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Occurrence of Lipoteichoic Acid in Oral Streptococci

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The heterogeneous bacterial group known as oral streptococci was screened for the presence of cellular polyglycerolphosphate-containing lipoteichoic acid. This compound was detected in phenol extracts of lyophilized cells by an immunoassay in which polyglycerolphosphate-specific monoclonal antibody was used. Polyglycerolphosphate-containing lipoteichoic acid occurred in all 86 strains of oral streptococci examined except the *Streptococcus mitis* and *Streptococcus oralis* strains. This confirms the findings of Rosan (B. Rosan, Science 201:918–920, 1978) and Hamada et al. (S. Hamada, J. Mizuno, S. Kotani, and M. Torii, FEMS Microbiol. Lett. 8:93–96, 1980), is consistent with the results of the taxonomic study of oral streptococci performed by Kilian et al. (M. Kilian, L. Mikkelsen, and J. Henrichsen, Int. J. Syst. Bacteriol. 39:471–484, 1989), who emended the descriptions of *Streptococcus sanguis*, *S. oralis*, and *S. mitis*, and reflects the phylogenetic relationship among *S. mitis*, *S. oralis*, and *Streptococcus pneumoniae*.

The term lipoteichoic acid (LTA) has been used to describe a group of closely related macroamphiphiles which are synthesized and primarily located in the cytoplasmic membranes of various gram-positive bacteria (5, 48). Historically, LTA has been structurally characterized by an unbranched linear polymer of 1,3-linked glycerol phosphate residues covalently attached to a terminal hydrophobic region comprised of fatty acids. Variations may occur in the length of the polyglycerolphosphate (PGP) chain, which usually does not exceed 40 residues, in the presence of glycosyl substituents and D-alanine on C-2 of the glycerol moiety, and in the structure of the glycolipid anchor. Thus, it is now common to use the term LTAs to indicate this heterogeneity. For reviews see references 10 and 38.

Recently, Fischer (12) has redefined LTAs as macroamphiphiles that contain alditolphosphates as integral parts of the hydrophilic chain. This more liberal definition allows for the inclusion of compounds in which the repeating unit contains glycosyl residues. These polyglycosylalditolphosphate-containing LTAs are very much less common than the classic PGP-containing LTAs (PGP-LTAs). The wider definition was thought to be necessary to distinguish LTAs from macroamphiphiles which do not contain phosphate residues as an integral part of the repeating structure of the polar moiety and which Fischer (12) refers to as lipoglycans.

Not all gram-positive microorganisms synthesize a form of LTA in its broader sense, and current evidence suggests that the organisms in which it is not present instead elaborate a lipoglycan. This may imply that these two classes of compounds could have similar functions, although the nature of the functions is by no means clear. This division between LTA- and lipoglycan-producing microorganisms, as defined by Fischer (12), appears to follow the broad taxonomic line based on guanine-plus-cytosine (G+C) contents of DNAs in that LTA synthesis seems to occur predominantly in members of the low-G+C-content *Clostridium-Bacillus* branch of the gram-positive eubacteria rather than in members of the high-G+C-content actinomycete branch as defined by Fox et al. (15). However, to date, only a relatively few species belonging to a small number of bacterial genera have been examined.

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Both PGP- and non-PGP-LTA-synthesizing species are represented within the oral streptococci, and since Schleifer et al. (35), Ruhland and Fiedler (34), and, more recently, Sutcliffe (37) have suggested that LTAs may have value as chemotaxonomic markers, it is clearly of interest to know the distribution of amphiphile types within these species.

Previously, Rosan (32) and Hamada et al. (17) showed that *Streptococcus mitis* and *Streptococcus sanguis* biotype II (also previously known as biotype B) do not synthesize PGP-LTA. However, since these studies there have been profound changes in the classification of oral streptococci, including emended descriptions of some species and descriptions of a number of new species, such as *S. mitis* and *S. sanguis* (for a review see reference 19). Therefore, it is appropriate at this juncture to reexamine the oral streptococci to determine which species synthesize PGP-LTA.

