

substituting specific primers for each oral bacterial species during the second PCR

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Identification of mutans streptococci by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified 16S ribosomal RNA genes

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Mutans streptococci are frequently isolated from dental plaque and carious lesions. These bacteria have been identified by conventional methods such as biochemical and serologic tests followed by the isolation of colonies on the mutis salivarius agar which are sometimes inconsistent. Recently, species specific polymerase chain reaction (PCR) has been reported to rapidly identify *Streptococcus mutans* and *Streptococcus sobrinus*. However, in the case of identification and classification into several species, e.g. within the group of mutans streptococci consisting of seven species, the identification using species specific PCR seems somewhat inefficient because of need for the development and preparation of specific primers for each species. Therefore, in this study we developed a simple method using restriction fragment length polymorphism analysis of PCR amplified 16S ribosomal RNA genes (16S rRNA genes PCR RFLP) for the identification of seven different species included in the group of mutans streptococci. We amplified 16S rRNA gene sequences from genomic DNA samples by PCR using universal primers and digested the PCR products with the restriction endonucleases *HpaII* and *HaeIII*. *HpaII* produced six RFLP patterns for eight reference strains since the patterns for *S. sobrinus*, *Streptococcus downei* and *Streptococcus ferus* were similar. RFLP patterns produced with *HaeIII* could separate these three species. Furthermore, the RFLP patterns predicted from the 16S rRNA gene sequences in the GenBank database agreed with the actual RFLP patterns produced in the present study. The 16S rRNA sequence comparisons can be used to identify oral mutans streptococci; however, the identification by sequencing is sometimes difficult in large scale studies and for small laboratories. Therefore, 16S rRNA genes PCR RFLP using *HpaII* and *HaeIII* could be an alternative method for the identification of mutans streptococci and may be applicable for large scale studies on the cariogenicity of mutans streptococci.

Key words *Streptococcus mutans*,
Streptococcus sobrinus, PCR RFLP, 16S
rRNA genes

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Mutans streptococci, in particular *Streptococcus mutans* and *Streptococcus sobrinus*, have been thought to be associated with human dental caries because they

have frequently been isolated from dental plaque where they produce large amount of acids and extracellular polysaccharides which promote dental caries (7, 8).

Mutans streptococci are usually subdivided into species level, e.g. *S. mutans*, *S. sobrinus*, *Streptococcus criceti*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus*

macacae Streptococcus rattii following the isolation of colonies on mutis salivarius agar. The method for species identification of mutans streptococci has been based on conventional tests including metabolic and enzymatic activities (1-14) but it is time consuming and labor intensive and some times inconsistent. Recently species specific polymerase chain reaction (PCR) based upon dextranase (2, 4, 10) and glucosyltransferase (9) gene sequences have been developed and successfully applied for the identification of *S. mutans* and *S. sobrinus*. When attempting to identify several species of mutans streptococci as in our study however the identification using species-specific primers seems somewhat inefficient due to the preparation of different primer sets for each bacterial species and the requirement of large numbers of PCR comprising the reaction with each primer set. We have therefore developed a simple method using PCR RFLP for the identification of seven different species included in the group of mutans streptococci.

Material and methods

Bacterial strains and culture conditions

We used the following reference strains of mutans streptococci in our laboratory: *S. mutans* NCTC10449^T (serotype c, T=type strain), Ingbritt (serotype c), GS5 (serotype c), NCIB11723 (serotype c), MT8148 (serotype c), LM7 (serotype e) and MT6229 (serotype f); *S. sobrinus* OMZ176 (serotype d) and 6715 (serotype g); *S. criceti* GTC 242^T (serotype a); *S. downei* GTC 632^T (serotype h); *S. ferus* GTC 279^T (serotype c); *S. macacae* GTC 538^T (serotype c) and *S. rattii* GTC 245^T (serotype b).

In addition 16 clinical isolates (12 *S. mutans* and 4 *S. sobrinus*) were obtained from our departmental collection. Identification of the clinical strains used in this study was confirmed by species specific PCR based on the described dextranase gene sequence (2, 4). Briefly the dextranase genes were amplified by PCR with the *S. mutans* specific primers SD1 and SD2 and with the *S. sobrinus* specific primers SOF14 and SOR1623 (2, 4). The primer sequences were SD1: 5' TAT GCT GCT ATT GGA GGT TC 3', SD2: 5' AAG GTT GAG CAA TTG AAT CG 3', SOF14: 5' TGCTATCTT TCCCTA GCA TG 3' and SOR1623: 5' GGT ATT CGG TTT GAC TGC 3'. Amplification by PCR and agarose gel electrophoresis proceeded as described below. The predicted PCR products of *S. mutans* and *S. sobrinus* were 1272 bp and 1610 bp respectively.

All of the strains were incubated on Fastidious Anaerobe Agar (FAA Lab M Bury UK) plates supplemented with 5% rabbit blood (Nippon Bio Test Laboratories Tokyo Japan) at 37 C for 3 days in an anaerobic glove box (Model AZ Hard Hirasawa Tokyo Japan) containing 80% N₂, 10% H₂ and 10% CO₂.

16S rRNA genes PCR RFLP

We extracted DNA from 3 day old cultures using either the InstaGene Matrix kit (Bio Rad Laboratories Richmond CA) or the GFX genomic blood DNA purification

kit (Amersham Pharmacia Biotech Inc Piscataway NJ) according to the instructions provided by the manufacturers. The 16S rRNA gene sequences were amplified by PCR using the universal primers 8UA and 1492R (12) and *Taq* DNA polymerase (HotStarTaq Master Mix Qiagen GmbH Hilden Germany) according to the manufacturer's instructions. The primer sequences were 8UA: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' TAC GGG TAC CTT GTT ACG ACT T 3'. Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals Ohtsu Shiga Japan) programmed as

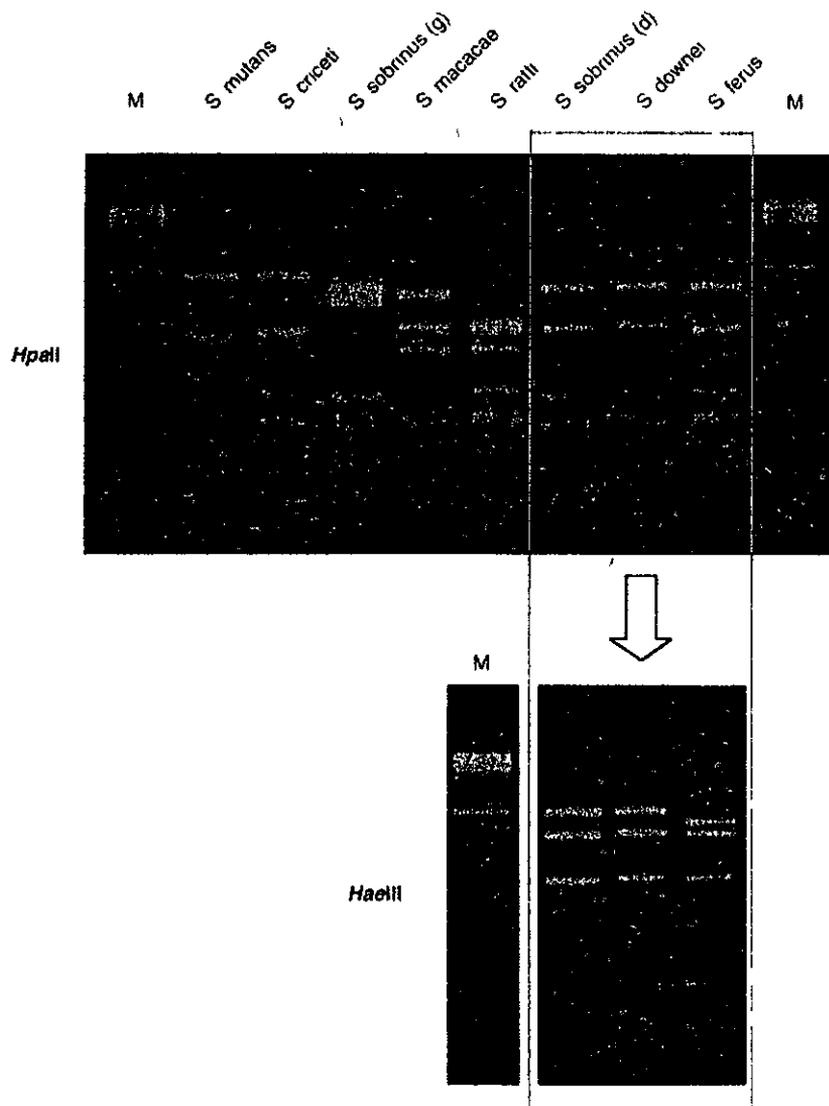


Fig 1 Scheme for identification of mutans streptococci by 16S rRNA genes PCR RFLP profiles. Lanes: *S. mutans* NCTC 10449^T, *S. criceti* GTC 242^T, *S. sobrinus* (g) 6715, *S. macacae* GTC 538^T, *S. rattii* GTC 245^T, *S. sobrinus* (d) OMZ176, *S. downei* GTC 632^T, *S. ferus* GTC 279^T. M: Molecular size markers (100 bp DNA Ladder Invitrogen Corp). *Hpa*II produced six RFLP patterns for eight reference strains, in which the patterns for *S. sobrinus* (serotype d), *S. downei* and *S. ferus* were similar. RFLP patterns with *Hae*III could differentiate between the three species.

follows 15 min at 95 C for initial heat activation and 35 cycles of 1 min at 94 C for denaturation 1 min at 60 C for annealing and 1.5 min at 72 C for extension and 10 min at 72 C for a final extension. The PCR products were purified using the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech). Purified 16S rRNA genes were individually digested with *HpaII* or *HaeIII* (New England Biolabs Inc. Beverly, MA) according to the manufacturer's instructions. Digestion products were separated on 2% or 4% NuSieve 3.1 Agarose (BioWhittaker Molecular Applications Inc. Rockland, ME) gels in Tris borate EDTA buffer (100 mM Tris, 90 mM Borate, 1 mM EDTA, pH 8.4) stained with ethidium bromide and photographed under UV light. The molecular size marker was 100 bp DNA Ladder (Invitrogen Corp. Carlsbad, CA).

16S rRNA gene sequencing

The PCR product of *S. sobrinus* 6715 described above was sequenced using the Thermo Sequenase Cy5 Dye Terminator Kit and an ALF Express II automated DNA sequencer (Amersham Pharmacia Biotech). Primers 8UA and 1492R were used to sequence both strands and DNA data were analyzed using the DNASIS program (Hitachi Software Engineering Co. Ltd. Tokyo, Japan). The GenBank accession number of the 16S rRNA genes obtained in this study is AF439398 (*S. sobrinus* 6715). Other accession numbers used in this study are as follows: *S. mutans* NCTC 10449, AJ243965; *S. sobrinus* NCTC 12279, AJ243966; *S. criceti* NCTC 2720, X58305; *S. downei* NCTC 11391, X58306; *S. ferus* KOMA81, AF336367; *S. macacae* NCTC 11558, X58302; and *S. rattii* NCDO 2723, X58304. We compared the calculated theoretical RFLP patterns of mutans strepto-

cocci based on the GenBank database sequences and those obtained in the present study.

