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Teichoic Acids from Chemostat-Grown Cultures of *Streptococcus mutans* and *Lactobacillus plantarum*

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We examined the effect of growth conditions in chemostat culture on the quantity and composition of the cell wall teichoic acids of *Streptococcus mutans* BHT and *Lactobacillus plantarum* NCIB 7220 and the membrane lipoteichoic acid from *S. mutans* Ingbritt. With the cell wall teichoic acids, which are covalently linked to peptidoglycan, the amount of teichoic acid is independent of the growth conditions employed. However, the extent of glucosyl substitution of the polymer from *L. plantarum* was dependent on growth conditions. *S. mutans* Ingbritt lipoteichoic acid, on the other hand, was little affected by growth conditions in terms of composition or serological activity, but the amount produced was markedly affected by changes in growth conditions.

A number of studies of organisms in continuous culture have shown that environmental conditions can affect both the surface properties and physiological characteristics (5–8, 10) of bacteria. In our studies on oral bacteria, generation time, pH of growth, and the nature of the limiting carbohydrate nutrient used have been shown to have marked effects on the production of cellular and extracellular lipoteichoic acid (LTA) by *Streptococcus mutans* strains BHT (15, 26) and Ingbritt (13, 14), *Lactobacillus salivarius* (20), and other oral lactobacilli and streptococci (16, 32). LTA is not covalently associated with other cell wall or cell membrane components and thus is readily excreted in a fully acylated or deacylated form by a variety of streptococci and lactobacilli (18, 19, 28). Cell wall teichoic acids and polysaccharides, on the other hand, are generally held to be in covalent linkage to wall peptidoglycan (29). Studies on *S. mutans* Ingbritt (23) revealed a phenotypic stability of both peptidoglycan and associated cell wall polysaccharide under conditions of carbohydrate limitation during continuous culture at different generation times and pH. Strains of *L. casei* subsp. *rhamnosus* showed that, in some cases, the relative proportions of the two cell wall polysaccharides were subject to variation with changes in growth conditions. The composition of the polysaccharides remained stable while the amount varied, associated with changes in immunogenicity and enzyme lysis of whole organisms (A. J. Wicken, A. Ayres, L. K. Campbell, and K. W. Knox, submitted for publication).

Bacterial cell wall polysaccharides are generally heteropolymers, and their biosynthesis depends on polymerization of repeating sequences of different sugar moieties. Cell wall teichoic acids, on the other hand, contain a polyglycerol or polyribitol phosphate backbone to which carbohydrate substituents may be attached subsequently and in a random manner (11, 31). Whereas the biosynthesis of the polyglycerophosphate backbone of LTAs follows a different route from that of wall teichoic acids (31), carbohydrate substitution of the polymer is probably also a secondary event. This basic difference between the mode of biosynthesis of cell wall polysaccharides on the one hand and teichoic acids and LTAs on the other hand allows for the greater possibility of a phenotypic variation in the composition of the latter polymers as a response to environmental change.

In this study we have examined the yields and composition of two cell wall teichoic acids and an LTA from different organisms under a variety of different growth conditions. *L. plantarum* NCIB 7220 has a wall ribitol teichoic acid substituted with glucosyl groups (24). *S. mutans* BHT contains a wall glycerol teichoic acid with a relatively high degree of galactosyl substitution as well as a negatively charged wall polysaccharide with rhamnose and glucose as the major components (2, 30). The LTA from *S. mutans* Ingbritt has a relatively low degree of glucosyl substitution, and this organism lacks a wall teichoic acid (13, 14, 23).

MATERIALS AND METHODS

Organisms and cultural conditions. The strains of *S. mutans* (BHT and Ingbritt) and *L. plantarum* (NCIB 7220) were those used in previous studies (14, 15, 20), and in the case of *S. mutans* strains both chemostat and batch-grown organisms were available for this study. *L. plantarum* was grown in batch and continuous culture at constant dilution rate but at different controlled pH values in the dialyzed medium and by procedures described previously (20). In continuous culture the dilution rate (*D*) is related to the generation time by the formula: generation time = ln 2 (0.69)/*D*.

Preparation of cell walls. Cell walls of *S. mutans* BHT and *L. plantarum* were prepared by mechanical disruption of organisms followed by treatment with boiling sodium dodecyl sulfate (3). This procedure has been shown previously to remove LTA from cell wall preparations without loss of covalently linked polymers (24).

