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Phenotypic Stability of the Cell Wall of *Streptococcus mutans* Ingbritt Grown Under Various Conditions

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Quantitative analyses of cell walls from *Streptococcus mutans* Ingbritt grown under carbohydrate limitation in the chemostat showed that growth conditions had no statistically significant effect on the composition of polysaccharide, peptidoglycan, or the proportion of polysaccharide in the cell wall. Lysis of cell wall preparations with a muramidase supported this conclusion and further indicated that there was little difference in their overall structure. In contrast, there was a consistent difference between the rates of lysis by this enzyme of organisms grown in 0.2% glucose and 0.5% glucose. Extremes of pH or dilution rate essentially did not influence the immunogenicity of type *c* antigen in whole organisms irrespective of whether the carbohydrate source was glucose or sucrose. However, differences were found in the immunogenicity of lipoteichoic acid under similar circumstances. The results indicated there was an inherent phenotypic stability in the cell walls of *S. mutans* Ingbritt despite changes in pH, generation time, and carbohydrate source, and that any changes that did occur were probably due to associated cell-surface components.

The serological identification of strains of *Streptococcus mutans* has received considerable attention because of their relation to dental caries (2-4, 21, 25, 29). Most studies have assumed that the serological properties of organisms growing in plaque with a mean generation time of 12 to 24 h (8, 11) would be similar to the properties of organisms growing in batch culture under laboratory conditions. However, the surface properties of bacteria can be profoundly influenced by the growth conditions (8, 9), specific examples being the effect of phosphate limitation on teichoic acid formation (9), the effect of generation time on the toxicity of gram-negative bacteria (23, 24), and the immunogenicity of surface components of lactobacilli (17), as well as the effect of generation time, pH, and carbohydrate source on the production of lipoteichoic acid (LTA) by *S. mutans* strains BHT (14) and Ingbritt (13). In contrast, it was recently reported that the production of exopolysaccharide by *Azotobacter vinelandii* is unaffected by most environmental conditions (15).

Serological type *c* strains of *S. mutans* generally predominate in plaque (2, 25, 29). The type *c* antigen, isolated from both strain Ingbritt (22) and strain GS5 (31) is a cell wall polysaccharide that contains rhamnose as its major component and glucose as its immunodominant component. The possible effect of growth conditions on the properties of this antigen was indicated from studies by Ellwood (8) which showed that chemostat-grown organisms of an Ingbritt strain had a higher rhamnose-to-glucose ratio than batch-grown cells and were more effective in immunizing monkeys against dental caries.

The present study describes the properties of organisms obtained from cultures of *S. mutans* Ingbritt grown in a chemostat at different generation times and dilution rates and with different carbohydrate sources (13). Comparing cell wall preparations for their chemical composition, rates of solubilization by dilute acid or enzymes, as well as their serological reactivity, indicated there was very little variation imparted by these growth conditions. However, studies on the immunogenicity of LTA and type *c* antigen and the rates of enzymatic lysis of whole organisms provided evidence that overall phenotypic variation could occur under certain circumstances.

MATERIALS AND METHODS

Organism. *S. mutans* Ingbritt was kindly supplied by B. Krasse, University of Göteborg, Sweden. This

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strain shows some differences in characteristics (13) from the strain of Ingbritt employed by Ellwood, which had been reisolated from a carious lesion in a monkey (8).

Growth conditions. Organisms grew in continuous culture under anaerobic conditions and at constant pH with either glucose or sucrose as the limiting nutrient. Details of the growth conditions were described previously (13, 14). The culture fluids flowing from the chemostat were collected at 4°C and routinely harvested at 24-h intervals by centrifugation. Cells were washed twice with cold 0.85% NaCl solution and stored frozen at −20°C.

From the previous studies, where the culture fluids had been examined for extracellular bacterial components (13), organisms were available that had been grown under the following conditions: (i) in 0.2% glucose at pH 6.0 and dilution rates (D) from 0.05 h^{-1} to 0.5 h^{-1}; (ii) in 0.5% glucose at pH 6.0 and pH 7.5 and at dilution rates from 0.05 h^{-1} to 0.5 h^{-1}; (iii) in 0.5% glucose at D = 0.1 h^{-1} and at pH values from 5.5 to 7.5; (iv) in 0.5% sucrose at pH 6.0 and at different dilution rates; and (v) in 0.5% sucrose at D = 0.1 h^{-1} and at different pH values.