In this study we report on the occurrence of PGP-LTA in 86 strains of oral streptococci representing all of the currently recognized species isolated from humans in this group.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study had been identified previously on the basis of DNA-DNA hybridization data obtained by the S1 nuclease method (47) or the results of biochemical tests, including a panel of phenotypic tests described by Beighton et al. (3).

For chemical extraction, strains were grown overnight anaerobically in 20 ml of Todd-Hewitt broth (Oxoid, Ltd., Basingstoke, Hants, United Kingdom). Cells were harvested by centrifugation, transferred to microcentrifuge tubes in 1 ml of distilled water, pelleted by centrifugation, lyophilized, and stored at −20°C.

Preparation of bacterial phenol extracts. Lyophilized cells, prepared as described above, were resuspended in 0.5 ml of distilled water to which an equal volume of 90% (wt/vol) phenol was added. The mixture was heated at 70°C with frequent mixing for 1 h before the aqueous phase was separated by centrifugation and removed. The phenol phase was then reextracted with 0.5 ml of distilled water, and the two aqueous phases were pooled, dialyzed against water, lyophilized, and stored at −20°C.

Production of monoclonal antibody. Splenocytes specific for PGP-LTA were induced by immunizing BALB/c mice with a cocktail comprising equal quantities of *Streptococcus sobrinus* HG961, HG962, HG970, and HG977, all of which were fresh isolates as described by De Soet et al. (8). Cultures of each strain were washed and diluted in phosphate-buffered saline (PBS) to an optical density at 600 nm of 1.0 before storage at −20°C. The mice were injected intravenously with 100 μl of the microbial mixture; this was followed 2 weeks later and at two weekly intervals with two intraperitoneal injections consisting of 100 μl of microbial mixture supplemented with Freund's complete adjuvant. Four days prior to fusion, the mice were given a booster consisting of an additional 100 μl of the microbial mixture.

Splenocytes were isolated and fused with myeloma cells as described previously (7). Hybridomas were screened by testing culture fluids for antibody ac-

tivity with a direct enzyme-linked immunosorbent assay (ELISA) against either whole intact cells of *S. sobrinus* or a crude glucosyltransferase-LTA mixture prepared as described by Umesaki et al. (39) by using *S. sobrinus* HG459. Further screening for hybridomas that produced antibodies specific for PGP was performed with a direct ELISA, using plates coated with deacylated cardiolipin produced by hydrolysis of cardiolipin (Sigma, Poole, United Kingdom) in 15% ammonium hydroxide as described by Courtney et al. (6). All hybridoma cell lines which produced antibodies to PGP were subcloned twice, and the monoclonal antibody activity was tested with the ELISA against LTA isolated from *Streptococcus mutans* and *S. sanguis*. Cross-reactivity with other antigens was determined by probing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels blotted onto positively charged nylon membranes as described below. Monoclonal antibodies for experimental purposes were produced by using a continuous dialyzing system, as described by van Raamsdonk et al. (40).

Screening bacterial extracts for PGP-LTA. Bacterial extracts were screened for PGP-LTA by performing a competitive ELISA (23) in which LTA extracted from *S. sanguis* NCTC 7863^T (T = type strain) with phenol (21) and purified by hydrophobic interaction chromatography (14) was used as the standard.

Briefly, the wells of Immunol 1 microtiter plates (Dynatech Laboratories, Inc., McLean, Va.) were coated with antigen by treating each well with 75 μl of a standard LTA solution (5.0 $\mu g \cdot ml^{-1}$) in PBS at 4°C overnight. Some wells were not treated with standard LTA and served as controls for nonspecific interactions, which were blocked by incubating all wells with 150 μl of 10% (vol/vol) fetal calf serum (Gibco, Paisley, United Kingdom) in PBS containing 0.05% (wt/vol) Tween 80 (PBST). LTA standards were prepared from a stock solution (10 $\mu g \cdot ml^{-1}$) in PBS by serial twofold dilution.