Extraction of wall polymers. Cell wall teichoic acids were extracted from purified walls with cold 10% trichloracetic acid (TCA) essentially as described previously (24, 33). Cell walls were suspended in cold 10% TCA (10 mg/ml) and stirred vigorously at 4°C for 24 h. Walls were recovered by centrifugation, the extraction was repeated twice more, and the extracts were combined. LTAs were extracted from freeze-dried whole organisms with hot aqueous 45% phenol (33). Autoclave extraction of *S. mutans* BHT cell walls was carried out as described previously (3).

Purification of wall polymers. Wall teichoic acid extracts were dialyzed to remove TCA, freeze-dried, and fractionated by upward flow on columns (40 by 2.6 cm) of agarose gel (AcA44; LKB-Produkter, Bromma, Sweden) in 0.2 M ammonium acetate (pH 6.9) at a flow rate of 20 ml/h. The aqueous phase of phenol extracts of LTA was dialyzed and treated with DNase and RNase as described previously (33) before freeze-drying and fractionation by upward flow on columns (40 by 2.6 cm) of AcA22 agarose gel in 0.2 M ammonium acetate (pH 6.9) at a flow rate of 20 ml/h. DEAE-Sephacel (Pharmacia, Uppsala, Sweden) chromatography was carried out on columns (22 by 2.6 cm) with linear gradients of 0 to 1.0 M or 0.25 to 0.75 M NaCl in 0.05 M imidazole-hydrochloride buffer (pH 6.5) at a flow rate of 20 ml/h. Eluants from all columns were monitored continuously for extinction at 206 and 280 nm (Uvicord III, LKB-Produkter). Approximately 4-ml fractions were collected, and samples were analyzed for organic phosphorus and hexose. Fractions containing teichoic acid or LTA were pooled, dialyzed, and freeze-dried.

***N*-Acetylmuramidase digestion of cell wall residues.** TCA-extracted cell walls were dialyzed to remove residual TCA and freeze-dried. A portion of the dry residue (25 mg) was suspended in 0.02 M Tris-hydrochloride buffer (pH 6.8) and incubated at 37°C for 3 h with 250 μg of *M*-1-*N*-acetylmuramidase (kindly supplied by K. Yokogawa, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan; 37). The clear digest was dialyzed against distilled water (three times) and freeze-dried.

Chemical procedures. Hydrolysis of purified extracts was carried out in 2 N HCl in sealed tubes at 100°C for 3 h. Samples for chromatographic analysis were dried in vacuo over P_2O_5 and NaOH; samples for quantitative analysis were neutralized immediately with 2 N NaOH. Paper chromatography of hydrolysates and appropriate standards was carried out in the following systems: propan-1-ol–aqueous ammonia (specific gravity 0.88)–water (6:3:1, vol/vol), (12), Whatman no. 4 paper, ascending, for polyols and polyolphosphates; butan-1-ol–pyridine–water (6:4:3, vol/vol) (17), Whatman no. 1 paper, descending, for neutral and amino sugars. Spray reagents were those described previously (34). Glucose was determined by glucose oxidase (4), galactose was determined by galactose dehydrogenase (27), rhamnose was determined by the procedure of Gibbons (9), and phosphate was quantitated by the procedure of Ames and Dubin (1).

Serological procedures. Quantitative precipitin curves were established as previously described (22). Antisera used were available from previous studies. Antisera 497 and 499 were prepared against chemostat-grown *L. plantarum* whole organisms and were specific for the α-D-glucosyl substituents of the ribitol teichoic acid (20). Antiserum 565 was prepared against *S. mutans* Ingbritt whole organisms (14) and is reactive with the homologous LTA. Antiserum 647 was prepared (35) against *L. casei* NCTC 6375 LTA, which is devoid of glycosyl substitution, and the serum is specific to the polyglycerophosphate backbone of LTA.

