JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 5247–5257
0021-9193/87/115247-11$02.00/0

Vol. 169, No. 11

Genetic Relationships among the Oral Streptococci

MARION N. GILMOUR,[1] THOMAS S. WHITTAM,[2] MOGENS KILIAN,[3] AND ROBERT K. SELANDER[1]*

Department of Biology, University of Rochester, Rochester, New York 14627[1]; Department of Biology, Pennsylvania State University, University Park, Pennsylvania 16802[2]; and Department of Oral Biology, Royal Dental College, Aarhus, Denmark[3]

Received 11 June 1987/Accepted 17 August 1987

Genetic relationships and species limits among the oral streptococci were determined by an analysis of electrophoretically demonstrable variation in 16 metabolic enzymes. Fifty isolates represented 40 electrophoretic types, among which the mean genetic diversity per locus was 0.857. Mannitol-1-phosphate dehydrogenase was not detected in isolates of the sanguis species complex, and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were absent in species of the mutans complex. Clustering from a matrix of Gower's coefficient of genetic similarity placed the 40 electrophoretic types in 10 well-defined groups corresponding to the *Streptococcus* species *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus*, *S. ferus*, *S. oralis* (*mitior*), two distinct assemblages of *S. sanguis* strains, and two subdivisions of "*S. milleri*." The assignments of isolates to these groups were the same as those indicated by DNA hybridization experiments, and the coefficient of correlation between genetic distance estimated by multilocus enzyme electrophoresis and genetic similarity indexed by DNA hybridization was −0.897 ($P < 0.001$) for 50 pairwise combinations of isolates. *S. ferus*, which is widely believed to be a member of the mutans complex, was shown to be phylogenetically closer to species of the sanguis complex.

Despite the considerable effort of microbiologists to classify the oral streptococci, the systematic relationships of these medically important microorganisms remain inadequately known. Included in the oral streptococci are species of the mutans complex, which are major etiological agents of dental caries (36), and species of the sanguis complex, which have been implicated in a variety of systemic diseases, including bacteremia and endocarditis (20, 51), as well as in caries and gingivitis (2, 61, 64).

Few groups of bacteria better illustrate the inadequacy of phenotypic variation as a basis for systematics. Studies of biochemical and physiological traits either have failed to detect the existing species diversity or have yielded conflicting pictures of the phylogenetic relationships of the species (3, 11, 39). Serological classifications are unsatisfactory because antigenic determinants tend not to be species specific and are mediated by an unknown but probably small number of genetic loci; antigenic drift is a further complication (15, 16, 32). As Kilian et al. (42) have pointed out, additional taxonomic confusion has arisen as a result of the arbitrary grouping of strains to establish pragmatic guidelines for identification (25).

In recent years, major advances in understanding of species limits and phylogenetic relationships have been achieved through hybridization of total cellular DNA. Among strains formerly assigned to the single species *Streptococcus mutans* on the basis of phenotypic characters (24), six distantly related species are now recognized (16); and the genetic relationships indicated by DNA-DNA hybridization have been confirmed by DNA-rRNA hybridization (54). This work has stimulated renewed interest in biotypic and other phenotypic variation (15), leading to the discoveries that *Streptococcus ferus* is not cariogenic in animal models (29) and that there is interspecific variation in the molecular mechanism of cellular attachment to tooth surfaces (31).

More recently, DNA hybridization of strains of the sanguis complex has revealed a high level of genetic diversity associated with the occurrence of five or more species (16, 23, 43).

Although DNA hybridization has been of great value in defining species limits and relationships among the oral streptococci, it has not provided information on genetic diversity and relationships within species. Moreover, the genetic distances determined by DNA hybridization may be distorted because they were derived by comparing isolates with only a small number of reference strains rather than being determined for all pairs of isolates (58). These limitations are overcome through the use of multilocus enzyme electrophoresis (55), which provides information on allelic variation at individual genetic loci and estimates of genetic distances for all pairs of isolates. This technique has recently been used to study genetic structure in several types of bacteria, including *Escherichia coli* (56), *Legionella* spp. (57), *Haemophilus influenzae* (49), *Bordetella* spp. (50), and *Neisseria meningitidis* (7).

Electrophoretically demonstrable variation in enzyme structure has had some use as an adjunct character in taxonomic research on the oral streptococci (5, 60, 62), but population-genetic studies have not previously been undertaken. We here report the results of the application of multilocus enzyme electrophoresis to the study of intraspecific and interspecific relationships among the oral streptococci. The interspecific relationships revealed by this technique correlate well with those indicated by DNA hybridization experiments, but our analysis indicates that *S. ferus*, which is currently assigned to the mutans species complex, is phylogenetically closer to species of the sanguis complex.

MATERIALS AND METHODS

Isolates. This study was based on a collection of 57 *Streptococcus* isolates, representing *S. mutans*, *S. rattus*, *S. sobrinus*, *S. cricetus*, and *S. ferus* of the mutans complex

* Corresponding author.

and strains of the sanguis complex received as *S. sanguis*, *S. mitior*, *S. mitis*, *S. oralis*, and *S. milleri* (Table 1). These isolates included 30 that were coded by one of us (M.K.) for analysis in Rochester, N.Y., 12 that were used by Coykendall in DNA hybridization experiments (12, 13, 19), and 7 that were duplicates of strains obtained from various laboratories.

After our analysis of the 57 strains was completed, three additional isolates of *S. ferus* (8S1, 4S1, and 5T1, obtained from A. L. Coykendall) were examined.

Isolates were stored at −70°C, and cultures were derived from single colonies on anaerobically (95% N_2 and 5% CO_2) incubated blood agar plates. Short-term stock cultures were grown on a medium modified from one recommended by I. L. Shklair (personal communication): Todd-Hewitt broth (Difco Laboratories) supplemented with 0.1% glucose; 0.5% lactalbumin hydrolysate (Difco), 0.07% agar, and 0.15% $CaCO_3$.

Serotyping. Isolates of the mutans complex were grown in Todd-Hewitt broth supplemented with 50 mM potassium phosphate and 0.8 mM $MgSO_4$. Cells were harvested, washed twice in phosphate-buffered saline (pH 7.2), and allowed to react with appropriate dilutions of specific antibodies obtained from the National Institute for Dental Research; reactions were assessed by fluorescence microscopy at 1,000× magnification. As a control, each isolate was tested with several specific antisera (26).