Samples (75 μl) of standard LTA or streptococcal extract dissolved in 1.0 ml of PBS were incubated with an equal volume of PGP-LTA-specific monoclonal antibody OMVU13 diluted 1:1,000. The wells were washed five times with PBST before addition of 75 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Sigma) diluted to the recommended working strength. The wells were then washed a minimum of 10 times with PBST before the phosphatase substrate (Sigma) was added. Optical densities at 405 nm were determined with a Titertek Multiscan model MCC/340 MK II instrument (Flow Laboratories, Sittingbourne, United Kingdom). Antibody dilutions were prepared in PBST containing fetal calf serum, and all preparations were incubated for 1 h at 37°C.

All LTA measurements, including measurements for standards, were determined in quadruplicate. Values that were more than twice the background value were considered positive for the presence of LTA.

Immunoblotting. Lyophilized bacterial cell phenol extracts or lyophilized whole cells from 20-ml cultures were treated with Laemmli sample buffer (100°C, 5 min), separated by SDS-PAGE (7.5% [vol/vol] acrylamide gel), electrophoretically blotted onto a positively charged nylon membrane (Zeta-Probe; Bio-Rad, Hemel Hempstead, United Kingdom), and probed with OMVU13. The blots were developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) (BCIP)-nitroblue tetrazolium (Cambridge Bio/Science, Cambridge, United Kingdom) following incubation with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma). Nonspecific interactions were blocked by using PBST containing 5% (wt/vol) skim milk powder, which was also used to prepare all antibody dilutions. All preparations were incubated for 60 min at room temperature.

RESULTS AND DISCUSSION

Monoclonal antibody OMVU13 recognized the glycerol-phosphate-glycerol epitope of deacylated cardiolipin, PGP-LTA isolated from *S. mutans* 292A, *Lactobacillus casei* 267, and *Enterococcus hirae* 283/1, which was kindly supplied by W. Fischer, PGP-LTA isolated from *S. sanguis* NCTC 7863^T by us, and commercially available LTA from *S. mutans* (Sigma) in an ELISA (data not shown). Immunoblotting of SDS-PAGE-separated phenol extracts of representatives of each species studied revealed a cell component from each strain that had a mobility identical to that of standard PGP-LTA isolated from *S. sanguis* NCTC 7863^T and little evidence of cross-reactions with other extracted compounds (data not shown). The PGP-LTA migrated close to but not coincident with the leading edge of the electrophoresis gel and exhibited some heterogeneity in PGP chain length, which is a feature of this compound (29). Sometimes small amounts of faster-migrating material were detected, which may have been LTA degradation products or extracted cardiolipin. Taken together, our results show that OMVU13 recognizes PGP and detects PGP-LTA in cell phenol extracts.

A total of 86 strains, representing all of the recognized oral species of streptococci isolated from humans, including most type strains and a variety of other reference and wild strains, were examined for the presence of PGP-LTA by using OMVU13. The results are shown in Table 1 together with the origins, sources, and identities of the strains. All of the strains except those belonging to *Streptococcus oralis* and *S. mitis* were PGP-LTA positive.

Although PGP-LTA has been recognized as an important gram-positive antigen for some time and has been associated with a variety of host-bacterium interactions, the number of bacterial species in which it has been positively identified remains limited (11). In the human mouth, PGP-LTA has been implicated in the adhesion of *Streptococcus pyogenes* to pharyngeal epithelial cells (2) and the adhesion of oral commensal streptococci to tooth surfaces (22). This compound has also been found to promote bone resorption in tissue culture (20), to induce severe inflammatory responses in rat periodontium (1), to activate complement by both the classical (28) and alternate pathways (9), and to activate macrophages (36). The polyanionic nature of PGP-LTA permits cation binding, and Rose et al. (33) have suggested that this compound may function as a calcium buffer in dental plaque, thereby affecting the availability of calcium ions and possibly the dissociation equilibria of tooth mineral. From the oral perspective, it should be particularly interesting to establish which oral streptococci elaborate this polymer, since the streptococci are numerically dominant in the oral cavity.