Preparation of cellular components and cell walls. Cell walls were prepared by mechanical disruption of organisms followed by treatment with boiling sodium dodecyl sulfate (5). Type *c*-specific polysaccharide was obtained from strain Ingbritt by autoclaving cell wall (5) and from *S. mutans* GS5 by formamide extraction (31). LTA was isolated from organisms by extraction with hot aqueous phenol followed by column chromatography (13).

Quantitative analyses of purified cell walls. Amino acids and amino sugars in purified walls were determined using a JEOL model JLC-6AH automated amino acid analyzer (JEOL Inc., Cranford, N.J.). Samples were hydrolyzed for 18 h in 6 N HCl at 105°C and prepared as previously described (30). Phosphorus was measured by the method of Chen et al. (6).

Sugars were quantitated by gas-liquid chromatography. Samples (1 to 5 mg) were hydrolyzed in sealed ampoules with 1 N H_2SO_4 for 8 h at 100°C. After hydrolysis, the samples were cooled and opened, and 1.0 ml of a mannitol solution of a known concentration (usually 5.5 μmol ml^{-1}) was added as an internal standard. The hydrolysates were neutralized with $BaCO_3$ and centrifuged, and the supernatants were lyophilized. Hydrolysates were converted to their trimethylsilyl derivatives using TRI-SIL (Pierce Chemical Co., Rockford, Ill.) according to the instructions in the Pierce *Handbook of Silylation* (26). The derivatives were separated on glass columns (2 m by 4 mm) packed with SE-30 Ultraphase (3%) on Chromosorb W (HP) 80/100 mesh (Pierce) on a Packard model 803 gas liquid chromatograph (Packard Instrument Co., Downers Grove, Ill.). The operating temperature was 165°C (isothermal), the carrier gas (nitrogen) flow rate was 30 cm^3 min^{-1}, and a hydrogen flame ionization detector was used. Peaks were quantitated using an Autolab Minigrator electronic digital integrator (Spectra-Physics).

Dilute acid hydrolysis of cell wall. Cell wall preparations were heated at 60°C in 0.1 N H_2SO_4 for appropriate periods, and the rates of release of soluble rhamnose and hexose were estimated (5).

Lysis of cells and cell wall preparations by muramidase. Mutanolysin and the M-1 enzyme preparation from *Streptomyces globisporus* 1829, which contains an *N*-acetylmuramidase, were kindly supplied by K. Yokogawa, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan (32).

For detailed studies with the M-1 enzyme, appropriate suspensions of cells in water were dispersed by mild sonication for 15 min (Bransonic 12, Bransonic Cleaning Equipment Co., Shelton, Conn.). A 1.9-ml volume of suspension was diluted with 2.0 ml of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (0.02 M, pH 7.0, at 37°C), and the enzyme was added (10 μg per 0.1 ml of water). Experiments on lysis of cell wall were performed on 1.5 mg of wall per 1.0 ml of water to which was added 2.0 ml of sodium acetate buffer (0.1 M, pH 5.4) and 10 μg of enzyme per 0.1 ml of water. The mixture was transferred to a preheated (37°C) 1-cm cuvette and placed in the heated cuvette holder of a spectrophotometer (Beckman model 25, Beckman Instruments Inc., Fullerton, Calif.). The temperature was maintained at 37°C, and the decrease in absorbance at 600 nm was recorded. Linear decrease in absorbance was measured and corrected for any decrease that occurred in the absence of enzyme.

The amount of enzyme employed was shown from preliminary experiments to give a readily measurable rate of lysis over a 5-min interval. Preliminary experiments also showed that the rate of lysis was independent of cell concentration within the absorbance range of 0.30 to 0.67 and independent of cell wall concentration over the range of 0.75 to 3.0 mg per 4 ml of reaction mixture. One preparation of cells, namely those grown in 0.5% glucose at D = 0.4 h^{-1}, was assayed each day as the standard for cell lysis, and the corresponding cell wall preparation was similarly assayed. Experiments were carried out in quadruplicate, and the mean rate of lysis was expressed as a percentage of the rate of lysis of the appropriate standard.

