 1, dialyzed native culture fluid; 2, retentate from an Amicon XM300 membrane, diluted to original volume; 3, diffusate from 2; 4, retentate obtained after passing 3 through an Amicon XM50 membrane and diluting to original volume; 5, diffusate from 4; 6, culture fluid reconstituted by mixing 2 and 3; 7, Amicon XM300 diffusate reconstituted by mixing 4 and 5; 8, culture fluid reconstituted by mixing 2, 4, and 5.*

The individual results at $D = 0.1\ h^{-1}$ are also given in Fig. 8 and showed an average recovery of 93%. The results at $D = 0.2\ h^{-1}$ (data not shown) showed a similar total recovery, with 22 to 30% of material being retained by ultrafiltration.

The results indicate that the cell extract contains a greater proportion of XM300 retentate than does the culture fluid and that the amount in the culture fluid also varies with growth conditions, particularly at different dilution rates. Because the LTA component in the retentate contains associated deacylated LTA (from immunoelectrophoresis), the results do not allow for a direct comparison of the relative amounts of LTA and deacylated LTA. However, a comparison of the ratio of rocket heights for retentate and diffusate can provide useful information by indicating the proportion of the higher-molecular-weight fraction in cell extracts and culture fluids. From the results in Fig. 8, it can be calculated that this ratio is 1.7 to 2.7:1.0 for the cell extracts but 0.2 to 1.5:1.0 for culture fluid. The greatest variation is found with culture fluid from organisms grown at pH 6.0, where the ratio is greater than 1:1 for slow-growing organisms ($D = 0.05$ to 0.1 h^{-1}) and 0.3 to 0.5:1.0 for faster-growing organisms ($D = 0.30$ to 0.69 h^{-1}).

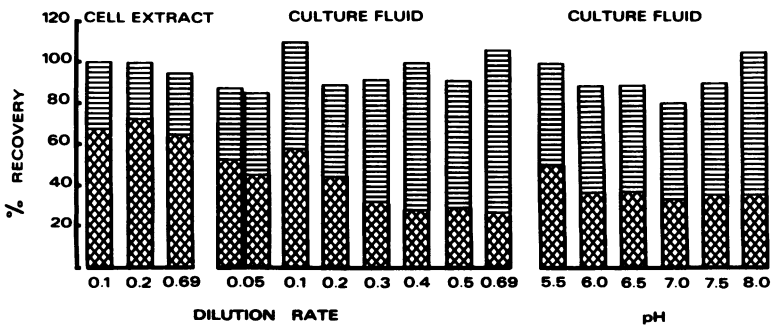

FIG. 8. *Effect of growth conditions on the relative amounts of serologically reactive material in the retentate and diffusate after ultrafiltration through an Amicon XM300 membrane. The results for retentate (hatched bars) and diffusate (horizontal bars) are expressed as a percentage of the rocket height obtained with unfractionated material. Cultures were grown either at pH 6.0 and different dilution rates or at a dilution rate of $0.1\ h^{-1}$ and different pH values.*

DISCUSSION

These studies have shown that *S. mutans* BHT produces significant though varying amounts of extracellular LTA when grown in a chemostat at different generation times and pH values. Two methods have been employed for measuring the amounts of extracellular material, the semiquantitative modification of the hemagglutination procedure, which detects only LTA, and rocket immunoelectrophoresis, which detects both LTA and deacylated LTA. The possibility of applying rocket immunoelectrophoresis to the quantitation of acidic polysaccharides such as LTA had been indicated by the studies of Owen and Salton (24) on succinylated lipomannan from *Micrococcus lysodeikticus*. It was also shown that the lipomannan could be quantitated by rocket affinoelectrophoresis employing a lectin (24), and this technique was subsequently shown to be applicable to the estimation of streptococcal LTA (23).

For each of the serological procedures employed in the present study, the estimation has been carried out routinely on dialyzed culture fluid in which the native LTA is associated, presumably in micelles, with protein, polysaccharide (22), and deacylated LTA. Partial purification of the complex by extraction with hot aqueous phenol did not cause a detectable change in serological activity as estimated by the two methods, indicating that these methods can be applied to culture fluid.