It is also pertinent to reexamine LTA synthesis in view of the recent advances made in the taxonomy of this difficult and heterogeneous group of bacteria (26). Although PGP-LTA synthesis was found to be widespread among the species examined, strains of both *S. mitis* and *S. oralis* were exceptional in this respect, which supports the results of previous studies of PGP-LTA synthesis in oral streptococci (17, 32) and is consistent with the results of more detailed taxonomic studies which have shown that these organisms are closely related to *Streptococcus pneumoniae*. Thus, *S. mitis*, *S. oralis*, and *S. pneumoniae* form a small cluster, which exhibits 99% 16S rRNA sequence homology, within a larger cluster termed the mitis group, which also contains *Streptococcus gordonii*, *S. sanguis*, and *Streptococcus parasanguis* (25). Currently, 16S rRNA sequence data is not available for *Streptococcus crista*.

There have been some reports suggesting the possibility of atypical behavior with regard to PGP-LTA synthesis in some strains of *S. oralis*. Thus, Vickerman and Jones (42), using rabbit polyclonal antibody, identified PGP-LTA in strain C5, which had been received as *S. oralis* but which had also been shown to resemble *S. gordonii* phenotypically (41). Our data confirm the identity of strain C5 as a *S. gordonii* strain and also confirm that this organism produces PGP-LTA (Table 1).

Ohkuni et al. (31), using a polyclonal antiserum to LTA prepared in rabbits by injecting whole cells of *E. hirae*, detected cross-reacting material in culture supernatants of 4 of 17 *S. oralis* strains and 5 of 13 *S. mitis* strains in a study of the role of oral streptococci in the etiology of Kawasaki disease. However, Ohkuni et al. (31) also detected PGP-cross-reacting material in the culture supernatant of *S. mitis* NCTC 12261^T, which was not detected in phenol extracts of cells in the present study, and also failed to detect PGP-LTA in the culture supernatants of *S. sanguis* ATCC 10556^T (= NCTC 7863^T) and *S. gordonii* NCTC 7865^T.

The close phylogenetic relationship among *S. oralis*, *S. mitis*, and *S. pneumoniae* (25) is reflected in the cell wall compositions of these organisms. Kilpper-Bälz et al. (27) have shown that *S. oralis* walls characteristically contain choline, and

TABLE 1. Streptococcal strains used in this study, their sources, and their abilities to synthesize PGP-LTA