Fractionation of cell wall lysate. A suspension of 50 mg of cell wall from batch-grown *S. mutans* Ingbritt in 12 ml of Tris-hydrochloride buffer (0.01 M, pH 7.0) was incubated with 250 μg of M-1 *N*-acetylmuramidase for 19 h at 37°C with shaking. After centrifuging (25,000 × *g*, 1 h, 5°C), the supernatant was dialyzed against distilled water and then freeze-dried.

The lysate which in duplicate experiments represented approximately 70% of the cell wall was fractionated on a diethylaminoethyl-Sephadex column (25 by 0.9 cm) (Pharmacia Fine Chemicals AB, Uppsala, Sweden) following a procedure similar to that previously employed with lysates of *Lactobacillus casei* cell wall (18). Forty 3-ml fractions eluted with Tris-hydrochloride buffer (0.05 M, pH 7.8) were analyzed for hexose content, and appropriate eluates were pooled to yield fraction A which represented 33% of the material applied to the column. Fraction B, representing 43% of the material, was obtained by the subsequent elution of the column with Tris-hydrochloride buffer containing 0.1 M NaCl.

Fractions were analyzed for rhamnose (10), total hexose (7), and hexosamine content after hydrolysis (18).

Serological methods. Antisera to organisms grown under different conditions were prepared by intravenously injecting male New Zealand white rabbits of the same age (approximately 4 months, median weight = 3 kg) with a suspension of heat-killed organisms in 0.85% NaCl at 3- to 4-day intervals by the following schedule: 0.2, 0.5, 1.0, and 1.5 ml of suspension with absorbance of 1.0 at 600 nm in a 1-cm cell, followed by 1.0 and 1.25 ml of suspension with absorbance of 2.0. Rabbits were bled 6 days after the last injection, and antisera were examined for their reactivity with type-specific polysaccharide (5) and LTA (13) from *S. mutans* Ingbritt by the quantitative precipitin method (19). Statistical analysis by the Student *t* test was carried out to determine the significance of differences in detectable antibody level. The antiserum employed in the comparative study on the serological reactivity of cell wall lysates was prepared against organisms grown at pH 6.0 and $D = 0.5\ h^{-1}$ (rabbit 532).

RESULTS

Quantitative analysis of cell wall preparations. Cell walls prepared from organisms grown in 0.2 and 0.5% glucose at pH 6.0 and at dilution rates from 0.05 h^{-1} to 0.5 h^{-1} were analyzed for their component carbohydrates, amino acids, and total phosphorus. The results (Tables 1 and 2) suggest that there were only minor differences in composition. This was confirmed by a statistical analysis of the results by an analysis of variance which showed that there were no significant differences at different dilution rates.

TABLE 1. *Composition of the cell wall of S. mutans Ingbritt grown at pH 6.0 in 0.2% glucose at different dilution rates*

Component	Amt (μmol mg^{-1}) in cell wall at D of:					
	0.05[a]	0.1	0.2	0.3	0.4	0.5
Rhamnose	2.08	1.88	1.80	2.05	1.89	1.76
Glucose	0.80	0.80	0.81	0.86	0.84	0.82
Glucosamine	0.40	0.38	0.38	0.42	0.33	0.41
Muramic acid	0.29	0.25	0.26	0.24	0.24	0.26
Alanine	1.15	1.18	1.14	1.14	1.15	1.20
Lysine	0.40	0.39	0.38	0.39	0.36	0.40
Glutamic acid	0.36	0.36	0.34	0.32	0.34	0.37

[a] Dilution rate in $hours^{-1}$

TABLE 2. *Composition of the cell wall of S. mutans Ingbritt grown at pH 6.0 in 0.5% glucose at different dilution rates*

Component	Amt (μmol mg^{-1}) in cell wall at D of:					
	0.05[a]	0.1	0.2	0.3	0.4	0.5
Rhamnose	1.87	1.80	1.51	1.97	1.92	1.76
Glucose	0.84	0.83	0.79	0.85	0.79	0.81
Glucosamine	0.38	0.40	0.38	0.43	0.43	0.43
Muramic acid	0.24	0.31	0.26	0.28	0.27	0.25
Alanine	1.18	1.21	1.18	1.17	1.36	1.21
Lysine	0.41	0.38	0.42	0.40	0.41	0.43
Glutamic acid	0.37	0.38	0.37	0.36	0.36	0.37

[a] Dilution rate in $hours^{-1}$

A similar comparison of cell wall preparations from organisms grown at $D = 0.1\ h^{-1}$ in 0.5% glucose and at pH 5.5, 6.5, and 7.5 also gave results that were not significantly different.