RESULTS

Cell wall glycerol teichoic acid of *S. mutans* BHT. *S. mutans* BHT was grown in continuous culture with limiting (0.5%) glucose at constant pH (6.0) with different dilution rates and at constant dilution rate (0.1 h^{-1}) with different pH values (15). Purified cell wall preparations were obtained from cultures representative of the various conditions, namely, D = 0.033, 0.10, and 0.69 h^{-1} at pH 6.0 and D = 0.10 h^{-1} at pH 7.0 and 8.0. Analyses of the cell wall for phosphorus and galactose, two of the components of the wall teichoic acid, are given in Table 1. Galactose is also a component, together with rhamnose, of a cell wall polysaccharide, and representative preparations were therefore analyzed for their rhamnose content. The constant amount of rhamnose (Table 1) suggests that the amount of the cell wall polysaccharide remains constant in continuous culture. Analyses of batch-grown organisms showed a higher rhamnose but not galactose content.

Extraction of the cell wall preparations with TCA followed by column chromatography yielded teichoic acid preparations which were free of polysaccharide as shown by the absence of rhamnose. The yield of teichoic acid was 21 to 26% of the cell wall (Table 1), and quantitative analyses indicated constancy in the amounts of the phosphorus and galactose components. The percentage recovery of galactose and phosphorus in these preparations was 61 to 71% and 65 to 69%, respectively, of the totals in the cell wall (Table 1). This indicated that these components are present in another cell wall polymer.

Fractionation of an autoclave extract of purified *S. mutans* BHT cell walls on DEAE-Sephacel separated two phosphorus-containing fractions. Fraction I, which eluted with 0.36 M

NaCl, had a much higher hexose-to-phosphorus ratio than fraction II, which eluted with 0.43 M NaCl. A comparison of the areas of peaks in the phosphorus elution curve showed that approximately 1/3 of the total phosphorus was in fraction I and 2/3 was in fraction II. Both fractions were further purified by rechromatography separately on DEAE-Sephacel. Paper chromatography of acid hydrolysates of fraction II showed it to be the glycerol teichoic acid, and rhamnose was not present in this fraction. Fraction I contained rhamnose, galactose, galactosamine, and phosphorus and had a phosphorus-to-rhamnose molar ratio of 1.0:4.6.

Cell wall ribitol teichoic acid from *L. plantarum* NCIB 7220. Organisms were grown in continuous culture with limiting (1%) glucose at $D = 0.45\ h^{-1}$ and pH values from 5.4 to 7.4. These conditions were chosen to complement an earlier study in which the effects of different dilution rates at constant pH were studied (20). Cell wall preparations were analyzed for glucose and phosphorus, both of which are components of the wall ribitol teichoic acid. Glucose is also a component of the membrane LTA, but previous studies have shown that this teichoic acid is not present in cell wall preparations purified by extraction with sodium dodecyl sulfate (24). The results of the cell wall analyses (Table 2) show that both the absolute amount of glucose and the molar proportion relative to phosphorus increased with increasing pH of growth. The same trend was shown with the analyses of the isolated glucosyl-ribitol teichoic acid (Table 2). The recovery of teichoic acid from the different cell wall preparations was 19 to 26% and accounted for essentially all of the glucose present in the wall. Glycerol or glycerophosphates were not detected in acid hydrolysates. However, the recovery of phosphorus was only 50 to 67%, indicating that a significant proportion of the wall phosphorus is not in teichoic acid or is present in a nonextracted, non-glucosylated polymer.

To investigate the nonextracted phosphorus-containing material, purified cell walls from organisms batch-grown in dialyzed MRS medium were extracted with TCA. The extracted teichoic acid after purification by gel chromatography was obtained in a yield of 22% (dry weight) of the wall and had a molar ratio of phosphorus to glucose of 1.00:0.70. A total of 65% of the total cell wall phosphorus was present in the extracted teichoic acid fraction, and a further 25% was found in the cell wall residue remaining after extraction. A portion of the residue was solubilized by digestion with *N*-acetylmuramidase and fractionated on DEAE-Sephacel to yield a single, broad, phosphorus-containing fraction that eluted between 0.3 and 0.5 M NaCl.