Preparation of lysates. Cultures were grown in a buffered tryptone-based broth containing 0.5% tryptone (Difco), 0.25% yeast extract (Sigma Chemical Co.), 20 mM DL-threonine (Sigma), 0.6 mM $MgSO_4$, 0.03 mM $MnSO_4$, 0.08 mM NaCl, 25 mM NH_4HCO_3, 1.0 M L-cysteine hydrochloride, and 0.4% glucose in 40 mM potassium phosphate buffer. (Threonine was added to weaken the cell walls [8].) The glucose, bicarbonate, cysteine, and phosphate buffer solutions were sterilized individually and added to double-strength tryptone-yeast extract-salts base (pH 7.2) just before inoculation. Flasks containing 200 ml of broth were inoculated with 0.2 to 0.5 ml of 10- to 12-h anaerobic cultures grown in Todd-Hewitt broth supplemented with 0.1% glucose and 0.15% $CaCO_3$; the flasks were closed with silicone bungs and incubated aerobically. Cultures were harvested between late log and early stationary phase, as estimated from spectrophotometrically determined growth curves (46). Because the activity of some enzymes was weak in extracts made from acidic cultures, sterile KOH was added as needed to maintain a pH higher than 5.1 during the late phase of growth.

Cells were harvested by centrifugation and suspended in 2 ml of a 40 mM potassium phosphate buffer solution (pH 7.5) that contained 3 mM dithiothreitol, 10 mM L-cysteine hydrochloride, 1 mM EDTA, and 500 μg of bovine serum albumin per ml (10). Glass beads (5 μm in diameter) were added (37), and the suspension was sonicated (Branson Sonifier cell disruptor, model 200, with microtip) for 4 min at 50% pulse, with dry ice-methanol cooling. After centrifugation at 20,000 × *g* for 20 min at 4°C, the supernatant was removed and stored at −70°.

Electrophoresis and enzyme assays. Electrophoretic conditions were as described by Selander et al. (55), except that voltages were decreased to prevent loss of activity of certain enzymes. Buffer systems A (Tris-citrate, pH 8.0), B (Tris-citrate, pH 6.7), and D (lithium hydroxide, pH 8.3) were used as follows for the 16 enzymes assayed. Buffer system A at 120 V: aldolase (ALD), leucine amino peptidase (LAP), phenylalanyl leucine peptidase (PLP), leucylglycyl glycine peptidase (LGP), nucleoside phosphorylase (NSP), 6-phosphogluconate dehydrogenase (6PG), glucose-6-phosphate dehydrogenase (G6P), and mannose phosphate isomerase (MPI). Buffer system B at 120 V: adenylate kinase (ADK), NAD-dependent glyceraldehyde phosphate dehydrogenase (GP1), and mannitol-1-phosphate dehydrogenase (M1P). Buffer system D at 310 V: hexokinase (HEX), indophenol oxidase (IPO), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), and NADP-dependent glutamate dehydrogenase (GD2).

The stains used to demonstrate enzyme activities were described by Selander et al. (55), except for ALD, which was stained with 50 ml of the Co^{2+}-K^+-cysteine buffer solution of Groves et al. (35), to which was added fructose-1,6-bisphosphate (Sigma), 100 mg; glyceraldehyde-3-phosphate dehydrogenase (Sigma), 50 U; triose phosphate isomerase (Sigma), 100 U; sodium arsenate, 100 mg; and quantities of NAD, dimethylthiazol tetrazolium, and phenazine methosulfate as described by Selander et al. (55).

Electromorphs (mobility variants) of each enzyme were numbered in order of decreasing anodal mobility (55). In addition, the absolute distance traveled by each mobility variant relative to that of the fastest electromorph of a given enzyme was determined by measuring adjacent bands on several gel slices; the mean distances between each pair of variants were summed sequentially from the fastest allele, which was assigned a value of 1.

Electromorphs were equated with alleles at the corresponding structural gene locus. We presume that the structural gene loci are located on the chromosome rather than on plasmids because all isolates showed activity for 13 of the enzymes; all isolates of the sanguis complex showed activity for G6P and 6PG, and all isolates of the mutans complex demonstrated activity for M1P. Isolates of species of the mutans complex reportedly either lack plasmids or carry only small plasmids that occur at a frequency of 10% and have no known physiological or phenotypic correlates apart from a bacteriocin profile (6).

Each isolate was characterized by its profile of alleles at the 16 enzyme loci assayed, and distinctive allelic combinations, representing multilocus genotypes, were designated electrophoretic types (ETs) (55).

Statistical methods. Genetic diversity at a locus among ETs or isolates was calculated as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the *i*th allele and *n* is the number of ETs or isolates (55). Mean genetic diversity per locus (*H*) is the arithmetic average of *h* values for all loci.

Three types of coefficients were used to estimate genetic relationships between pairs of isolates or ETs: unweighted genetic distance (*D*) (55), weighted genetic distance (D_w) (57), and Gower's coefficient of similarity (S_G) or distance ($1 - S_G$) (33, 59). Clustering of multilocus genotypes from matrices of genetic distance or similarity was performed by the average-linkage method (59).

RESULTS

ETs. Comparison of the electrophoretic mobilities of the 16 enzymes in the 57 isolates (Table 1) revealed a total of 40 distinctive profiles of electromorphs (ETs) (Table 2). In general, isolates of the same strain designation received from different laboratories were of the same ET; thus, for example, three cultures of *S. mutans* 10449 were genotypically identical, representing ET 1 (Table 1). In such cases, data for only one isolate were included in the statistical analyses. There were, however, several sets of isolates of the same

TABLE 1. Properties of 57 isolates of oral streptococci representing 40 ETs[a]