The total phosphorus content of cell wall preparations was 0.19 to 0.26 μmol mg^{-1}, values which are comparable to those for batch-grown *S. mutans* Ingbritt (5) and other batch-grown strains (1).

Dilute acid hydrolysis of cell wall. Previous studies on cell wall preparations from batch-grown strains of *S. mutans* showed that there were variations in the rate of release of cell wall polysaccharide on hydrolysis in 0.1 N H_2SO_4 at 60°C (5). To examine whether growth conditions affected the rate of release of wall polysaccharide, a similar experiment was carried out with cell wall preparations from strain Ingbritt grown in 0.5% glucose at different dilution rates and pH values. A comparison of the results obtained on analyzing the soluble fraction after hydrolysis for 1 h at 60°C indicated that 26 to 28% of the total rhamnose was released from cell walls of organisms grown at $D = 0.05$ to $0.2\ h^{-1}$ at pH 6.0 and at $D = 0.1\ h^{-1}$ and pH 5.5 to 6.5. However, higher values, 38 to 40%, were obtained for preparations from organisms grown at $D = 0.3$ to $0.4\ h^{-1}$ and pH 6.0 and at $D = 0.1\ h^{-1}$ and pH 7.5.

Enzymatic lysis of organisms and cell wall preparations. The rate of lysis of batch-grown *S. mutans* Ingbritt by mutanolysin was compared with that for batch-grown *S. mutans* BHT, which is known to be readily lysed by the enzyme and which was used as the standard organism in the original studies describing the enzyme's properties (12, 32). During the initial 5 min of incubation at 37°C, the rate of lysis of strain Ingbritt by mutanolysin (10 μg ml^{-1}) at pH 7.0 was 65% of that for strain BHT.

For more detailed studies, organisms grown in continuous culture at pH 6.0 with 0.2 and 0.5% glucose were examined for their relative rates of lysis by the M-1 *N*-acetylmuramidase derived from mutanolysin. The results (Table 3) showed that, although there were differences in the rates of lysis for organisms grown at different dilution rates, the major difference was the consistently greater rate of lysis for organisms grown at the lower glucose level, where differences were statistically significant ($P < 0.01$). However, these differences were no longer apparent when cell wall preparations were studied for the rate of lysis (Table 3).

A similar comparison of organisms grown in 0.5% glucose at $D = 0.1\ h^{-1}$ and at different pH

values (5.5, 6.5, 7.5) yielded results that did not differ significantly from each other.

Serological reactivity of cell wall lysates. After the completion of the experiments on cell wall described in the previous experiments, the four samples of the same cell wall preparation were pooled, and incubation was continued at 37°C for a further 18 to 24 h. After centrifugation to remove residual cell wall, the soluble fraction was dialyzed against 0.85% NaCl. The lysates were diluted in 0.85% NaCl to give a nominal concentration of 50 μg of lysed cell wall per ml, and appropriate amounts of lysate (5 to 20 μg) were analyzed by the quantitative precipitin method against 67 μl of antiserum 532 to *S. mutans* Ingbritt. From the resultant quantitative precipitin curves, the maximum amount of antibody precipitable from 1 ml of antiserum could then be calculated. The results (Table 4) show that variations in pH, dilution rate, and glucose content of the medium have only a minor effect on the serological reactivity of the solubilized fraction of cell wall.

The specificity of the reaction between antiserum and the type *c*-specific polysaccharide component of the lysed wall was confirmed (22, 31) by showing that 75 μmol of methyl-α-D-glucoside inhibited by 34% the reaction between 67 μl of antiserum 532 and a typical lysate of cell wall prepared from chemostat-grown organisms (0.5% glucose, pH 6.0, $D = 0.2\ h^{-1}$).