TABLE 1. Analysis of cell walls and isolated cell wall teichoic acid from *S. mutans* BHT

Conditions of growth		Cell wall				Teichoic acid					
		µmol/mg			Mole ratio, P:Gal:Rha	µmol/mg		Mole ratio, P:Gal	% Yield purified teichoic acid	% Total P in teichoic acid fraction	% Total Gal in teichoic acid fraction
D (h^{-1})	pH	P	Gal	Rha		P	Gal				
0.033	6.0	0.59	0.91		1.00:1.54	1.82	2.69	1.00:1.48	21	65	62
0.10	6.0	0.61	0.98	1.01	1.00:1.60:1.66	1.85	2.76	1.00:1.49	22	67	60
0.69	6.0	0.60	1.01		1.00:1.68	1.70	2.58	1.00:1.51	24	68	61
0.10	7.0	0.65	0.93	1.04	1.00:1.43:1.60	1.68	2.53	1.00:1.51	26	67	71
0.10	8.0	0.66	1.10	1.14	1.00:1.67:1.73	1.82	2.69	1.00:1.48	25	69	61
Batch culture	No pH control	0.62	0.97	1.45	1.00:1.56:2.34	1.70	2.56	1.00:1.51	24	66	63

TABLE 2. Effect of pH of growth at $D = 0.45\ h^{-1}$ on the ribitol teichoic acid from *L. plantarum* NCIB 7220

Growth pH	Cell wall µmol/mg P	Cell wall µmol/mg Glc	Cell wall Mole ratio, P:Glc	Teichoic acid µmol/mg P	Teichoic acid µmol/mg Glc	Teichoic acid Mole ratio, P:Glc	% Yield of wall	% Total P in teichoic acid fraction	% Total Glc in teichoic acid fraction
5.5	1.25	0.42	1.00:0.34	3.40	2.09	1.00:0.61	22	60	109
6.0	1.19	0.43	1.00:0.36	3.15	2.08	1.00:0.66	19	50	92
6.6	1.06	0.50	1.00:0.47	2.69	2.04	1.00:0.76	23	58	94
6.9	1.15	0.59	1.00:0.51	2.72	2.42	1.00:0.89	26	61	106
7.4	1.17	0.58	1.00:0.50	2.55	2.27	1.00:0.89	25	55	98

Paper chromatography of acid hydrolysates of this fraction showed the presence of hexosamine, glycerol, and traces of glycerol phosphates and anhydroribitol. Ribitol phosphates or neutral sugars were not detected.

The cell wall glucosyl-ribitol teichoic acid of *L. plantarum* NCIB 7220 is the group D antigen of lactobacilli, with glucose being the immunodominant component (24). Examination of the reactivity of two grouping antisera with ribitol teichoic acid from organisms grown at pH 5.5 and 7.4 (Fig. 1) showed that the preparation obtained from cells grown at the higher pH, which has a higher glucose content (Table 2), reacted much more strongly. For comparison, results are included for a ribitol teichoic acid preparation from batch-grown organisms available from a previous study (20), which contained an intermediate amount of glucose (phosphorus/glucose molar ratio, 1.00:0.65) and gave intermediate values in reacting with grouping antisera.

LTA from *S. mutans* Ingbritt. In contrast to *S. mutans* BHT and *L. plantarum*, *S. mutans* Ingbritt does not contain a cell wall teichoic acid (23). This strain was chosen for chemical examination because serological studies indicated an effect of growth conditions on the relative amounts of LTA produced under different growth conditions. Organisms grown in continuous culture with limiting glucose, fructose, or sucrose at different dilution rates and different pH values were available from previous studies (13, 14). Some representative samples were chosen (Table 3) on the basis that they spanned the range of values for LTA detectable by rocket immunoelectrophoresis of cell extracts. LTA was extracted from a known weight of organisms with hot aqueous phenol and purified by column chromatography. The yields differed

