Relatedness and Classification of *Streptococcus mutans* and "Mutans-like" Streptococci

K. H. SCHLEIFER*, R. KILPPER-BÄLZ*, J. KRAUS*, and F. GEHRING+

Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstraße 21, D-8000 München 2, Federal Republic of Germany; and +Bakt. Labor am Lehrstuhl Exptl. Zahnheilkunde der Universitäts- und Poliklinik für Zahn-, Mund- und Kiefernkrankheiten, Würzburg, Federal Republic of Germany

*The "mutans-like" streptococci can be separated into five species (*Streptococcus mutans, S. rattus, S. sobrinus, S. cricetus, *and* S. ferus*) that belong to the same rRNA homology cluster. New valuable chemical characters for differentiation of the five species – such as peptidoglycan type, presence of cell wall teichoic acid, and cell wall sugar composition – are described. The peptidoglycan type and the cell wall sugar composition can be determined by rapid procedures.*

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Introduction.

Streptococcus mutans was described by Clarke (1924), but it was not mentioned as an independent species in the 8th edition of **Bergey's Manual** (Deibel and Seeley, 1974). Serological studies showed that *S. mutans* strains were heterogeneous and could be subdivided into seven distinct serogroups (Bratthall, 1970; Perch *et al.*, 1974). A classification of *S. mutans* strains on the basis of physiological and biochemical tests was proposed by Facklam (1974) and Shklair and Keene (1974, 1976). Studies of Coykendall 1971, 1974) and Coykendall *et al.* (1976) showed that five genospecies exist within the *S. mutans* group and led finally to the proposal (Coykendall, 1977 and 1983) of four new species (*S. cricetus*, *S. ferus*, *S. rattus*, and *S. sobrinus*) that resemble *S. mutans* but that are genetically and biochemically distinguishable.

In the present study, nucleic acid hybridization, chemical and biochemical studies have been carried out on type strains and some other strains of the mutans group of streptococci in an attempt to clarify their taxonomic position.

Materials and methods.

The organisms included in this study are listed in Tables 1 and 2. They were cultivated in CASO-bouillon* without aeration, and, if not otherwise stated, at 35°C, and were harvested at the end of logarithmic growth. Lysis of cells and determination of DNA base composition (mol % G+C) were carried out as described by Kilpper-Bälz *et al.* (1982). Isolation of nucleic acids, DNA-DNA (nitrocellulose filter method), and DNA-rRNA hybridization experiments were performed as described previously (Kilpper *et al.*, 1980; Kilpper-Bälz and Schleifer, 1981). 2,8 H^3-adenine† was used for *in vivo* labeling of both DNA and rRNA. Qualitative sugar composition and peptidoglycan types of cell walls were determined according to Schleifer and Kandler (1967a and 1972). Rapid screening methods were applied for the determination of cell wall sugars (Staneck and Roberts, 1974) and peptidoglycan types (Schleifer and Kandler, 1972) from whole cells. Growth with and without aeration was determined in CASO-bouillon at 35°C. Growth without aeration was also determined in CASO-bouillon at 30°C, 35°C, 40°C, and 45°C. Production of H_2O_2 was determined as follows: Strains were cultivated in CASO-bouillon with aeration at 35°C and harvested. To 1 ml of the clear medium were added 0.1 - 0.2 ml of a solution consisting of 0.9 ml 0.1 M potassium phosphate buffer (pH 7.0), 0.1 ml 2% (w/v) aqueous 4-aminoantipyrine‡, 0.2 ml 2% (w/v) N, N-dimethylaniline‡, and 0.01 ml peroxidase (500 U/ml)§. A red color appears if H_2O_2 is present in the medium. The chemical composition of cell wall teichoic acid was determined as previously described (Endl *et al.*, 1983). Bacitracin sensitivity was tested as described by Shklair and Keene (1974).

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*Merck, Darmstadt, FRG
†New England Nuclear, Dreieich, FRG

Results.

(1) *Nucleic acid hybridization studies.* – Results of the DNA-DNA hybridization studies (Fig.) demonstrate that all strains within one species show DNA homology values above 90%. Average homology values of 45% were found between *S. mutans* and *S. rattus* strains, on the one hand, and between *S. sobrinus* and *S. cricetus* on the other. Lower overall DNA sequence similarity (about 25%) was