Results

The RFLP profiles generated by *HpaII* and *HaeIII* digestion are shown in Fig 1. These profiles agreed with the predicted restriction profiles as described in Table 1. *HpaII* produced six RFLP patterns (A to F, Table 1) for eight reference strains in which the patterns for *S. sobrinus* OMZ176 (serotype *d*), *S. downei* and *S. ferus* were similar (Fig 1). In contrast, RFLP patterns obtained by *HaeIII* could distinguish the three species (Fig 1). *HaeIII* produced five patterns (I to V, Table 1) in which the patterns for *S. criceti* and *S. downei* were similar (RFLP pattern II, Table 1) and also the patterns for *S. ferus* and *S. rattii* were similar (RFLP pattern V, Table 1). Thus, the combination of the results of 16S rRNA genes PCR RFLP using *HpaII* and *HaeIII* allowed the eight mutans streptococci to be distinguished from one another (Fig 1, Table 1).

The profiles obtained using *HpaII* and *HaeIII* of six laboratory strains of *S. mutans* (i.e. MT8148 (serotype *c*), Ingbritt (serotype *c*), GS5 (serotype *c*), NCIB11723 (serotype *c*), LM7 (serotype *e*) and MT6229 (serotype *f*)) and 12 clinical isolates of *S. mutans* corresponded to those of type strain NCTC 10449 (serotype *c*) (Fig 2A, B, lanes 1 to 11).

Clinical isolates of *S. sobrinus* were assigned to either serotype of *S. sobrinus* the profiles with *HpaII* of Tohoku Ss 1 and Tohoku Ss 2 were identical to that of *S. sobrinus* 6715 (serotype *g*) (Fig 2A, lanes 12 and 13) and those of MKS4A and MKS100A were the same as that of *S. sobrinus* OMZ176 (serotype *d*) (Fig 2A, lanes 14 and 15).

Discussion

In this study, 16S rRNA gene PCR RFLP analysis was used to differentiate the respective reference strains of mutans streptococci. The single digestion with *HpaII* differentiated *S. mutans*, *S. criceti*, *S. macacae*, *S. rattii* and *S. sobrinus* (serotype *g*) and the profiles with *HaeIII* could help separate *S. downei*, *S. ferus* and *S. sobrinus* (serotype *d*) (Fig 1). Therefore, the combination of *HpaII* and *HaeIII* can clearly differentiate all of the species included in the group of mutans streptococci (Fig 1). The theoretical and actual RFLP patterns for *HpaII* and *HaeIII* agreed (Table 1), supporting the reliability of the method used in this study.

The RFLP patterns of seven laboratory strains (5 serotype *c*, 1 serotype *e* and 1 serotype *f*) as well as 12 clinical strains of *S. mutans* generated by *HpaII* or *HaeIII* were identical (Fig 2). However, the profiles of *S. sobrinus* OMZ176 (serotype *d*) and 6715 (serotype *g*) generated by *HpaII* differed (Fig 1) although they were identical using *HaeIII* (Fig 1). We sequenced the 16S rRNA genes of *S. sobrinus* 6715 (serotype *g*) in this study (GenBank accession number AF439398) and confirmed that the calculated RFLP patterns based on the sequence (Table 1) were accordance with the RFLP patterns of *S. sobrinus* 6715 (Fig 1) and clinical isolates (Fig 2). These results show that the method using *HpaII* could differentiate those serotypes of *S. sobrinus*.

Species specific PCR for *S. mutans* and *S. sobrinus* is considered a reliable means of their identification (2, 4, 9-11). We introduced species specific PCR based on the dextranase gene to initially identify clinical isolates of *S. mutans* and *S. sobrinus* and confirmed the results corresponded to those of the 16S rRNA genes PCR RFLP in this study. Therefore, as an

Table 1. 16S rRNA genes PCR RFLP profiles of mutans streptococci with *HpaII* and *HaeIII*

Species	Strains	Serotype	<i>HpaII</i>		<i>HaeIII</i>		GenBank accession numbers
			PCR RFLP profiles	Predicted DNA fragment sizes ^b	PCR RFLP profiles	Predicted DNA ^b fragment sizes	
<i>Streptococcus mutans</i>	NCTC 10449 ^T	<i>c</i>	A	564 304 288 130 120	I	505 457 318	AJ243965
<i>Streptococcus criceti</i>	GTC 242 ^T	<i>a</i>	B	564 303 163 130 125 120	II	598 456 274 126	X58305
<i>Streptococcus sobrinus</i>	6715	<i>g</i>	C	488 439 163 133 119	III	596 457 272	This study (AF439398)
<i>Streptococcus macacae</i>	GTC 538 ^T	<i>c</i>	D	441 315 249 130 122 120	IV	596 456 318 126	X58302
<i>Streptococcus rattii</i>	GTC 245 ^T	<i>b</i>	E	315 303 249 163 130 125 120	V	505 456 274 126	X58304
<i>Streptococcus sobrinus</i>	OMZ176	<i>d</i>	F	489 317 163 130 125 120	III	598 457 273	AJ243966
<i>Streptococcus downei</i>	GTC 632 ^T	<i>h</i>	F	490 315 163 130 125 120	II	597 456 274 126	X58306
<i>Streptococcus ferus</i>	GTC 279 ^T	<i>c</i>	F	490 304 163 130 125 120	V	505 457 274 126	AF336367

^a16S rRNA genes PCR RFLP profiles were obtained in this study (Fig 1)
^bUnique predicted DNA fragment sizes (at least 100 bp long) are underlined

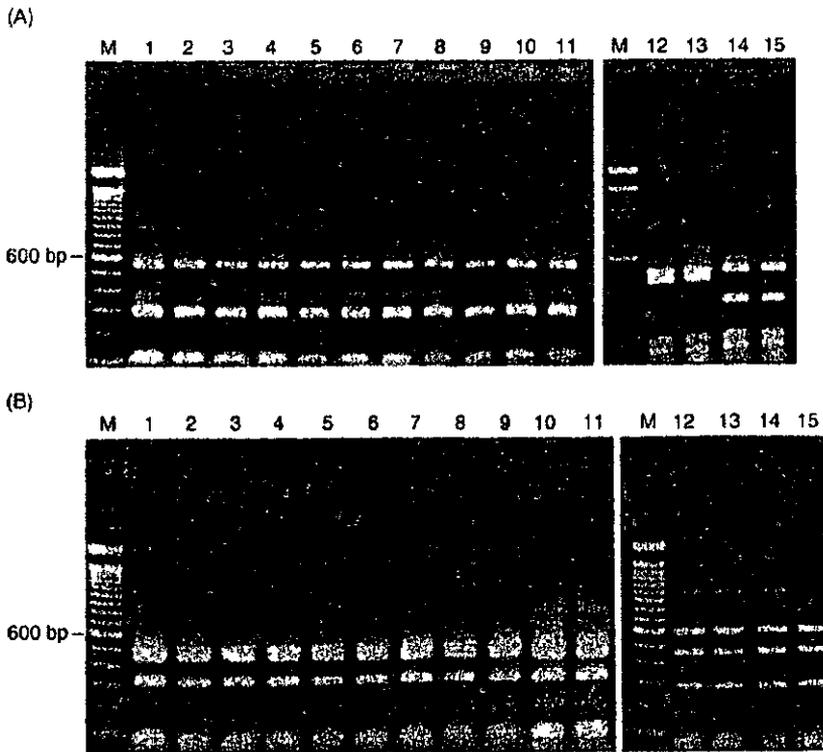


Fig 2 PCR amplified 16S rRNA genes from *S. mutans* and *S. sobrinus* strains digested with *HpaII* (panel A) and *HaeIII* (panel B) Lanes 1 *S. mutans* NCTC 10449T (c) 2 *S. mutans* Ingbritt (c) 3 *S. mutans* GS5 (c) 4 *S. mutans* NCIB 11723 (c) 5 *S. mutans* MT8148 (c) 6 *S. mutans* LM7 (e) 7 *S. mutans* MT6229 (f) 8 *S. mutans* Tohoku Sm 1 9 *S. mutans* Tohoku Sm 2 10 *S. mutans* Tohoku Sm 3 11 *S. mutans* Tohoku Sm-4 12 *S. sobrinus* 6715 (g) 13 *S. sobrinus* Tohoku Ss 1 (g) 14 *S. sobrinus* OMZ176 (d) 15 *S. sobrinus* MKS4A (d) M Molecular size markers (100 bp DNA Ladder Invitrogen Corp)

alternative method the 16S rRNA gene PCR RFLP using *HpaII* and *HaeIII* is reliable and useful for the identification of *S. mutans* and *S. sobrinus*

In addition to the method described here has the ability to identify not only *S. mutans* and *S. sobrinus* but also other mutans streptococci e.g. *S. criceti*, *S. downei*, *S. ferus*, *S. macacae* and *S. rattii* (Fig 1 Table 1) without the development and preparation of specific primers for each streptococcal species. Furthermore it was confirmed that RFLP profiles using *HpaII* and *HaeIII* of other oral streptococcal species including *Streptococcus anginosus*, *Streptococcus australis*, *Streptococcus constellatus*, *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus sanguinis* and *Streptococcus vesicularis* obtained from the Genbank database (data not shown) could differentiate all of the oral streptococcal species including mutans streptococci.

This method requires only universal primers for bacterial 16S rRNA genes a molecular clock of evolution and widely

used in studies of bacterial taxonomy (13). Although 16S rRNA sequence comparisons can be used to identify the clinical isolates the identification by sequencing is sometimes difficult in large scale studies and for small laboratories because it is time consuming and expensive. Therefore PCR RFLP analysis based on 16S rRNA genes could be an alternative method for the bacterial identification and may be applicable for the identification and comparison of a wide range of bacterial species.

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Biochemical and functional properties of a pyruvate formate-lyase (PFL)-activating system in *Streptococcus mutans*

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Takahashi Abbe S Abe K Takahashi N Biochemical and functional properties of a pyruvate formate lyase (PFL) activating system in *Streptococcus mutans*
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Streptococcus mutans has an oxygen sensitive enzyme pyruvate formate lyase (PFL) which is a key enzyme in anaerobic sugar fermentation. We have shown that *S mutans* has an activating system including a PFL activating enzyme (PFL-activase) and an electron transport system; the latter transfers an electron from NADPH to PFL-activase as occurs in *Escherichia coli*. NADPH was a physiological electron donor for the electron transport system and as little as 0.02 mM NADPH activated over 80% of PFL of *S mutans*. The optimum pH of the PFL activating system was around 6.8 whereas the optimum of the *E coli* system is at alkaline pH. In addition, small dialyzable molecules in cell free extracts participated in keeping PFL active in *S mutans*. These results suggest that in dental plaque under anaerobic conditions where sugar supply is often limited or pH frequently falls below neutrality *S mutans* always keeps PFL active through the activating system.

