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Quantitative and Qualitative Characterization of Mutans Streptococci in Saliva and in the Dentition

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Academic Dissertation

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Cover photo: Mutans streptococci on mitis salivarius bacitracin agar (MSB). To the left, colony representing *Streptococcus sobrinus.* In the middle and to the right, colonies representing *Streptococcus mutans.*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals, and on some unpublished results.

I. Alaluusua S, Grönroos L, Kleemola-Kujala E. *Streptococcus mutans*, not detected? Oral Microbiol Immunol 1989: 4: 176-177.

II. Grönroos L, Mättö J, Saarela M, Luoma A-R, Luoma H, Jousimies-Somer H, Pyhälä L, Asikainen S, Alaluusua S. Chlorhexidine susceptibilities of mutans streptococcal serotypes and ribotypes. Antimicrob Agents Chemother 1995: 39: 894-898.

III. Alaluusua S, Mättö J, Grönroos L, Innilä S, Torkko H, Asikainen S, Jousimies-Somer H, Saarela M. Oral colonization by more than one clonal type of mutans streptococcus in children with nursing-bottle dental caries. Arch Oral Biol 1996: 41: 167-173.

IV. Grönroos L, Alaluusua S. Site-specific oral colonization of mutans streptococci detected by arbitrarily primed PCR fingerprinting. In press.

V. Alaluusua S, Grönroos L, Zhu X, Saarela M, Mättö J, Asikainen S, Fukushima K. Production of glucosyltransferases by clinical mutans streptococcal isolates as determined by semiquantitative cross-dot assay. Arch Oral Biol 1997: 42: 417-422.

VI. Grönroos L, Saarela M, Mättö J, Tanner-Salo U, Vuorela A, Alaluusua S. Mutacin production by *Streptococcus mutans* may promote transmission of bacteria from mother to child. Infect Immun 1998: 66: 2595-2600.

ABBREVIATIONS

AP-PCR	arbitrarily primed polymerase chain reaction
ATCC	American Type Culture Collection
BHI broth	brain heart infusion broth
bp	base pair
CFU	colony forming units
CHX	chlorhexidine
DMF	decayed, missing, filled
dmf	for deciduous teeth decayed, missing, filled
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GTF	glucosyltransferase
IDH	Institute of Dentistry, Helsinki
IPS	intracellular polysacharide
LB broth	Luria-Bertani broth
MAb	monoclonal antibody
MIC	minimal inhibitory concentration
MS agar	mitis salivarius agar
MSB agar	mitis salivarius agar with sucrose and bacitracin
n	number
NaF	sodium fluoride
NCCLS	National Committee for Clinical Laboratory Standards
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA fingerprinting
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SD	standard deviation
SDS	sodium dodecyl sulfate
0.5xTBE buffer	45 mM Tris, 45 mM boric acid, 1 mM EDTA
TE buffer	10 mM Tris-HCl, 1 mM EDTA
Tris	tris(hydroxymethyl)aminomethane
TSA	trypticase soy agar
TSB	trypticase soy broth
UV	ultraviolet

INTRODUCTION

The mutans streptococci comprise a group of seven species, of which *Streptococcus mutans* and *Streptococcus sobrinus* are the predominant species isolated from human saliva and dental plaque (Loesche, 1986). Experiments with gnotobiotic hamsters revealed these to be the main initiator microorganisms in dental caries disease (Fitzgerald and Keyes, 1960). Dental caries is a common infectious disease world-wide. The aetiology of the disease is multifactorial, life habits and mutans streptococcus infection being the most important factors (Johnson, 1991; Bratthall, 1997). In the disease process, the calcified tissues of the tooth are demineralized and the organic substance is broken down.

In the western world, the prevalence of caries disease has declined, but 5 - 20% of the population age groups remain at high risk (Paunio, 1993; Bolin, 1997; Watt and Sheiham, 1999). In developing countries, the rate of dental caries is rising, and because more than 80% of the world's children live in these countries, dental caries disease is considered to be a major public health problem (Cirino and Scantlebury, 1998). Dental caries disease causes many people to experience a great deal of continuous discomfort through impaired function and aesthetics as well as inconvenient treatment. Dental caries may even lead to life-threatening infections, and the costs for operative dental treatment are significant both for individuals and society. Therefore, a need exists to identify individuals at risk for the disease, and to target preventive measures and active treatment for these individuals. Because mutans streptococci are the main initiator microorganisms in dental caries disease, individuals heavily colonized by the bacteria were earlier thought to automatically be at high risk for the disease, but it became evident that on the individual level the caries risk rate could not be accurately predicted on the basis of how heavily a child or adolescent was infected (Alaluusua, 1993; van Houte, 1993; Tenovuo, 1997). Thus, research on the colonization pattern and the virulence traits of mutans streptococci is essential.

The hypothesis inspiring this study was that individual oral isolates of mutans streptococci can be qualitatively different, expressing varying degrees of virulence characteristics, and that some strains are more likely to be associated with a high probability of transmission between individuals and/or dental caries disease than are others. Previous reports have shown that the oral cavity of an individual can be colonized by one or by multiple clonal types of mutans streptococci (Bowden and Hamilton, 1998). Using a large study population

and numerous intra-individual mutans streptococcal isolates, we wanted to clarify this clonal diversity. The goal of this study was to improve on the basic information needed for research and for preventive dental treatment approaches.

REVIEW OF LITERATURE

General bacteriological aspects of mutans streptococci

Historical background

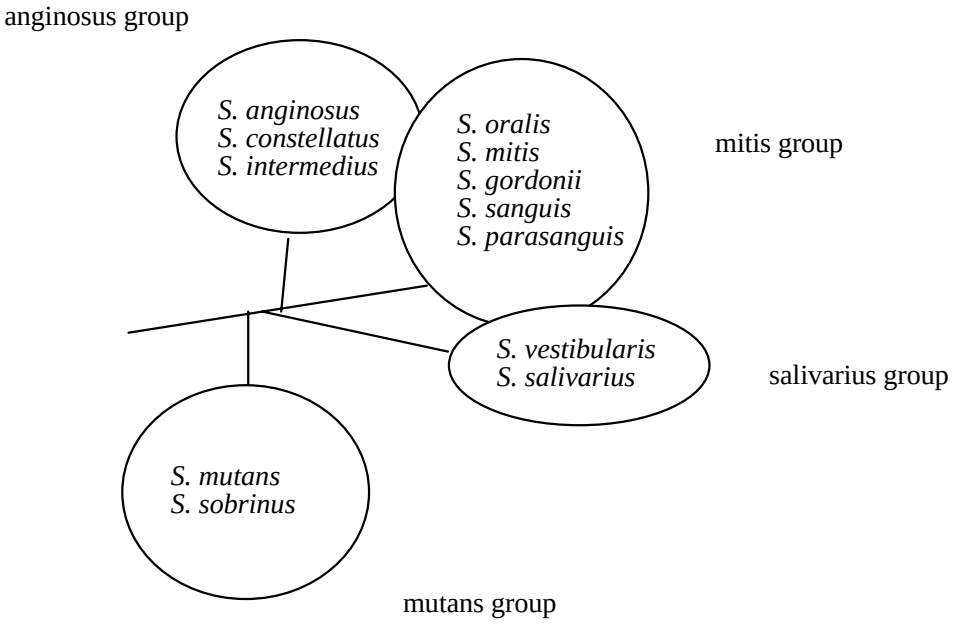
Streptococcus mutans was first described by J. Kilian Clarke in 1924. Kilian Clarke, a microbiologist, had performed his study supported by a research grant from the Dental Diseases Committee of the Medical Research Council. The money for the grant had been raised by British dentists amongst themselves, to support dental research. His task was to study the microbiology of dental caries disease. In deep dentin caries lesions, he found a small, chained coccobacillus which was more oval than spherical in shape. He suggested that these microorganisms were mutant streptococci and called them *Streptococcus mutans* (Clarke, 1924). Clarke tried to prove the association of these streptococci with dental caries disease, but since other researchers did not support his hypothesis, interest in *S. mutans* waned. In the 1960s, the recently developed method of gnotobiotic animal research stimulated studies on the microbiology of dental caries disease, and *S. mutans* was convincingly connected to dental caries disease (Hamada, 1986a).

Taxonomy

According to the classification in Bergey's Manual of Determinative Bacteriology, 9th ed. (Holt et al., 1994), the genus *Streptococcus* includes the pyogenic, oral and anaerobic groups of streptococci, as well as a group of other streptococci. The cells are spherical or ovoid, 0.5-2.0 μm in diameter, occurring in pairs or chains when grown in liquid media, and stain Gram-positive. Streptococci require nutritionally rich media for growth. The metabolism is fermentative, producing mainly lactate but no gas. The streptococci are catalase-negative, and they commonly attack red blood cells, with either greenish discoloration (α-hemolysis) or complete clearing (β-hemolysis). Optimum temperature for growth is 37°C, and growth is usually restricted to 25-45°C. Streptococci constitute a major population in the oral cavity, with several different species colonizing the various ecological niches of the mouth. Some of the species exhibit Lancefield serological group antigens. Differentiation between the

pyogenic, oral and anaerobic groups may be laborious, and combined information is needed for classification (Holt et al., 1994).

Fig. 1. Streptococci commonly found in the human mouth; phylogenetic relationships among groups (information from Russell, 2000).



The oral group has sometimes been named the viridans streptococci, referring to the partial clearing of the erythrocytes around the colony. However, the terms are not interchangeable, as some species classified as viridans streptococci are not detected in the oral cavity. The current classification of the oral streptococci places the bacteria into four species groups; the anginosus, mitis, mutans and salivarius groups (Fig. 1). The classification is based on chemotaxonomic and genotypic data, especially DNA-DNA base pairing and 16S rRNA gene sequence analysis (Whiley and Beighton, 1998). The mutans group includes *S. mutans*, *S. sobrinus, Streptococcus cricetus, Streptococcus rattus, Streptococcus downeii* and *Streptococcus macacae.* Although the phylogenetic position of *Streptococcus ferus* has yet to be determined by 16S rRNA sequencing, other data indicate that *S. ferus* also belongs to the mutans group (Whiley and Beighton, 1998). The mutans streptococci represent eight serotypes (Maiden et al., 1992; Whiley and Beighton, 1998).

Table 1. Characteristics of the mutans streptococci group[a]

	mol% G+C	Serotype	Major cell wall polysacharide constituents[b]
S. mutans	36-38	*c, e, f*	Rha, Glc
S. rattus	41-43	*b*	Rha, Gal, Gro
S. sobrinus	44-46	*d, g*	Rha, Glc, Gal
S. cricetus	42-44	*a*	Rha, Glc, Gal
S. downei	41-42	*h*	ND
S. macacae	35-36	*c*	ND
S. ferus	43-45	*c*	Rha, Glc[c]

[a]Information from Maiden et al., 1992; Whiley and Beighton, 1998.

[b]Abbreviations: Rha, rhamnose; Glc, glucose; Gal, galactose; Gro, glycerol; ND, not determined.

[c]*S. ferus* included in the mutans group by DNA-DNA hybridization, but not by multilocus enzyme electrophoresis.

S. mutans cells are about 0.5-0.75 μm in diameter. *S. mutans* occurs in pairs or in short- or medium-length chains, without capsules. Under acid conditions in broth and on some solid media, these cocci may form short rods 1.5-3.0 μm in length. Rod-shaped morphology may be evident on primary isolation from oral specimens. *S. sobrinus* are about 0.5 μm in diameter. *S. sobrinus* occurs in pairs and in chains. The word sobrinus means male cousin on mother's side and refers to the "distant relationship" between this species and *S. mutans. S. rattus, S. ferus* and *S. cricetus* are about 0.5 μm in diameter, occurring in pairs or chains (Hardie, 1986).

The differentiation within the mutans streptococci is based on differences in biochemical reactions (Table 2) and physicochemical surface properties (van der Mei et al., 1991), and on use of molecular techniques (Whiley and Beighton, 1998).

Table 2. Differential characteristics of the mutans streptococci group[a]

Characteristic	Species						
	S. mutans	*S. rattus*	*S. sobrinus*	*S. cricetus*	*S. downei*	*S. macacae*	*S. ferus*
Growth in air	Δ	Δ	Δ	Δ	+	w	Δ
Growth at:							
10°C	-	-	-	-	ND	ND	-
45°C	Δ	Δ	Δ	Δ	-	-	-
Growth with:							
6.5% NaCl	-	-	Δ	Δ	-	-	-
Hemolysis	γ	ND	γ or α	γ	ND	α	ND
Fermentation of:							
mannitol	+	+	+	+	+	+	+
sorbitol	+	+	Δ	+	-	+	+
raffinose	+	+	Δ	+	-	+	-
inulin	+	+	Δ	Δ	+	-	+
melibiose	Δ	+	-	ND	ND	ND	ND
salicin	+	+	-	+	+	ND	+
trehalose	+	+	Δ	+	+	+	ND
Hydrolysis of:							
arginine	-	+	-	-	-	-	-
esculin	+	+	Δ	Δ	-	+	+

Production of:							
hydrogen peroxide	-	-	+	-	-	-	-
α-galactosidase	Δ[b]	ND	-	ND	ND	ND	ND
β-glucosidase	+	ND	-	ND	ND	ND	ND
Voges-Proskauer test	+	+	+	+	+	ND	Δ
Bacitracin resistance	+	+	+	-	-	-	-
Primary host	human	rat, human	human	hamster, human, wild rat	monkey	monkey	wild rat

---

Δ, 11-89% of strains are positive

+, 90% or more of strains are positive

-, 90% or more of strains are negative

w, weak growth

ND, not determined

α, alpha-hemolysis, greenish discoloration on blood agar

γ, gamma-hemolysis, no clearing

[a]Information from Beighton et al., 1991; Maiden et al., 1992; Holt et al., 1994; Whiley and Beighton, 1998.

[b]According to Beighton et al., 1991, *S. mutans* strains that do not ferment melibiose neither produce α-galactosidase.