‡Sigma St. Louis, MO, USA
§Boehringer, Mannheim, FRG

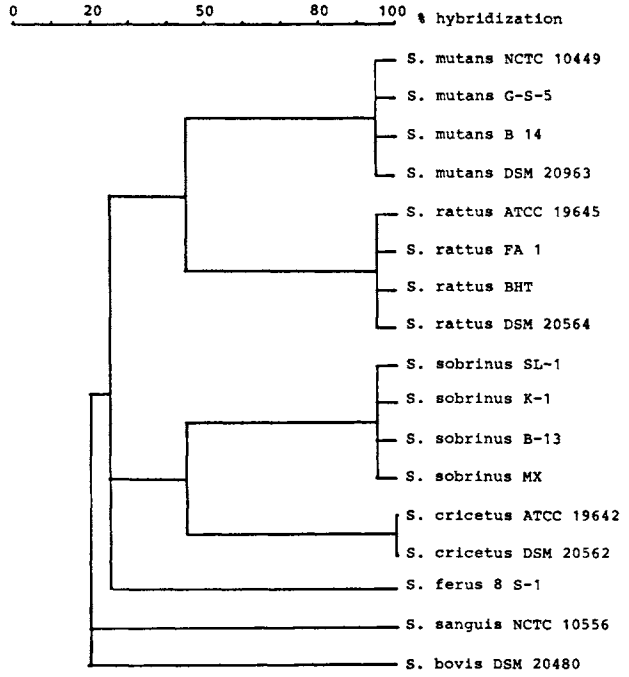

Fig. 1 – Dendrogram based on DNA-DNA hybridization studies. Hybridization was carried out under optimum conditions (25°C below the melting point of DNA).

found among strains of the two groups: *S. mutans/S. rattus* and *S. sobrinus/S. cricetus*. *S. ferus* showed 25% hybridization with both groups. Homology values between these five species and other streptococci were significantly lower (<20%).

Results of DNA-rRNA hybridization studies between labeled 23 S rRNA of *S. mutans* ATCC 25175 and of *S. sobrinus* B 13, respectively, and filter-bound DNA of other streptococci confirm the significant genetic relationship of "mutans-like" streptococci. $\Delta T_{m(e)}$ values below 4°C were found between *S. mutans*, *S. rattus*, *S. sobrinus*, *S. cricetus*, and *S. ferus*. The $\Delta T_{m(e)}$ values with all the other streptococci investigated were higher (Table 2).

(2) *Cell wall components and DNA base composition.* – Peptidoglycan-type, cell wall teichoic acid, cell wall sugar, and DNA base composition of the organisms investigated are listed in Table 1. Strains belonging to *S. mutans* or *S. rattus* contained in their peptidoglycan an interpeptide bridge consisting of two or three alanine residues. They differed, however, in containing glucose (*S. mutans*) or galactose (*S. rattus*) as additional cell wall sugar in addition to rhamnose (Hardie and Bowden, 1974). Moreover, only strains of *S. rattus* exhibited a glycerol-containing cell wall teichoic acid. The structure of the cell wall teichoic acid of *S. rattus* BHT (ATCC 19645) was studied in detail and was described as glycerol teichoic acid with a relatively high degree of galactosyl substitution (Vaught and Bleiweis, 1974; Bleiweis *et al.*, 1976; Wicken *et al.*, 1982). DNA base composition was different: 36 - 38 mol% GC in *S. mutans* and 42 - 43 mol% GC in *S. rattus*. These differences in the DNA base composition were also reported by Dunny *et al.* (1972) and Coykendall (1977). *S. ferus* showed the peptidoglycan type Lys-Ala_{2-3}. Its cell wall did not contain teichoic acid, but glucose and rhamnose were found as com-

TABLE 1
ORIGIN OF BACTERIAL STRAINS USED, THEIR DNA BASE COMPOSITION (MOL% GUANINE PLUS CYTOSINE), AND CHARACTERISTIC COMPONENTS OF THEIR CELL WALLS