Key words: pyruvate formate lyase activating system, pyruvate formate lyase activating enzyme, PFL-activase, *Streptococcus mutans*, pyruvate formate lyase activation, inactivation, oxygen sensitive.

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Streptococcus mutans is considered a major acidogenic and cariogenic agent among the microorganisms (13) in dental plaque where the environment is anaerobic (8, 9, 16). Oral streptococci including *S mutans* have a pyruvate formate lyase (PFL) which is a key enzyme in anaerobic sugar fermentation (17, 22, 25). The active form of PFL (PFL_A) is extremely oxygen sensitive; that is, its activity is lost irreversibly by exposure to air (19, 22).

It has been reported that an oxygen sensitive PFL_A also exists in *Escherichia coli* and that the enzyme has a free radical which participates in the enzyme activity (11, 23). PFL of *E coli* is interconverted between PFL_A and a reversible inactive form (PFL_R) that does not have the radical (10, 11, 23). PFL_R is converted into PFL_A by an activating system containing a flavodoxin system and a PFL

activating enzyme (PFL-activase) (11). PFL-activase catalyzes the free radical formation on PFL in the presence of *S*-adenosyl-L-methionine, pyruvate and flavodoxin reduced by flavodoxin oxidoreductases in the flavodoxin system (11, 23).

The *pfl* gene and the *act* gene encoding PFL and PFL-activase respectively have been identified in *S mutans* (26, 27). In addition, there have been reports that *S mutans* PFL is activated by reduced methylviologen (MV) or reduced ferredoxin (from *Clostridium pasteurianum*) as a reducing agent in addition to *S*-adenosyl-L-methionine and pyruvate *in vitro* under strictly anaerobic conditions (22, 27). There is, however, only limited information available about the PFL activating system of *S mutans*. Thus, in this study we attempted to investigate the biochemical

and functional properties of the PFL activating system of *S mutans*.

Material and methods

Bacterial strain and preparation of cell free extract

S mutans NCIB 11723 (4) was grown in a modified D87 medium under strictly anaerobic conditions in an anaerobic glove box (atmosphere: N₂ 80%, H₂ 10%, CO₂ 10% type NHC) as described previously (2, 25). The medium contained galactose as a carbon source because PFL in cell free extracts of galactose grown cells exhibits higher activity (2, 25) and is activated more easily than in extracts from glucose grown cells in this study. The cells were then harvested in the early logarithmic growth phase (optical density at 660 nm 0.9–1.1) and stored at –20 °C in another anaerobic glove

box (atmosphere N₂ 90% H₂ 10% type NH) as described previously (25). The cells were disrupted by ultrasonic treatment for 10 min under strictly anaerobic conditions (25). The supernatant fluid was filtered through a membrane filter (pore size 0.45 µm type Dismic 25cs Toyo Roshi Tokyo Japan). This sample was designated cell free extract.

Unless otherwise noted, all of the following procedures were carried out under strictly anaerobic conditions in a type NH anaerobic glove box as described previously (2, 19, 22).

Inactivation of PFL in cell free extract

The cell free extract was dialyzed against 1 mM dithiothreitol (DTT) in 40 mM potassium phosphate buffer (PPB, pH 7.0) (DTT buffer) for 4 h at 4°C. For the anaerobic inactivation of PFL, the dialyzed sample was incubated for 60 or 120 min at 35°C. For the aerobic inactivation of PFL by exposure to air, a part of the sample was brought out from the anaerobic glove box and then exposed to a steady stream of air for 5 min at 0°C. Then the aerobically inactivated sample was returned to the anaerobic glove box after evacuating the air from it 4 times (2). To extensively eliminate the last traces of oxygen from the sample, 8 mM glucose and 16 U/ml glucose oxidase were added. Incubation of PFL with glucose and glucose oxidase showed no significant effect on the activation of PFL that had been kept under anaerobic conditions.

Preparation of PFL_R

The untreated cell free extract was concentrated by ultrafiltration (pore size mol wt 100 000). The concentrated sample was diluted with DTT buffer and then concentrated again. These procedures were repeated 6 times to remove most of the contaminating PFL activase and other small molecules (less than mol wt 100 000). To remove PFL activase completely, the sample was applied to a reactive blue 2 agarose column (1.76 cm² by 8.0 cm Sigma Chemical Co. St. Louis, Mo) equilibrated with DTT buffer.

Purification of PFL activase

The untreated cell free extract was filtered through a first ultrafiltration (pore size mol wt 100 000 Millipore Tokyo Japan) to remove PFL whose molecular weight is about 180 000 (22). The filtrate was then concentrated by a second ultrafiltration (pore

size mol wt 10 000 Millipore) after which the sample was diluted with DTT buffer. The diluted sample was then concentrated again to remove small molecule contamination (less than mol wt 10 000). Seven milliliters of the sample supplemented with ammonium sulfate (final concentration 500 mM) and 3 morpholinopropane sulfonic acid (MOPS) NaOH buffer (pH 7.5 final concentration 100 mM) were applied to a phenylsepharose CL 4B column (1.76 cm² by 7.4 cm Amersham Pharmacia Biotech Tokyo Japan) equilibrated with 500 mM ammonium sulfate in 100 mM MOPS NaOH buffer. The column was successively washed with the same solution and then PFL activase was eluted from the column using a stepwise dilution of ammonium sulfate and MOPS NaOH buffer. Before being chromatographed, Fe(SO₄)₂(NH₄)₂ (final concentration 2 mM) and DTT (final concentration 1 mM) were placed in all fraction tubes to protect the enzyme.

Assay of enzyme activity

PFL activity

The activity of PFL_A was assayed spectrophotometrically by recording the change in absorbance at 340 nm at 30°C (UV spectrophotometer model UV 160A Shimadzu Co. Kyoto Japan). The standard PFL assay mixture contained 20 mM sodium pyruvate, 0.08 mM Co A, 1 mM NAD, 6 mM sodium DL malate, 2 mM DTT, 1.4 U of citrate synthase (pig heart EC 4.1.3.7) per ml, 13.8 U of malate dehydrogenase (pig heart EC 1.1.1.37) per ml and PFL in 100 mM PPB (final pH 7.6) (25).

PFL activase activity (activation of PFL_R)

For the assay of PFL activase activity, the dialyzed cell free extract or purified PFL activase and PFL_R were first incubated with the following activating mixture (final pH 6.8) at 30°C: 0.6 mM S-adenosyl-L-methionine, 2.9 mM DTT, 10 mM sodium pyruvate or oxamate, 1 mM reduced MV or NADPH, 2 mM Fe(SO₄)₂(NH₄)₂ and 5 mM MgCl₂ in 100 mM PPB. PFL activity was then measured in the standard PFL assay mixture.

Molecular size determination

The apparent molecular size of the native PFL activase was estimated by gel chromatography on a Superose 12 HR 10/30 (Amersham Pharmacia Biotech) column equilibrated with 0.1 M KCl in 40 mM PPB (pH 7.0) at a flow rate of 1 ml/min.

Chromatofocusing

The chromatofocusing column (0.39 cm² by 13 cm Polybuffer exchanger PBE 94 Amersham Pharmacia Biotech) was equilibrated with 25 mM ethanolamine HCl buffer (pH 9.40). After pre-running with 5 ml of elutant (polybuffer 96 diluted 1:10 and adjusted to pH 6.02 with HCl), 0.5 ml of enzyme solution (0.53 mg protein) was applied to the column. The column was subsequently run with elutant at a flow rate of 40 cm/h.

Other analytical methods

The protein concentration of the cell free extract was estimated by the biuret method (12) and that of the purified PFL activase samples by a dye binding method (Bio Rad Laboratories Richmond, CA).

Results and discussion

Interconversion of PFL in untreated and dialyzed cell free extract

Under strictly anaerobic conditions, PFL_A in untreated cell free extract of *S. mutans* kept its activity after incubation for 2 h at 35°C. This observation indicates that PFL_A in untreated cell free extracts is stable at 35°C, similar to the enzyme in intact *S. mutans* cells (20). However, in the dialyzed cell free extracts, the PFL activity decreased gradually during incubation (Fig 1(I), open bars). These results suggest that small dialyzable molecules are involved in maintaining PFL activity.

The anaerobically lost activity was restored to near the original level when the dialyzed cell free extract was incubated with the activating mixture at 30°C (Fig 1(I), closed bars).

After 5 min exposure of the dialyzed cell free extract to the atmospheric oxygen, no PFL activity was detected even though oxygen had been excluded from the extract by evacuation treatment and by incubation with the glucose oxidase system (data not shown). On the other hand, when the extract was anaerobically inactivated and then exposed to air, the activity was partly recovered by anaerobic incubation with the activating mixture (Fig 1(II), hatched bars B and C). The more the enzyme had been anaerobically inactivated, the more activity was anaerobically restored after exposure to air (Fig 1, open bars and hatched bars). A similar amount of PFL activity was regained by the activation, whether the extract was exposed once to air or not (Fig 1a-c and hatched bars A-C).

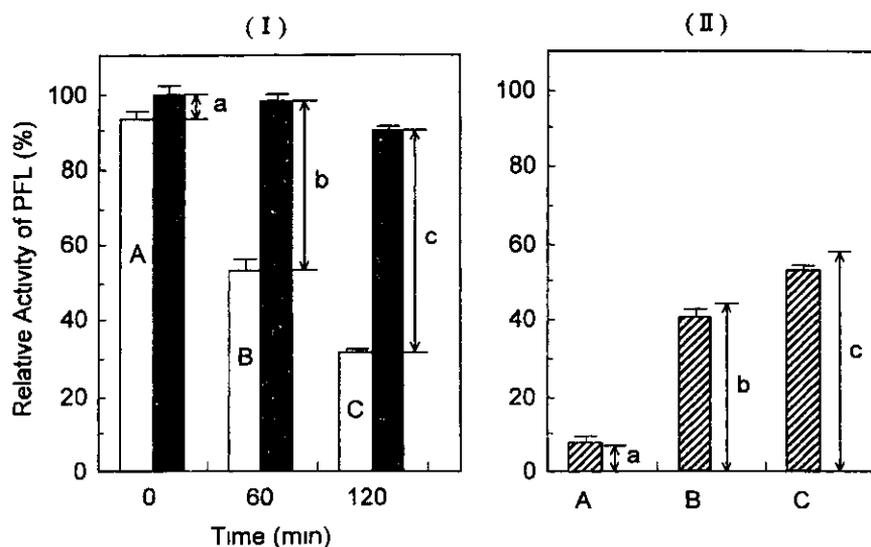


Fig 1 Interconversion of PFL in the dialyzed cell free extract (I) All experiments were carried out in the NH type anaerobic glove box. Open bars: PFL activity after inactivation at 35 °C for 0, 60 or 120 min. Closed bars: PFL activity after incubation with the activating mixture containing pyruvate and reduced MV at 30 °C for 120 min. (a, b, c) PFL activity regained by the activation determined by subtracting PFL activity before activation from that after activation. (II) Hatched bars: inactivated samples (A–C) of (I) that were exposed to air and then activated by incubation with the activating mixture containing pyruvate and reduced MV at 30 °C for 120 min under strictly anaerobic conditions. (a, b, c) as for (a, b, c) of (I). The mean activity (7.15 U/mg of protein) of PFL in the dialyzed cell free extract after activation was regarded as 100%. Bars represent the averages (\pm SD) of three different experiments.