Epidemiology

Epidemiological studies show that human populations world-wide carry mutans streptococci (Loesche, 1986). Mutans streptococci have been demonstrated in nearly all subjects in populations with high, low and very low prevalence of caries (Carlsson, 1988). These microorganisms are harboured by 33-75% of 4-year-old children (Carlsson et al., 1975; Alaluusua and Renkonen, 1984; Köhler et al., 1988; Caufield et al., 1993), 80-90% of adolescents, and virtually all adults (Loesche, 1986; Carlson, 1988; Alaluusua et al., 1990). Of the serotypes, *c / e / f* (representing *S. mutans)* and *d / g* (representing *S. sobrinus)* have been detected in humans with high frequency. Some reports of finding serotypes *a (S. cricetus)* and *b (S. rattus)* have also been published (Loesche, 1986; van der Mei et al., 1991). Serotype *c* is predominant in plaque and saliva samples from humans (Loesche, 1986). In samples from Finnish children, 75-90% of *S. mutans* isolates are serotype *c*, 10-20% serotype *e*, and only a few percent represent serotype *f* (Alaluusua et al., 1989, 1994). *S. sobrinus* is not as prevalent as *S. mutans* and is usually detected together with *S. mutans* (Loesche, 1986). The prevalence of *S. sobrinus* has been reported to range between a very low frequency and 30% in different populations. In selected subject groups, a higher frequency of *S. sobrinus* has been reported (Lindquist, 1991). Only a few studies report finding *S. sobrinus* as the sole mutans species, and subjects who harbour both *S. mutans* and *S. sobrinus* tend to have higher salivary mutans streptococcal counts than subjects harbouring *S. mutans* alone (Köhler et al., 1988).

Culture methods and species identification

Mutans streptococci are facultative anaerobes and their optimal growth is at 37°C (Ma and Marquis, 1997).

On MS agar, *S. mutans* colonies are small, raised, irregularly margined and adherent, while *S. sobrinus* colonies are surrounded by a zooglea with a gelatinous consistency (Hamada & Slade, 1980b). On sucrose-containing agar, most strains of *S. mutans* produce colonies of about 1 mm in diameter, with beads, droplets or puddles containing soluble extracellular polysaccharide (Hardie, 1986). On blood agar incubated anaerobically for two days, *S. mutans* colonies are white or gray, circular or irregular, 0.5-1.0 mm in diameter, sometimes tending to adhere to the surface of the agar (Hardie, 1986).

For primary isolation of mutans streptococci, the most frequently used medium is mitis salivarius bacitracin (MSB) agar (Gold et al., 1973), which is composed of mitis salivarius agar with sucrose, bacitracin and potassium tellurite. MSB agar is selective for *S. mutans, S. sobrinus* and *S. rattus.* TYCSB agar contains TYC agar (trypticase, yeast extract and cystine) with sucrose and bacitracin (van Palenstein-Helderman et al., 1983). GSTB agar contains a basal GS agar (trypticase and yeast extract) with glucose, sucrose, bacitracin and potassium tellurite (Tanzer et al., 1984). TSY20B agar contains trypticase soy agar, yeast extract, sucrose and bacitracin (Schaeken et al., 1986). MSKB medium is composed of mitis salivarius agar, sorbitol, kanamycin sulfate, bacitracin and potassium tellurite (Kimmel and Tinanoff, 1991). Differences in culture result when different media are used (Little et al., 1977; Jordan, 1986; Schaeken et al., 1986; Dasanayake et al., 1995). Little et al. (1977) found that the use of blood-sucrose media produced the highest recoveries of mutans steptococci as compared with the selective media MSB and the Carlsson medium. Schaeken et al. (1986) also reported that MSB agar is inhibitory to mutans streptococci, especially *S. sobrinus*, finding higher recoveries on TYCSB agar. Dasanayake et al. (1995) found higher counts on MSB agar than on GSTB agar.

In addition, chair-side methods for detection and enumeration of mutans streptococci have been developed. In the Dentocult SM® dip slide (Orion Diagnostica, Espoo, Finland), a special slide was coated with mitis salivarius agar containing 20% sucrose (Alaluusua et al., 1984). After inoculation of the slide with saliva, two discs containing bacitracin (5 μg) were placed on the agar surface, and the growth density of mutans streptococci was scored after incubation at 37°C for 48 h in an atmosphere created by CO_2-generating tablets that were placed into the cover tubes of the slides. This type of chair-side slide had a short shelf-life because of the mitis salivarius-sucrose agar. The Dentocult-SM® Strip Mutans test was introduced in 1989 (Jensen and Bratthall, 1989). When performing this test, a bacitracin disc is added to the broth at least 15 min before use. After the test subject has chewed a piece of paraffin for at least 1 min, a plastic strip is turned around in the mouth. The strip is withdrawn through closed lips such that a thin layer of saliva remains on the strip. The strip is closed in the cover tube with selective broth and incubated at 37°C for 48 h. After incubation, the strip with attached colonies is compared with the model chart for counts 0 to 3. These strips can be dried and stored for long periods. For validation of method, these chair-side techniques have been thoroughly compared with conventional selective agar plate

culture. The comparison yields a good correlation between the methods with regard to detection of mutans streptococci, as well as counts on agar and score values (Alaluusua et al., 1984; Jensen and Bratthall, 1989).

A number of other chair-side culture methods for mutans streptococci exist, including methods using the adherence ability of isolates and using tooth picks or wooden spatulas for sampling (Matsukubo et al., 1981; Jordan et al., 1987; Bratthall et al., 1996).

The identification of mutans streptococci is based on distinctive colonial morphology on selective and nonselective agar, Gram staining, distinctive cell shape on light microscopy, specific growth characteristics, and sugar fermentation and enzymatic patterns. The identification scheme used in the present study is presented in Table 2. The scheme is based on information in Bergey's Manual of Determinative Bacteriology (9th ed., 1994) and on additional information on phenotypic properties. *S. mutans* isolates can also be identified by the commercial biochemical test system API 20 Strep (Bio Mérieux, Marcy-l'Étoile, France).

Methods for direct detection: monoclonal antibodies and DNA probes

Monoclonal antibodies (MAbs) directed against specific species of mutans streptococci, and also against cell markers and enzymes have been developed. The MAbs have been used in a number of studies on mutans streptococci, for detection in epidemiological studies and in studies on microbial mechanisms (de Soet et al., 1987, 1990a, 1990b; Takei et al., 1992; Fukushima et al., 1993; Shi et al., 1998).

A DNA probe for detection of *S. mutans* was developed by using glucosyltransferase B gene (*gtfB*) and fructosyltransferase gene (*ftf*) fragments, enabling detection without culture (Smorawinska and Kuramitsu, 1992). The dextranase gene *(dexA)* has also been used to construct a DNA probe for detection of *S. mutans* (Ida et al., 1998). For detection of *S. sobrinus*, a DNA fragment in the dextranase gene (SSB-3) has been suggested to be useful (Ida et al., 1999).

Typing of mutans streptococci

Typing of isolates is applied in epidemiological studies to determine bacterial occurrence and modes of transmission. Typing of isolates is also performed for evaluation of whether certain strains are associated with specific clinical disease conditions and to characterize the heterogeneity of infection, i.e., whether subjects are colonized by one or multiple types of the microorganism. Evaluation criteria for typing methods include typeability (ability to give an outcome for every isolate included), reproducibility (ability to give the same result when repeating the analysis) and discriminatory power (ability to differentiate between unrelated strains) (Arbeit, 1999). Two main types of epidemiological typing systems for microorganisms are available, the phenotypic and the genotypic methods.

Phenotyping

Traditional methods of characterizing bacterial isolates have relied on measurement of characteristics expressed by the microorganisms, such as bacteriocin production and sensitivity to bacteriocins, serotype, biochemical properties, antibiotic resistance and bacteriophage type (Maslow and Mulligan, 1996).

Bacteriocin typing

One of the first epidemiological typing systems for oral streptococci was bacteriocin typing (Kelstrup et al., 1970). Bacteriocins are proteinaceous substances produced by the bacteria that inhibit the growth of other, mostly closely related, bacteria. The typing is performed by measuring the inhibiting effect on bacterial growth of certain indicator strains, and by measuring the sensitivity of the bacteria to be typed to bacteriocins from other strains (Jack et al., 1995). Heterogeneity among strains of mutans streptococci within one individual was first shown by bacteriocin typing (Kelstrup et al., 1970).

Serotyping

Using Ouchterlony immunodiffusion, Bratthall demonstrated five serological groups of mutans streptococci (Bratthall, 1970). A total of eight serotypes were subsequently recognized (Perch et al., 1974; Beighton et al., 1981). The classification is based on cell-wall carbohydrate antigen (Table 1). Serotyping by immunodiffusion, immunofluorescence or immunoelectrophoresis has been widely applied for typing of mutans streptococci.

Biotyping

In 1974, Shklair and Keene divided mutans streptococci into five biotypes (a-e) on the basis of fermentation characteristics, arginine hydrolysis and bacteriocin sensitivity, and they reported that these biotypes corresponded with the serotypes reported in 1970. Their later scheme (Shklair and Keene, 1976) also includes serotypes *f* and *g*.

Among other phenotypic methods are cellular fatty acid analysis, whole-cell protein analysis and multilocus enzyme electrophoresis (MEE). MEE is based on the relative electrophoretic mobility of metabolite cellular enzymes. MEE has been successfully applied in studies with many organisms, but only one report using MEE in strain identification of mutans streptococci has been published (Gilmour et al., 1987). Phenotypic typing is, in most cases, relatively inexpensive to perform and typeability of mutans streptococcal isolates is high, however, reproducibility and discriminatory power are usually somewhat poorer (Arbeit, 1999).

Genotyping

For isolate fingerprinting, molecular typing methods have a higher discriminatory ability and reproducibility since these methods do not examine the gene expression but rather the DNA of the microorganisms to be studied (Arbeit, 1999; Olive and Bean, 1999). Among these typing methods are plasmid analysis, restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) (including ribotyping), pulsed field gel electrophoresis (PFGE) and arbitrarily primed polymerase chain reaction (AP-PCR).

Plasmid analysis

Plasmids are extrachromosomal circles of DNA that encode many properties, including antimicrobial resistance, many virulence traits and hydrocarbon metabolism (Madigan et al., 1997a). Plasmid analysis was the first DNA-based technique applied in epidemiological studies on mutans streptococci (Caufield et al., 1982). Because plasmids are infrequently detected in mutans streptococci, in only 5% of strains (Hamada and Slade, 1980b), plasmid analysis is not applicable to typing of these bacteria.

Restriction endonuclease analysis (REA)

In restriction endonuclease analysis (REA), bacterial chromosomal DNA is cut with a restriction endonuclease and separated by gel electrophoresis. The restriction endonucleases are enzymes that cut the DNA chain at specific recognition sequences. The restriction enzymes are nowadays synthetically fabricated, but were originally isolated from bacteria, with their original function being defence against other bacteria. After separation by gel electrophoresis, gels are stained with ethidium bromide and detected under UV light, whereby the banding patterns obtained for different strains are compared. Often the process results in fingerprints with many bands, thus the interpretation of the REA profiles can be complicated. REA has been applied for evaluation of relatedness of mutans streptococcal isolates (Caufield and Walker, 1989; Kulkarni et al., 1989).

Restriction fragment length polymorphism (RFLP), including ribotyping

After cleaving the chromosomal DNA of the microorganisms to be studied, the separation products can be labelled with either DNA or RNA probes in the Southern blot technique (Southern, 1975). The use of a probe derived from the *Escherichia coli* ribosomal operon was introduced by Grimont and Grimont in 1986. They had discovered that variations of the genes encoding ribosomal ribonucleic acid (rRNA), and variations in sites flanking those loci, could serve as a means of typing strains since ribosomal sequences are highly conserved. In ribotyping an isolate, after the gel electrophoresis of the cleaved DNA, the fragments are hybridized with the rRNA probe. When detecting the hybrids, every fragment containing a ribosomal gene will be highlighted. The banding patterns obtained in ribotyping include only a small number of bands, thus rendering comparison of fingerprints among isolates easier than comparing REA patterns. The term ribotyping was introduced in 1988 (Stull et al., 1988), and ribotyping of mutans streptococci has been applied since 1993 (Saarela et al., 1993) mainly in studies on transmission of mutans streptococci and stability of infection (Alaluusua et al., 1994).

Pulsed field gel electrophoresis (PFGE)

In pulsed field gel electrophoresis, a variation of agarose gel electrophoresis, the orientation of the electric field across the gel is changed periodically ("pulsed"), thus larger bacterial DNA fragments can be analysed than by REA (Arbeit, 1999). PFGE is considered the "gold standard" of molecular typing methods, with excellent discriminatory power and

reproducibility (Arbeit, 1999; Olive and Bean, 1999). This method has not been applied for typing of mutans streptococci.

Arbitrarily primed polymerase chain reaction (AP-PCR)

Of the genotyping methods thus far applied to mutans streptococci, AP-PCR is perhaps the least laborious (Olive and Bean, 1999). AP-PCR can be performed with a very small sample volume. For the polymerase chain reaction, the template is annealed to one or more short primers (typically 9-10 bp) at low stringency. Amplification results in an array of DNA fragments, often termed random amplified polymorphic DNA (RAPD), that can be resolved by gel electrophoresis. AP-PCR requires no previous knowledge of the DNA to be analysed (Welsh and McClelland, 1990; Williams et al., 1990). A limitation of the method is that it is very sensitive to even minor variations in technical factors such as temperature, Mg^{2+} concentration and polymerase source. Interlaboratory comparison of typing results is impeded by only a fair reproducibility (Arbeit, 1999), and only isolates processed simultaneously and fingerprints obtained concomitantly can be compared. This fair reproducibility is, however, complemented by a good discriminatory power (Arbeit, 1999). AP-PCR typing has been shown to be well applicable to typing of mutans streptococci (Saarela et al., 1996; Li and Caufield, 1998).

Primary acquisition and transmission of mutans streptococci

Normally, before birth the foetus is sterile. The inoculation of the human oral cavity starts with the first tactile contacts with the mother and other persons present at the parturition, and contact with air and equipment. The oral cavity of the toothless child contains only epithelial surfaces and the first colonizers are species not requiring a nonshedding surface. Early colonizers include some streptococci, *Veillonella, Actinomyces, Fusobacterium* and a few Gram-negative rods (Könönen et al., 1994, 1999). *Streptococcus salivarius* is among the first permanent colonizers, colonizing the dorsum of the tongue in the edentulous infant (Socransky and Manganiello, 1971). In the first months, most of the detected strains are transient colonizers. *Streptococcus sanguis* and the mutans streptococci are stably colonized only after the first tooth has erupted (Berkowitz et al., 1975b).