Species	Strain	Origin	Serotype‡	Biotype§	G+$C_{mol\%}$	Type of peptidoglycan	Cell wall sugar	Cell wall teichoic acid
S. mutans	NCTC 10449	National Collection of Type Cultures, London, UK	c,e,f	"c"	38.2	Lys-Ala_{2-3}	Glc,Rha	–
	G-S-5	Grenier, E.M., University of Michigan, Ann Arbor, USA	c	"c"	37.3	Lys-Ala_{2-3}	"	–
	LM-7	"	e	"e"		Lys-Ala_{2-3}	"	–
	B14	Bratthall, D., University of Göteborg, Sweden	e	"e"	36.2	Lys-Ala_{2-3}	"	–
	ATCC 25175 (type strain)	American Type Culture Collection, Rockville, MD, USA			37.9	Lys-Ala_{2-3}	"	–
	DSM 20963	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG				Lys-Ala_{2-3}	"	–
S. rattus	ATCC 19645 (type strain)	Grenier, E.M.	b	"b"	42.0	Lys-Ala_{2-3}	Gal,Rha	+
	BHT	Bratthall	b	"b"	42.9	Lys-Ala_{2-3}	Gal,Rha	+
	FA 1	Coykendall, A.L., School of Dental Medicine, Farmington, CT, USA	b	"b"	42.3	Lys-Ala_{2-3}	"	+
	DSM 20564	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG				Lys-Ala_{2-3}	"	+
S. sobrinus	K-1	Fitzgerald, R., VA Hospital, Miami, USA		"d"	44.8	Lys-Thr-Ala	Glc,Gal,Rha	–
	B-13	Hardie, J.M., London Hospital Medical College, UK	d		43.7	Lys-Thr-Ala	"	–
	SL-1 (type strain)	Coykendall, A.L.	d,g	"d"	45.1	Lys-Thr-Ala	"	–
	MX	Hardie, J.M.	d		44.8	Lys-Thr-Ala	"	–
S. cricetus	ATCC 19642 (type strain)	American Type Culture Collection, Rockville, MD, USA	a	"a"	42.7	Lys-Thr-Ala	"	–
	OMZ 61	Hardie, J.M.	a	"a"		Lys-Thr-Ala	"	–
	HS6	Coykendall, A.L.	a			Lys-Thr-Ala	"	–
	DSM 20562	Deutsche Sammlung von Mikroorganismen, Göttingen, FRG			43.0	Lys-Thr-Ala	"	–
S. ferus	8S-1 (type strain)	Coykendall, A.L.	c		44.2	Lys-Ala_{2-3}	Glc,Rha	–

‡Bratthall (1970), Perch *et al.* (1974), and Coykendall *et al.* (1976).
§Shklair and Keene (1976).

ponents of cell-wall-bound polysaccharide. Moreover, we could confirm Coykendall's (1977) observation that the DNA base composition of *S. ferus* is significantly higher than that of *S. mutans* (Table 1).

S. cricetus and *S. sobrinus* contained glucose, galactose, and rhamnose as cell wall sugars (Hardie and Bowden, 1974), the peptidoglycan type Lys-Thr-Ala, and no cell wall teichoic acid. Analysis of these data indicates that cell wall composition is a valuable characteristic of the separation and identification of the "mutans-like" streptococci. Since preparation of cell walls is rather time-consuming, simple and rapid procedures using whole cells were tested to obtain data on peptidoglycan type and cell wall sugar composition. The rapid screening method described by Schleifer and Kandler (1972) was applied for the determination of the peptidoglycan type. Comparison of two-dimensional paper chromatograms of total hydrolysates (6N HCl, 105°C, 6hr) of trichloroacetic-acid-extracted cells was sufficient to distinguish *S. mutans*/*S. rattus*/*S. ferus*, on the one hand, from *S. sobrinus*/*S. cricetus*, on the other hand. The chromatograms of the latter organisms always revealed the characteristic, rather acid-stable dipeptide N^6-threonyllysine (Schleifer and Kandler, 1967b). The cell wall sugars could be identified from whole cells by applying the method of Staneck and Roberts (1974). The latter method provided sufficient information to separate *S. rattus* from *S. mutans* and *S. ferus*.

(3) *Simple physiological tests for the differentiation of* S. mutans, S. rattus, S. ferus, S. sobrinus, *and* S. cricetus. – Perch *et al.* (1974) reported that strains of serogroups *d* and *g* which have been classified as *S. sobrinus* by Coykendall (1977, 1983) produce H_2O_2. Using a different method, we could confirm their data and demonstrate that none of the studied strains of *S. mutans*, *S. rattus*, *S. ferus*, or *S. cricetus* excreted H_2O_2 in traceable amount into the medium. Moreover, we could corroborate the finding of Coykendall (1977) that only *S. rattus* can grow at 45°C, whereas representatives of other species stopped growth at temperatures above 40°C. All strains studied, with the exception of *S. cricetus*, could grow aerobically in culture fluid. Thus, the failure of *S. cricetus* to grow under aerobic conditions in bouillon is a valuable criterion for the identification of *S. cricetus*. Another possibility to identify different species within the *S. mutans* species group was the bacitracin sensitivity test. In contrast to strains of *S. cricetus* and *S. ferus*, those of *S. mutans*, *S. rattus*, and *S. sobrinus* were not inhibited by bacitracin.