These results indicate that the PFL activating system of *S mutans* is resistant to oxygen because the system is still active after exposure to air and confirmed that PFL_A is extremely sensitive to and inactivated irreversibly by oxygen and that PFL_R is resistant to oxygen. The oxygenolytic cleavage of a subunit of PFL_A has been reported (3, 14, 23). The subunit of *S mutans* PFL_A may be cleaved by oxygen as occurs in *E coli* (23).

Purification of PFL activase

Before purification of PFL activase, PFL_R was prepared for use as a substrate for PFL activase. Most of the PFL_A in the cell free extract was converted into PFL_R during the removal of small molecules by ultrafiltration (mol wt 100 000) at 4 °C. All the contaminated PFL activase combined strongly with reactive blue 2 agarose but PFL_R did not. PFL_R was eluted immediately after void fractions from the reactive blue 2 agarose column with DTT buffer.

For the purification of PFL activase, the sample containing the enzyme was applied to the phenylsepharose column as described in material and methods. The enzyme was then eluted from the column by 100 mM ammonium sulfate in 100 mM MOPS NaOH buffer after stepwise dilution of ammonium sulfate and MOPS

NaOH buffer. PFL activase was purified 30 fold overall and the specific activity of the enzyme was 105 ± 17 U/mg of protein (the mean of the results from 3 different experiments \pm SD). The activity of the purified PFL activase in phenylsepharose fractions supplemented with Fe(SO₄)₂(NH₄)₂ and DTT was relatively stable at 4 °C. However, after further concentrating the fractions by ultrafiltration (pore size mol wt 10 000), desalting by gel chromatography or dialysis, PFL activase was inactivated. For these reasons, it was difficult to carry out further purification of the enzyme.

Biochemical and functional properties of *S mutans* PFL activase

The molecular size of the native PFL activase was determined to be about 40 kDa by gel chromatography while the enzyme of *E coli* is 34 kDa (5). The isoelectric point of *S mutans* PFL activase was 7.80, whereas that of the *E coli* enzyme is 5.75 (5).

The optimum pH for PFL activation in *S mutans* was around 6.8 (Fig 2) while the activation of *E coli* PFL has been carried out at alkaline pH (pH 7.6–7.7) (5, 10). The activity of the *S mutans* PFL activating system at pH 7.7 was only 20% of the maximal activity (Fig 2). It is

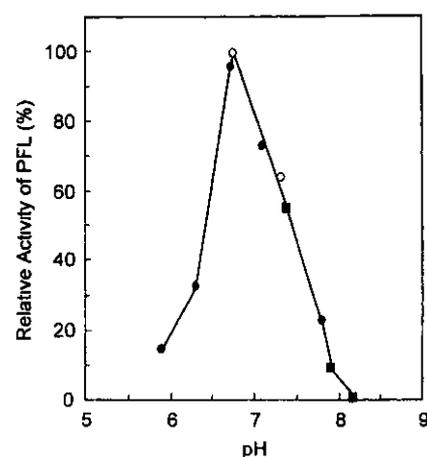


Fig 2 Effect of pH on the activation of *S mutans* PFL. The buffers of the activating mixture were used as follows: (●) PPB, (○) HEPES, (■) Tris-HCl.

reasonable that the *E coli* activating system functions efficiently at alkaline pH because *E coli* lives in the large intestine under alkaline conditions. In contrast, the optimum pH of the *S mutans* activating system was weakly acidic (Fig 2). This may be advantageous to *S mutans* for living in dental plaque where pH frequently falls below neutrality.

PFL activase in the dialyzed cell free extracts activated PFL in the presence of NADPH as well as in the presence of reduced MV in the activating mixture (Table 1). NADPH was more effective for PFL activation than NADH and as little as 0.02 mM NADPH activated over 80% of PFL_R (Table 1). These results imply that NADPH participates in the PFL activation as a physiological reducing agent *in vivo*. Reduced benzylviologen, reduced hydrosulfite or oxidized MV did not activate PFL_R at all. In contrast to the PFL activase in cell free extracts, the purified enzyme activated PFL on incubation with reduced MV but not with NADPH in the activating mixture (Table 1). These results indicate that an electron does not directly transfer from NADPH to PFL activase and strongly imply that the dialyzed cell free extract of *S mutans* contained an electron transport system which transfers an electron from NADPH to PFL activase. It has been reported that in *E coli*, flavodoxin reduced by two kinds of flavodoxin oxidoreductases is involved in PFL activation as electron donor and one of the reductases is NADPH dependent (11). The electron transport system for PFL activation in *S mutans* is still unknown though *S mutans* is reported to have a flavoprotein (6, 15).

Table 1 Effect of reduced MV NADPH or NADH on the activation of *S mutans* PFL

Reducing agents	Conc (mm)	Relative activity of PFL (%)	
		Cell free extract	Phenylsepharose fractions
Reduced MV	1.0	100	100
NADPH ^b	0.02	84.1	— ^d
	0.05	94.2	— ^d
	0.1	99.0	— ^d
	0.2	100	— ^d
	0.5	101	— ^d
	1.0	101	0.00
NADH ^b	2.0	101	0.00
	0.5	39.7	— ^d
	1.0	60.0	— ^d
	2.0	69.6	— ^d

When reduced MV was used for PFL activation instead of NADPH pyruvate was used instead of oxamate in the activating mixture

^bWhen NADPH or NADH was used for activation instead of reduced MV oxamate was used instead of pyruvate in the activating mixture

PFL activity after 90 min activation with the activating mixture containing reduced MV is regarded as 100%

^dNot tested

PFL activase in cell free extracts activated PFL on incubation with pyruvate or oxamate in the activating mixture (Fig 3A). The purified activase also fully activated PFL in the presence of pyruvate but scarcely activated PFL in the presence of oxamate (Fig 3B). The PFL activation by PFL activase purified from *S mutans* indicates that the presence of pyruvate is absolutely required. On the other hand, oxamate is a competent substitute for pyruvate in the activation of

PFL by the activase purified from *E coli* (10, 11).

We have shown that *S mutans* has an oxygen resistant PFL activating system including PFL activase and an electron transport system. The latter transferred an electron from NADPH to PFL activase. We have also shown that pyruvate is necessary to activate PFL of *S mutans* and that addition of $\text{Fe}(\text{SO}_4)_2(\text{NH}_4)_2$ with DTT into phenylsepharose fractions protected the activity of PFL activase. We have reported

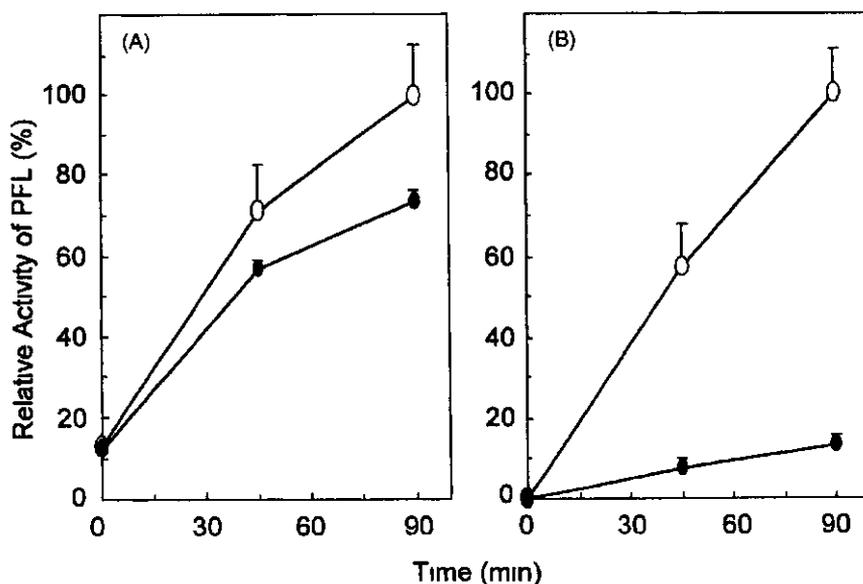


Fig 3 Effect of pyruvate or oxamate on the activation of *S mutans* PFL with the activating mixture containing reduced MV (A) PFL activase in cell free extract (B) Purified PFL activase in phenylsepharose fractions (A, B) The PFL activity after 90 min activation with the activating mixture containing pyruvate was regarded as 100%. The mean value (\pm SD) of three different experiments is shown (○) the activating mixture containing pyruvate instead of oxamate (●) the activating mixture containing oxamate instead of pyruvate

that *S adenosyl L methionine* is indispensable for PFL activation of *S mutans* similar to PFL activation in *E coli* (22). For activation of *S mutans* PFL, therefore, it is considered that an electron is transferred from activase to PFL in the presence of pyruvate, *S adenosyl L methionine* and $\text{Fe}(\text{SO}_4)_2(\text{NH}_4)_2$ as occurs in *E coli*, the differences being the isoelectric point of the activase and the optimum pH of the system.

In addition, the small dialyzable molecules that are involved in maintaining PFL activity of *S mutans* could be NADPH, pyruvate, $\text{Fe}(\text{SO}_4)_2(\text{NH}_4)_2$ and/or *S adenosyl L methionine*, all of which are indispensable for PFL activation.

Implication of PFL activating system in physiology of *S mutans*

In the present study, we showed the presence of a PFL activating system consisting of PFL activase and an electron transport system in *S mutans*. PFL catalyzes the first step of the conversion of pyruvate into formate, acetate, and ethanol, and the enzyme plays several important roles for *S mutans* (1, 2, 18, 22, 24, 25). When a little sugar is supplied (between meals, especially during sleeping hours) or a slowly metabolizable sugar such as galactose is fermented, *S mutans* is able to obtain sufficient energy and acetyl CoA through the PFL pathway under anaerobic conditions (1, 24, 25). In addition, *S mutans* is able to utilize sugar alcohols (sorbitol, mannitol, etc.) frequently contained in fruits, which have more hydrogen atoms than glucose or sucrose (1, 2, 18). *S mutans* can efficiently re-oxidize surplus NADH derived from sugar alcohols through the PFL pathway.

It has been reported that the intracellular NADPH pool of *S mutans* during glucose depletion is kept at levels similar to those found during glucose metabolism under strictly anaerobic conditions (7). In addition, pyruvate for the PFL activation is supplied continuously through intracellular polysaccharide metabolism under starvation (21). Deep layers of dental plaque, which favor *S mutans*, are expected to give rise to highly anaerobic conditions (8, 9, 16). Furthermore, the activating system of *S mutans* activated PFL efficiently under weakly acidic conditions. Thus, the activating system can always keep PFL active and allow the organism to be equipped with full metabolic activity. For these reasons, *S mutans* can produce acids continuously in dental plaque where sugar supply is often limited or pH frequently falls

below neutrality. This could be one of the reasons *S. mutans* is highly cariogenic.