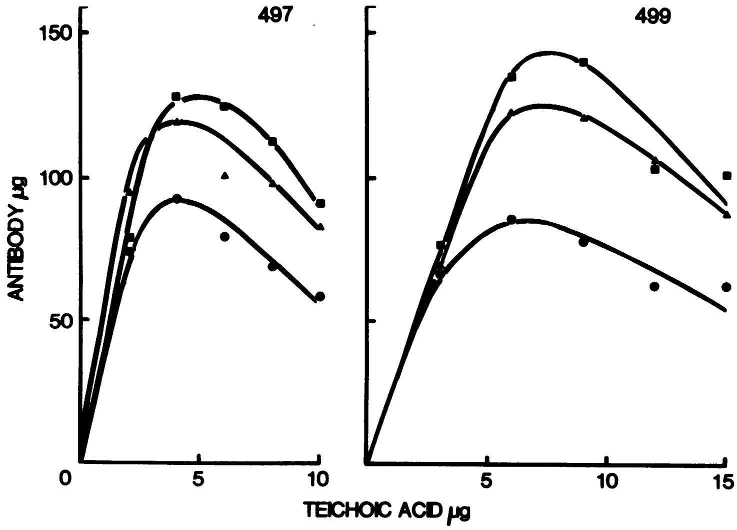

FIG. 1. Precipitation of *L. plantarum* NCIB 7220 ribitol teichoic acid preparations by antisera obtained by injecting rabbits with chemostat-grown organisms (20): antiserum 497 (0.05 ml), prepared against organisms grown at $D = 0.05\ h^{-1}$, and pH 6.0 and antiserum 499, (0.15 ml) prepared against organisms grown at $D = 0.5\ h^{-1}$ and pH 6.0. Symbols: ribitol teichoic acid from batch-grown organisms in complex medium (▲); ribitol teichoic acid from chemostat-grown organisms at $D = 0.45\ h^{-1}$, pH 5.5 (●) and pH 7.4 (■).

considerably (Table 3). Glucose was the only sugar detectable by paper chromatography of acid hydrolysates, and quantitative analyses showed that the molar ratio of phosphorus to glucose for the seven different preparations ranged from 1.00:0.11 to 1.00:0.21 (Table 3).

The previous studies on *S. mutans* Ingbritt, which employed rocket immunoelectrophoresis against cross-reacting backbone-specific antiserum to *L. casei* LTA (13, 14) required that differences in rocket height were primarily due to differences in the amount of LTA and that differences in sugar substitution were not sufficient to influence the results. To obtain confirmation, preparations of LTA from organisms grown in sucrose at pH 6.0 and $D = 0.10$, 0.30, and 0.50 h^{-1} were examined by the quantitative precipitin method for their reaction with antiserum to *L. casei* LTA (antiserum 647). The three preparations behaved similarly; the maximum amount of antibody precipitated was 0.96 to 0.98 mg/ml of serum. The three preparations also reacted similarly with homologous antiserum (565) obtained by injection of whole organisms of *S. mutans* Ingbritt ($D = 0.1$ h^{-1}, pH 7.5); the maximum amount of antibody precipitated was 0.40 to 0.47 mg/ml of serum.

DISCUSSION

Studies on the two cell wall teichoic acid types, a glycerol teichoic acid in *S. mutans* BHT and a ribitol teichoic acid in *L. plantarum*, show that growth conditions have little effect on the total amount of teichoic acid present in these cell walls (Tables 1 and 2). *S. mutans* BHT glycerol teichoic acid has a relatively high degree of galactosyl substitution (2), and in this case changes in growth condition did not induce changes in the degree of secondary substitution of the glycerophosphate backbone of the polymer (Table 1). Earlier analyses of cell walls of *S. mutans* BHT (2, 6, 30) by other workers indicated that not all of the cell phosphorus could be accounted for by a glycerol teichoic acid (wall or membrane). Non-teichoic acid phosphorus has been shown to be associated with the galactose- and rhamnose-containing polysaccharide of these cell walls. This phosphorylated polysaccharide, in containing approximately 1/3 of the cell wall galactose and all of the rhamnose, would amount to approximately 22% by weight of the cell wall. This amount is comparable to that found for the teichoic acid fraction and, like the latter, is not affected markedly by growth conditions. A phosphorus-containing cell wall polysaccharide has been previously reported as the group G antigen of *L. salivarius* (21), and from its electrophoretic properties an anionic polysaccharide may also be present in group N streptococci (36). Phosphorylated polysaccharides have not been reported from other strains of *S. mutans*, and it is interesting that in an earlier study (3) strain BHT showed a much greater release of wall polysaccharide after autoclaving in saline than did other *S. mutans* strains. This difference was attributed to the ionizable phosphate moieties of the wall teichoic acid of strain BHT influencing the hydrogen ion concentration. It can be postulated that the presence of phosphate moieties within the polysaccharide itself would have an even greater effect on the ease of covalent bond breakage during autoclaving.