The minor differences that did occur in the reactivities of wall lysates could derive from differences in structure of the type polysaccharide or differences in the size of the products of wall lysis. The two fractions obtained on column chromatography of lysates of wall from batch-grown organisms were compared by the quantitative precipitin method. The results (Fig. 1) show that fraction A, which was eluted first, is only half as effective in precipitating antibody as fraction B. The type-specific polysaccharides from strains Ingbritt and GS5 each precipitated 1.1 mg of antibody per ml, a value slightly greater than that for fraction A. Fractions A and B were analyzed for rhamnose, hexose, and total hexosamine. The molar ratios of rhamnose to hexose were very similar, being 1.99:1.00 and 2.07:1.00, respectively, and close to the previously reported values (22, 31). However, the molar ratio of rhamnose to hexosamine was 1.48:1.00 for fraction A and 3.00:1.00 for fraction B.

TABLE 3. *Relative rates of hydrolysis of organisms and cell wall preparations by M-1 N-acetylmuramidase*

D (h^{-1})	Relative rate of hydrolysis:			
	Organisms[a]		Cell wall[b]	
	Glucose (0.5%)	Glucose (0.2%)	Glucose (0.5%)	Glucose (0.2%)
0.05	145	202	96	79
0.10	125	225	106	114
0.20	111	220	72	114
0.40	100	188	100	
0.50	72	175	119	128

[a] Results are the mean of quadruplicates and are expressed relative to that for organisms grown at pH 6.0 in 0.5% glucose at $D = 0.4\ h^{-1}$.

[b] Results are the mean of quadruplicates and are expressed relative to that for the cell wall preparation from organisms grown at pH 6.0 in 0.5% glucose at $D = 0.4\ h^{-1}$.

TABLE 4. *Serological reactivity of enzymatic lysates of cell wall preparations from organisms grown under different conditions*

Conditions of growth			Antibody precipitated[a] (mg/ml of serum)
pH	Glucose (%)	D (h^{-1})	
6.0	0.2	0.05	1.74
6.0	0.2	0.20	1.82
6.0	0.2	0.50	1.69
6.0	0.5	0.05	1.71
6.0	0.5	0.20	1.82
6.0	0.5	0.50	1.69
5.5	0.5	0.10	1.50
6.5	0.5	0.10	1.54
7.5	0.5	0.10	1.54

[a] Maximum amount of antibody precipitable from 1 ml of serum as calculated from results of quantitative precipitation with *S. mutans* Ingbritt antiserum.

Immunogenicity of *S. mutans* Ingbritt whole organisms. Tables 5 and 6 summarize the results of the mean antibody titers for LTA and type *c*-specific polysaccharide immunogens from organisms grown on glucose or sucrose under a variety of chemostat conditions. Differences in the LTA antibody levels were significant for organisms grown on glucose at pH 5.5 compared with those at pH 7.5 ($P < 0.02$), but nonsignificant for cells grown on sucrose (Table 5). In contrast, antibody levels detected against type *c* polysaccharide showed significant differences ($P < 0.02$) with change of pH of growth only when the organisms were grown in sucrose but not glucose (Table 5). At constant pH and variable dilution rate (Table 6), significant differences ($P < 0.05$) were found in LTA antibody levels for organisms grown at $D = 0.05\ h^{-1}$ compared with organisms grown at $D = 0.5\ h^{-1}$ in sucrose but not glucose. No significant differences were found for serotype *c* polysaccharide antibodies for organisms grown on either carbon source.

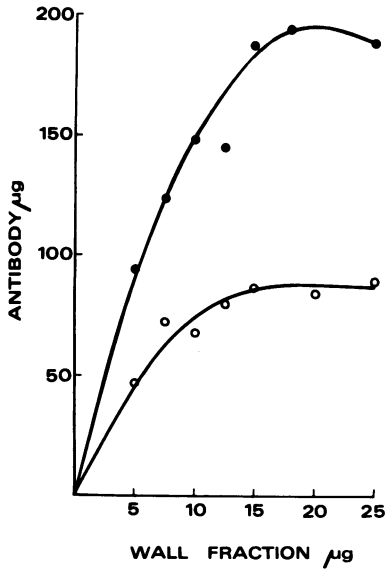

FIG. 1. *Differences in the size of the products of wall lysis as determined by the quantitative precipitin method. For details see text. Symbols: ○, fraction A; ●, fraction b.*

TABLE 5. *Immunogenicity of the polysaccharide and LTA components of organisms grown at D = 0.1 h^{-1} and at different pH values*

Growth conditions		No. of rabbits injected	Mean antibody level[a] (mg/ml of serum)	
Carbohydrate source (0.5%)	pH		Polysaccharide	LTA
Glucose	5.5	10	0.75 ± 0.40	0.15 ± 0.10
	7.5	10	0.82 ± 0.26	0.28 ± 0.12
Sucrose	5.5	8	1.34 ± 0.52	0.17 ± 0.16
	7.5	8	0.53 ± 0.31	0.15 ± 0.09

[a] Antibody level was determined by the quantitative precipitin method by reaction of antisera with the relevant components isolated from batch-grown organisms. Results are quoted ± standard deviation.