Strain	Sender(s)[a]	Method used to identify[b]	Source	PGP-LTA
Streptococcus crista strains				
CC5A	Handley	DNA	Dental plaque	+
AK1	Handley	Phenotype	Throat	+
CR3	Handley	DNA	Dental plaque	+
CR311^T (= NCTC 12479^T)	Handley	DNA	Periodontal abscess	+
Streptococcus gordonii strains				
H44	LHMC	Phenotype	Dental plaque	+
NCTC 10231	NCTC	DNA		+
NCTC 3165	NCTC	DNA	Pyorrhea	+
H730	LHMC	Phenotype	Dental plaque	+
NCTC 7868	NCTC	DNA	Strain Challis	+
NCTC 7865^T	NCTC	DNA	SBE[c]	+
M5	Rosan	DNA	Dental plaque	
GPF1	Carlsson	Phenotype	Dental plaque	+
F90A	Colman	DNA	Strain Perryer	+
C5	Vickerman	Phenotype	Oral cavity	+
Streptococcus sanguis strains				
AC59	LHMC	Phenotype	Dental plaque	+
P695	LHMC	Phenotype	Dental plaque	+
KPE2	Carlsson	DNA	Dental plaque	+
PC3829	LHMC	Phenotype	Dental plaque	+
AC3137	LHMC	Phenotype	Dental plaque	+
PC3821	LHMC	Phenotype	Dental plaque	+
HPC1	Carlsson	DNA	Dental plaque	+
NP506	LHMC	Phenotype	Dental plaque	+
NCTC 7863^T	NCTC	DNA	SBE	+
SK96	Ranke	DNA	Dental plaque	+
Streptococcus parasanguis strains				
FW213	Handley	DNA	Unknown	+
MGH143	Handley	DNA	Urine	+
EF3711	LHMC	DNA	Dental plaque	+
UC 4989	Handley	DNA	Throat	+
85-81	CDC	DNA	Blood	+
2156-81A	CDC	DNA	Blood	+
SS897	CDC	DNA	Throat	+
SS898^T (= ATCC 15912^T)	CDC	DNA	Throat	+
Streptococcus mutans strains				
161	Perch	Phenotype	Blood	+
KPSK2	Carlsson	Phenotype	Human mouth	+
B48	LHMC	Phenotype	Dental plaque	+
OMZ 175	Guggenheim	Phenotype	Dental plaque	+
4177	Perch	Phenotype	Blood	+
NCTC 10449^T	NCTC	Phenotype	Carious dentine	+
Streptococcus sobrinus strains				
SL-1^T (= ATCC 33478^T)	Coykendall	Phenotype	Dental plaque	+
OMZ 65	Guggenheim	Phenotype	Dental plaque	+
279	Perch	Phenotype	Human teeth	+
B13	Edwardsson	Phenotype	Dental plaque	+
B542	LHMC	Phenotype	Dental plaque	+
Streptococcus constellatus strains				
NCTC 10714	NCTC	DNA	Throat	+
NCTC 11063	NCTC	DNA	Throat	+
NCTC 5389	NCTC	DNA	Unknown	+
Streptococcus intermedius strains				
NCDO 2227^T (= ATCC 33397^T)	NCDO	DNA	Unknown	+
UNS 35	Unsworth	DNA	Brain abscess	+
Streptococcus anginosus strains				
NCTC 10713^T	NCTC	DNA	Throat	+
NMH 10	Wren	DNA	Perforated ulcer	+
G5:3	Mejare	DNA	Dental plaque	+
2236-81	CDC	DNA	Blood	+
PC4890	LHMC	DNA	Dental plaque	+
NCTC 11062	NCTC	DNA	Dental root canal	+
KR 687	Ruoff	DNA	Axillary abscess	+
KR 455	Ruoff	DNA	Urine	+
Streptococcus mitis strains				
NCTC 10712	NCTC	DNA	Sputum	−

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TABLE 1—*Continued*

Strain	Sender(s)[a]	Method used to identify[b]	Source	PGP-LTA
PP53	Carlsson	DNA	Dental plaque	−
NS51^T (= NCTC 12261^T)	Carlsson	DNA	Human oral cavity	−
K208	Colman	DNA	Abscessed tooth	−
Streptococcus vestibularis strains				
OP81	Carlsson	DNA	Dental plaque	+
MM1^T (= NCTC 12166^T)	LHMC	DNA	Vestibular mucosa	+
JW3	LHMC	DNA	Vestibular mucosa	+
Streptococcus salivarius strains				
M36	LHMC	DNA	Dental plaque	+
H53	LHMC	Phenotype	Dental plaque	+
A385	LHMC	DNA	Dental plaque	+
NCTC 8606	NCTC	DNA		+
H50	LHMC	Phenotype	Dental plaque	+
GSS2	Carlsson	Phenotype	Saliva	+
B242	LHMC	Phenotype	Dental plaque	+
KPS1	Carlsson	Phenotype	Dental plaque	+
B128	LHMC	Phenotype	Dental plaque	+
P511	LHMC	Phenotype	Dental plaque	+
T267	LHMC	Phenotype	Dental plaque	+
M236	LHMC	Phenotype	Dental plaque	+
ATCC 9759	ATCC	Phenotype		+
NCTC 8618^T	NCTC	DNA	Throat	+
Streptococcus oralis strains				
LVG1^T (= NCTC 11427^T)	Carlsson	DNA	Vestibular mucosa	−
B88	LHMC	Phenotype	Dental plaque	−
M254	LHMC	Phenotype	Dental plaque	−
M524	LHMC	Phenotype	Dental plaque	−
OS51	Carlsson	DNA	Saliva	−
GPD1	Carlsson	Phenotype	Dental plaque	−
OPA1	Carlsson	DNA	Dental plaque	−
H362	LHMC	Phenotype	Dental plaque	−
PC1467	LHMC	DNA	Dental plaque	−
NCTC 7864	NCTC	DNA	SBE	−