In general, the acquisition of microorganisms by the human body is by transmission directly from one host to another, or indirectly by means of another living agent (vector). Pathogens

can also be transmitted by inanimate objects and disease vehicles such as food and water (Madigan et al., 1997b). Saliva is regarded as the most important vehicle of transmission of mutans streptococci via physical contact (Duchin and van Houte, 1978; Köhler and Bratthall, 1978) or use of shared objects, e.g. spoons and forks (Bratthall, 1997); mutans streptococci can be recovered from a metal plate 24-48 h after inoculation (Köhler and Bratthall, 1978). The mother is considered to be the most important source of infection for the child, the first indication of this obtained by using a phenotypic typing technique of *S. mutans*, bacteriocin typing (Berkowitz and Jordan 1975a; Masuda et al., 1985). Li and Caufield (1995) detected by molecular typing of mutans streptococcal isolates a homology of strains in 71% of mothers and their children, and Kozai et al. (1999) found that 51.4% of mutans streptococcal genotypes found in children were also identified in their mothers. Fathers and infants have a far lower strain match (Rogers, 1981; Davey and Rogers, 1984; Kulkarni et al., 1989; Li and Caufield, 1995, 1998). Only Kozai et al. (1999) found an abundant similarity of strains in fathers and children, 31.4% of strains detected in children were in agreement with those in their fathers. The time period when most children gain mutans streptococci in their oral flora is when the primary teeth are erupting, i.e., between 8 months and 3 years of age (Caufield et al., 1993). The probability of colonization with mutans streptococci is high when inoculation with mutans streptococci is frequent and microbial cell count is at least 10^5 per ml saliva (Berkowitz et al., 1981). Another suggested prerequisite for early colonization is that the baby's diet includes frequent intake of refined carbohydrates (Alaluusua, 1991a). Recently, it has been shown that mothers can diminish the probability of transmitting mutans streptococci to their children by using xylitol chewing gum (Söderling et al., 2000). The exact mechanism of action regarding xylitol is unknown. The sugar substitute xylitol is not fermented by mutans streptococci into cariogenic acid end-products, and in the oral environment, xylitol presumably selects for mutans streptococci with a weakened virulence (Trahan, 1995). It has previously been shown that in mothers harbouring high numbers of mutans streptococci in their saliva, preventive measures aimed at decreasing mutans streptococcal counts also result in decreased caries counts in their children, even after 15 years (Köhler et al., 1982, 1983, 1994; Bratthall, 1997).

Occurrence of mutans streptococci in the oral cavity

The primary habitat of *S. mutans* and *S. sobrinus* is the human dentition. In addition, after mutans streptococci have colonized the dentition, they can be detected in saliva, on the tongue, on oral mucous membranes, on denture surfaces and on surfaces of orthodontic appliances. When a mutans streptococcal strain has achieved stable colonization of the oral cavity, the strain usually persists for a long time period (Alaluusua et al., 1994). Mutans streptococci can also be detected in faeces (Hamada et al., 1980a), but otherwise, very few reports on finding mutans streptococci outside the oral cavity are available. The organism has been detected in endocarditis (Parker and Ball, 1976) and in purulent eyes of a neonate (Reeder et al., 1985).

Dentition

The first tooth erupts at the age of about 8 months (± 2 months) (Kreiborg 1991), and only after that can there be stable colonization by mutans streptococci. Mutans streptococci can occur in the dentition on the tooth surfaces in dental plaque and in carious enamel or dentin.

In enamel caries, the subsurface tissue is invaded by microorganisms. The identification of the bacteria invading dental enamel is as yet incomplete, but in experiments with gnotobiotic rats, in combination with scanning electron microscopy, *S. mutans* and *S. sobrinus* have been shown to be able to invade the enamel (Luoma et al., 1987; Seppä et al., 1989).

In carious dentin, the number of bacteria recovered per mg of dentin has been reported to be higher in superficial layers of the lesion as compared with deeper layers, with regard to both mutans streptococci and total bacterial count (Edwardsson, 1974; Hoshino, 1985). Many bacterial species are present, but no evidence suggests that a specific combination of organisms acting in symbiosis are involved in the decomposition of dentin (Edwardsson, 1974).

The dental plaque consists of cells (about 70% of the overall volume) and plaque matrix (Jenkins, 1978). The water content ranges between 80% and 85%. Of the dry weight, 40-50% is protein, 13-17% carbohydrate, 10-14% lipid and 10% ash. The concentrations of calcium and phosphate are quite variable, but typical figures are calcium 8 μg per mg and

inorganic phosphate (as P) 16 μg per mg (Jenkins, 1978). Plaque maximum thickness on smooth surfaces is 300 μm, on approximal surfaces 5 mm and in fissures 2 mm (Sissons, 1997). The microbial composition of dental plaque differs both qualitatively and quantitatively from the bacterial communities of other oral surfaces, and the microflora also varies over time. Plaque microorganisms exhibit a high genetic heterogeneity, with 30 to 300 species represented (Sissons, 1997). This conglomerate of bacteria and interbacterial substances can be regarded as a prototype for a biofilm, a bacterial community. The biofilm forms a dynamic link between the microbial flora, the tooth surfaces, and the different constituents of saliva, with the microorganisms living in symbiosis and antibiosis with each other (Costerton and Lewandowski, 1997). Survival of oral bacteria is enhanced by dental plaque formation (Bowden and Hamilton, 1998).

The frequency of isolation of mutans streptococci in dental plaque has been reported to be site-dependent, such that the isolation frequency steadily increases from approximal surfaces of mandibular incisors, approximal surfaces of maxillary incisors, approximal surfaces of molars, to fissures of molars (Loesche, 1986). *S. mutans* and *S. sobrinus* show a somewhat similar colonization pattern in the dentition, but *S. sobrinus* is more frequently isolated from posterior than anterior teeth. In subjects harbouring both *S. mutans* and *S. sobrinus*, the species colonize buccal surfaces in comparable numbers, but on all other surfaces, *S. mutans* is predominant (Lindquist, 1991).

Saliva

Saliva is a complex mixture of several components (Whelton, 1996). Whole saliva (oral fluid) is formed primarily from salivary gland secretions, but also contains gingival fluid, desquamated epithelial cells, bacteria, leucocytes, and possibly food residues, blood and viruses (Dawes, 1996; Whelton, 1996). Saliva is essential for maintenance of healthy oral tissues; it coats the oral mucosa and protects against irritation, forms an ion reservoir for tooth remineralization, functions as a buffer, aids in swallowing, exerts antimicrobial action, participates in pellicle formation and enzymic digestion of starch with amylase, and also participates in taste sensation by acting as a solvent (Whelton, 1996). Antimicrobial components in saliva include immunoglobulins, lysozymes, lactoferrins, salivary peroxidases, myeloperoxidases, histatins, amylases and anionic proteins (Bowen, 1996; Tenovuo, 1998). Moreover, organic components in saliva, especially mucous glycoproteins,

function as nutrients for many oral bacteria (Bowen, 1996). Salivary proteins can be degraded by proteases produced by, for example, *S. mutans* and *S. sanguis* (Bowen, 1996).

The bacterial content of saliva is estimated to approach 10^9 bacteria per ml (Bowen, 1996). Saliva helps to control invasion of the mouth by microorganisms, and lack of saliva results in increased numbers of bacteria in the mouth. Saliva can act as a selective medium for bacterial growth, but continuously repeated swallowing results in clearing of bacteria (Bowen, 1996).

Salivary mutans streptococcal counts rarely exceed 10^7 CFU per ml. A highly significant correlation has been demonstrated between the salivary numbers of mutans streptococci and their prevalence in the dentition, both in terms of the number of tooth surfaces colonized and the level of infection of tooth surfaces (Duchin and van Houte, 1978; Lindquist, 1991).

Clinical illness connected with mutans streptococci

Dental caries - a multifactorial disease

Dental caries disease includes a breakdown of enamel, the hardest material in the human body, and a subsequent breakdown of the underlying dentin. The disease is the most prevalent of the chronic diseases affecting the human race. The aetiology of dental caries disease is multifactorial in that simultaneous participation of multiple factors is required for caries to occur (Tanzer, 1992). The original still-prevailing theory explaining the disease process implicates carbohydrates, oral microorganisms, and acids as the main factors in the caries process. This acidogenic theory (or Miller's chemoparasitic theory, 1902) states that "Dental decay is a chemico-parasitic process consisting of two stages, the decalcification of enamel, which results in its total destruction, and the decalcification of dentin, as a preliminary stage, followed by dissolution of the softened residue. The acid which affects this primary decalcification is derived from the fermentation of starches and sugar lodged in the retaining centers of the teeth." Miller isolated many microorganisms from the human oral cavity, some acidogenic and some proteolytic. Miller believed that caries is caused by a variety of microorganisms. Two other theories explaining the disease process are the proteolytic theory and the proteolysis-chelation theory (Shafer et al., 1983).

Today, mutans streptococci are considered to be the main aetiological microorganisms in caries disease, with lactobacilli and other microorganisms participating in the disease progression (Tanzer, 1992). Occasionally, some other microorganisms have been traced as initiator microorganisms. Severe dental caries has been induced in hyposalivated rats infected with *Lactobacillus fermentum* (Ooshima et al., 1994). Acidogenesis at a low pH has also been reported for a group of non-mutans streptococci (van Houte et al., 1991).

Nursing caries

When the caries disease has a very fast progression it is called rampant caries. A specific form of rampant caries involves the dentition of very young children. The condition includes a demineralization and cavitation of the labial surfaces of the maxillary primary incisors, followed by involvement of the first primary molars (Ripa 1988). The disease onset is between 1 and 2 years of age. A variety of names have been given to the condition; nursing caries, nursing bottle caries, early childhood caries, baby bottle tooth decay and baby bottle caries (Curzon and Pollard, 1994; Tinanoff, 1997). The prevalence of nursing caries in different populations varies between 1% and 80% of preschool children, the proportion of affected children being very low in many western societies (Curzon and Pollard, 1994; Milnes, 1996). Some reports have been made on increasing incidence of this condition in western countries (Duperon, 1995). In Finland, the prevalence can be estimated at about 1%, exact figures being unavailable (Paunio, 1993; Alaluusua, 1999). The aetiology of this condition is also considered multifactorial. In most cases, the affected child has frequently received fermentable carbohydrates as a drink when going to sleep, combined with a lack of toothbrushing with fluoride toothpaste (Curzon and Pollard, 1994). Moreover, the combination of frequent breast-feeding and low additional fluoride use are considered to be contributing factors in the process of nursing caries (Hallonsten et al., 1995; Weerheijm et al., 1998). In children with nursing caries, mutans streptococci can be so dominant that the plaque above the caries lesions consists almost entirely of these organisms (van Houte et al., 1982).

Mutans streptococci in caries prediction

The fact that dental caries is a world-wide disease requiring vast economic resources and causing a great deal of discomfort has called upon attempts aimed at developing an accurate

screening method for detection of the 5-20% of subjects comprising the high-caries-risk group (Pienihäkkinen, 1987; Roeters, 1994; Hausen, 1997). Because mutans streptococci are considered to be the predominant pathogens of dental caries disease, individuals heavily colonized by mutans streptococci were thought to automatically be at high risk for caries. Indeed, in young children, early mutans streptococcal colonization on tooth surfaces has been recognized as an indicator of later high scores of decayed, missing and filled surfaces in deciduous teeth (dmfs index) (Alaluusua and Renkonen, 1983; Köhler et al., 1984, 1988; Jokela, 1997). However, it has become evident that, although prevalence of the infection is indicative of the disease status on a population level, when it comes to an older child or adult, on the individual level, the caries risk rate cannot be accurately predicted on the basis of how heavily the subject is colonized by mutans streptococci (Stecksen-Blicks, 1985; Alaluusua et al., 1987, 1990; Alaluusua, 1993; Alanen et al., 1994; Hausen, 1997).

Virulence factors of mutans streptococci

The term virulence describes the capacity of a parasite (a microorganism) to cause disease to its host, the organism it lives on or in. The property is quantitative and expresses the degree of pathogenicity, the ability to inflict damage to the host. The relationship between host and parasite is dynamic and depends on their individual characteristics and their interrelationship as well as on external factors. Virulence consists of bacterial properties required in the interaction between host and parasite, factors that promote the entry, colonization and growth of the pathogen within the host, including those required for opposing host defences and for nutrient acquisition (Madigan, 1997b). The virulence factors of microorganisms can be studied by the classical approach, by isolating bacteria from healthy and diseased subjects and comparing the phenotypic properties of these microorganisms. During the last two decades, approaches utilized in virulence studies have also included molecular biology methods, techniques for the manipulation of DNA *in vitro* (Hensel and Holden, 1996). Studies on the expression of the modified bacterial chromosome can subsequently be extended to cell culture and animal experiments.

As regards the mutans streptococci, properties that affect their ability to cause dental caries disease are virulence factors promoting their colonization and survival in the biofilm, the dental plaque, that covers the tooth surfaces. Recognized virulence factors of mutans streptococci are adhesin-like cell surface proteins, acid tolerance, acid production, and

production of glucosyltransferases, mutacin and intracellular polysaccharides (Kuramitsu, 1993). In addition to the recognized virulence factors, other properties of the microorganism may influence virulence. One of the suggested virulence factors is the proteolytic activity of mutans streptococci (Homer et al., 1990; Harrington and Russell, 1994; Jackson et al., 1997). *S. mutans* has been shown to produce two extracellular proteases, possibly metalloproteases, capable of degrading both gelatin and collagen-like substrates (Harrington and Russell, 1994). Currently under debate is whether metalloproteases detected in connection with mutans streptococci are produced by the microorganism or whether they are host-derived (Tjäderhane et al., 1998). Regarding the sIgA protease activity of mutans streptococci, the presiding view is that the organism itself does not produce this protease (Marcotte and Lavoie, 1998). Many oral streptococci do produce sIgA protease, which impairs the host defence by cleaving the secretory IgA present, and apparently mutans streptococci benefit from protease produced by the primary colonizers (Marcotte and Lavoie, 1998). Another trait enabling survival is the ability of mutans streptococci to rapidly adapt to the environment by microbial genetics phenomena; this property has been suggested to be an essential element in the dominance of *S. mutans* in cariogenic dental plaque (Burne et al., 1997; Burne, 1998). As a rule, the ability of cells to take up exogenous DNA, the regulation of natural genetic competence in bacteria, is dictated by nutritional conditions and cell-to-cell signalling (Solomon and Grossman, 1996).

Factors affecting adherence ability

The mutans streptococci synthesize extracellular polysaccharides from sucrose to increase their stickiness. Surface proteins of *S. mutans* also participate in adherence. In *S. sobrinus*, the adherence is probably primarily mediated by extracellular polysaccharides, with a minor influence by surface proteins (Gibbons et al., 1986). In addition to the microbial properties, host factors may affect adherence, and salivary components can function as receptors in oral pellicles for microbial adhesion to host surfaces (Scannapieco, 1994).