Comparison of the immunogenicity of glucose- to sucrose-grown organisms under the same growth conditions showed no significant differences for type *c*-specific polysaccharide. However, differences in the immunogenicity of LTA were observed in cells grown at pH 6.0 and $D = 0.5\ h^{-1}$ ($P < 0.05$) as well as pH 7.5 and $D = 0.1\ h^{-1}$ ($P < 0.05$).

DISCUSSION

The results of several studies with chemostat-grown organisms have indicated that growth conditions can have a profound effect on the surface properties of bacteria (8, 9). The cell surface is composed of the peptidoglycan, with covalently joined carbohydrate polymers, associated membrane-derived components, particularly proteins and LTA, and, in some instances, capsular polysaccharides. The methods for preparing cell wall generally remove components which are not in covalent linkage with peptidoglycan, and to this extent the cell wall, as an operational fraction, can be distinguished from the cell surface.

The present study on the cell wall of *S. mutans* Ingbritt examined preparations by a number of procedures aimed at detecting differences in composition or structure. The quantitative analyses are very similar to those reported for cell wall from batch-grown cultures of other strains of serotype *c* (1) and account for the major part of the cell wall. Calculations based on the molecular weight of the components, assuming acetylated hexosamines, and on the phosphate content, would indicate that approximately 85 to 90% of the different cell wall preparations has been recovered, although this value falls to 75 to 80% if allowance is made for water of hydrolysis. A comparison of the results also leads to the conclusion that the growth conditions that were examined did not have a significant effect on the composition of the polysaccharide and peptidoglycan nor the proportion of polysaccharide in the cell wall. Although the differences in the amounts of the rhamnose and glucose components are not statistically significant, the differences, when compounded, result in a variation in the rhamnose-to-glucose ratio from 2.6:1.0 to 1.9:1.0. In a previous study (20) in which different methods of analysis were used, differences in the rhamnose-to-glucose ratio were interpreted as indicating that some variation in structure was occurring at different gen-

TABLE 6. *Immunogenicity of the polysaccharide and LTA components of organisms grown at pH 6.0 and at different dilution rates (D)*

Growth conditions		No. of rabbits injected	Mean antibody level[a] (mg/ml of serum)	
Carbohydrate source (0.5%)	D (h^{-1})		Polysaccharide	LTA
Glucose	0.05	5	0.34 ± 0.10	0.25 ± 0.14
	0.50	5	0.47 ± 0.41	0.23 ± 0.34
Sucrose	0.05	7	0.60 ± 0.31	0.15 ± 0.04
	0.50	8	0.34 ± 0.13	0.10 ± 0.05

[a] Antibody level was determined by the quantitative precipitin method by reaction of antisera with the relevant components isolated from batch-grown organisms. Results are quoted ± standard deviation.

eration times; these differences would now appear not to be significant. Differences have, however, been noted in the rhamnose-to-glucose ratio of wall from chemostat- and batch-grown cultures of *S. mutans* Ingbritt, although the extent of the difference was not given (8).

Analyses of the composition of cell wall do not necessarily distinguish between differences that could occur in the structure of the peptidoglycan and polysaccharide components, and accordingly additional studies were performed. Changes in the degree of cross-linking of the peptidoglycan chains could be expected to influence the rate of enzymatic lysis as has been observed with a micrococcus grown in synthetic and complex media (16). Heterogeneity of cell wall structure is indicated by results of column chromatography of *S. mutans* wall lysates, but any differences between the rates of enzymatic lysis of cell wall preparations from organisms grown at different dilution rates and different pH values are only minimal, and would indicate that there may be little difference in their overall structure. In contrast, there is a major and consistent difference between the rates of lysis of organisms grown in 0.2% glucose and 0.5% glucose, presumably due to the presence of associated cell surface components. Differences in such associated components probably account for the wide differences in relative rate of lysis of clinical isolates of serotype *c S. mutans* strains by mutanolysin (12).