Genetic group	ET	Species[a]	Taxon	Serotype[b]	Isolate identification[c]	Source[d]
I	1	*S. mutans*		c	SK28 (NCTC 10449^T) (= ATCC 25175)	Kilian (NCTC)
				c	10449* (NCTC 10449)	Coykendall
				c	10449* (NCTC 10449)	Eisenberg
	2			f	OMZ175	Coykendall
	3			e	V100	Shklair
	4			c	SK30 (GS5)	Kilian (Gibbons)
				c	GS5*	Eisenberg
	5			e	LM7	Eisenberg
				e	"AHT"* (LM7)	Eisenberg
	6			c	SK20 ("*S. mutans* complex")	Kilian (Perch)
II	7	*S. rattus*		b	FA-1 (= ATCC 19645^T)	Coykendall
				b	SK29* (FA-1)	Kilian (Perch)
				b	FA-1*	Marquis
				b	BHT	Coykendall
				b	BHT*	Eisenberg
	8			b	PIG107	Shklair
				b	PIG130	Shklair
III	9	*S. sobrinus*		d/g	6715	Coykendall
				d/g	6715-15*	Eisenberg
	10			d/g	TEA	Shklair
	11			d/g	K1R	Coykendall
	12			d/g	B13	Shklair
	13			d/g	SL1 (= ATCC 33478^T)	Coykendall
	14			d/g	SL1	Marquis
IV	15	*S. cricetus*		a	AHT	Coykendall
				a	E49	Shklair
				a	HS1	Shklair
	16			a	HS6 (= ATCC 19642^T)	Coykendall
				a	OMZ61	Coykendall
V	17	*S. ferus*		c	8S1 (= ATCC 33477^T)	Coykendall
	18			c	HD3	Coykendall
VI	19	*'S. milleri''*			SK87	Kilian[e]
VII	20	*S. sanguis* genotype 1, sp. nov.	Taxon 1		SK120 (*S. oralis* PB179) (Carlsson KPE1, group I:B)	Kilian (Sneath)
			Taxon 1		SK121 (colonial variant of SK120)	Kilian (Sneath)
	21		Taxon 2		SK3 (*S. sanguis* ATCC 10558) (= NCTC 7865)	Kilian (ATCC)
	22		Taxon 3		SK8 (*S. sanguis* S7)	Kilian (Liljemark)
	23		Aberrant		SK42 (from *Macaca fascicularis*)	Kilian[e]
	24		Taxon 1		SK6 (group H, ATCC 12396)	Kilian (ATCC)
	25		Taxon 3		SK51 (*S. mitis*, NCTC 3165^T) (= ATCC 33399)	Kilian (NCTC)
VIII	26	*S. oralis* (*mitior*)	Taxon 1		SK170	Kilian[e]
	27		Taxon 1		SK10 (*S. sanguis*, NCTC 7864) (= ATCC 10557)	Kilian (NCTC)
	28		Taxon 1		SK111	Kilian[e]
	29		Taxon 1		SK23 (*S. oralis*, NCTC 11427^T) (= PB182; Carlsson LVG1, group 1A)	Kilian (Sneath)
	30		Taxon 3		SK96	Kilian[e]
	31		Aberrant		SK38	Kilian[e]
	32		Taxon 1		SK107	Kilian[e]
	33		Taxon 3		SK103	Kilian[e]
IX	34	*S. sanguis* genotype 2	Taxon 4		SK112	Kilian[e]
	35		Taxon 4		SK49	Kilian[e]
	36		Taxon 1		SK76	Kilian[e]
			Taxon 1		SK77	Kilian[e]
	37		Aberrant		SK80	Kilian[e]
	38		Taxon 1		SK1 (*S. sanguis*, ATCC 10556^T) (= NCTC 7863)	Kilian (ATCC)
			Taxon 1		SK108 (*S. oralis*, PB177) (= Carlsson KPE2, group I:B)	Kilian (Sneath)
	39		Aberrant		SK82	Kilian[e]
X	40	*"S. milleri"*			SK65	Kilian[e]
					SK73	Kilian[e]

[a] Species names and taxon assignments of most of the isolates in groups VI to X are from M. Kilian, L. Mikkilsen, and J. Henrichsen (in preparation). For groups VII to IX, we have also used the designations of Coykendall (14): VII, *S. sanguis* genotype 1; VIII, *S. mitior*; and IX, *S. sanguis* genotype 2.

[b] Serotypes of isolates in groups VI to X will be reported by Kilian et al. (in preparation).

[c] SK code numbers were assigned by Kilian. ^{T}, Type strain. Duplicate strains not used in analyses are marked (*).

[d] NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection.

[e] Newly isolated by Kilian (unpublished data).

TABLE 2. Allele profiles for 16 enzyme loci in 40 ETs *Streptococcus* spp.