Acknowledgments

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Xylitol inhibition of anaerobic acid production by *Streptococcus mutans* at various pH levels

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Miyasawa H Iwami Y Mayanagi H Takahashi N Xylitol inhibition of anaerobic acid production by *Streptococcus mutans* at various pH levels
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Xylitol inhibits the glycolysis and growth of *Streptococcus mutans*. We studied the inhibitory effect of xylitol on the acid production of *S. mutans* at several pH levels under the strictly anaerobic conditions found in the deep layer of dental plaque. Xylitol inhibited the rate of acid production from glucose and changed the profile of acidic end products to formate–acetate dominance with a decrease in the intracellular level of fructose 1,6-bisphosphate and an intracellular accumulation of xylitol 5-phosphate (X5P). These results were notable at pH 5.5–7.0 but were not evident at pH 5.0. Since the activity of phosphoenolpyruvate phosphotransferase for xylitol was greater at higher pH, it is suggested that xylitol could be incorporated more efficiently at higher pH and that the resultant accumulation of X5P could inhibit the glycolysis of *S. mutans* more effectively.

Key words *Streptococcus mutans*, xylitol, glycolysis, pH, anaerobic

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Xylitol is widely used as a non-cariogenic sugar substitute because it is not fermented by oral bacteria (7). In addition, in long-term consumers, xylitol decreases the number of mutans streptococci and their ratio both in dental plaque (7, 10, 16, 22, 27, 32) and in saliva (2, 16, 17, 33).

Xylitol inhibits the growth of mutans streptococci in the presence of glucose, mannose, lactose, sorbitol and mannitol as a carbon source *in vitro* (1, 6, 14, 21, 30, 31) and inhibits the growth of *Streptococcus mutans* selectively in the mixed culture using a chemostat (3, 20). Xylitol also inhibits the acid production from glucose by resting cells of *S. mutans* (30). It has been supposed that xylitol is incorporated into the bacterial cells as xylitol 5-phosphate (X5P) and that the X5P inhibits the enzyme activity of sugar metabolism, resulting in the inhibition of both bacterial growth and acid production (28). In addition, this futile cycle, in which X5P is dephosphorylated to xylitol with waste of PEP potential, can also retard the growth of *S. mutans* (28).

In previous studies (30), the inhibitory effect of xylitol on the glycolysis of *S. mutans* has been investigated at neutral pH. When dietary sugars are taken into the oral cavity, the sugars are degraded into acids by dental plaque bacteria and subsequently the dental plaque pH decreases rapidly to about 4 (9, 23). Therefore, we studied the effect of pH on the xylitol inhibition of acid production by *S. mutans* and its biochemical mechanism. In addition, we conducted the experiments on xylitol inhibition under strictly anaerobic conditions as reported previously (5, 19) since the interior of dental plaque is strictly anaerobic (13) and the glycolysis of dental plaque bacteria under anaerobic conditions differs from that under aerobic conditions (26, 34).

Material and methods

Bacterial strain and growth conditions

S. mutans NCTC 10449 was kindly provided by Prof. L. Trahan (Université Laval

Quebec, Canada) (29) and used throughout this study. This strain was characterized as xylitol sensitive by him. The strain was inoculated in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) under strictly anaerobic conditions in an anaerobic chamber (N₂ 80%, H₂ 10%, CO₂ 10%, NHC type Hirasawa Works, Tokyo, Japan) and incubated at 35 °C overnight. The cell culture was transferred into a complex medium containing 1.7% tryptone (Difco), 0.3% yeast extract (Difco), 85 mM NaCl and 11 mM glucose as described previously (30) and incubated overnight at 35 °C. The cell culture was again transferred into the same complex medium (5% inoculum size) and grown at 35 °C. The bacterial cells were harvested by centrifugation (7000 × g for 15 min at 4 °C) at an early logarithmic phase of growth (optical density at 660 nm [OD₆₆₀] = ca 0.3) under the anaerobic conditions described previously (25). Bacterial purity was regularly confirmed by culturing on blood agar plates.

Acid production from glucose in the presence of xylitol

The following experiments were conducted in a different type of anaerobic chamber (N₂ 90% H₂ 10% NH type Hirasawa Works) The cells were washed twice with cold 2 mM potassium phosphate buffer (pH 7.0) plus 150 mM KCl and 5 mM MgCl₂ and suspended in the same buffer The optical density at 660 nm of the cell suspension was adjusted to 3.5 (1.9 mg [dry weight of cells] per ml)

The cell suspensions were agitated with a magnetic stirrer at 35 °C The reaction was started by addition of the mixture containing 10 mM glucose and 0, 30, 60 or 120 mM xylitol to the cell suspensions The rate of acid production by the cells was monitored at pH 7.0 to 5.0 by an automatic pH titrator (model AUT 211S Toa Electronics Ltd Kobe Japan) with 50 mM KOH The rate of acid production at 2 min after addition of glucose or glucose-xylitol mixture was calculated as mol proton per min per mg dry weight of cells

Before and after incubation for 10 min the cell suspensions (0.9 ml) were sampled and mixed immediately with 0.1 ml of 6 N perchloric acid The mixtures were filtered (pore size 0.20 µm ADVANTEC polypropylene Toyo Roshū Ltd Tokyo Japan) and the cell free filtrates were diluted with 0.2 N hydrochloric acid and stored at 4 °C for the assay of acidic end products

Analysis of acidic end products

Acidic end products lactic acetic formic and pyruvic acids were quantified with a carboxylic acid analyzer (model EYELA S 3000X Tokyo Rikakikai Co Ltd Tokyo Japan) as described previously (25)

Assays of glycolytic intermediates and xylitol 5 phosphate (X5P)

After incubation for 2 min the cells were collected by passing the reaction mixture through a membrane filter (pore size 0.45 µm Acrodisc polyethersulfone Pall Gelman Laboratory Ann Arbor MI) Glycolytic intermediates and X5P in the cells were immediately extracted by addition of 0.6 N perchloric acid and neutralized with 5 M K₂CO₃ in air The neutralized extracts were stored at 4 °C for the assay of the glycolytic intermediates and X5P except for 3-phosphoglycerate (3PG) which was stored at -20 °C

The glycolytic intermediates in the cell extracts were determined enzymatically at 35 °C by a modification of the enzymatic

method by Minakami et al (18) using a dual wavelength spectrophotometer (model 557 Hitachi Ltd Tokyo Japan) at 340 nm

X5P in the cell extracts was determined by a modification of the enzymatic method by Supp (24) The assay mixture for X5P contained an aliquot of sample in dephosphorylation buffer (pH 8.5 0.5 M Tris HCl containing 1 mM EDTA 0.625 mM MgCl₂) and 1.1 mM NAD at 35 °C First xylitol in the assay mixture for X5P was converted into xylulose by the addition of 4.2 U/ml polyol dehydrogenase (EC 1.1.1.14 sorbitol dehydrogenase sheep liver Roche Diagnostics Indianapolis IN) and the increase in NADH was monitored at 340 nm After the reaction reached a plateau 50 U/ml alkaline phosphatase (EC 3.1.3.1 calf intestine Roche Diagnostics) was added to the reaction mixture for the conversion of X5P into xylulose The increase in NADH along with the conversion of X5P into xylulose through xylitol was monitored at 340 nm The amount of X5P was estimated as the increase of NADH in this reaction

Phosphoenolpyruvate phosphotransferase system (PEP PTS) for glucose and xylitol

The PEP PTS activity was estimated by a modification of the method of Kornberg & Reeves (15) The cells were harvested washed twice as described above and stored at -20 °C After being thawed the cells were suspended in 2 mM potassium phosphate buffer (pH 7.0) with 150 mM KCl and 5 mM MgCl₂ (OD⁶⁶⁰ = ca 5.0) Toluene was added at a final concentration of 1% to the cell suspension and mixed vigorously for 1 min After centrifugation (1200 × g for 3 min) the cells were suspended in the same buffer (OD⁶⁶⁰ = ca 5.0) The activity of PEP PTS for glucose or xylitol at pH 5.5-7.0 was estimated from the decrease of NADH in the reaction mixture containing 0.1 mM NADH 53 µg of dry weight of cell/ml 1 mM phosphoenolpyruvate 11 U/ml lactate dehydrogenase (EC 1.1.1.27 rabbit muscle Roche Diagnostics) 100 mM potassium phosphate buffer plus acetate buffer (pH 7.0-5.5) and 10 mM glucose or 30 mM xylitol at 35 °C The decrease of NADH was monitored spectrophotometrically at 340 nm

Statistical methods

Differences in the rate of acid production were analyzed by the Mann-Whitney U test Differences in the profile of glycolytic

intermediates were analyzed by the Mann-Whitney U test and Dunn test using the ratio of glycolytic intermediate levels in the presence of xylitol to those in the absence of xylitol (Intermediate level^{G + X} / Intermediate level^G) Differences in the amount of X5P at pH 7.0 and pH 5.0 were analyzed by the Newmann-Keuls test

Results

Inhibitory effect of xylitol on acid production at different pH levels

The acid production by *S. mutans* from glucose was decreased in the presence of 30 mM xylitol (Fig 1) At pH 7.0 to 5.5 the inhibition was significant ($P < 0.05$) with inhibition rates of 23.1 ± 3.8% to 28.5 ± 3.5% On the other hand at pH 5.0 the xylitol inhibition diminished to the half (13.2 ± 3.8%) - a non significant decrease When xylitol concentration increased the pH dependency of xylitol inhibition was similar The acid production rate in the presence of 60 and 120 mM xylitol decreased by 31.3 ± 5.9% and 40.8 ± 7.9% at pH 7.0 while at pH 5.0 the rate decreased by 23.1 ± 3.7% and 33.1 ± 9.9% respectively

Addition of xylitol changed the profile of acidic end products (Table 1) In the absence of xylitol at pH 7.0 approximately 50% of the total amount of acidic end products of *S. mutans* was lactic acid In

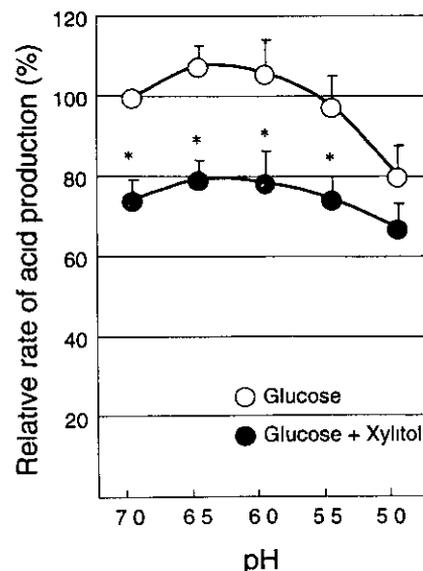


Fig 1 Acid production rate from glucose by the cells of *S. mutans* in the presence and absence of xylitol 10 mM glucose (○) 10 mM glucose plus 30 mM xylitol (●) Significant difference between the acid production rates in the presence and absence of xylitol ($P < 0.05$) Vertical bars indicate standard deviations from six independent experiments