TABLE 2
DNA-rRNA HYBRIDIZATION STUDIES BETWEEN ^{3}H-LABELED 23S rRNA FROM *S. mutans* ATCC 25175 AND FROM *S. sobrinus* B13 AND FILTER-BOUND DNA FROM DIFFERENT STREPTOCOCCI

Filter-bound DNA	^{3}H-labeled 23S rRNA from *S. mutans* ATCC 25175 $T_{m(e)}$‖	$\Delta T_{m(e)}$¶	*S. sobrinus* B13 $T_{m(e)}$‖	$\Delta T_{m(e)}$¶
S. mutans ATCC 25175 (type strain)	77.6	0	74.2	3.8
S. mutans B14	77.1	0.5	74.1	3.9
S. rattus ATCC 19645 (type strain)	74.3	3.3	74.0	4.0
S. rattus BHT	74.2	3.4	74.2	3.8
S. sobrinus B13	73.7	4.0	78.0	0
S. sobrinus MX	73.8	3.9	77.7	0.3
S. cricetus ATCC 19642 (type strain)	73.7	3.9	74.6	3.4
S. ferus 8-S-1 (type strain)	n.d.	n.d.	74.0	4.0
S. salivarius ATCC 13419	73.0	4.6	73.6	4.4
S. sanguis DSM 20066	72.6	5.0	73.4	4.6
S. thermophilus DSM 20479	72.3	5.3	73.2	4.8
S. bovis DSM 20480 (type strain)	72.0	5.6	73.2	4.8
S. equinus ATCC 9812 (type strain)	71.3	6.3	72.3	5.7
S. lactis DSM 20481 (type strain)	70.6	7	71.0	7
S. faecalis DSM 20376	69.2	8.4	69.1	8.9
E. coli K-12	57.6	20	58.3	19.7

‖$T_{m(e)}$, temperature (°C) at which 50% of the bound rRNA is eluted from the filters.
¶ $\Delta T_{m(e)}$, difference between $T_{m(e)}$ of homologous and heterologous DNA-rRNA hybrids.

Discussion.

The results of the present study support the proposal of Coykendall (1977, 1983) to differentiate the mutans group of streptococci into five species: *S. mutans*, *S. rattus*, *S. sobrinus*, *S. cricetus*, and *S. ferus*. DNA-DNA hybridization values, obtained under optimal hybridization conditions, of 25%-45% between their representatives justify the separation into different species (Schleifer and Stackebrandt, 1983). Strains of the same species are very closely related, displaying DNA homology values above 90%. The dendrogram of relationship derived from DNA-DNA hybridization studies of *S. mutans* and related species (Fig. 1) reveals three main branches: one consisting of *S. mutans* and *S. rattus*, one of *S. sobrinus* and *S. cricetus*, and one of *S. ferus*. They separate at a level of 25% homology, which indicates that they are different from each other, but not as different as they are from other streptococcal species, where DNA homology was below 20%. Results of DNA-rRNA hybridization studies confirmed this view. Low $\Delta T_{m(e)}$ values between *S. mutans*, *S. rattus*, *S. sobrinus*, *S. cricetus*, and *S. ferus* demonstrate a relationship. Among the other streptococci investigated, *S. salivarius* (ATCC 13419) appears to be the closest relative to the mutans group of streptococci. Data obtained from comparative oligonucleotide sequencing of 16S rRNA are in accord with these results (Seewaldt *et al.*, manuscript in preparation). Biochemical and physiological properties also demonstrate the natural unit of the "mutans-like" streptococci. However, a set of different tests provides their separation and identification (Table 3). Besides well-known characteristics – such as growth at 45°C, growth under anaerobic conditions, excretion of H_2O_2, bacitracin sensitivity, and DNA base composition (mol % G+C) – new chemotaxonomic characteristics

TABLE 3
SET OF BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF THE DIFFERENT SPECIES WITHIN THE *S. MUTANS* SPECIES GROUP

Species	Interpeptide Bridge of Peptidoglycan	Cell Wall Teichoic Acid	Cell Wall Sugar Composition	Growth at 45°C	Aerobic Growth in Culture Fluid	Excretion of H_2O_2	Bacitracin Sensitivity	G+C Content mol %
S. mutans	Lys-Ala_{2-3}	–	Glc,Rha	–	+	–	–	36-38
S. rattus	Lys-Ala_{2-3}	Glycerol	Gal,Rha	+	+	–	–	42-43
S. sobrinus	Lys-Thr-Ala	–	Glc,Gal,Rha	–	+	+	–	44-45
S. cricetus	Lys-Thr-Ala	–	Glc,Gal,Rha	–	–	–	+	43
S. ferus	Lys-Ala_{2-3}	–	Glc,Rha	–	+	–	+	44

(peptidoglycan type, cell wall teichoic acid, and cell wall sugar composition) are now also available for the correct identification and classification into five distinct species of "mutans-like" streptococci. Rapid screening techniques for the determination of peptidoglycan type and cell wall sugar composition from whole cells were successfully tested in the present study. Additional characteristics [such as production of ammonia from arginine by *S. rattus*, the failure of *S. sobrinus* to ferment raffinose (Coykendall, 1977), or various biochemical tests (Kral and Daneo-Moore, 1981)] for the differentiation of "mutans-like" streptococci are also helpful.

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