Genetic group	Species or taxon name	ET	Allele at the indicated enzyme locus[a]:														
			ADK	HEX	PGI	MPI	IPO	PGM	ALD	NSP	LAP	PLP	LGP	GD2	GP1	M1P	G6P	6PG
I	*S. mutans*	1	12	9	1	13	8	7	5	14	8	14	12	4	7	7	NU	NU
		2	12	6	1	15	7	7	7	9	10	16	13	4	7	7	NU	NU
		3	12	6	1	17	7	7	6	12	9	15	13	4	7	8	NU	NU
		4	12	9	1	16	8	11	6	16	8	15	13	4	7	9	NU	NU
		5	12	9	4	14	8	7	5	19	9	15	11	4	7	6	NU	NU
		6	12	9	1	14	8	7	5	19	8	15	11	4	7	6	NU	NU
II	*S. rattus*	7	3	3	3	19	5	5	4	15	3	16	10	5	6	5	NU	NU
		8	3	3	3	18	5	5	4	15	1	16	9	5	6	5	NU	NU
III	*S. sobrinus*	9	1	9	7	1	6	9	3	24	2	3	4	3	5	2	NU	NU
		10	1	9	7	1	6	9	2	22	2	2	4	3	5	2	NU	NU
		11	1	9	5	2	6	9	3	23	2	3	4	3	5	2	NU	NU
		12	1	9	6	1	6	10	2	20	2	1	4	3	5	2	NU	NU
		13	1	9	5	3	6	9	3	21	1	3	4	3	5	3	NU	NU
		14	4	9	5	3	6	9	3	21	1	3	4	3	5	3	NU	NU
IV	*S. cricetus*	15	13	4	4	4	9	8	3	18	7	5	9	6	4	4	NU	NU
		16	13	4	4	4	9	8	3	18	7	5	9	7	4	4	NU	NU
V	*S. ferus*[b]	17	4	2	2	12	2	2	1	8	6	6	11	1	1	1	NU	NU
		18	4	1	2	12	2	2	1	4	6	6	12	1	1	2	NU	NU
	4S1	A	4	2	2	12	2	3	1	1.5	6	6	13	1	1	2	NU	NU
	5T1	B	4	2	2	12	2	2	1	3	3	7	11	1	1	2	NU	NU
VI	"*S. milleri*"	19	5	9	2	3	2	5	3	17	8	12	2	2	3	NU	7	6
VII	*S. sanguis*	20	5	9	2	9	1	2	3	7	5	13	7	2	4	NU	5	2
	genotype 1	21	9	8	2	5	2	3	3	3	9	7	7	2	4	NU	7	2
		22	10	9	2	10	3	2	2	2	11	4	7	1	3	NU	7	2
		23	10	9	2	9	3	2	2	2	11	4	7	2	4	NU	7	2
		24	11	9	2	9	2	2	2	8	9	11	7	2	3	NU	6	2
		25	9	9	2	8	3	3	2	1	9	4	8	2	4	NU	4	3
VIII	*S. oralis*	26	7	5	2	10	4	2	3	7	5	4	5	2	2	NU	2	2
	(*mitior*)	27	8	5	2	10	4	2	3	9	5	4	5	2	3	NU	2	2
		28	6	5	2	10	4	2	2	8	5	4	5	2	3	NU	2	2
		29	8	5	2	10	4	2	2	5	5	2	5	1	3	NU	2	4
		30	7	5	2	5	4	2	2	11	4	4	6	1	5	NU	2	5
		31	7	5	2	7	4	2	3	10	7	8	3	2	3	NU	2	5
		32	8	5	2	5	4	3	3	5	8	8	2	2	2	NU	2	5
		33	7	5	2	6	4	1	2	8	12	5	6	2	4	NU	1	5
IX	*S. sanguis*	34	5	7	2	9	2	4	3	14	14	6	7	1	3	NU	5	4
	genotype 2	35	2	7	2	8	2	5	3	14	13	6	7	1	4	NU	5	4
		36	7	7	3	4	3	5	2	10	11	9	2	1	3	NU	5	4
		37	9	6	2	4	3	6	3	6	9	10	6	1	3	NU	5	4
		38	9	4	2	6	4	5	3	8	9	9	1	1	3	NU	5	1
		39	9	4	3	4	3	5	3	13	11	6	5	1	3	NU	3	2
X	"*S. milleri*"	40	2	10	3	11	4	7	2	12	11	10	14	1	3	NU	8	5

[a] Alleles are numbered consecutively in order of decreasing anodal mobility; NU, null (no enzyme activity).

[b] Three additional isolates of *S. ferus* were obtained after the analysis for this study was completed. These isolates (8S2, 4S1, and 5T1) represent two additional ETs, A and B; 8S2 was genotypically identical to ET 17.

strain designation that varied in electrophoretic profile. For example, two cultures received as *S. sobrinus* SL1 differed in mobility at the ADK locus and therefore represented different, albeit closely related, ETs (ET 13 and 14). One of two isolates received as *S. cricetus* AHT was ET 15, but the other was genotypically and serotypically identical to *S. mutans* strain LM7, representing ET 5. (Marked phenotypic variation among cultures of strain AHT from various laboratories has been noted previously and attributed to labeling errors [53].) We also received three other isolates that, according to multilocus genotype and more conventional characters, had been misidentified.

After electrophoretically identical replicate strains were eliminated, there were 50 isolates representing 40 ETs (Table 2). Twenty-four isolates, representing 18 ETs, belonged to species of the mutans complex, and 26 isolates, representing 22 ETs, were members of the sanguis complex.

For purposes of statistical analysis, we have treated *S. ferus* as a member of the mutans complex, following taxonomic convention, although our analysis strongly sug-

TABLE 3. Number of alleles and genetic diversity (h) at 16 enzyme loci among ETs of oral streptococci

Enzyme locus	ETs of mutans complex (n = 18)		ETs of sanguis complex (n = 22)		All ETs (n = 40)		No. (%) of alleles shared between complexes
	No. of alleles	h	No. of alleles	h	No. of alleles	h	
ADK	5	0.804	8	0.879	13	0.926	0
HEX	6	0.686	7	0.797	10	0.794	3
PGI	7	0.876	2	0.247	7	0.696	2
MPI	12	0.954	9	0.896	19	0.950	2
IPO	6	0.837	4	0.697	9	0.865	1
ALD	7	0.856	2	0.519	7	0.712	2
NSP	14	0.974	14	0.952	24	0.971	2
LAP	8	0.902	9	0.866	14	0.918	4
PLP	8	0.889	11	0.883	16	0.932	3
LGP	6	0.837	9	0.840	14	0.921	0
GD2	6	0.791	2	0.519	7	0.791	1
GP1	5	0.784	4	0.593	7	0.814	2
M1P	9	0.902	—[a]		—		—
G6P	—		8	0.818	—		—
6PG	—		6	0.758	—		—
Means[b]							
13 loci	7.5	0.849	6.8	0.726	12.2	0.857	1.9 (15.8)
14 loci	7.6	0.853	—		—		
15 loci	—		6.8	0.734	—		

[a] —, No enzyme activity.
[b] Data were compiled as follows: 13 loci, all loci excluding M1P, G6P, and 6PG; 14 loci, all loci excluding G6P and 6PG; and 15 loci, all loci excluding M1P.

gests that its closest relatives are members of the sanguis complex.

Single-locus diversity. Activity of 13 of the 16 enzymes assayed was demonstrated in all isolates, and all 13 enzymes were polymorphic (Table 3). For each of the remaining three enzymes, activity was shown by members of only one of the two species complexes. M1P was present and polymorphic for nine alleles in species of the mutans complex but was not detected in species of the sanguis complex. Conversely, neither G6P nor 6PG was detected in species of the mutans complex, but both G6P and 6PG were active and polymorphic in the sanguis complex, being represented by eight and six alleles, respectively.

Estimates of genetic diversity among the 40 ETs are presented in Table 3 for the 14 enzyme loci that could be scored in the mutans complex and the 15 scorable loci in the sanguis complex, as well as for the 13 loci that could be assayed in isolates of both species complexes. NSP was the most variable locus, with 24 alleles and a genetic diversity (h) of 0.971. PGI was the least variable locus, with only seven alleles each and an h of 0.696.