Table 1 Acidic end products from 10 mM glucose (G) and 10 mM glucose plus 30 mM xylitol (G + X) at pH 7.0, 6.0 and 5.0

pH	Substrate	Acidic end products		
		Lactate	Acetate	Formate
7.0	G	1.16 ± 0.21 (44.7 ± 0.9) ^b	0.56 ± 0.18 (21.3 ± 2.8)	0.88 ± 0.13 (34.0 ± 1.9)
	G + X	0.40 ± 0.04 (17.3 ± 0.4)	0.72 ± 0.15 (31.0 ± 2.1)	1.19 ± 0.13 (51.7 ± 1.7)
6.0	G	1.49 ± 0.39 (50.8 ± 1.8)	0.58 ± 0.26 (19.4 ± 3.2)	0.87 ± 0.22 (29.8 ± 1.4)
	G + X	0.58 ± 0.13 (22.4 ± 0.7)	0.75 ± 0.27 (28.9 ± 2.8)	1.26 ± 0.28 (48.7 ± 2.1)
5.0	G	1.41 ± 0.37 (61.7 ± 1.1)	0.32 ± 0.08 (13.9 ± 0.1)	0.55 ± 0.11 (24.4 ± 1.2)
	G + X	0.91 ± 0.17 (41.4 ± 0.2)	0.46 ± 0.10 (21.2 ± 0.3)	0.82 ± 0.15 (37.5 ± 0.1)

Amounts of acidic end products (mean ± standard deviation μmol/mg cells) obtained from three independent experiments

^bRelative amounts of acidic end products (mean ± standard deviation %) obtained from three independent experiments

the presence of xylitol however the proportion of lactic acid decreased while that of formic and acetic acids increased. The proportion of lactic acid increased as the reaction pH was lowered.

Effect of xylitol on the profile of glycolytic intermediates

When *S. mutans* was metabolizing glucose a large amount of fructose 1,6 biphosphate (FBP) and small amounts of glucose

6 phosphate (G6P) and fructose 6 phosphate were detected in the metabolic intermediates of glycolysis (Fig 2). In the presence of xylitol however the level of FBP was decreased ($P < 0.05$) and a significant amount of X5P was detected in the cells (Fig 2). Among the other intermediates an increased level of glyceraldehyde 3 phosphate (G3P) and a decreased level of the other intermediates were also observed although these changes were not significant.

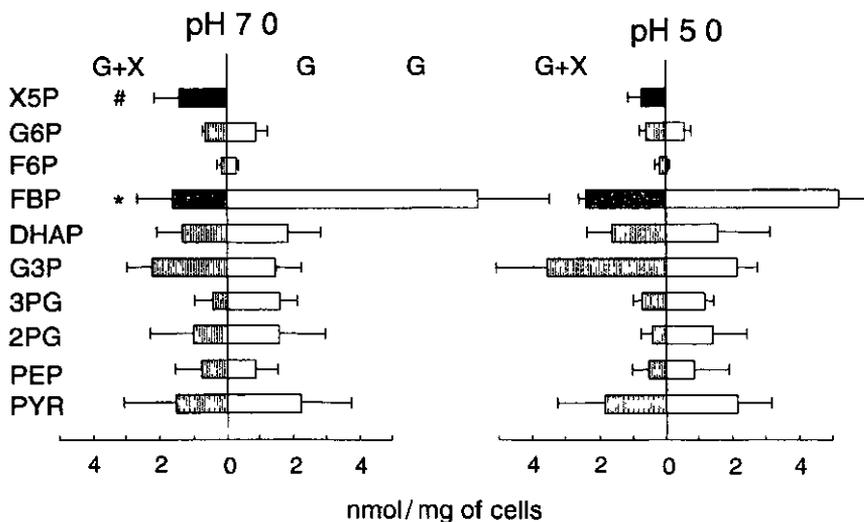


Fig 2 Glycolytic intermediates and xylitol 5 phosphate (X5P) 2 min after addition of glucose (10 mM) or glucose (10 mM) xylitol (30 mM) mixture to *S. mutans* cells. Glycolytic intermediates in the absence of xylitol (G □) and in the presence of xylitol (G + X) X5P (■). The left pair shows intermediates at pH 7.0 and the right at pH 5.0. G6P = glucose 6 phosphate, F6P = fructose 6 phosphate, FBP = fructose 1,6 biphosphate, DHAP = dihydroxyacetone phosphate, G3P = glyceraldehydes 3 phosphate, 3PG = 3 phosphoglycerate, 2PG = 2 phosphoglycerate, PEP = phosphoenolpyruvate, PYR = pyruvate. Significant difference between the intermediate level in the presence and absence of xylitol ($P = 0.05$). Significant difference between the amounts of X5P at pH 7.0 and at pH 5.0 ($P < 0.05$). Horizontal bars indicate standard deviations from four independent experiments.

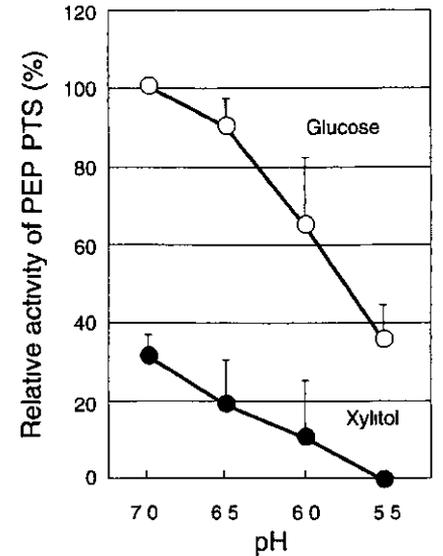


Fig 3 PEP PTS activities for glucose and xylitol of *S. mutans*. Glucose (10 mM ○) and xylitol (30 mM ●). Vertical bars indicate standard deviations from three independent experiments.

Both the decrease of FBP and the accumulation of X5P in the presence of xylitol were more evident at pH 7.0 than at pH 5.0. The FBP level at pH 7.0 decreased by 78.6% while at pH 5.0 the level decreased by 54.4% ($P = 0.05$). The amount of X5P accumulated at pH 7.0 was about twice as high as that at pH 5.0 ($P < 0.05$).

PEP PTS activity for glucose and xylitol

S. mutans showed PEP PTS activity for glucose and xylitol (Fig 3). The activity of both decreased as the reaction pH was lowered. At pH 5.5 no xylitol dependent activity was found though glucose dependent activity was still detected.

Discussion

This study showed clearly that under anaerobic conditions the acid production of *S. mutans* from glucose was inhibited by xylitol (Fig 1) as previously reported under aerobic conditions (6, 30). Furthermore it was found that *S. mutans* produced less lactate and more formate and acetate in the presence of xylitol (Table 1). This study also indicated that these effects of xylitol were evident at pH 5.5–7.0 but not at pH 5.0.

The analysis of metabolic intermediates in this study revealed the accumulation of X5P and the decreased level of FBP in the presence of xylitol (Fig 2). Together with the detection of PEP PTS activity for xylitol responsible for the uptake of xylitol

and its phosphorylation to X5P (Fig 3) this finding supports the speculation of Trahan (28) that *S mutans* transports xylitol as X5P and consequently the X5P inhibits phosphoglucose isomerase (PGI) and phosphofructokinase (PFK) the glycolytic enzymes for the conversion of G6P to FBP resulting in the decrease in the intracellular level of FBP and in the rate of glycolysis

Furthermore the effect of xylitol on glycolytic intermediates was influenced by environmental pH the X5P level was higher and the FBP level lower at pH 7.0 than at pH 5.0 (Fig 2) In this study the PEP PTS activity for xylitol was found to be higher at pH 7.0 (Fig 3) Therefore xylitol could be incorporated as X5P more efficiently at pH 7.0 resulting in the greater accumulation of X5P and the subsequent strong inhibition of glycolysis at pH 7.0

At pH 5.0 there was no PEP PTS activity for xylitol (Fig 3) but a small accumulation of X5P (Fig 2) and a slight inhibition of xylitol (Fig 1) were observed This discrepancy could be due to the fact that the intracellular pH of glycolyzing cells is usually higher than that of extracellular pH At an extracellular pH of 5.0 the intracellular pH of glycolyzing *S mutans* is reported to be 5.5–6.0 (4, 8, 12)

The intracellular level of G6P also appeared to decrease at pH 7.0 in the presence of xylitol (Fig 2) suggesting that xylitol and/or X5P might inhibit the uptake system of glucose In the intermediates after FBP the level of G3P increased and the levels of the other intermediates decreased This might indicate that X5P inhibits glyceraldehyde 3 phosphate dehydrogenase involved in the conversion of G3P to 3PG

S mutans degrades glucose into pyruvate via the Embden–Meyerhof pathway The pyruvate is further converted into lactate by lactate dehydrogenase (LDH) which requires FBP as an activator Thus when the intracellular level of FBP is high enough LDH is active (34) When the FBP level is low and subsequently LDH is not activated the pyruvate has to turn to formate and acetate through the other metabolic pathway involving pyruvate formate-lyase (PFL) Therefore the end product shift to formate–acetate dominance observed in the presence of xylitol (Table 1) is considered to be due to the decrease of intracellular FBP levels (Fig 2)

In addition the optimal pH of FBP dependent LDH is 5.5–6.3 while PFL is the most active at pH 7.5 and does not require metabolic intermediates as activators (11) These properties of LDH and PFL along with the lower FBP level ob-

served in the presence of xylitol at pH 7.0 (Fig 2) could enhance the end product shift to formate–acetate dominance around neutral pH (Table 1)

It has been reported that when *S mutans* cells are grown at pH 5.0 in a continuous culture they are repressed to synthesize enzyme II a component of PEP PTS (31) These cells are probably unable to incorporate xylitol via PEP PTS and consequently are unaffected by xylitol In addition we showed that *S mutans* with a significant activity of PEP PTS for xylitol can also minimize the xylitol inhibition at pH 5.0 through inactivation of the xylitol PEP PTS These observations suggest that xylitol inhibits the acid production and growth of *S mutans* most efficiently around neutral pH where PEP PTS for xylitol is synthesized and active

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Fermentation of Five Sucrose Isomers by Human Dental Plaque Bacteria

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Key Words

Acid production, *Actinomyces*, α -D-Glucosyl-D-fructoses, Palatinose, Sucrose, Sucrose isomers

leucrose. We therefore conclude that in human dental plaque there are significant numbers of bacteria that are able to ferment sucrose isomers.