Glucosyltransferases and fructosyltransferases

Glucosyltransferases (GTFs) and fructosyltransferases (FTFs) catalyse the synthesis of water-soluble and water-insoluble glucan and fructan polymers from sucrose (Loesche, 1986). The nucleotide sequences of *gtf* genes from different oral streptococci comply with

the same basic pattern, and the GTFs are approximately 1500 amino acids long (Russell, 1994). Streptococcal GTFs have two common functional domains. The amino-terminal portion, the catalytic domain, is responsible for the cleavage of sucrose, and the carboxyl-terminal portion, the glucan binding domain, is responsible for glucan binding (Colby and Russell, 1997). *S. mutans* produces at least one FTF and three GTFs (Sato and Kuramitsu, 1986; Russell, 1994). GTF-I and GTF-SI enzymes, products of *gtfB* and *gtfC* genes, primarily catalyse the synthesis of water-insoluble glucans, whereas GTF-S, the product of *gtfD*, mainly catalyses the synthesis of water-soluble glucans. *S. sobrinus* has four *gtf* genes (Russell, 1994). Of the four GTFs of *S. sobrinus*, GTF-I produces water-insoluble glucans, while the other three produce water-soluble glucans (Hanada et al., 1993). The three *gtf* genes from *S. mutans* have been thoroughly assessed by comparative sequence analysis, revealing that interstrain differences of *gtfB* and *gtfD* are limited, but *gtfC* exhibits significant interstrain variability (Fujiwara et al., 1998).

The *in vitro* effects of the GTFs have been extensively studied; however, few studies with clinical strains have been performed to date.

Surface proteins

S. mutans cells express a predominant surface protein called P1 (or I/II, B, IF, SR or PAc), which functions in the binding of mutans streptococci to human salivary pellicle-coated surfaces (Crowley et al., 1999). The apparent functional equivalent to this protein is the spaA protein of *S. sobrinus* (Kuramitsu, 1993).

Acidogenicity and acid tolerance

The mutans streptococci ferment many different sugars, and they appear to metabolize sucrose to lactic acid more rapidly than other oral bacteria. This is thought to be related to the multitude of enzyme systems catalysing the reactions of transport and metabolism of sucrose expressed by these organisms (Kuramitsu, 1993). These metabolic reactions render the dental plaque acidic in the presence of a fermentable carbon source, and the acid tolerance of the mutans streptococci enables them to continue metabolisms even at low pH. It has been demonstrated that strains of mutans streptococci are more acid tolerant than all other bacteria examined, with the exception of lactobacilli (Loesche, 1986). An inducible

property exists in mutans streptococci which permits adaptation to acidic environments (Hamilton and Buckley, 1991; Birkhed et al., 1993). This property of acid tolerance (or acidurance) appears to be connected with the membrane-associated H^+(proton)-translocating ATPase of these organisms (Bender at al., 1986).

Mutacin production

Many bacteria produce bacteriocins, i.e. antibacterial peptides, to interfere with the growth of other closely related microorganisms (Jack et al., 1995). Bacteriocins are ribosomally synthesized and usually require extensive posttranslational modification for activity. The genes involved in the synthesis and modification of bacteriocins are often carried by a plasmid or a transposon (Madigan et al., 1997a). Bacteriocins are frequently named according to the bacterial species producing them; bacteriocin produced by mutans streptococci is called mutacin. Mutacin production is usually not plasmid encoded (Caufield et al., 1990). When bacteriocin activity is plasmid encoded, the plasmid generally also confers bacteriocin immunity to the microorganism (Jack et al., 1995). Some bacteriocins also have a commercial value. Nisin, which is produced by *Lactococcus lactis* strains, has been used for more than 30 years as a preservative in the food industry (Jack et al., 1995).

Several mutacins have been purified and biochemically characterized (Fukushima et al., 1982; Ikeda et al., 1982; Hamada et al., 1986b; Loyola-Rodriguez et al.,1992; Novak et al. 1994; Chikindas et al., 1995). In replacement experiments, strains producing increased amounts of mutacin have been shown to colonize more easily (van der Hoeven and Rogers, 1979; Hillman et al., 1987).

Production of intracellular polysaccharides

Most strains of *S. mutans* and *S. sobrinus* produce intracellular iodine-staining polysaccharides (IPS) from sucrose, which, according to results from experiments with rats, may contribute to their virulence (Kuramitsu, 1993). Because of this intracellular polysaccharide storage, these cariogenic bacteria have the ability to continue fermentation in the absence of exogenous food supplies (Loesche, 1986).

Control of mutans streptococci by chlorhexidine (CHX)

The traditional preventive treatment approaches for dental caries disease include dietary counselling on reduced intake of refined sugars, oral hygiene instruction and topical application of fluoride. To aid these preventive measures, antimicrobial preparations, such as chlorhexidine (CHX), can be used to reduce the numbers of mutans streptococci colonizing an individual (Emilson and Fornell, 1976; Twetman and Petersson, 1999). The outcome of all these measures, including CHX use, is largely dependent on patient compliance.

Chlorhexidine, developed in the 1950's, is a bisbiguanide that typically exerts its action on Gram-positive bacteria, particularly on mutans streptococci (Hennessey, 1973; Emilson, 1994; Järvinen et al., 1995). Clinically, the reduction in mutans streptococcal counts not only reduces the caries increment, but may also reduce the probability of transmission of mutans streptcocci from mothers to their young children (Emilson, 1994; Köhler and Andréen, 1994). The antibacterial action of CHX is based on its adsorption on bacterial surfaces. CHX can be administered in solutions, dental gels, varnish or even chewing tablets (Luoma, 1992; Nuuja, 1992). The combination of CHX with sodium fluoride is a more potent inhibitor of acidogenic streptococci than either CHX or fluoride alone (Luoma, 1972; McDermid et al., 1985). The inhibiting effect of CHX on plaque formation has been reported to persist in human subjects even upon 2 years of continuous use (Gjermo and Eriksen, 1974). Surfaces which are heavily infected by mutans streptococci are more rapidly recolonized after antimicrobial treatment. In practice, posterior teeth are recolonized more readily than other tooth surfaces (Lindquist, 1991). Adverse effects of CHX are rare, but both IgE-mediated local responses and anaphylactic shock reactions may occur (Ebo et al., 1998).

CHX resistance

Bacterial resistance to antiseptics and disinfectants may be an inherent or acquired property of an organism. Antimicrobial resistance can evolve through acquisition of genetic material, such as plasmids or transposons, or by mutation. Thus far, laboratory tests have failed to conclusively demonstrate the possibility of "training" organisms to become chlorhexidine-resistant (Russell and Day, 1993). Many bacterial species, e.g. *Proteus* and *Providencia*, have an intrinsic chlorhexidine resistance (Russell and Day, 1993).

AIMS OF THE STUDY

For improvement of high-caries-risk subject selection and for planning of preventive dental treatment approaches, more basic information is required on the virulence characteristics and colonization patterns of mutans streptococci. To this end, two longitudinal and two cross-sectional studies were planned to assess the quantitative and qualitative characteristics of oral mutans streptococci. The classical approach chosen consisted of isolating and characterizing strains from healthy and diseased subjects, and comparing the culture and characterizing results with clinical findings. The microbial typing methods included the molecular biology methods of ribotyping and AP-PCR typing combined with phenotypic methods.

The specific aims of this study were:

1. to follow the detection of oral mutans streptococci in subjects in whom these microorganisms were initially not detected,

2. to study the number of clonal types of mutans streptococci detected within one individual, to compare clonal types between individuals, and in young children, to compare the intra-individual clonal diversity of mutans streptococci with clinical caries status,

3. to study a possible site-specificity of mutans streptococcal infection within the oral cavity of an individual, and to draw inference from this for sampling techniques,

4. to examine the *in vitro* differences in chlorhexidine (CHX) susceptibility of mutans streptococcal isolates of different serotypes and genotypes, to elucidate whether mutans streptococci have evolved CHX resistance as compared with earlier reports, to examine the *in vivo* changes in CHX susceptibility of isolates and in levels of salivary mutans streptococci after 1 year of periodic use of low-concentration CHX-NaF gel in an instructed trial, and to examine the stability of the infection during the treatment, and

5. to study the glucosyltransferase production and mutacin activity of individual fingerprinted strains isolated from mothers and their young children, and to compare these results on phenotypic properties with the event of transmission of strains from mother to child and to clinical caries status of the child.

MATERIALS AND METHODS

Subjects and trial conditions

The study subjects comprised a total of 402 participants in two longitudinal studies and two cross-sectional studies. A more detailed analysis was performed using mutans streptococcal isolates from 24 adolescents, 40 mothers with their 1.5- to 3-year-old children (one twin pair) and seven 3- to 7-year-old children.

Adolescents in follow-up on colonization of mutans streptococci (study I and unpublished results)

A group of 182 adolescents aged 12 to 17 years were invited to participate in a longitudinal study on salivary levels of mutans streptococci. Since their birth the subjects had been annually attending a longitudinal study on facial development and oral health at the Department of Pedodontics and Orthodontics, Institute of Dentistry, University of Helsinki (Haataja et al., 1976; Nyström, 1982; Nyström et al., 1990; Könönen and Nyström, 1993) (Table 3).

Table 3. Adolescents over an 8-year follow-up on colonization of mutans streptococci

	12- to 17-year-old adolescents invited to participate (n=182)
Sampling I:	Chair-side test for mutans streptococci (n=**146**); mutans streptococci not detected in 24 subjects
Sampling II:	Annual chair-side test for mutans streptococci (n=23)
Sampling III:	Annual chair-side test for mutans streptococci (n=24)
Sampling IV:	Annual chair-side test for mutans streptococci (n=24), complemented by conventional agar plate culture of pooled plaque samples (n=10)
Sampling V:	Five years later, chair-side test and conventional agar plate culture for detection of mutans streptococci (n=22)

After excluding subjects who had been on antibiotics or chlorhexidine during the last two months, and those who had fixed orthodontic appliances, a salivary sample was obtained from 146 subjects. The subjects were annually sampled and examined clinically for decayed, missing and filled teeth during the next three years. At the first sampling, 24 subjects (8 girls, 16 boys) had undetectable levels of mutans streptococci as assessed by a chair-side dip-slide method (Dentocult-SM®). In 10 of these 24 adolescents, on the fourth sampling occasion, the salivary examination was complemented by an examination of mutans streptococci in plaque. The results from this 3-year follow-up with four sampling occasions

are presented in study I. Eight years after the first microbiological examination, salivary samples from 22 of these 24 subjects were assessed for mutans streptococci using a chair-side method complemented by conventional agar plate culture, and clinical examinations were performed. The results from the 8-year follow-up examination are presented in this thesis.

Mothers and their children in a preventive treatment programme (II, III, V, VI and unpublished results)

The study population for study II and a portion of the subjects in studies III, V and VI were participants in a preventive programme that targeted pregnant women at a community health centre in Espoo, Finland. A total of 153 mothers were screened before childbirth for mutans streptococci in saliva by a commercial strip method (Dentocult-SM STRIP MUTANS test), and subjects with Dentocult-SM STRIP MUTANS test scores of 2 and 3 (corresponding to counts of •100 000 CFU/ml, according to the manufacturer) (n=105) were randomly selected for the test group (n=75) or control group (n=30). The mothers in the test group brushed their teeth periodically with a gel that contained 0.3% CHX digluconate and 0.2% NaF, pH 5.8. Mothers' gel use occurred when the child was between 6 months and 3 years of age, with the gel being used twice a day for the first 10 days of each month. The control mothers were treated according to the regular prophylaxis protocol provided for all pregnant women at the health centres in Espoo, without periodic CHX-NaF gel use. The children were screened at the ages of approximately 18 and 36 months for the colonization of mutans streptococci (Table 4). At the samplings, the subjects were examined clinically for decayed, missing and filled teeth.

Table 4. Mothers and their children in a preventive treatment programme using CHX-NaF gel

Sampling I:	Mothers screened for mutans streptococci in saliva before childbirth by Dentocult-SM STRIP MUTANS test (n=**153**)		
	Mothers with test scores of 2 and 3 (n=105)		
	test group (n=75)		control group (n=30)
	Mothers included for 3 additional samplings,		
	when child about		
Sampling II:	- 6 months		
Sampling III:	- 18 months		
Sampling IV:	- 36 months		
	Samples from children at about		
(n=53)	- 18 months		(n=27)
(n=47)	- 36 months		(n=15)

In study II, 24 mothers from the test group and 10 mothers from the control group were included for examination of the effect of CHX on salivary mutans streptococcal levels, and for testing of CHX susceptibility of their mutans streptococcal isolates. The CHX study was started when the preventive programme was still ongoing, and those subjects who had already attended three sampling occasions were also included in the CHX study.

In study III, on occurrence of multiple clonal types of mutans streptococci in children, and study V, on GTF production by mutans streptococci, isolates from six mother-child pairs were included; three from the test group and three from the control group. The mother-child pairs included were those in the test and control groups who were first found culture-positive regarding mutans streptococci in the child.

In study VI, on mutacin production by *S. mutans*, isolates from 13 mother-child pairs were included; eight from the test group and five from the control group. Seven of the children harboured mutans streptococci, and six children did not. The mother-child pairs with mutans streptococci not detected in the child were chosen at random from among the mothers included for ribotyping of isolates in the CHX susceptibility study.

Nursing-caries children and their mothers (III, V, VI)

A group of six 1.5- to 3-year-old children with nursing caries and their mothers participated in studies III, V and VI.

In study III, the number of clonal types of mutans streptococci detected in plaque from the six children with nursing caries were compared with the number of clonal types detected in six age-matched caries-free children of the preventive treatment programme.

Mutans streptococcal isolates from these children and their mothers were also studied for production of glucosyltransferases (V).

In study VI, mutacin production by mutans streptococcal isolates from the nursing-caries children and their mothers was studied. We also included mutans streptococcal isolates from the twin brother of one of the children.

Caries-active children referred for dental treatment under general anaesthesia (IV)

Seven children referred to the Helsinki University Dental Clinic for treatment under general anaesthesia were enrolled in a study on the intra-individual clonal diversity of mutans streptococci in caries-active children. The children were 3 to 7 years of age and had a mean number of 7.6 decayed teeth (range, 3 - 15). Their general health was good and they were not on medication.

The study protocols were approved by the Ethics Committee of the Institute of Dentistry, University of Helsinki, or by the Ethics Committee of the Espoo Health Centre. Informed consent was obtained from the subjects or their parents.

In study VI, we included 39 mutans streptococcal isolates from our IDH (Institute of Dentistry, Helsinki) collection. Of these, 38 isolates had been obtained from five initially 5-year-old children who had attended a longitudinal study on dental health up to the age of 12 years (Alaluusua et al., 1989). These five children were caries-free or had a moderate caries activity (dmfs range 0-8, mean 1.1). For mutacin activity testing, a clinical strain from the IDH collection exhibiting very distinct antibacterial activity was used as a control. This strain had originally been isolated from the mouth of a Japanese child (Alaluusua et al., 1991b).