There was greater genetic diversity among ETs of the mutans complex than among ETs of the sanguis complex (Table 3). For the 13 enzyme loci common to ETs of both complexes, there were, on average, 7.5 alleles per locus in the mutans complex, and the mean genetic diversity (H) among ETs was 0.849. The comparable values for the sanguis complex were 6.8 alleles per locus and an H of 0.726. For the 13 common loci, H for the 40 ETs was 0.857.

The greater diversity among ETs of the mutans complex than among those of the sanguis complex was largely attributable to unusually high diversity values (h) for four enzyme loci, PGI, IPO, ALD, and GD2; for these loci, the numbers of alleles were 7, 6, 7, and 6, respectively, for the mutans complex, but only 2, 4, 2, and 2, respectively, for the sanguis complex.

The difference between the two species complexes in level of interspecific heterogeneity is illustrated in Fig. 1, which shows, for all pairs of ETs within each complex, the number of loci at which different alleles occurred (mismatches). In this analysis, each ET was compared with itself and with every other ET within its complex (total number of comparisons, 18 by 18 for the mutans complex and 22 by 22 for the sanguis complex). The frequency distribution for the mutans complex was bimodal; 73% of the pairs of ETs had 13 or 14 mismatched loci, and 27% of the pairs had fewer than 11 mismatches. In contrast, the distribution of mismatches was unimodal for the sanguis complex; 88% of the paired ETs had dissimilar alleles at 7 to 15 loci, and 12% had 5 or fewer mismatches.

Distribution of alleles within and between species. The average number of alleles at the 13 loci scorable in all isolates was 12.2 (range, 7 to 24), and the number of alleles per locus shared between species of the mutans and sanguis complexes ranged from 0 to 3, with an average of 1.7, or 13.9%.

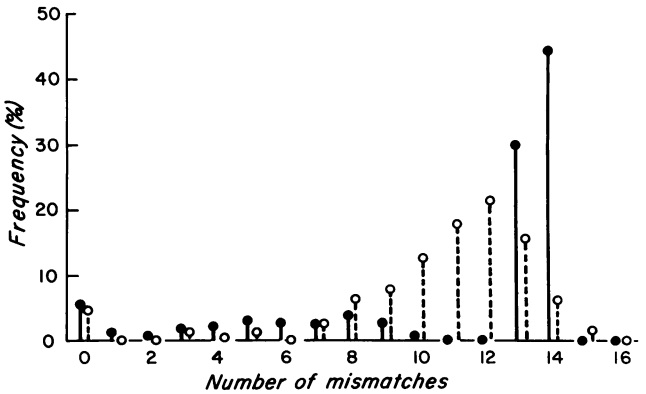

FIG. 1. Frequency distribution of numbers of enzyme loci at which unlike alleles (mismatches) occurred in pairwise comparisons of ETs. Symbols: ●, mutans complex; ○, sanguis complex.

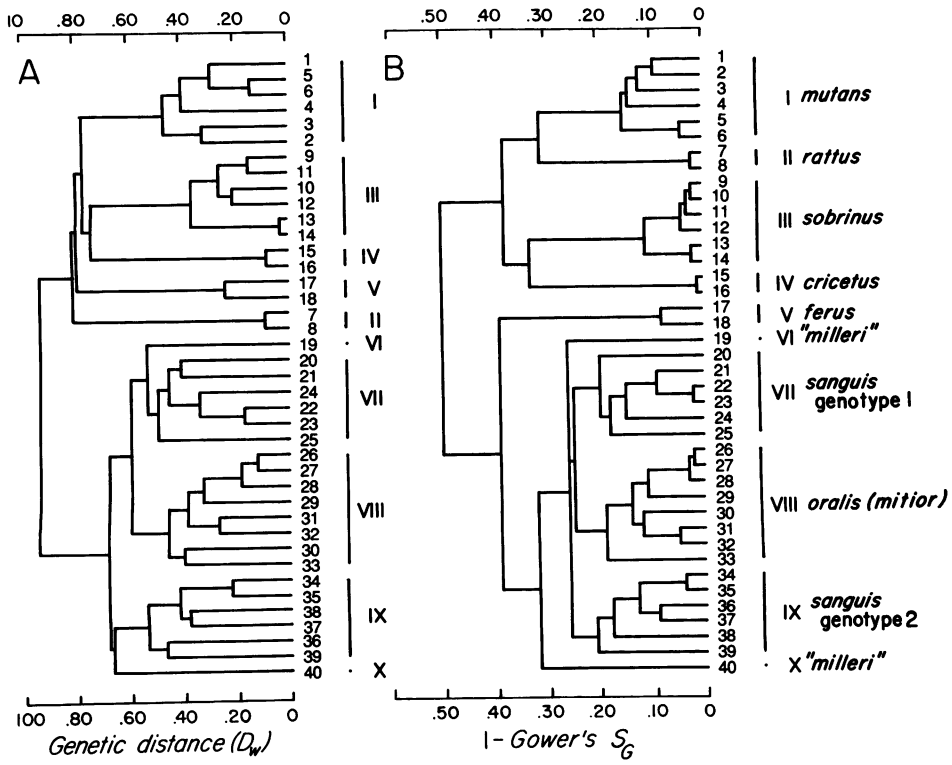

FIG. 2. Genetic relationships among 40 ETs of oral streptococci. The two dendrograms were generated by the average-linkage method of clustering from matrices of genetic distance based on allele profiles for 16 enzyme loci. (A) Weighted mismatch genetic distance coefficient (D_w); (B) Gower's distance coefficient ($1 - S_G$).

Species of the mutans complex had few common alleles. Indeed, for six loci (GD2, PGM, IPO, MPI, NSP, and GP1), no alleles were shared among species. In general, the ranges of electrophoretic mobilities of enzymes were much smaller within species than among species, and this pattern was especially marked in the case of the mutans complex.

Multilocus genotypic relationships. The simplest of the three coefficients used to estimate genetic relationships between pairs of ETs was unweighted genetic distance (D), which is the proportion of loci at which a pair of ETs has dissimilar alleles (i.e., the proportion of mismatches) (55). The second coefficient, weighted genetic distance (D_w), was similar to D, but the contribution of each locus was weighted inversely by its genetic diversity (h) in the total sample of ETs, thereby giving greater weight to differences at less variable loci than to those at highly polymorphic loci (57). An unsatisfactory aspect of the use of these matching coefficients in analyzing the present data stems from the lack of expression of some enzymes in certain strains; in these cases, the absence of activity of an enzyme was attributed to the presence of a "null" allele at a genetic locus that, in reality, may have been absent altogether from the genome.