As determined by the hemagglutination procedure, organisms in continuous culture at pH 6.0 produced the greatest amounts of extracellular LTA when growing at the slow generation times of 7 and 14 h (D = 0.1 and 0.05 h^{-1}, respectively). The results for rocket immunoelectrophoresis of cell extracts and extracellular material have reinforced this conclusion. Although there were some variations of detectable cellular material at different dilution rates and pH 6.0, there were far greater differences in the amounts of extracellular material. Thus, the ratio of extracellular to cellular material was 8.3:1.0 at D = 0.5 h^{-1} and 1.5:1.0 at D = 0.69 h^{-1}. The earlier studies using batch-grown organisms grown in complex medium with excess glucose gave a ratio of 11:1, as determined by the quantitative precipitin method (22). However, a more valid comparison of batch- and chemostat-grown organisms is provided in the present study where the ratio for organisms grown in dialyzed medium at pH 6.0 with limited glucose was 2:1.

The pH of growth also influences the amounts of serologically reactive material. This was particularly evident from the results of rocket immunoelectrophoresis on cultures grown at D = 0.1 h^{-1} and pH values between 5.5 and 8.0, where there were marked variations in the amounts of both cellular and extracellular material. However, the differences for extracellular material were greater so that the ratio of extracellular to cellular material varied from 9:1 at pH 6.0 to 15:1 at pH 7.5 and then dropped to 2:1 at pH 8.0.

A comparison of the results obtained by the two serological procedures shows some disparity, and this could most readily be accounted for by the presence of varying amounts of deacylated LTA, which would not be detected by the hemagglutination procedure. Quantitation of the proportions of LTA and deacylated LTA could not be achieved because separation by gel filtration or membrane filtration was incomplete. Also, quantitation of LTA and deacylated LTA by serological procedures is restricted by their different reactivities in the quantitative precipitin method (20, 21) and on rocket immunoelectrophoresis. Qualitative differences in the appearances of the rockets for LTA and deacylated LTA are apparent, but in addition there is the observation that phenol-extracted cellular LTA gives a rocket height that is only one quarter of that given by a comparable weight of the teichoic acid component derived by alkaline deacylation. However, other studies in progress (unpublished data) indicate that the differences in reactivity of native extracellular LTA and its deacylation derivative may not be as great.

Despite these limitations placed on the interpretation of results, a comparison of the relative amounts of higher- and lower-molecular-weight fractions obtained by XM300 filtration provides useful information, because the greater proportion of the LTA is present in the retentate, and LTA is the component with the greater biological potential. First, a comparison of cellular and extracellular products indicates that there is a greater proportion of high-molecular-weight material in the cell extracts, an observation which is consistent with the studies of Shockman and co-workers (16, 17). Secondly, the proportion of XM300 retentate in culture fluids was influenced more by the dilution rate than by the pH of growth. With regard to potential biological properties, it is significant that the amount of XM300 retentate was greater for slower-growing organisms where there is already a much greater total amount of extracellular material. The sum effect is that the culture fluid from organisms growing at pH 6.0 and D = 0.05 h^{-1} contained nine times as much high-molecular-weight material as the culture fluid from organisms growing at D = 0.69 h^{-1}.

The results of these studies on *S. mutans* BHT show that LTA and its deacylated derivative are present as extracellular products under

conditions of growth that are relevant to those in the oral cavity where the pH of dental plaque would generally be below 7.0 and bacteria probably grow at two to three generations per day (8, 9, 11). Because of the important biological properties of LTA (32, 33), it can therefore be envisaged that its production by plaque bacteria would contribute to their pathogenic potential. *S. mutans* BHT may be regarded as an atypical strain because it produces such large amounts of extracellular LTA in both batch (22) and continuous culture, and for this reason the production of this component by other oral bacteria has also been examined. By comparable procedures, evidence has been obtained (unpublished data) for the production of significant though lesser amounts of extracellular LTA by *S. mutans* strains AHT and Ingbritt and by *L. fermentum*; however, *L. casei* and *Lactobacillus plantarum* produce only very small amounts. More detailed studies with *S. mutans* Ingbritt and *L. fermentum* have also provided evidence for deacylated LTA in the culture fluid.

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