Microbiological examination

Sampling

Paraffin-stimulated salivary samples were obtained from the adolescents (I), the mothers (II, III, V, VI) and the children referred for dental treatment under general anaesthesia (IV) by collecting saliva into receptacles over a 5-min period while the subject was chewing a piece of paraffin. From the mothers participating in the preventive programme, saliva was collected on four occasions: before childbirth, when each child was 6 months old, one year later and finally when the child was about 3 years old. In the test group, salivary samples were taken 7 to 11 days after the CHX-NaF gel usage period.

From 10 of the adolescents in follow-up (I), on the fourth sampling occasion, two pooled plaque samples were collected. One sample was collected with a sharp sterile dental explorer from the occlusal surfaces of the premolars and molars of an upper and the opposite lower quadrant teeth. Another sample was collected from the corresponding approximal contacts by using dental floss (Butler, unwaxed floss), which was drawn through the approximal contacts and moved buccolingually 4 to 5 times to and fro in the contact area. Samples from the seven nursing-caries children and the children in the preventive programme were obtained by taking a pooled plaque sample with a sterile explorer. Follow-up samples also included, when possible, those taken with dental floss from one or two approximal contact sites between a first and second primary molar.

The children in the preventive programme were sampled on two occasions, at about 18 months and 36 months of age. First, plaque samples were obtained from 80 children (53 in test group and 27 in control group), and on the second occasion, from 62 children (47 test and 15 control). At least one sample was obtained from 83 children (56 test and 27 control), and both samples were obtained from 59 children (44 test and 15 control).

From the children referred for dental treatment under general anaesthesia, separate plaque samples were gathered from a total of 13 caries-free sites, from a caries-free buccal surface or from a caries-free occlusal surface, with a sterile excavator. In addition, the superficial and deep layers of dentin caries lesions were separately sampled, with two or three lesions for each child. After the superficial layer was sampled with a sterile excavator, the cavity walls were cleaned using sterile absorbent cotton pellets, first for 1 s by a pellet dipped in 0.5% sodium hypochlorite, and then for another 1 s by a pellet dipped in 3% hydrogen peroxide. Finally, the cavity was rinsed with water. After this cleaning, more carious dentin was removed by a high-speed bur with water cooling, and the deepest layers of carious dentin were removed with a sterile excavator into the transport medium. A total of 19 superficial-layer and 18 deep-layer samples were obtained from the seven children.

Samples from the mothers and their children, and saliva samples from adolescents were transported to the laboratory in VMGA III transport medium (Möller, 1966). Samples from children treated under general anaesthesia and plaque samples from adolescents, which were cultured within two hours, were transported in small vials containing 20% skim milk and glass beads (Sutter et al., 1984), or thioglycollate, respectively.

Culture and isolation

A chair-side method for detection of mutans streptococci was used for salivary samples from the adolescents in longitudinal follow-up. On the four first sampling occasions, the culture was by Dentocult SM® dip slide (Orion Diagnostica, Espoo, Finland) (Alaluusua et al., 1984). The fifth salivary sample was cultured by a strip method, Dentocult-SM STRIP MUTANS® test (Orion Diagnostica, Espoo, Finland) (Jensen and Bratthall, 1989).

Of the samples transported to the laboratory in VMGA III transport medium, skim milk or thioglycollate, tenfold dilutions were made in peptone water. Appropriate 10^0 to 10^5 dilutions were plated on agar. In the longitudinal study on adolescents, on the fourth sampling occasion, samples from 10 subjects were cultured on TSY20B agar (Schaeken et al., 1986) for detection of mutans streptococci. The agar plates were incubated at 37°C for 4 days in Gas Pack. Samples from the mothers and their children, the adolescents on the fifth sampling occasion and the children treated under general anaesthesia were cultured on mitis

salivarius bacitracin (MSB) agar (Gold et al., 1973) for mutans streptococci, and incubated at 37°C for 48 h in candle jars.

For total streptococcal count, appropriate dilutions of samples were cultured on mitis salivarius (MS) agar. Plates were incubated at 37°C for 48 h in candle jars. In addition, dentin samples from the children treated under general anaesthesia were cultured on Brucella base blood agar for total anaerobe count. Plates were incubated at 37°C in an anaerobe chamber for 7 days.

Colonies of mutans streptococci on agar were examined with a dissecting microscope and identified by distinctive colonial morphology, complemented by Gram staining, biochemical tests and tests for growth requirements (Table 2). Selected isolates were tested with the API 20 Strep system (Bio Mérieux, Marcy-l'Étoile, France). All isolates from the caries-active children treated under general anaesthesia were tested for fermentation of melibiose.

From the mothers and children in the preventive programme, at each sampling time, at least four *S. mutans* isolates and four *S. sobrinus* isolates, if available, were picked and tested. From the nursing-caries children and their mothers, 3 - 13 isolates from each sample were included. From the children treated under general anaesthesia, 9 - 29 isolates from each sample, if available, were included. Among the isolates obtained, a total of 505 isolates from the mothers, 115 isolates from their children and 598 isolates from the children treated under general anaesthesia were included for genotyping and/or assessment of phenotypic properties.

In addition to the bacterial isolates obtained from the study subjects, as previously stated, we included 39 mutans streptococcal isolates from our IDH collection (VI).

Isolates were stored at -70°C in skim milk until tested.

Typing of isolates

Phenotyping of mutans streptococci

For typing of isolates, the phenotypic expression of selected isolates was studied regarding serotype and mutacin activity.

Serotyping

Rabbit antisera raised against mutans streptococcal reference strains MT8148 (*S. mutans* serotype *c*), MT703R (*S. mutans* serotype *e*), OMZ175 (*S. mutans* serotype *f*), B13 (*S. sobrinus* serotype *d*) and 6715 (*S. sobrinus* serotype *g*), kindly provided by S. Hamada, Osaka University, were used to characterize the selected isolates by their carbohydrate antigens, by Ouchterlony immunodiffusion (Hamada et al., 1980a). Antisera against serotypes *d* and *g* showed cross-reactions, and serotype-specific antisera were prepared by absorption with cross-reactive strains (Hamada et al., 1978). Antigen extracts were prepared by autoclaving the cells (Rantz and Randall, 1955).

Bacteriocin typing

In study VI, 107 isolates from the mothers and their children were bacteriocin (mutacin) typed by assessment of their mutacin activity. The procedure is described in more detail in the section on testing for virulence attributes.

Genotyping of mutans streptococci

Of the genotyping methods available, ribotyping and AP-PCR typing were chosen for determination of clonality.

Ribotyping

A total of 110 isolates from the mothers and 81 isolates from their children were ribotyped as part of this study.

DNA extraction
Chromosomal DNA of isolates was isolated by the method of Ushiro et al. (1991).

Restriction endonuclease digestion and gel electrophoresis
Chromosomal DNA of isolates (2-3 μg) was digested to completion with the restriction endonuclease *Hin*dIII (Promega, Madison, WI) according to the manufacturer's instructions. In two cases, where two unrelated mothers were found to harbour identical ribotypes by analysis with *Hin*dIII, the isolates were also ribotyped with *Eco*RI. The restriction fragments were separated by electrophoresis through 0.9% (wt/vol) agarose gels, and lambda DNA (marker III; Boehringer Mannheim GmbH, Germany) was used as the molecular size marker for gel electrophoresis.

Preparation of probe for ribotyping
The recombinant plasmid pKK3535, consisting of the *rrnB* rRNA operon of the *E. coli* chromosome and the cloning vector pBR322 (Brosius et al., 1981), was used as a probe for ribotyping. It was possible to use the whole plasmid as a probe because the cloning vector region did not hybridize with the chromosomal DNA of mutans streptococci. The *E. coli* strain DH5α with the plasmid pKK3535 (kindly provided by P. A. Lawson, London Hospital Medical College, London) was cultured in LB broth supplemented with 50 μg/ml ampicillin,

and plasmid DNA was isolated by standard techniques. The plasmids were labelled by the random primed labelling method with digoxigenin.

Southern blotting, hybridization and detection of DNA-DNA hybrids
The DNA restriction fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim GmbH) and fixed by UV irradiation at 302 nm for 4 min. Fragments were hybridized to the recombinant plasmid pKK3535. Hybridization, as well as labelling and detection, was performed using the non-radioactive DIG DNA labelling and detection kit (Boehringer Mannheim GmbH).

Ribotypes were considered identical when they exhibited the same numbers and sizes of hybridizing fragments. Strains with closely resembling hybridization patterns were always compared after running the digests in the same gel. Any one band difference was considered discriminatory.

Arbitrarily primed polymerase chain reaction (AP-PCR) typing

A total of 598 mutans streptococcal isolates from the children referred for dental treatment under general anaesthesia were included for typing by the arbitrarily primed polymerase chain reaction method.

Template preparation
Templates for PCR reaction were crude cell lysates, prepared by the DNA isolation method of Bollet *et al*. (1991), but without treatment with phenol and chloroform. In brief, bacterial cells were harvested from a 5-ml BHI culture inoculated with one colony and incubated at 37°C for 48 h. Cells were washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated with sodium dodecyl sulphate (SDS) in TE buffer, 3% final concentration, at 65°C for 30 min. After being heated with SDS, bacterial cells were harvested and treated in a microwave oven at 490 W for 2 min 30 s. The pellets were dissolved in 250 µl TE buffer, and the supernatants diluted to 1:50 in sterile water served as templates for the PCR reaction, immediately after cell lysis or after overnight freezing.

PCR amplification and resolving the amplicons
PCR amplification was performed with single-stranded 10-mer oligonucleotide primer OPA-05 5'-AGGGGTCTTG-3'. Reaction mixture and PCR cycle were as described by Saarela et al. (1996). Amplification was performed in 50 µl reaction volumes containing 2 µl of the 1:50 water dilution of the lysis supernatant as the template; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 4 mM $MgCl_2$; 0.2 mM of each deoxynucleoside triphosphate (Pharmacia Biotech., Piscataway, NJ); 0.4 µM primer OPA-05 and 2.5 U Ampli-Taq® DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR performed with sterile water only as the template served for the negative control and was included in every amplification. As a method reference, a clinical control strain was repeatedly included in the whole process from lysis to gel electrophoresis. The amplification was performed in a thermocycler (Perkin-Elmer Cetus) with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C, 1 min), annealing (36°C, 2 min) and extension (72°C, 2 min). The final extension step was at 72°C for 5 min. The AP-PCR products were resolved by electrophoresis through 1% (wt/vol) TBE agarose gels (Boehringer GmbH, Mannheim, Germany) with 0.5 µg/ml ethidium bromide. Gels were photographed under UV light. A 1 kbp DNA ladder (Gibco BRL Life Technologies, Gaithersburg, MD) served as a molecular size marker in the gel.

Analysis of AP-PCR banding patterns
The AP-PCR fingerprints were analysed by side-by-side visual comparison, as suggested in a recent study (Burr and Pepper, 1997). Only isolates processed simultaneously, starting with lysing of cultures up to electrophoresis in the same gel, were used for the final judgement of similarity or dissimilarity. Fingerprints were considered similar when all major bands were identical, and minor bands had no more than two differences. Any repeatable difference regarding the strong bands was considered discriminatory.

Combining phenotyping and genotyping

In study VI, in two cases, two mutans streptococcal isolates from unrelated mothers had had similar ribotype profiles. However, the mutacin activity profiles of these isolates were not identical, and by combining the results of ribotyping and bacteriocin typing, these isolates could be differentiated.

Chlorhexidine susceptibility testing

In study II, the minimum inhibitory concentrations (MICs) of chlorhexidine (CHX) for 379 clinical mutans streptococcal isolates derived from the mothers participating in the preventive programme were determined by the agar dilution method, according to the standards of the National Committee for Clinical Laboratory Standards (NCCLS, 1991).

For CHX susceptibility testing, strains were cultured from frozen stocks on Brucella base blood agar in 5% CO_2 for 48 h. Approximately 20 colonies were transferred to 5 ml of Todd-Hewitt broth and grown for 24 h. Suspensions were adjusted to 0.5 on the McFarland turbidity standard and further diluted to 1:10 in sterile broth. With a multipoint inoculator, a final inoculum of approximately 10^4 CFU per spot was delivered onto Mueller-Hinton blood agar supplemented with 5% defibrinated sheep blood (NCCLS, 1991). The CHX concentration in agar varied from 0.03 to 128 μg/ml, and plates were prepared with 20% CHX digluconate stock solution. The plates were read after incubation at 35°C in 5% CO_2 for 48 h. The following eight mutans streptococcal laboratory strains, kindly provided by S. Hamada, Osaka University, were inoculated on each plate: *S. mutans* MT 8148 (serotype *c*), *S. mutans* LM 7 (*e*), *S. mutans* OMZ 175 (*f*), *S. sobrinus* B13 (*d*), *S. sobrinus* 6715 (*g*), *S. sobrinus* MT 6223 (*g*), *S. cricetus* E49 (*a*) and *S. rattus* FA-1 (*b*). *Staphylococcus aureus* ATCC 25923 and ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Streptococcus pyogenes* ATCC 19615 from the ATCC collection were also included as controls. In order to facilitate the intra-individual comparison, the strains from one subject were always tested at the same time on the same series of Mueller-Hinton agar. A difference in MIC values in one plating series was interpreted when the results differed more than one twofold dilution step. Generally, the acceptable reproducibility of the test is within one twofold dilution of the actual endpoint (NCCLS, 1991).

Examination of virulence factors

Glucosyltransferase activity and mutacin production by part of the isolates were studied in detail. Preliminary tests on other suggested virulence factors were also performed.

Glucosyltransferase (GTF) activity

The mutans streptococcal isolates included for glucosyltransferase activity testing were derived from six nursing-caries children and their mothers, and six caries-free children of the preventive programme and their mothers, before the commencement of CHX-NaF gel brushing. The isolates represented all distinct ribotypes detected in the subjects. The total number of isolates included for GTF activity testing was 44. Among these isolates were five derived from test-group mothers of the preventive programme.

Production of monoclonal antibodies

The monoclonal antibodies P72, P32, P4 and B17 were prepared as described by Oschiai et al. (1990) and Fukushima et al. (1993). These antibodies react monospecifically with GTF-I, GTF-SI and GTF-S obtained from *S. mutans*, and GTF-I from *S. sobrinus*, respectively. The specificities and potencies of these monoclonals were confirmed by enzyme immunoassay (Fukushima et al., 1993, 1994).

Extraction of extracellular and cell-bound GTFs

The isolates were cultured in Todd-Hewitt broth, supplemented with 56 mM glucose, at 37°C for 18 h in candle jars. Extracellular GTF was precipitated using a saturated ammonium sulphate solution. Cell-associated GTF was extracted by the method of Hamada et al. (1989), with several modifications, using urea. The extraction supernatants were pooled and subjected to testing as the sample containing total GTFs.