The third measure employed was Gower's coefficient of similarity (S_G), which permits the combined use of qualitative and quantitative characters (33, 59). The coefficient was calculated as

$$S_G = \frac{1}{v} \sum_{k=1}^{v} s_{ijk}$$

where s_{ijk} is the difference in mobility between electromorphs, expressed either as absolute distance on gels or as the difference in mobility rank scores, for enzyme k between ETs i and j, and v is the total number of enzymes compared over all ETs. For quantitative characters, $s_{ijk} = 1 - (x_i - x_j)/R_k$, where x_i and x_j are the mobility values for the kth enzyme in ETs i and j, respectively, and R_k is the range of values for the kth enzyme in the sample. Because the scores for quantitative characters were normalized by ranging, the maximum contribution to the coefficient is the same for each enzyme, being independent of variation in degree of polymorphism among loci. For qualitative characters, s_{ijk} equals 1 for matches and 0 for mismatches.

In our analysis, the G6P, 6PG, and M1P loci were scored at multistate qualitative characters, with the absence of enzyme activity considered a separate state, and were combined in Gower's coefficient with the 13 other loci, for which allelic variation was treated as a quantitative character based on the relative mobility of electromorphs. For comparison with the other two measures of genetic relationship, which are distances, Gower's measure was also expressed as a distance, $1 - S_G$.

Estimates of genetic relationships among ETs, derived from the weighted coefficient of genetic distance, D_w, are shown in Fig. 2A. At a genetic distance of about 0.55, there were 10 major branches, each of which included from 1 to 8 of the 40 ETs. (For the sake of convenience, we refer to each of these subdivisions as a "group," although two of them consisted of single ETs.) Groups I to V included ETs of the mutans complex exclusively, and groups VI to X included ETs of the sanguis complex exclusively. Intergroup genetic distances were, on average, much greater for the mutans

complex than for the sanguis complex. Groups I (ETs 1 to 6), II (ETs 7 and 8), III (ETs 9 to 14), IV (ETs 15 and 16), and V (ETs 17 and 18) correspond to the species *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. ferus*, and *S. rattus*, respectively. Within the sanguis complex, group VI includes a single isolate (SK87) identified as *S. milleri*, and groups VII to IX were composed of isolates of the species *S. sanguis*, genotype 1; *S. oralis* (*mitior*); and *S. sanguis*, genotype 2, respectively (14). Group X consisted of a single ET that was represented by two isolates (SK65 and SK73), both of which were identified as *S. milleri*.

The relationships of ETs indicated by the unweighted coefficient of genetic distance (*D*) were closely similar to those estimated by D_w, although there were several minor differences in the relative relationships of ETs in groups VII and IX.

A dendrogram of ETs generated from a matrix of the pairwise values of Gower's coefficient of distance ($1 - S_G$) is shown in Fig. 2B. (Rank scores of electromorphs were used in calculating the coefficients for this dendrogram, but a dendrogram based on coefficients calculated from the absolute distance between electromorphs was essentially identical to the one shown.) The 40 ETs were separated into 10 well-defined genetic groups at a distance of about 0.21, and the ET compositions of the groups were the same as that in the dendrogram based on D_w. Again, there was greater diversity among ETs of groups I to V than among ETs of groups VI to X.

The genetic relationships among species estimated by $1 - S_G$ and D_w differed in several respects. The dendrogram based on Gower's coefficient indicated that groups I (*S. mutans*) and II (*S. rattus*) were more similar to each other than to any other group, and the same was true for groups III (*S. sobrinus*) and IV (*S. cricetus*). Additionally, groups I and II were, by this measure, also more closely related to groups III and IV than to group V (*S. ferus*). In contrast, the results obtained with D_w indicated that all five groups of ETs of the mutans complex were about equally distant from each other. The two dendrograms also differed in the estimated relationships among some groups of the sanguis complex. For example, group X was shown to be somewhat more distantly related to all other groups of the complex with $1 - S_G$ than with D_w, but the coefficient used had little effect on estimated distances among groups VII, VIII, and IX.

The coefficient of correlation between matrices of D_w and $1 - S_G$ for all pairs of ETs was 0.860 ($P < 0.001$). The relationship between values of the two coefficients was curvilinear, with a disproportionately greater increase in $1 - S_G$ at the larger distances (Fig. 3).

The two dendrograms also differed in the indicated relationship of group V, *S. ferus*, to the other groups. With $1 - S_G$ (Fig. 2B), *S. ferus*, which is widely believed to be a member of the mutans complex, was shown to be more closely related to ETs of the sanguis complex than to those of the mutans complex. But with D_w (Fig. 2A), *S. ferus* was more closely allied with ETs of the mutans complex than with those of the sanguis complex. This difference between the two dendrograms results from two factors: the presence of M1P and the absence of G6P and 6PG among ETs of *S. ferus*, *S. mutans*, *S. rattus*, *S. sobrinus*, and *S. cricetus* and the considerable sharing of electromorphs of the remaining 13 enzymes by ETs of *S. ferus* and those of the sanguis complex (Table 2). For these 13 loci, the frequency of electromorph sharing relative to the total numbers of possible comparisons was 17% between *S. ferus* and the sanguis complex ETs but only 1.2% between *S. ferus* and the mutans

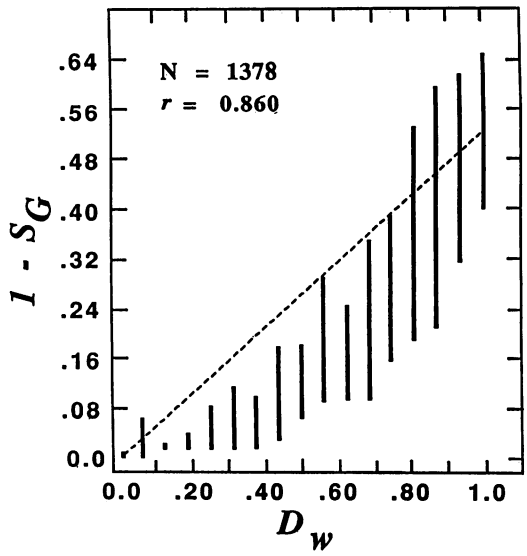

FIG. 3. Relationship between estimates of genetic distance between pairs of ETs based on the proportion of allele mismatches (D_w) and Gower's distance coefficient ($1 - S_G$). The line connects the origin and the mean of $1 - S_G$ at a maximum of D_w. Bars represent the range of $1 - S_G$ values for discrete values of D_w.