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Abstract

Sucrose has five structural isomers: palatinose, trehalulose, turanose, maltulose, and leucrose. Although these isomers have been reported to be noncariogenic disaccharides which cannot be utilized by mutans streptococci, there is no information about their fermentability by other bacteria in dental plaque. The purpose of the present study was to examine whether these isomers were fermented by predominant bacteria in human dental plaque. Clinical bacterial isolates obtained from dental plaque from 3 children aged 22 months to 50 months (146 strains) were inoculated into 3 ml of peptone yeast extract (PY medium) containing glucose for 1 day, then an aliquot of 20 μ l of culture medium was inoculated into 1 ml of PY medium containing 1% (w/v) of the respective test carbohydrates. After incubation for 1 day, the pH values and the optical density at 660 nm of the cultures were measured. Fermentation ability was measured by pH ≤ 5.5 , growth by an OD₆₆₀ of ≥ 0.5 . Of the clinical isolates, 33% fermented palatinose, and 69% of these were *Actinomyces* species. All of the palatinose fermenting bacterial strains fermented trehalulose, 25% fermented turanose, 70% fermented maltulose, and 23% fermented

leucrose. We therefore conclude that in human dental plaque there are significant numbers of bacteria that are able to ferment sucrose isomers.

Sucrose [α -D-glucopyranosyl (1 \rightarrow 2)- β -D-fructofuranoside] has an α -D-glucoside linkage between a glucose and a fructose unit and five isomeric α -D-glucosyl-D-fructoses. These isomers are palatinose [α -D-glucopyranosyl (1 \rightarrow 6)-D-fructofuranose], trehalulose [α -D-glucopyranosyl (1 \rightarrow 1)-D-fructofuranose], turanose [α -D-glucopyranosyl (1 \rightarrow 3)-D-fructofuranose], maltulose [α -D-glucopyranosyl (1 \rightarrow 4)-D-fructofuranose], and leucrose [α -D-glucopyranosyl (1 \rightarrow 5)-D-fructofuranose].

In order to reduce the incidence of dental caries, sucrose has been replaced by sweeteners, some of which are sucrose isomers. It has been reported that these sucrose isomers are not utilized by oral mutans streptococci as substrates in acid production [Ohta and Takazoe, 1983; Ooshima et al., 1983, 1991; Sasaki et al., 1985; Takazoe et al., 1985; Ziesentz et al., 1989; Minami et al., 1990] or in glucan synthesis [Ohta and Takazoe, 1983; Minami et al., 1990] and, except for turanose, these isomers were observed to inhibit sucrose-dependent cellular adherence of *Streptococcus sobrinus* MT8148 [Minami et al., 1990].

Dental plaque consists of numerous species of microorganisms in addition to mutans streptococci. Among

these bacterial strains *Actinomyces Lactobacillus* and non mutans streptococci are the predominant genera in dental plaque [Hoshino et al 1989 Milnes et al , 1993] Other acidogenic bacteria such as *Propionibacterium Bifidobacterium* and *Peptostreptococcus* are also associated with further development of dentin caries [Hoshino et al 1984] Most of these bacteria are able to metabolize sucrose as well as glucose and fructose to acid and may contribute to acid production in dental plaque tooth surface demineralization and the subsequent initiation and progression of dental caries However there is little information available on the fermentability of the five sucrose isomers by these bacteria The purpose of the present study was to examine whether these five isomers are fermented by the predominant bacteria in human dental plaque

Materials and Methods

Carbohydrates

Sucrose glucose and fructose were purchased from Wako Pure Chemical Co (Osaka Japan) Isomers of sucrose were purchased from the following sources palatinose and trehalulose from Wako Pure Chemical Co leucrose and turanose from Fluka Chemie GmbH (Buchs Switzerland) and maltulose from Tokyo Kasei Organic Chemicals Co (Tokyo Japan)

Bacterial Strains

Clinical bacteria (146 isolates) were isolated from dental plaque on the buccal smooth surfaces of the maxillary first or second deciduous molars of 3 children (ages 22 months 46 months and 50 months) using methods described previously [Uematsu and Hoshino 1992 Sato et al 1993] Briefly plaque samples were suspended in 1 ml of sterilized 40 mM potassium phosphate buffer (pH 7.0) and dispersed with a glass homogenizer Serial 10 fold dilutions (0.1 ml each) were spread on the surface of brain heart infusion blood (sheep) agar (BHI blood agar) plates [Holdeman et al 1977] and incubated at 37 °C for 7 days in an anaerobic glove box (model AZ Hard Hirasawa Tokyo) in an atmosphere of 80% N₂ 10% H₂ and 10% CO₂ After 7 days of incubation all colonies from plates with less than 100 colonies were subcultured Altogether 146 strains were isolated as predominant bacteria and identified according to the VPI manual [Holdeman et al 1977] supplemented with information described in *Bergey's Manual of Systematic Bacteriology* [Kandler and Weiss 1986 Moore et al 1986 Scaal 1986 Scaidovi 1986]

All of the *Actinomyces* strains including reference strains used in this study were classified into *Actinomyces georgiae Actinomyces gerencsiae Actinomyces naeslundii* genospecies 1 and *A. naeslundii* genospecies 2 by PCR RFLP analysis as described previously [Sato et al 1998 Matsuyama et al 2000]

Fermentation of Palatinose

Bacterial strains were inoculated onto BHI blood agar plates and incubated at 37 °C for 3 days in an anaerobic glove box After incubation bacterial strains were inoculated into 3 ml of peptone yeast

extract (PY media) [Holdeman et al 1977] containing glucose and incubated for 1 day and a 20 µl aliquot of each culture was inoculated into 1 ml of PY media containing filter sterilized 1% (w/v) test carbohydrates palatinose sucrose glucose and fructose After incubation at 37 °C for 1 day the pH value of each culture was measured using a pH meter (model HM 35V Toa Japan) and optical density at 660 nm was determined using a spectrophotometer (model U 3200 Hitachi Japan) Postincubation cultures with a pH value of ≤ 5.5 were considered positive for acid production while those having an optical density of ≥ 0.5 at 660 nm were considered positive for growth

Fermentation of Trehalulose Turanose Maltulose and Leucrose

Bacterial strains that were able to ferment palatinose were then further investigated using the methods described above for their ability to ferment trehalulose turanose maltulose and leucrose as well as sucrose glucose and fructose

Furthermore representative strains of human *Actinomyces* species (*Actinomyces israelii* ATCC 10102 *A. georgiae* ATCC 49285 *A. gerencsiae* ATCC 23860 *Actinomyces meyeri* ATCC 35568 *A. naeslundii* genospecies 1 ATCC 12104 *A. naeslundii* genospecies 2 WVU 627 and *Actinomyces odontolyticus* ATCC 17929) were also investigated for their ability to ferment the sucrose isomers sucrose glucose and fructose

Results

Identification of Dominant Bacteria in Dental Plaque

A total of 146 strains (30, 20 and 96 strains from samples A, B and C respectively) were isolated from dental plaque on buccal smooth surfaces as predominant bacteria Table 1 shows the predominant bacterial species of the dental plaque samples Ninety six strains (66%) were identified as *Actinomyces* which constituted the major portion of isolates In all cases *Actinomyces* species constituted the majority (50–71%) of isolates In addition *Micromonas micros* (*Peptostreptococcus micros*) *Propionibacterium* species and *Veillonella parvula* were found in sample A and *Lactobacillus* species *Streptococcus* species and *V. parvula* were found in sample C (table 1)

Acid Production from Palatinose by Clinical Isolates

Of the 146 isolated strains 48 (33%) were able to ferment palatinose and exhibited a culture pH of ≤ 5.5 along with positive growth (table 1) In samples A, B and C the proportions of palatinose fermenting strains were 27% (8 strains) 50% (10 strains) and 31% (30 strains), respectively These 48 strains included *A. georgiae* (1 strain) *A. gerencsiae* (4 strains) *A. naeslundii* genospecies 1 (17 strains), *A. naeslundii* genospecies 2 (11 strains) *Lactobacillus crispatus* (2 strains) *Lactobacillus salivarius* (5 strains) *Propionibacterium abidum* (1 strain) *Propionibacterium acidipropionica* (1 strain) and *Streptococcus*

Table 1 Palatinose fermentation by predominant bacteria isolated from human dental plaque

Bacterial strains	Number of strains palatinose positive/total			
	sample A	sample B	sample C	total
<i>Actinomyces georgiae</i>		1/1		1/1
<i>Actinomyces gerencseriae</i>	3/3	1/1		4/4
<i>Actinomyces naeslundii</i> genospecies 1	2/4	7/9	8/9	17/22
<i>Actinomyces naeslundii</i> genospecies 2	2/2	1/1	8/57	11/60
<i>Actinomyces odontolyticus</i>	0/5			0/5
<i>Actinomyces</i> spp	0/1	0/1	0/2	0/4
<i>Bifidobacterium magnum</i>	0/1			0/1
<i>Lactobacillus brevis</i>		0/3		0/3
<i>Lactobacillus crispatus</i>			2/4	2/4
<i>Lactobacillus salivarius</i>			5/5	5/5
<i>Lactobacillus</i> spp			0/1	0/1
<i>Micromonas micros</i>	0/3			0/3
<i>Propionibacterium abidum</i>	0/1		1/1	1/2
<i>Propionibacterium acidipropionica</i>	1/1			1/1
<i>Propionibacterium propionicum</i>	0/3	0/2		0/5
<i>Streptococcus intermedius</i>			6/6	6/6
<i>Streptococcus mitis</i> biovar 1	0/1			0/1
<i>Streptococcus parasanguinis</i>	0/1			0/1
<i>Streptococcus salivarius</i>			0/4	0/4
<i>Streptococcus sanguinis</i> biovar 2		0/1		0/1
<i>Veillonella parvula</i>	0/4		0/7	0/11
Not identified		0/1		0/1
Total	8/30	10/20	30/96	48/146

Palatinose positive indicates that media pH of postincubation cultures were below 5.5 and that optical density of the cultures was more than 0.5 at 660 nm

intermedius (6 strains) These bacteria also fermented sucrose, glucose and fructose, and showed positive growth with these carbohydrates. The palatinose fermenting *Actinomyces* species were isolated in all the samples while the palatinose fermenting *Lactobacillus* and *S. intermedius* strains were isolated in only sample C (table 1).

Fermentation of Sucrose Isomers by Palatinose Fermenting Bacteria

Forty eight palatinose fermenting strains were tested for their ability to ferment trehalulose, turanose, maltulose and leucrose, but 8 strains were lost during the experimental process. Table 2 shows the number of bacterial strains that were able to ferment the sucrose isomers and the pH values of the media containing these isomers. All of the strains fermented trehalulose as well as palatinose, sucrose, fructose and glucose, and lowered the culture pH below 5.5.

Ten of the 40 strains fermented turanose. These 10 strains included *A. naeslundii* genospecies 1 (5 strains), *A.*

naeslundii genospecies 2 (3 strains), *L. salivarius* (1 strain) and *P. abidum* (1 strain). None of the *A. georgiae*, *A. gerencseriae*, *L. crispatus*, *P. acidipropionica* and *S. intermedius* strains were able to ferment turanose.