Cross-dot assay

The assay was as described by Tomita et al. (1996), with slight modifications. For the assay, a Clear Blot Membrane-P (Atto, Tokyo) was assembled into the cross-dot apparatus. In the apparatus, diluted enzyme preparations were allowed to react with monoclonal antibodies P72, P32, P4 or B17. The staining was by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin and 0.05% 4-chloro-1-naphtol. The results were developed by a Densitograph (AE-6920-MLR, Atto, Tokyo). Results were marked with 1-7 plus signs, corresponding to positive reactions with twofold dilutions of the enzyme preparation from 2^0 to 2^6.

Mutacin production

In study VI, the isolates included in evaluation of mutacin activity were selected from the mother-child material such that every distinct ribotype was represented in duplicate. When ribotypes were shared by mother and child, four isolates exhibiting the same ribotype, if available, were selected, two from the mother and two from the child. The material also included isolates obtained at follow-up. From the IDH collection, we included 38 isolates that originated from a follow-up study of 5-year-old children. In total, 145 clinical mutans streptococcal isolates were included for mutacin activity testing. In addition, a clinical strain exhibiting very distinct antibacterial activity was used as a control in the stab culture assay on each plate.

Stab culture technique

The mutacin activity of the mutans streptococcal clinical isolates was tested by a modification of the deferred antagonism method of Fredericq, the stab culture method (Hamada and Ooshima, 1975).

For mutacin activity testing, the following 14 stock culture strains were utilized as indicator strains: *S. mutans* MT 8148 (serotype *c*), *S. mutans* LM 7 (*e*), *S. mutans* OMZ 175 (*f*), *S. sobrinus* 6715 (*g*), *S. sobrinus* B13 (*d*), *S. rattus* FA-1 (*b*), *S. sanguis* ATCC 10556, ST3, ST202 and B220, *S. oralis* ATCC 10557, *S. gordonii* ATCC 10558, *S. salivarius* HHT and *S. pyogenes* SV. Serving as a control in the stab culture assay, one clinical strain exhibiting very clear antibacterial activity was used on every plate.

The strains were grown from the frozen stocks on Mitis Salivarius agar, and about 20 colonies were transferred to 4 ml of Brain Heart Infusion broth and cultured overnight in candle jars. The strains were inoculated into Trypticase soy agar (TSA) (BBL, 2% agar) with a 0.6-mm-thick needle. A clinical mutans streptococcal control strain was included on each plate. After a 48-h culture at 37°C in candle jars, the stab cultures were overlaid with 0.5 ml (about 10^7 CFU) of an overnight Trypticase soy broth (TSB) culture of the indicator strain suspended in soft TSA (4 ml, 0.8% agar). After overnight incubation at 37°C, the diameter of the inhibition zone was measured. The isolate was recorded as mutacin-active if the diameter was 4 mm or greater. A difference in mutacin activity values was interpreted when the size of the inhibition zone diameter differed by more than 4 mm. In order to facilitate intra-individual comparison, the strains from one subject were always tested in duplicate at the same time on the same series of TSA. For interindividual comparisons, the experiment was performed at least twice, and the mean size of inhibition zones was calculated. The surface pH of the TSA inside the inhibition zone was measured with a Ross Combination Flat Surface Electrode, model 8135 (Orion Research Inc, Boston, MA) before covering the surface with the soft indicator agar. The surface pH on the agar after a 48-h culture in a candle jar did not fall below 6.0 around any of the stab-inoculated strains.

Statistics

Because the salivary mutans streptococcal counts, typing results, and GTF and mutacin production results did not follow a normal distribution, either non-parametric statistical methods were used to evaluate differences between groups, or comparisons were made after logarithmic transformation. Throughout the studies, the differences between salivary mutans streptococcal counts of the subject groups were evaluated with the Mann-Whitney *U* test. In the study on the effects of chlorhexidine on mutans streptococci (II), analysis of variance was used for testing differences in bacterial counts between group means at baseline and post-treatment after logarithmic transformation. Bacterial counts within groups on different sampling occasions were compared by Scheffé's test. When results followed a normal distribution, the Mann-Whitney *U* test and Student's *t*-test were used to compare the serotype distribution and MICs of CHX for isolates from CHX-treated subjects and control subjects at three samplings.

In the study on clonal diversity of mutans streptococci in children with nursing caries (III), the differences in frequencies of ribotypes between nursing-caries children and caries-free children was evaluated using Fisher's exact probability test. The association between the number of ribotypes and the proportion of mutans streptococci in plaque was studied by the Kruskal-Wallis test. In the study on intra-individual clonal diversity of mutans streptococci (IV), the frequency distribution of AP-PCR types of mutans streptococci in different sites within the same mouth was compared with the chi-square test.

Ribotyping and mutacin typing were used to study the transmission of mutans streptococci between mothers and their children. A strain was considered transmitted if representative isolates from a mother and her child had similar ribotype profiles. Furthermore, in study VI, to be considered transmitted, strains must also have been identical in mutacin typing. The odds ratio was used to estimate the likelihood of ribotype matching between family members.

In the study on glycosyltransferase production by clinical mutans streptococcal isolates (V), relative amounts of GTFs of isolates obtained from participant groups were compared by the Mann-Whitney *U* test. In the study on mutacin production by *S. mutans* (VI), the Mann-Whitney *U* test was used to analyse differences between the sizes of inhibition zone

produced against single indicator strains by isolates derived from different subject groups. When the numbers of indicator strains inhibited by transmitted strains and nontransmitted strains were compared, the equality of medians was tested by Fisher's exact test. The total inhibitory activity of isolates among subject groups was compared by the Wilcoxon signed-rank test for mean inhibition zone sizes.

$P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

General culture and fermentation results

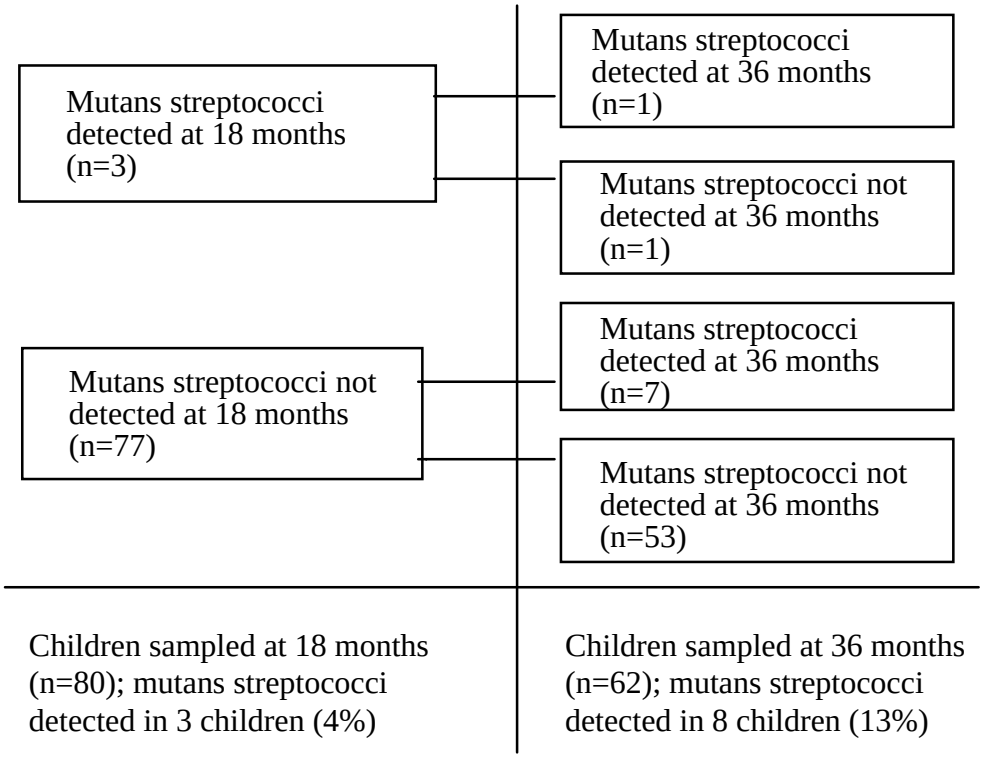
Detection of mutans streptococci

In study I, 24 adolescents initially had undetectable levels of salivary mutans streptococci as assessed by a chair-side dip-slide method. During the three-year follow-up, mutans streptococci were readily detected using the dip-slide in 15 of these subjects in at least one of the annual samples. On the fourth sampling occasion, saliva samples were supplemented with plaque samples, but this did not increase the isolation frequency. Five years after the fourth sampling occasion (8-year follow-up), saliva samples from 22 of the subjects were tested by using the chair-side strip method Dentocult-SM STRIP MUTANS® test and conventional culturing. Mutans streptococci were detected in 11 subjects. In two samples, the number of mutans streptococci was low, in eight samples moderate and in only one sample high. MSB agar plate culture of saliva samples revealed mutans streptococci in samples from two subjects in whom the strip method did not detect mutans streptococci. In the 8-year follow-up, mutans streptococci were not detected in four subjects on any of the five sampling occasions, neither in saliva, nor in plaque. A fifth subject in whom mutans streptococci could not be detected also existed, but this subject had been on antibiotics recently before sampling, and thus, his results were excluded. At the final sampling, the ages of the four subjects were 22, 23, 23, and 24 years, and their DMF indices were 0, 0, 5 and 7, respectively. In these four subjects with five negative sampling results over an eight-year period, no new manifest caries lesions had developed. The 23-year-old subject with DMF 0 had one white spot lesion at the eight-year check-up. Because the DMF indices of all subjects were not zero, it is possible that mutans streptococci had at some time been present and then disappeared. It is also possible that the fillings that had been made earlier had not been made because of actual caries lesions but were prophylactic fillings. The possibility still remains as well that mutans streptococci actually were present in these subjects, but in such low numbers that they went undetected. Molecular biology methods are more sensitive than conventional selective agar plating of samples, and it is possible, even probable, that mutans streptococci would have been detected in more subjects using DNA probes or PCR detection (Smorawinska and Kuramitsu, 1992; Rupf et al., 1999). Yet another possibility is that the initiator microorganism for dental caries disease in these subjects was a microorganism other

than mutans streptococci, e.g. non-mutans streptococci (van Houte et al., 1991; Tanzer, 1992).

Mutans streptococci were found in all but three of the follow-up salivary samples (n=232) from mothers who participated in the preventive treatment study, as assessed by the chair-side strip method complemented by conventional selective agar plate culture. One of the negative samples was obtained during antibiotic treatment of the subject and was excluded from analysis. Another was obtained from a mother in the test group before commencement of CHX-NaF brushing, and the third was from a mother in the control group on the fourth sampling occasion. The occasional negative finding of mutans streptococci in adults suggests that the colonization temporarily drops below the detection level of the method. In the follow-up of children in the same study, 10 of the 83 children sampled harboured mutans streptococci in their plaque, six in the test group and four in the control group (Tables 4 and 5).

Table 5. Detection of mutans streptococci in children of the preventive treatment programme, sampled at about 18 months and 36 months.



Seven children were culture-negative for mutans streptococci at the first sampling, but at the second sampling tested positive. One culture-positive child contributed a sample only at the first sampling, and one child harboured mutans streptococci at both samplings. Another child had a positive sample at first sampling at 16 months, but a negative sample at 36 months. In this child, the colonization may have been transient, or at the second sampling, mutans streptococci may have been present but in numbers under the detection level (II, III, V and VI).

In the preventive study, only 12% of the 1.5- to 3-year old-children were colonized by mutans streptococci. This is a low percentage as compared with earlier results from other populations. Caufield et al. (1993) reported finding 75% of 31-month-old children to be culture-positive. Alaluusua and Renkonen (1983) reported mutans streptococci in plaque samples in 31% of 3-year-old children, and Pienihäkkinen and Jokela (1995) detected mutans streptococci in plaque by the strip method in 25% of 2-to 3-year-old children. The differences between the studies may be partly due to differences in sampling methods. As the levels of mutans streptococci are low during the first years of the child's life, the amount of plaque sample obtained and the dilutions used may cause differences in prevalence figures. When comparing our test and control groups, however, the results are comparable because samples from both groups were taken and handled in the same way. It is also noteworthy that the preventive treatment programme at the Health Centre in Espoo, at the time of the experiment, was strongly orientated to prevention of early mutans streptococcal colonization by providing initial information to all parents well before childbirth and supporting information for the first years of the child's life.

The seven 1.5- to 3-year-old nursing-caries children all harboured mutans streptococci. Similarly, all seven 3- to 7-year-old children treated under general anaesthesia (IV) harboured mutans streptococci. Among the 55 samples obtained from children treated under general anaesthesia, mutans streptococci were detected by culture on MSB agar in 40 samples. No mutans streptococci were detected in 5 out of 13 samples from caries-free sites, in 1 out of 19 samples from the superficial layer of caries lesions, or in 9 out of 18 samples from deep layers of lesions. All five salivary samples obtained exhibited mutans streptococci upon culture. These findings support earlier reports of a higher isolation frequency in superficial layers of caries lesions as compared with deep layers or caries-free sites (Edwardsson, 1974). In deep layers, other species are more prevalent (Tanzer, 1992). Counts

of mutans streptococci obtained by culture in the candle jar and in the anaerobe chamber were at the same level, indicating that the strains present were aerotolerant. It has previously been suggested that strains may be obligate anaerobes on first isolation, and only later become facultative (Pulliam et al., 1980).

Proportion of mutans streptococci of total streptococcal count

The proportion of mutans streptococci of the total streptococcal count in the plaque samples from caries-free 1.5- to 3-year-old children varied between <1% and 3.3% (median <1%), whereas in nursing-caries children, the proportion varied between 8.3% and 98% (median 30%). In the 3- to 7-year-old caries-active children, the corresponding proportion in salivary samples varied between 0.09% and 8.2% (median 0.5%). The mean proportion of mutans streptococci of total streptococcal count in the culture-positive samples from caries-free sites, from superficial layers of caries lesions and from deep layers of caries lesions was 1.1% (range, < 0.01% to 4%), 14.7% (range, 0.16% to 93%) and 10% (range, 0.05% to 27%), respectively. This finding of significantly higher proportions of mutans streptococci in dental plaque in nursing-caries children concurs with the earlier studies of van Houte et al. (1982) and Ripa (1988) who reported higher percentages of mutans streptococci in dental plaque from nursing-caries children as compared with plaque from caries-free children or children with dental caries not conforming to the criteria stipulated for nursing caries.