With *L. plantarum* cell wall ribitol teichoic acid it has previously been reported (20) that the degree of glucosyl substitution varied from 0.57:1.00 to 0.75:1.00 (with respect to molar ratios to phosphorus) when the dilution rate of chemostat-grown organisms at pH 6.0 was changed from 0.05 to 0.5 h^{-1}. In this study, change in pH at constant dilution rate was shown, similarly, to affect the degree of secondary glucosyl substitution of the teichoic acid while its amount remained constant (Table 2).

TABLE 3. Analysis of LTA from *S. mutans* Ingbritt

Limiting carbohydrate	pH	D (h^{-1})	Extracted LTA yield (mol/g of cells)	Mole ratio, P:Glc	Serological estimation[a]
Glucose	6.0	0.10	13	1.00:0.21	1.0
	7.5	0.50	ND[b]	1.00:0.18	2.4
Fructose	6.0	0.50	119	1.00:0.15	6.0
Sucrose	6.0	0.10	13	1.00:0.21	0.8
	6.0	0.30	39	1.00:0.12	1.6
	6.0	0.50	88	1.00:0.11	3.3
	7.5	0.10	34	1.00:0.16	3.6

[a] Relative amount of LTA in phenol extracts as determined by quantitative rocket immunoelectrophoresis (data from reference 14).

[b] ND, Not done.

As shown in Fig. 1, the level of glucosyl substitution as a response to pH of growth is reflected in serological reactivity with antisera to the teichoic acid, an effect similar to that noted earlier for chemostat-grown cells at various generation times but constant pH (20).

As with *S. mutans* BHT, not all of the cell wall phosphorus of *L. plantarum* could be accounted for by extracted ribitol teichoic acid, but all of the wall glucose was present in this fraction. The bulk of the remaining phosphorus was associated with a fraction obtained from the solubilized cell wall residue that had a chemical composition consistent with the glycerophosphate-*N*-acetyl hexosamine linkage units that have been reported to join wall teichoic acids to peptidoglycan (31). The presence of traces of anhydroribitol in acid hydrolysates of this fraction also suggests some residual ribitol teichoic acid which is non-glucosylated. As with *S. mutans* BHT, this method of purification of cell walls would remove contaminating LTA such that the latter would be an unlikely source of the glycerophosphate in the extracted cell wall residue. In any event, TCA extraction would remove LTA, if present, from cell walls, and no trace of glycerol or glycerophosphates were found in the ribitol teichoic acid extracts.

Wide variations in the amounts of LTA produced by *S. mutans* Ingbritt as a result of growth under different conditions of pH, dilution rate, or limiting carbohydrate carbon source have been reported (13, 14). In such studies quantitation of LTA by its reactivity with glycerophosphate backbone-specific antisera would be invalid if there were marked differences in the degree of carbohydrate subsitution of the LTA produced under different growth conditions; increasing glucosyl substitution of an LTA generally decreases reactivity with such cross-reacting antisera (25). In this study, extraction of LTA from *S. mutans* Ingbritt grown under a variety of growth conditions (Table 3) gave actual yields that were comparable to the relative amounts detected serologically in extracts by rocket immunoelectrophoresis (13, 14). Analysis of isolated and purified LTA preparations showed that growth conditions (carbohydrate source, pH, and dilution rate) had only a minimal effect on the degree of glucose substitution of the LTA (Table 3). This consistently low degree of glucosyl substitution validates the use of serological methods for quantitation of LTA in this organism, and as has been shown, the variation of glucosyl substitution that does occur has no quantitative effect on serological reactivity with anti-*S. mutans* Ingbritt antisera or cross-reactive *L. casei* antiserum.

From the results of these studies it would appear that the concept of phenotypic stability noted earlier for the cell wall of *S. mutans* Ingbritt (23) can be extended to *S. mutans* BHT and *L. plantarum* cell wall teichoic acids with respect to the amount of polymer synthesized. As shown with *L. plantarum*, the degree of secondary substitution can vary. Surface-associated polymers, such as the LTA of *S. mutans* Ingbritt, can vary widely in amount in response to changes in growth conditions while their structure with respect to secondary glycosidic substitution shows only minor variation. This offers further support for the earlier contention (13) that environmentally induced phenotypic changes in surface properties of oral microorganisms may be primarily due to changes in surface-associated components such as LTA or protein rather than in covalently linked cell wall polymers.

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