complex ETs. A dendrogram generated from D_w values based on the 13 common enzymes also indicated that *S. ferus* is more closely related to ETs of the sanguis complex than to those of the mutans complex. (Electromorph profiles of three additional isolates of *S. ferus* were identical or closely similar to those of the type strain 8S1 and strain HD3; Table 2). The activity pattern of enzymes M1P, G6P, and 6PG, therefore, weighted the values of D_w in such a way as to indicate a closer relationship of *S. ferus* to ETs of the mutans complex than to those of the sanguis complex.

The relationships among ETs within groups were also affected to various degrees by the nature of the coefficient used to estimate genetic distance. The effects were small for most ETs (e.g., *S. sobrinus*) but large for ETs 21, 30, and 36 of the sanguis complex.

Because the *S. sanguis*-like streptococci have been difficult to classify (38), the isolate composition of groups of the sanguis complex is of considerable interest. All three coefficients were similar in their allocation of isolates to groups. Group VII, which is an undescribed species (M. Kilian, L. Mikkilsen, and J. Hendrichsen, manuscript in preparation) (Table 1), included *S. sanguis* ATCC 10558, which was called *S. sanguis* genotype 1 by Coykendall and Gustafson (16); *S. sanguis* isolates S7, ATCC 12396, and SK42; the type strain of *S. mitis*, NCTC 3165 (= ATCC 33399); and *S. oralis* PB179. Group VIII, which was considered to represent *S. oralis* by Kilian et al. (in preparation) (Table 1), included *S. sanguis* ATCC 10557, which Coykendall and Gustafson (16) called *S. mitior*; the type strain of *S. oralis* (PB182), which was treated as a "species incertae sedis" in the ninth edition of *Bergey's* (38); and a group of strains isolated by Kilian. Group IX, which was assigned to *S. sanguis* by Kilian et al. (in preparation) (Table 1), included the type strain of *S. sanguis*, ATCC 10556 (*S. sanguis* genotype 2 of Coykendall and Gustafson [16]); *S. oralis* PB177; and a group of Kilian isolates. The distantly related groups VI and X both included isolates identified by Kilian as "*S. milleri*."

DISCUSSION

Multilocus enzyme electrophoresis has long been a standard technique in eucaryotic population genetics and systematics but has only recently been used to analyze the genetic structure of natural populations of bacteria (55, 56). Most previous studies of bacteria were concerned largely or wholly with intraspecific variation, whereas we have used the technique primarily to estimate degrees of relationship among species. Apart from an early study of several species of *Bacillus* (1), there appears to have been no previous application of multilocus enzyme electrophoresis to gram-positive organisms.

Correlation with results from DNA hybridization. The degree of correlation between genetic distances estimated from assessments of structural variation in primary gene products by multilocus enzyme electrophoresis and measures of total genomic divergence in nucleotide sequence, as indexed by DNA-DNA hybridization, is of considerable interest to systematists (55). Because DNA-DNA hybridization data are available for 50 pairwise combinations of the strains of oral streptococci we studied (12–14, 19, 43, 54, 65), we have been able to examine this relationship more closely than has hitherto been possible for bacteria.

The coefficients of correlation between percent relative binding ratio (RBR) of DNA and D_w and $1 - S_G$ were $r = -0.890$ ($P < 0.001$) and $r = -0.897$ ($P < 0.001$), respectively. (The sign of r is negative because RBR for DNA estimates similarity, whereas D_w and $1 - S_G$ are distance measures.) These results, together with those for several gram-negative bacteria (55), demonstrate that in general, multilocus enzyme electrophoresis and DNA hybridization yield similar estimates of genetic relatedness.

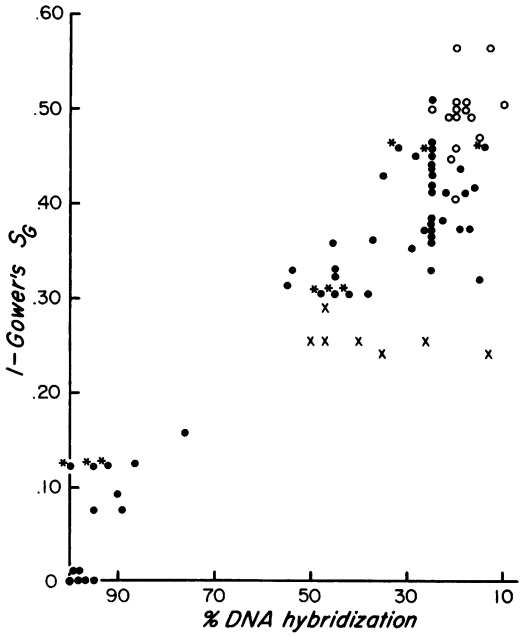

FIG. 4. Relationship between genetic distance estimated from Gower's coefficient and genetic similarity estimated from DNA hybridization for 50 pairwise combinations of isolates. Symbols: ●, mutans complex-mutans complex; ○, mutans complex-sanguis complex; ×, sanguis complex-sanguis complex. For three pairs of isolates, asterisks mark points representing replicate DNA hybridization experiments (see text).

Figure 4 illustrates the relationship between values of $1 - S_G$ and percent DNA hybridization for the 50 pairwise combinations of isolates. (The values at the origin, indicating identity for pairs of isolates, are 0 and 100, respectively, for the two measures.) The scatter of the scores in part reflects the large experimental error associated with DNA hybridization (21, 27, 28, 34, 63). The degree of variation among replicate and reciprocal DNA assays, which is illustrated by three data points marked with asterisks for each of three representative pairwise combinations of isolates, is similar to the range of 5 to 20% previously reported for experiments involving other types of bacteria (21, 22).