Detection of melibiose non-fermenting *S.mutans*

Among the 40 culture-positive samples from the 3- to 7-year-old caries-active children, a total of 598 mutans streptococcal isolates were included for further analysis. Melibiose fermentation is one of the biochemical tests used for distinguishing the various mutans streptococci. All isolates were included for testing; 557 were identified as *S. mutans* and 41 as *S. sobrinus*. Among the *S. mutans* isolates, 215 (39%) did not ferment melibiose. This percentage is higher than that (12%) reported earlier by Beighton et al. (1991). We also found that in the two subjects who harboured both melibiose-fermenting and melibiose-non-fermenting *S. mutans* isolates, the non-fermenters made up the majority of isolates. Thus, it can be questioned whether our finding of melibiose-negative isolates was random or suggestive of some property common to melibiose non-fermenters giving them a higher virulence and potential to survive. Earlier suggestions have been made that *S. mutans* isolates

unable to ferment melibiose are genetically related to each other in having a large chromosomal deletion including the multiple sugar metabolism (*msm)* operon which encodes many genes involved in the uptake and metabolism of a number of sugars including melibiose (Ushiro et al., 1991; Russell et al., 1992). Recently, however, it has been shown that the melibiose-negative phenotype can arise through more than one mechanism; the genotype and the phenotypic properties of these isolates can vary (Colby et al., 1995). So, as regards any possible significance of our present findings on increased amounts of melibiose non-fermenters, no final conclusions can be drawn before a comprehensive analysis of the genetic elements of the strains isolated has been performed.

Effects of chlorhexidine - sodium fluoride (CHX-NaF) gel brushing on salivary counts and transmission of mutans streptococci

The clinical effects of chlorhexidine-sodium fluoride (CHX-NaF) gel brushing on salivary counts of mutans streptococci was evaluated using follow-up samples from 24 mothers in the test group and 10 mothers in the control group (study II). For the comparison, samples were obtained on three occasions: before childbirth, when the child was about 6 months old and the CHX-NaF gel brushing was to commence in the test group, and when the child was about 18 months old and the periodic gel brushing had been ongoing for 1 year. The statistical analysis of the differences between bacterial counts in treatment and control groups indicated no significant effect of CHX gel brushing in the instructed trial on the salivary counts of mutans streptococci. These negligible effects of periodic low-concentration CHX brushing are comparable with earlier results (Emilson, 1994). When the gel is applied in trays, or when the brushing is done professionally, and the concentration of CHX in gel is 1%, a reducing effect is to be expected on salivary mutans streptococcal count (Emilson, 1994). The poor effect of CHX brushing in an instructed trial might be related to the bioavailability of the formulation when administered by the patient him/herself and also to the compliance of the patient.

The effect of CHX-NaF gel brushing on transmission of isolates from mother to child was also evaluated. Culture of dental plaque samples from the children revealed that mutans streptococci had been colonized in 6 out of 56 children in the test group and in 4 out of 27 children in the control group. The colonization frequencies in the two groups did not differ significantly from each other (Fisher's exact test, $p > 0.05$, ns) (unpublished result).

As a conclusion from the preventive programme, the periodic low-level CHX-NaF gel use did not significantly affect the salivary counts of mutans streptococci in the mothers or the colonization of mutans streptococci in their children. In earlier studies, when the salivary mutans streptococcal counts in mothers of young children have been significantly reduced by using 1% CHX gel in individual trays, controlled by culture of salivary samples, the probability of colonization of mutans streptococci in the child has been reduced as well (Köhler and Andréen, 1994). Use of CHX-NaF gel in standard trays by the mothers, without control of salivary mutans streptococcal counts, did not significantly reduce the colonization of mutans streptococci in their young children (Tenovuo et al., 1992). The difference in results can thus be attributed to differences in study set-up.

In the preventive programme study, the number of drop-out subjects was rather high. A 3-year sample was obtained from 47 children of the 75 mothers initially included in the test group, and from 15 children of the 30 mothers initially included in the control group. As the proportion of young inhabitants in the city of Espoo is quite high, and they change residence rather frequently, these low numbers could be anticipated.

The mutans streptococcal isolates derived from the preventive treatment programme were included for further studies on qualitative characteristics of mutans streptococci only after the effects of the periodic CHX-NaF gel use had been evaluated to be negligible.

Clonal diversity of mutans streptococci

The clonality of isolates was studied by ribotyping and AP-PCR typing. Before proceeding to genotyping by these molecular biology methods, mutans streptococcal isolates from the 1.5- to 3-year-old children and 40 mothers were phenotyped by serotyping. Serotyping revealed one, two, or three different types of mutans streptococci within the subjects. Among the 1.5- to 3-year-old children, 13 out of 14 children studied had serotype *c* isolates, 2 children had serotype *e* isolates and 3 had serotype *g* isolates. Two concomitant serotypes were detected in 3 children, the other children had only one serotype present on the actual sampling occasion. One caries-free child harboured serotype *c* isolates at first sampling and serotype *g* isolates at second sampling. In the 40 mothers included for further studies, 37

harboured serotype *c*, 13 serotype *g*, 11 serotype *e*, 1 serotype *f* and 1 serotype *d* strains. One serotype was detected in 20 mothers, with two and three concomitant serotypes found in 17 and 3 mothers, respectively (II, III, V, VI).

Total number of clonal types intra-individually

By ribotyping, one, two, three or four different clonal types of mutans streptococci were detected within each subject. Among the 19 mothers studied for clonal diversity, 6 mothers harboured one, 7 harboured two, 5 harboured three and 1 mother harboured four ribotypes. In the 1.5- to 3-year-old caries-free children, one ribotype was detected in 6 out of 7 children studied and two ribotypes in 1 child. In nursing-caries children, one ribotype was detected in 4 children, two in 2 children and three in 1 child.

In the seven 3- to 7-year-old caries-active children referred for dental treatment under general anaesthesia, by AP-PCR typing of 90, 74, 81, 53, 83, 103 and 114 isolates from the children, respectively, 2 children were found to harbour one, 3 children two, 1 child three and 1 child four different AP-PCR types of mutans streptococci. The culture method of samples did not alter the number of clonal types detected; culture in candle jar and in anaerobe chamber yielded the same AP-PCR types of mutans streptococci.

In our material, molecular biology methods revealed more types intra-individually than did phenotyping by serotyping, as has generally proven to be the case (Caufield and Walker, 1989; Alonso et al., 1993; Russell, 1994). In earlier studies, the number of clonal types detected within an individual has varied between one and four (Bowden and Hamilton, 1998). In this study, the increased number of isolates included for genotyping failed in detecting more clonal types; no more than four genotypes of mutans streptococci could be found within any individual.

Interindividual comparison

The mutans streptococcal isolates in studies III, V and VI, and isolates from seven mothers in study II, represented 52 different ribotypes. The isolates originated from 19 mothers and their 18-month- to 3-year-old children (one twin pair), and five originally 5-year-old children who had been studied in follow-up (Alaluusua et al., 1989). When comparing isolates from

unrelated subjects, identical ribotypes were detected in two cases. The isolates from these two pairs of mothers could not be distinguished by ribotyping with *Hin*dIII or with *Eco*RI. In comparison of ribotypes between mothers and their children, out of 14 mother-child pairs, identical ribotypes were detected in 9 pairs (64%).

In study IV, AP-PCR typing was performed for 598 isolates originating from seven children. Fourteen different genotypes were identified. By AP-PCR typing, isolates from one of the children, all representing the same AP-PCR type, could not be distinguished from isolates representing one out of two AP-PCR types detected in another child.

Our typing results confirmed earlier results of a high degree of genetic heterogeneity within mutans streptococci (Saarela et al. 1993; Bowden and Hamilton, 1998). Isolates from unrelated subjects that could not be distinguished by genotyping were detected in only two pairs of adults, where ribotyping failed to show a difference, and in one pair of children, where AP-PCR typing failed in discerning between isolates. The genotyping methods thus proved to be useful in transmission studies, especially in combination with phenotypic typing (VI).

Intra-individual site-specific evaluation: implications for sampling technique

In study IV, 598 mutans streptococcal isolates derived from 50 tooth-site samples and 5 salivary samples from the seven 3- to 7-year-old caries-active children were genotyped by AP-PCR typing. The tooth-site samples included 7, 5, 7, 6, 9, 9 and 7 samples from the subjects, respectively.

Two children harboured both *S. mutans* and *S. sobrinus* isolates. In one of the children, the difference in frequency distribution of *S. mutans* and *S. sobrinus* isolates at different sites was statistically significant**;** S. sobrinus constituted the majority of isolates in two out of three samples from superficial layers of caries lesions, but remained undetected in one caries lesion and in the one caries-free site sampled. By comparing the frequency distribution of AP-PCR types of *S. mutans* in different sites within the subjects, it was revealed that mutans streptococcal clones may selectively colonize specific hard-tissue sites; in three of the seven children, the distribution of AP-PCR types of *S. mutans* differed depending on tooth site.

Five children in study IV had contributed saliva samples. In two, one of the AP-PCR types detected in the tooth-site samples was not detected in the saliva sample. In these five children, 8 out of 10, or 80%, of all genotypes of mutans streptococci detected appeared in the saliva samples. The representativeness of tooth-site sampling was evaluated for all seven children. In the present sampling setting and with the present number of isolates included, when the first caries-free site sampled per child was considered, 6 out of 15, or 40%, of all mutans streptococcal genotypes were detectable. Including the first caries-free site sampled and both the superficial and the deep layer of the first caries lesion sampled revealed 11 out of 15, or 73%, of the genotypes. Including the second caries lesion sampled, as well, revealed all genotypes detected in the whole material. In conclusion, sampling of saliva is fairly efficient for mutans streptococcal strain isolation, but does not necessarily reveal all genotypes. When sampling for mutans streptococci for qualitative analysis, it is necessary to sample multiple sites of the dentition.

CHX susceptibility, GTFs and mutacin production of strains

When testing the clinical isolates for CHX susceptibility (II), *S. mutans* isolates were found to be more susceptible than *S. sobrinus* isolates, and analyses of laboratory strains also suggested that *S. mutans* is more susceptible than *S. sobrinus*, *S. cricetus* and *S. rattus*. Of the 379 clinical isolates studied, 50% were inhibited at 1 μg of CHX per ml, 90% were inhibited at 2 μg/ml, and all were inhibited at 4 μg/ml. Development of resistant strains during CHX-NaF gel use was not detected, and the serotype distribution of isolates remained unchanged by CHX-NaF gel use. Similarly, Kozai et al. (1991) reported that strains isolated after CHX varnish treatment were no more resistant to CHX than those isolated prior to treatment. When evaluating long-term CHX susceptibility, earlier studies have reported that mutans streptococci are inhibited at a CHX concentration of 1 to 4 μg/ml in agar (Meurman et al., 1989; Järvinen et al., 1993). The MIC values obtained in the present study were at the same level, and thus it can be concluded that mutans streptococci have not developed resistance to CHX over the years of treatment. When using antibiotics and biocides such as CHX, it is possible that resistant strains of commensal microorganisms and potential pathogens such as *Proteus mirabilis* are selected, but the clinical relevance of this possibility is still unclear (Russell et al., 1998).

In the analysis of GTF production by the strains in an 18-hr candle-jar culture in 56 mM glucose-supplemented Todd-Hewitt broth (V), all *S. mutans* isolates were shown to produce GTF-I and GTF-S, and all but two produced GTF-SI of *S. mutans*. All *S. sobrinus* isolates produced GTF-I of *S. sobrinus*. The relative amount of GTFs produced by serotype *c* and *e* isolates were often variable. No significant difference in production activity between serotypes *c* and *e* was found. Earlier studies on the environmental regulation of GTF production by *S. mutans* have shown that an addition of sucrose can increase the production up to twofold in batch culture (Hudson and Curtiss, 1990). Moreover, many other dietary components, such as various carbohydrates, milk and kappa casein, may also affect glucosyltransferase activity (Vacca-Smith and Bowen, 1995). In our study, the altering of the composition of the culture broth might have influenced detected levels of GTF production.

A result of the study on mutacin production of mutans streptococci (VI) was that the production of mutacins also varies among isolates. Of the 145 strains tested, 88% produced mutacin against more than 1 of the 14 indicator strains. The inhibition zone sizes for producer strains varied from 4 to 26 mm in diameter. On average, the isolates produced mutacins against 7 of the 14 indicator strains. *S. mutans* isolates showed more inhibitory activity than did *S. sobrinus* isolates. Four isolates, representing two ribotypes of *S. mutans* serotype *e*, were active against all indicator strains. *S. sobrinus* serotype *g* strains produced mutacins against only one or no indicator strain. On average, serotype *c* isolates produced mutacins against 8 indicator strains (range, 1 to 13), serotype *e* against 10 (range, 3 to 14) and serotype *f* against 6 (range, 3 to 6). The mutacin activity of isolates found in this material was at the same level as in earlier reports (Alaluusua et al., 1991b).

The stability of mutacin activity of clinical isolates was evaluated using samples in follow-up. The spectrum of mutacin activity of baseline and follow-up isolates of identical ribotypes within a subject was compared. The isolates were obtained at 1- to 7-year intervals (mean, 3.1; SD, 2.3) from 10 mothers and 5 initially 5-year-old children, producing a total of 19 comparable isolate pairs. The mutacin activity of the isolates initially and in follow-up was similar in 14 subjects, whereas only the isolates obtained from one child 5 years apart showed a difference in mutacin activity against two indicator strains. Thus, the results indicated that the bacteriocinogenecity of *S. mutans* strains is reasonably stable.

Intra-individual variation: strain specificity

Among the isolates included for CHX susceptibility testing were serotype *c* isolates from seven mothers that exhibited intra-individual variability in CHX susceptibility. By ribotyping these isolates, it was shown that if the CHX MICs for isolates from one subject were not the same (they differed by more than one dilution step), then the subject harboured two or three ribotypes. These findings suggest that each intra-individual strain presents unique susceptibility to CHX.

The analysis of GTF production of *S. mutans* strains revealed variability in the GTF-I, GTF-SI and GTF-S production of isolates exhibiting a different ribotype. Eight participants (three children and five mothers) harboured more than one ribotype of *S. mutans*: four had two ribotypes and four had three ribotypes. When comparing the production activity of GTFs of the intra-individual strains, eight out of the 16 comparable pairs had a difference in the relative amounts of one or two enzymes. As concerns mutans streptococcal ribotypes shared by a mother and her child, the production of the three GTFs of *S. mutans* was at the same level in five out of six mother-child pairs. In the exceptional pair, the difference may have been due to a technical problem, such as a failing culture of a strain. In most cases, the GTF production was ribotype-specific.