[a] Strains were received from the following sources: ATCC, American Type Culture Collection, Rockville, Md.; Carlsson, J. Carlsson, Dental School, University of Umea, Umea, Sweden; CDC, R. Facklam, Centers for Disease Control and Prevention, Atlanta, Ga.; Colman, G. Colman, Central Public Health Laboratory, Colindale, London, United Kingdom; Coykendall, A. Coykendall, School of Dental Medicine, University of Connecticut, Farmington, Conn.; Edwardsson, S. Edwardsson, Department of Oral Microbiology, School of Dentistry, University of Lund, Malmö, Sweden; Guggenheim, B. Guggenheim, Zahnarztliches Institut der Universitat Zurich, Zurich, Switzerland; Handley, P. Handley, Department of Cell and Structural Biology, Manchester University, Manchester, United Kingdom; LHMC, Department of Oral Microbiology, The London Hospital Medical College, London, United Kingdom; Mejare, B. Mejare, School of Dentistry, University of Lund, Malmö, Sweden; NCTC, National Collection of Type Cultures, Public Health Laboratory Service, Colindale, London, United Kingdom; Perch, B. Perch, State Serum Institute, Copenhagen, Denmark; Ranke, E. and B. Ranke, Bakteriologie Laboratorium Klinik, Universitat Hamburg, Hamburg, Germany; Rosan, B. Rosan, Department of Microbiology, School of Medicine, University of Philadelphia, Philadelphia, Pa.; Ruoff, K. Ruoff, Massachusetts General Hospital, Boston, Mass.; Unsworth, P. Unsworth, Central Public Health Laboratory, Colindale, London, United Kingdom; Wren, M. Wren, North Middlesex Hospital, London, United Kingdom.

[b] Strains were identified or identities were confirmed by DNA-DNA hybridization (DNA) or a panel of phenotypic tests (Phenotype). For descriptions of the DNA-DNA hybridization tests see references 18 (for *S. crista*), 45 (for *S. parasanguis*), 46 (for *S. vestibularis* and *S. salivarius*), 43 (for *S. salivarius*), 47 (for *S. anginosus*, *S. constellatus*, and *S. intermedius*), and 43 (for *S. gordonii*, *S. sanguis*, *S. mitis*, and *S. oralis*). For descriptions of the phenotypic tests see references 3 (for *S. crista*, *S. gordonii*, *S. sanguis*, *S. parasanguis*, *S. mitis*, *S. vestibularis*, *S. salivarius*, and *S. oralis*), 4 (for *S. mutans* and *S. sobrinus*), and 44 (for *S. constellatus*, *S. anginosus*, and *S. intermedius*).

[c] SBE, subacute bacterial endocarditis.

Gillespie et al. (16) have found a C-polysaccharide-like, choline-containing antigen in both *S. oralis* and *S. mitis* which is similar to the compound found in *S. pneumoniae*. Furthermore, *S. pneumoniae* has been shown to be unique in that it elaborates a teichoic acid and an LTA with an identical chain structure (13), and since some radiolabelled choline is incorporated into membrane components of *S. oralis* (24), it has been suggested that this species may in fact produce a choline-containing macroamphiphile similar to that of *S. pneumoniae*. It is, therefore, possible to speculate that members of this group share common structural LTA features.

This divergence in LTA type within a closely related group of streptococci is of considerable interest, not only to chemotaxonomists, but also to those who are pursuing the elusive function of bacterial macroamphiphiles. With this in mind, it may be significant that both *S. oralis* and *S. mitis*, non-PGP-LTA producers, are the major pioneer colonizers of cleaned tooth surfaces (30).

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