The polysaccharide component of cell wall is joined to peptidoglycan by a covalent linkage. In the case of *Lactobacillus casei* and *Lactobacillus fermentum*, the linkage is a phosphodiester bond between the polysaccharide and *N*-acetylmuramic acid, which is readily hydrolyzed by dilute acid and by autoclaving (5). The present studies have confirmed that the polysaccharide-peptidoglycan linkage in *S. mutans* Ingbritt is relatively stable to acid hydrolysis (5). Minor differences in the rate of release of polysaccharide are apparent, but, in the absence of information on the structure of the linkage between polysaccharide and peptidoglycan, interpretation of these differences is not possible.

Information on the structure of the polysaccharide component of type *c* strains of *S. mutans* is limited to the serological studies indicating the presence of terminal α-D-glucosyl residues, which are responsible for serological specificity (22, 31). Changes in the structure of the polysaccharide could result in differences in serological properties, and for this reason lysates of cell walls were examined. Such lysates were considered to be more representative of the cell wall than the relatively small amounts of polysaccharide released by dilute acid (5) or recovered after formamide extraction (31), where considerable degradation occurs. Cell wall lysates from organisms grown at different dilution rates, different pH values, and at two glucose concentrations showed only minor differences in their reaction with antiserum to whole cells of *S. mutans* Ingbritt, but this could have been due to differences in the products of lysis rather than differences in polysaccharide structure. The reaction of cells grown under different conditions with fluorescein-labeled antibody has also been studied, but no significant differences could be distinguished (D. Bratthall and L. K. Campbell, unpublished data).

Extremes of pH altered the immunogenicity of LTA in glucose-grown organisms in a manner predictable from the differences in the levels of LTA detected in these cells (13). However, cells cultured in sucrose failed to show these differences. Furthermore, sucrose-grown organisms which possessed far more detectable LTA when grown at high dilution rates compared to those grown at low dilution rates (13) gave significantly higher antibody titers to LTA for the slower growing organisms. Two explanations could account for this reversal in the predicted immunogenicity of LTA. Firstly, the LTA antigens could be masked by other cell surface components such as proteins, or, secondly, there could be changes in the structures of LTA such that the specificity of the antisera differed from that raised against batch-grown organisms. Chemical analyses of the LTAs obtained from organisms grown under these different conditions should help clarify this situation.

In all cases studied, the serotype *c* polysaccharide proved to be a stronger immunogen than the LTA, the best condition for the production of anti-polysaccharide antisera with low anti-LTA activity being with organisms grown in 0.5% sucrose at $D = 0.1\ h^{-1}$ and pH 5.5. Extremes of either pH or dilution rate essentially did not influence the immunogenicity of type *c* antigen in whole organisms irrespective of the carbohydrate source.

The results of these studies on the effect of dilution rate, pH, and carbohydrate source on the composition and properties of the cell surface of *S. mutans* Ingbritt indicate that the cell wall structure has not changed significantly and that any changes that do occur may be due to associated cell-surface components.

The conclusion that the cell walls of bacteria (as distinct from cell surface) are among those structures that are most susceptible to phenotypic change has generally been based on the effect of magnesium and phosphate on the syn-

thesis of teichoic acid and teichuronic acid by organisms growing in continuous culture (9). There have also been a number of studies with batch-grown cultures showing that changes in the medium composition can affect the cell wall, including the structure of peptidoglycan (16, 28) and polysaccharide (1) and the amounts of virulence-associated antigen (27). However, the present studies suggest that the polysaccharide-peptidoglycan complex is only slightly affected by changes in generation time and pH of growth. This is supported by the data of a recent paper showing that the peptidoglycan density in the sacculus of *Escherichia coli* remains constant despite different growth rates (33). In retrospect, this is not surprising as organisms in their natural environment, such as plaque, would be growing at different generation times and different pH values and there would be advantages in conserving the structure of the cell wall components. Thus, although phenotypic variability of the cell surface is well documented (8, 9, 13, 14, 17, 23, 24), there would appear to be phenotypic stability in the cell walls of *S. mutans* Ingbritt when subjected to carbohydrate limitation.

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