Identical ribotypes had similar mutacin activity profiles within a subject initially and in follow-up in all but two cases. To reach this conclusion, strains of an identical ribotype within a subject, isolated on the same occasion, were used. The data included 43 cases of two or more isolates of identical ribotype within a subject, obtained from the same sample. In 40 of these comparable cases, the sizes of the inhibition zones were identical, i.e., differed by less than 4 mm. With two isolate pairs, the sizes of the inhibition zones differed for one indicator strain. This difference for 1 indicator out of 14 fell within the range of accuracy for the method. One isolate pair differed in mutacin activity profile for four indicator strains. Thus, the mutacin activity of mutans streptococal isolates, defined by the stab culture method, was ribotype-specific in 42 out of 43 cases. Mothers and children shared 10 ribotypes, and the isolates representing these ribotypes exhibited a similar mutacin activity profile. Furthermore, all four isolates of the same ribotype, obtained from the twin brothers with nursing caries, showed an identical mutacin activity profile.

In conclusion, isolates that were similar as evaluated by genotypic methods had similar expression of phenotypic properties in most cases, and isolates that were genetically different differed also, in most cases, in their phenotypic properties. Differences between isolates derived from different sources were earlier suggested with regard to acid production (Köhler et al., 1995). The present finding of strain specificity of phenotypic properties of bacterial isolates supports the view that certain strains may more likely be associated with disease than others, by the inherited properties of the microorganism, not only by environmental modification of these properties.

Interindividual variation

As a rule, mutans streptococcal isolates from unrelated subjects could be differentiated by genotyping. The phenotypic properties showed insufficient variability for comprehensive differentiation between isolates. Identical ribotypes from related subjects, i.e. mother-child pairs, were in most cases similar in their phenotypic properties.

Effects of CHX-NaF-brushing on mutacin production

In the preventive study, the effect of CHX-NaF brushing on mutacin activity of clinical isolates was evaluated using samples in follow-up. Follow-up samples were obtained after 1 year of periodic CHX use for five subjects, and after 2.5 years of periodic CHX use for one subject. A total of seven identical ribotype pairs were compared, and for all pairs, the mutacin activity was identical in the initial and follow-up samples. In addition, in two cases, a shared ribotype of mutans streptococci obtained from a mother during CHX treatment and her child had identical mutacin activity profiles. These results indicate that periodic CHX administration by tooth brushing does not affect the phenotypic property of mutacin activity. By contrast, earlier *in vitro* studies have shown that CHX can affect phenotypic properties of oral streptococci, e.g. acid production and sugar transport (Marsh et al., 1983). However, the outcome of the present study, with periodic low-concentration CHX-NaF-gel brushing in an instructed trial is not comparable with *in vitro* results, as apparently variability exists in the bioavailability of different CHX preparations, administered in different modes (Emilson, 1994).

Clonal diversity of mutans streptococci in nursing caries

The results of study III on increased proportions of mutans streptococci of total streptococcal count in dental plaque of children with nursing caries was expected based on earlier reports (van Houte et al., 1982; Ripa, 1988). Further, in study III, providing that only clonal types detected simultaneously in a subject were considered, the number of clonal types detected was significantly higher in nursing-caries children as compared with caries-free children. This is contradictory to the finding of Kreulen et al. (1997), who, in family studies, found less clonal types of mutans streptococci in nursing-caries children than in siblings without nursing caries. The difference in findings may be the result of a difference in sampling technique; Kreulen et al. sampled only one tooth site for analysis, whereas we gathered pooled plaque samples. Because mutans streptococci were found to selectively colonize tooth sites, multiple sites should always be sampled for qualitative analysis of these bacteria.

Virulence factors studied and occurrence of nursing caries

In the study on glucosyltransferase activity of mutans streptococci, among the twelve 1.5- to 3-year-old children included, the six children with nursing caries harboured nine different *S. mutans* ribotypes and the six caries-free children harboured seven. Analysis using monoclonal antibodies revealed that isolates of the caries-free children produced more GTF-SI than isolates of the children with nursing caries. Regarding enzymes GTF-I and GTF-S, no significant difference between the groups was found. This finding of less GTF-SI produced by isolates from nursing-caries children was unexpected because inactivation of the *gtfC* gene, responsible for GTF-SI production, has been reported to drastically reduce adherence to smooth surfaces (Tsumori and Kuramitsu, 1997). Possibly, in the mouths of children with nursing caries, whose diets are rich in sucrose, special properties are not as important for a colonizing strain as in less favourable environments.

Mutacin activity was studied for isolates obtained from fourteen 1.5- to 3-year-old children, comprising 7 nursing-caries children and 7 caries-free children. The sample included a total of 17 distinct *S. mutans* ribotypes, with nine of these detected in the nursing-caries children. Overall, the ribotypes from nursing-caries children tended to inhibit more indicator strains than those detected in caries-free children, the former inhibiting a mean number of 10.2 indicator strains (SD 2.9), and the latter 9.2 indicator strains (SD 2.2). When comparing the

inhibition zone sizes against single indicators, the zones for all indicator strains were bigger for ribotypes originating in the oral flora of nursing-caries children than for ribotypes isolated from caries-free children, the difference being statistically significant for indicator strain *S. sobrinus* B13 (*b*) (Mann-Whitney U-test, $p < 0.04$) (unpublished results). Findings on mutacin activity of mutans streptococcal isolates from nursing-caries children have not been reported earlier. In a study by Alaluusua et al. (1991b) on older children aged 4 to 6 years, no association between caries experience and mutacin activity of mutans streptococci isolated was found.

Virulence factors studied and transmission of isolates from mother to child

Transmission of isolates between subjects can be supposed to have happened if isolates derived from the subjects are similar upon typing (Berkowitz and Jordan, 1975a; Li and Caufield, 1995). The methods with the highest discriminatory power are the genotypic typing methods (Arbeit, 1999). As concerns transmission studies between mothers and their young children, detection of microbial isolates with identical genotypes in mother and child has been considered to mean that the particular strain has been transmitted from the mother to her child, since at birth the child does not harbour any microorganisms (Könönen et al., 1992; Caufield et al., 1993). With older children and adults, a cross-sectional study does not allow for the determination of the direction of transmission, i.e., who is the source and who the recipient of the infecting organism.

In study VI, a statistical analysis was included for strain matching between mothers and children, and for evaluating the probability of transmission. Ribotyping and mutacin typing were used to study transmission. In the children who harboured mutans streptococci and in their mothers, a total of 36 ribotypes were detected. A ribotype match for mothers and children was found for 10 of the 36 ribotypes detected. The study also included isolates from 5 initially 5-year-old children, unrelated to each other and to the other subjects. When comparing ribotypes of these 5 children and the 19 unrelated adult women in the study (a total of 42 ribotypes), identical ribotypes were detected in two cases, where two mothers had the same ribotype. The odds ratio of the occurrence of shared identical strains, determined by ribotyping with one enzyme, in mothers and children living in the same household (10 out of 36) as compared with that in unrelated subjects not living in the same household (2 out of

42), was 7.69. When the results of ribotyping were complemented with the results of mutacin typing, no case of identical strains detected in epidemiologically unrelated persons remained. The presence of identical ribotypes in 9 of the 14 mother-child pairs indicated maternal transmission of mutans streptococci in 64% of mutans streptococcus culture-positive children. This result agrees with previous studies; Li and Caufield (1995) reported finding identical genotypes in 71% of mother-child pairs.

Transmitted mutans streptococcal strains produced the same levels of GTFs as strains that were not shared by mothers and their children, as assessed by the semiquantitative cross-dot assay using monoclonal antibodies P72, P32, P4 and B17 against GTF-I, GTF-SI and GTF-S from *S. mutans* and GTF-I from *S. sobrinus* (V). The impact of the mutans streptococcal glucosyltransferases in the early transmission of isolates from mother to child has not been extensively studied to date, and should undergo comprehensive examination to add vital information on the transmission process.

As regards the mutacin production of *S. mutans* isolates, mutacins may affect the transmission from mother to child (study VI). *S. mutans* isolates harboured by the mothers represented 35 distinct types, determined by ribotyping and mutacin typing. Eight of these had been transmitted to the child. The transmitted strains inhibited a mean of 10.6 indicator strains (SD 1.9; median, 10; range, 9 to 14), and nontransmitted strains inhibited a mean of 7.0 indicator strains (SD, 3.4; median, 6; range, 1 to 14); the difference was statistically significant (equality of medians tested by Fisher's exact test, $p < 0.002$). When the sizes of inhibition zone against single indicators were compared, for all indicator strains the zones were bigger for transmitted strains than for nontransmitted strains, the difference being statistically significant for 8 of the 14 indicator strains. The 8 *S. mutans* types that had been transmitted, compared with the 27 types that had been available from the mothers but had not been transmitted, showed significantly more mutacin activity (the mean zone sizes against the indicator strains were compared by the Wilcoxon signed-rank test, $p < 0.003$).

Five ribotypes representing *S. sobrinus* isolates harboured by the mothers inhibited only one or none of the indicator strains. The *S. sobrinus* mutacin production activity could not be linked to transmission.

Earlier, bacteriocins produced by mutans streptococci have been shown to enhance colonization in rats and adult humans (van der Hoeven and Rogers, 1979; Hillman et al., 1987). In this regard, our present finding that mutacins may promote transmission of *S. mutans* from mother to child clearly confirms results obtained two decades ago on enhanced colonization by bacteriocin production. Hillman et al. (1990; 1994) have tried to construct an effector strain for replacement therapy of mutans streptococci by using a strain with increased bacteriocin activity to render high-level colonization potential. Unfortunately, the deletion that rendered the low-level acid production proved to be lethal for the strain. However, the bacteriocin produced by the strain JH1000 clearly enhanced colonization in adults who already had a stable oral flora. As stated previously, bacteriocins are potent inhibitors of bacterial growth. Nisin, commercially prepared bacteriocin from *Lactococcus lactis*, has been used in dairy products as a preservative for decades (Jack et al., 1995). In dentistry, commercially prepared bacteriocins could perhaps aid in maintaining a nonpathogenic commensal oral microbial population. Experiments in beagle dogs by Howell et al. (1993) indicated that nisin in a mouthrinse has a clear antibacterial potential. Thus far, no microbial resistance or anaphylaxis problems have been reported for the bacteriocins.

As regards the clinical significance of the present findings on differences in the virulence characteristics and colonization pattern of oral mutans streptococci, the results are not as yet applicable to the selection of individuals at risk. The virulence of bacterial isolates, on the whole, is dependent on the environment. Molecular biology methods, such as PCR detection, can be developed and applied for selection of virulent strains once the genetic elements coding for a defined property have been recognized, but when evaluating the clinical significance, many factors must be considered, depending on the multifactorial aetiology of dental caries disease. The virulence attributes remain to be clarified; it is probable that in the future the adherence process during colonization (Kolenbrander and London, 1993), and the proteolytic activity of mutans streptococci (Homer et al., 1990; Harrington and Russell, 1994; Jackson et al., 1997) will attract an increasing interest. In any case, as the present study could identify differences between individual strains affecting the transmission of strains from mother to child, it seems realistic to anticipate that selection for virulent strains by molecular biology methods will be part of upcoming preventive treatment approaches. With regard to the possible intra-individual site-specific colonization pattern of mutans streptococci, our findings support the suggested site-directed antimicrobial varnish treatment of initial caries lesion sites (Twetman, 1998; Twetman and Petersson, 1999). In the

suggested treatment protocol the antimicrobial agent chlorhexidine is locally administered to eradicate the pathogenic mutans streptococci above the lesion, enabling colonization of a less acidogenic microflora at the site.

SUMMARY AND CONCLUSIONS

The present studies were performed for quantitative and qualitative characterization of oral mutans streptococci. For detection and isolation of mutans streptococci, saliva and/or plaque samples from 97 children, 146 adolescents and 159 adults were included. The subjects, participating in two cross-sectional studies and in two longitudinal studies, were also examined clinically for decayed, missing and filled teeth. Further, for qualitative analysis, bacterial isolates obtained from 64 individuals were subjected to genotyping and/or to phenotyping, in order to study the clonal diversity and site-specific colonization of intra-individual strains, and certain virulence factors of the organism. Phenotypical characterization included serotyping and determination of chlorhexidine (CHX) susceptibility, glucosyltransferase activity and bacteriocin (mutacin) activity of the isolates. In addition, the effects of periodic low-concentration CHX-NaF gel brushing in an instructed trial on salivary counts and transmission of mutans streptococci from mother to child was studied. Genotyping was performed by ribotyping 191 of the obtained isolates, and 598 isolates were fingerprinted by the AP-PCR method. Serotyping was performed for 661 isolates by Ouchterlony immunodiffusion using rabbit antisera. CHX susceptibility of 379 isolates was tested by the agar dilution method, and for testing of glucosyltransferase activity, using monoclonal antibodies in a semiquantitative cross-dot assay, 44 isolates were included. Mutacin production of 145 isolates was tested by the stab culture method, including 38 clinical mutans streptococcal isolates from our IDH (Institute of Dentistry, Helsinki) collection .

The main findings were as follows:

In cross-sectional studies of adolescents, when mutans streptococci are not detected by chair-side methods or by conventional agar plate culture methods of plaque and saliva, mutans streptococci may either be absent or present in numbers under the detection level. In this study, a longitudinal evaluation of adolescents with no mutans streptococci initially detected, revealed that only a few subjects remained culture-negative for the organism during follow-up.

The number of clonal types detected within a subject was from one to four, with saliva revealing 80% of the genotypes present. The isolates from related subjects were often

similar, but from unrelated subjects they were, in almost all cases, distinguished by genotyping. The children with nursing caries harboured more types of mutans streptococci than caries-free children. The colonization of genotypes tended to be site-specific, implying that sampling for intra-individual mutans streptococcal genotypes should include sampling of multiple sites.

The CHX susceptibility of mutans streptococcal isolates was ribotype-specific, and the susceptibility exhibited stability at follow-up. *S. sobrinus* isolates were somewhat more resistant to CHX than *S. mutans* isolates. In the past, no CHX resistance in mutans streptococci has appeared. In this study, periodic low-concentration CHX-NaF gel brushing did not significantly affect salivary counts of mutans streptococci, CHX susceptibility of isolates or transmission of isolates from mother to child.

Glucosyltransferase activity and mutacin production by mutans streptococcal isolates were also ribotype-specific. The mutacin activity of isolates was fairly stable, and mutacin production of *S. mutans* isolates was shown to have an impact on the probability of strain transmission from mothers to their children.

These results support the original study hypothesis that mutans streptococcal strains may differ in their phenotypic properties; CHX susceptibility, glucosyltransferase activity and mutacin production were strain-specific. Moreover, the results imply that *S. mutans* strains that produce increased amounts of mutacin may be more easily transmitted from mothers to their young children. Therefore, the role of mutans streptococci in human dental caries may be characterized by not only the quantity of the bacteria, but also the quality of strain(s) colonizing.

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