Twenty eight of the 40 strains fermented maltulose. These 28 strains included *A. georgiae* (1 strain), *A. gerencseriae* (4 strains), *A. naeslundii* genospecies 1 (6 strains), *A. naeslundii* genospecies 2 (10 strains), *L. crispatus* (1 strain), *L. salivarius* (4 strains), *P. acidipropionica* (1 strain) and *P. abidum* (1 strain).

Nine of the 40 strains fermented leucrose. These 9 strains included *A. georgiae* (1 strain), *A. gerencseriae* (4 strains), *A. naeslundii* genospecies 1 (1 strain), *A. naeslundii* genospecies 2 (2 strains) and *P. abidum* (1 strain).

All strains of *A. georgiae* and *A. gerencseriae* as well as 1 strain of *P. acidipropionica* used in this study fermented all the sucrose isomers except turanose. All strains of *S. intermedius* fermented palatinose and trehalulose, but did not ferment turanose, maltulose or leucrose. Fermentation of turanose, maltulose and leucrose

Table 2 Number of bacterial strains that were able to ferment glucose fructose sucrose and its isomers and their media pH values of postincubation cultures

Strains	Total number of strains	Number of strains giving pH values below 5.5							
		sucrose	palatinose	trehalulose	turanose	maltulose	leucrose	glucose	fructose
<i>Actinomyces georgiae</i>	1	1 (5.0)	1 (5.1)	1 (4.7)	0 (6.0)	1 (4.6)	1 (4.9)	1 (4.5)	1 (4.6)
<i>Actinomyces gerencsiae</i>	4	4 (4.7-4.6-4.8)	4 (5.0-4.6-5.2)	4 (4.7-4.3-5.1)	0 (6.0-5.9-6.2)	4 (4.6-4.5-4.7)	4 (5.3-5.1-5.4)	4 (4.5-4.4-4.5)	4 (4.6-4.4-4.8)
<i>A. naeslundii</i> genospecies 1	11	11 (4.8-4.5-5.5)	11 (5.2-4.7-5.5)	11 (5.3-4.8-5.5)	5 (5.6-5.1-6.0)	6 (5.6-4.9-6.4)	1 (6.0-5.0-6.4)	11 (4.7-4.5-5.1)	11 (4.8-4.5-5.3)
<i>A. naeslundii</i> genospecies 2	11	11 (4.6-4.5-4.8)	11 (4.8-4.5-5.5)	11 (5.0-4.8-5.2)	3 (5.6-4.9-5.9)	10 (5.1-4.7-5.9)	7 (5.8-5.5-6.1)	11 (4.7-4.5-4.9)	11 (4.8-4.5-5.1)
<i>Lactobacillus crispatus</i>	2	2 (4.8-4.6-4.9)	2 (5.0-4.6-5.0)	2 (5.3-5.0-5.5)	0 (5.9-5.8-5.9)	1 (5.6-5.2-5.9)	0 (6.0-5.9-6.0)	2 (4.8-4.7-4.8)	7 (4.9-4.6-5.2)
<i>Lactobacillus salivarius</i>	5	5 (4.6-4.5-4.6)	5 (4.8-4.7-4.8)	5 (4.8-4.7-4.9)	1 (5.7-5.3-5.9)	4 (5.3-4.7-5.8)	0 (5.8-5.7-5.9)	5 (4.5-4.5-4.7)	5 (5.0-4.7-5.2)
<i>Propionibacterium acidipropionica</i>	1	1 (4.7)	1 (4.6)	1 (4.4)	0 (6.0)	1 (4.7)	1 (5.3)	1 (4.6)	1 (4.5)
<i>Propionibacterium acidum</i>	1	1 (4.9)	1 (5.2)	1 (5.0)	1 (4.7)	1 (4.9)	0 (6.1)	1 (4.7)	1 (4.8)
<i>Streptococcus intermedius</i>	4	4 (4.3-4.2-4.4)	4 (4.7-4.6-4.7)	4 (4.8-4.8-4.9)	0 (6.3-6.2-6.3)	0 (6.2-5.9-6.4)	0 (6.5-6.0-6.6)	4 (4.0-4.0-4.0)	4 (4.2-4.1-4.2)
Total	40	40	40	40	10	28	9	40	40

Mean pH values and their ranges are shown in parentheses

Table 3 Fermentation of glucose fructose sucrose and its isomers by representative strains of human *Actinomyces*

Strains	Culture pH and growth							
	sucrose	palatinose	trehalulose	turanose	maltulose	leucrose	glucose	fructose
<i>A. georgiae</i> ATCC 49285	4.5 ^a + ^b	5.2 +	5.2 +	5.9 -	5.5 +	5.5 +	4.5 +	4.7 +
<i>A. gerencsiae</i> ATCC 23860	5.2 +	5.2 +	4.9 +	5.4 +	4.8 +	5.4 +	4.9 +	4.8 +
<i>A. israelii</i> ATCC 12102	4.3 +	4.8 +	4.9 +	4.7 +	4.6 +	5.2 +	4.2 +	4.5 +
<i>A. meyeri</i> ATCC 35568	4.6 +	5.2 +	5.1 +	5.6 -	5.6 -	5.7 -	4.6 +	4.6 +
<i>A. naeslundii</i> genospecies 1 ATCC 12104	4.7 +	5.5 +	5.2 +	5.6 -	5.5 +	5.7 -	4.8 +	4.5 +
<i>A. naeslundii</i> genospecies 2 WVU 627	4.7 +	5.4 +	5.2 +	6.1 -	5.3 +	6.0 -	4.6 +	5.3 +
<i>A. odontolyticus</i> ATCC 17929	5.0 +	6.1 -	6.2 -	6.1 -	6.2 -	6.2 -	5.3 +	5.0 +

^a Mean pH values were obtained from three independent experiments

^b Positive growth was indicated by optical density of more than 0.5 at 660 nm

by the strains of *A. naeslundii* genospecies 1 *A. naeslundii* genospecies 2 *L. crispatus* and *L. salivarius* used in this study was not consistent

Fermentation of Sucrose Isomers by Representative Strains of Human *Actinomyces*

As shown in table 3 *A. israelii* ATCC 10102 and *A. gerencsiae* ATCC 23860 fermented all of the sucrose isomers *A. georgiae* ATCC 49285 fermented all isomers

except turanose *A. naeslundii* genospecies 1 ATCC 12104 and *A. naeslundii* genospecies 2 WVU 627 fermented all isomers except for turanose and leucrose *A. meyeri* ATCC 35568 did not ferment turanose maltulose and leucrose while *A. odontolyticus* ATCC 17929 did not ferment any of the sucrose isomers

Discussion

Among the five isomers, palatinose has been most commonly used as a sugar substitute. No acid production from palatinose has been reported in experiments involving animal caries infected with mutans streptococci [Ooshima et al 1983, 1991, Sasaki et al 1985, Takazoe et al 1985].

In contrast to these results, it has been reported that acid production from palatinose varied among human individuals in vivo [Maki et al 1983]. Although acid production from palatinose was less than that observed from sucrose, acid production from palatinose in dental plaque suspensions was apparently observed. Several bacterial strains of *Streptococcus oralis*, *Lactobacillus casei* subspecies *alactosus*, *Lactobacillus oris*, *Lactobacillus acidophilus*, *Leuconostoc mesenteroides*, *Weissella paramesenteroides*, *Stomatococcus mucilaginosus* [Peltroche Llacsahuanga et al 2001], *Klebsiella pneumoniae* and *Fusobacterium mortiferum* [Thompson et al 2001a, b, Pikiş et al 2002] were recently found to ferment palatinose as an energy source for growth. However, most of these bacteria do not seem to be predominant in human dental plaque. We therefore focused on acid production from predominant oral bacteria isolated from human dental plaque.

The frequent isolation of *Actinomyces* in the present study was in agreement with previous reports of the dental plaque composition of children [Ikeda et al 1978, Boyar and Bowden 1985, Milnes et al 1993]. Out of all isolates in the present study, 33% of predominant plaque bacterial strains fermented palatinose. Among the palatinose-fermenting bacteria, *Actinomyces*, particularly *A. naeslundii* genospecies 1, constituted the major portion. Although isolates of *A. georgiae* and *A. gerencseriae* were few in number in dental plaque, all strains of *A. georgiae* and *A. gerencseriae* fermented palatinose.

The final culture pH with the palatinose fermenting bacteria was not lower than those containing sucrose, glucose and/or fructose media (table 2) and thus the acidogenicity and cariogenicity of palatinose seem to be weaker than those of sucrose, glucose and fructose. However, it is possible that catabolic enzymes for palatinose are induced and/or palatinose fermenting bacteria could be selected when microflora of dental plaque are frequently exposed to the sugar substitute. Such microbial adaptation and shift may enhance acid production from the sugar substitute. In fact, it has been reported that daily mouthrinses of palatinose resulted in slight increases in acid production of dental plaque in vivo [Topitsoglou et al 1984]. With regard to prevention of dental caries, it is thus necessary

to pay attention to microbial adaptation and shift in dental plaque microflora.

It has been reported that some bacteria including *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Weissella*, *Stomatococcus*, *Klebsiella* and *Fusobacterium* can utilize sucrose isomers as carbon sources [Peltroche Llacsahuanga et al 2001, Thompson et al 2001a, b, Pikiş et al 2002]. In addition to these studies, we showed in this study that palatinose fermenting oral strains of *Actinomyces* also utilized sucrose isomers as carbon sources (table 3). We believe that this is the first report that oral *Actinomyces* species are capable of fermenting sucrose isomers. We also demonstrated that oral *Actinomyces* species, except for *A. odontolyticus* ATCC 17929, fermented some of the sucrose isomers. Although these isomers were less acidogenic than sucrose in most cases, *A. gerencseriae* produced more acid from trehalulose and maltulose than from sucrose (table 3). Therefore, it is necessary to monitor the cariogenicity and acidogenicity of these isomers, since microbial shift may enhance acid production from these isomers.

K. pneumoniae and *F. mortiferum* have been reported to transport these five isomers through a phosphoenolpyruvate dependent phosphotransferase system, then hydrolyze them to glucose 6 phosphate and fructose [Thompson et al 2001a, b]. Among the five sucrose isomers, palatinose, trehalulose and maltulose were more likely to be fermented by oral *Actinomyces* species, while leucrose and turanose were less likely to be fermented (table 3). Although the transport and dissimilation systems for the isomers in *Actinomyces* are currently unknown, the present observations imply that the metabolic system for trehalulose and maltulose is common with that for palatinose and independent of that for leucrose and turanose.

In summary, 33% of predominant plaque bacterial strains fermented palatinose and the majority of palatinose fermenting bacteria was *Actinomyces*. In addition, among the palatinose fermenting bacteria, all strains fermented trehalulose, 25% of strains fermented turanose, 70% of strains fermented maltulose and 23% of strains fermented leucrose. We therefore conclude that in human dental plaque, there are significant numbers of bacteria that are able to ferment sucrose isomers.

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