For highly divergent pairs of ETs, genetic distances estimated by enzyme electrophoresis are generally smaller than those indicated by DNA hybridization. This disproportionality is in part attributable to variation in yields of heteroduplexes among similar and dissimilar DNAs, so that homologies may be overestimated among closely related strains and are likely to be underestimated among more distantly related isolates (27, 28). For example, the hybridization values of 99.7, 95, and 92% between the *S. mutans* strains 10449 and GS5 (for which $1 - S_G = 0.123$) probably overestimate homology. There was a 23% decrease in the yield of heteroduplexes from this pair of isolates when the renaturation temperature was increased by 8°C, indicating greater base-pair mismatching than in heteroduplexes formed by other pairs of strains with 85 to 98% homologies, for which yields were reduced by only 3 to 7% (12). In addition, considerable base-pair mismatching in heteroduplexes is expected as a consequence of differences in moles percent G+C content between isolates in various pairwise combinations of ETs. For example, *S. mutans* 10449 and *S. rattus* FA-1 (three data points at $1 - S_G = 0.305$ with RBR values ranging from 40 to 50%; Fig. 4) and the sanguis complex pair at 26% DNA homology differ by 4 and 6% G+C content, respectively (13, 43). At these levels of DNA homology (ca. 50%), yields of renatured heteroduplexes are profoundly affected by temperature (12, 21, 22, 41). Because both the ability to anneal and the thermostability of heteroduplexes decrease with increasing base-pair mismatching (63), homology between more distantly related strains is increasingly underestimated (22, 27, 28). In sum, the overall effects of renaturation temperature and other factors can account for the relatively large differences between the enzyme and DNA-DNA hybridization estimates of genetic relatedness for pairs of isolates of the sanguis complex, among which DNA homology values range from 13 to 35%.

Limitations of the multilocus enzyme technique (48, 55) should be recognized. Estimates of genetic distance must be based on large samples of structural gene loci to minimize error resulting from the large interlocus variance in evolutionary rate among genes. Variation at single loci (e.g., ALD [47]) clearly is not an adequate basis for estimating relationships among genomes. That the sample of 16 loci assayed in the present study is representative of structural genes in general and, hence, sufficient to provide reasonably accurate estimates of overall genomic relationships is strongly suggested by the similarity of results obtained to those derived from studies of genomic nucleotide sequence homology by DNA hybridization.

Relative advantages of various distance coefficients. In research on bacterial populations, D and, less frequently, D_w have generally been used to estimate genetic distance (55), but for studies of highly divergent species such as the oral streptococci, we prefer Gower's coefficient, which permits

both quantitative and qualitative treatment of electromorph mobilities. Use of simple matching coefficients requires no assumptions about relationships between the number, type, or location of amino acid substitutions and variation in the electrophoretic mobility of enzyme molecules. Because the variance in D or D_w is small for groups of ETs or strains with high proportions of mismatches, discrimination of differences in degree of relatedness between strongly divergent groups is limited. This may account for the fact that the four species *S. mutans*, *S. rattus*, *S. sobrinus*, and *S. cricetus* were found to be approximately equal in degree of relatedness with the D_w coefficient (Fig. 2A), whereas both DNA hybridization (14, 54) and Gower's coefficient indicated that *S. mutans* is more closely related to *S. rattus* and *S. cricetus* is allied with *S. sobrinus*. This observation, in conjunction with the overall curvilinear relationship between $1 - S_G$ and D_w (Fig. 3), indicates that additional information of use in determining interspecific relationships among distantly related forms can be obtained by use of the absolute or rank mobilities of electromorphs (52), as reflected in Gower's coefficient. Because the relationship between $1 - S_G$ and D_w is curvilinear, intraspecific relationships are estimated with greater sensitivity by the simple matching coefficient.

Species differences and recombination. Among genera of eucaryotes and in the bacterial genus *Legionella*, species often show low frequencies of sharing of electromorphs, and ranges of variation in electromorph mobility are generally smaller within species than between species (48, 57). For the oral streptococci, these characteristics are more clearly shown by species of the mutans complex than by those of the sanguis complex. If most enzyme polymorphisms are selectively neutral or nearly so (45), this finding implies either that the species of the mutans complex are older than those of the sanguis complex or that there has been less genetic recombination among species of the mutans complex through evolutionary time. These are not mutually exclusive possibilities. Genetic exchange by transformation among laboratory strains of *S. sanguis* and *S. oralis* (*mitior*) is well known, and many freshly isolated clinical strains of these species are competent (66), but isolates of *S. mutans* develop competence only under a relatively narrow range of conditions, and attempts to transform *S. sobrinus* have been unsuccessful (67). Certain transposons have been experimentally transmitted from clinical isolates of *S. sanguis* to other species of streptococci, and transposon incorporation into the genome of members of the mutans complex has also been reported (9). But the effectiveness of these elements in mediating exchange and chromosomal integration of genes among oral streptococci in natural populations is unknown.

Absence of G6P and 6PG activity in strains of the mutans complex. A lack of activity of G6P and 6PG in extracts from strains of the mutans complex correlates with the absence of a functional oxidative portion of the hexose monophosphate shunt in these organisms (4). If the loci for these enzymes are present in strains of the mutans complex, apparently they are not expressed, for extracts of isolates of these species failed to react with an antiserum raised to purified 6PG obtained from *S. faecalis* (4).

Relationships among forms of the sanguis complex. The division of ETs of the sanguis complex into five groups (Fig. 2B) is in accord with results obtained from examining an array of phenotypic and serological characteristics (Kilian et al., in preparation) and from DNA hybridization experiments. The degree of genetic dissimilarity between ETs of *S. sanguis* genotypes 1 and 2 (groups VII and IX, respectively) warrants specific separation and is consistent with the 40 to 60% RBR values derived from comparisons of isolates of the two genotypes by DNA hybridization (18). Our results showing that isolates of *S. oralis* (*mitior*) (group VIII; Fig. 2B) are specifically distinct also agree with those obtained by DNA hybridization (18, 43). However, the extensive genetic diversity within this group and *S. sanguis* genotypes 1 and 2 suggests that further division into genetic groups or subgroups will be required when additional strains are studied. This expectation is supported by a DNA hybridization study demonstrating that the isolates of *S. oralis* (*mitior*) represent two genetic groups (17). Representation of two highly distinctive genetic groups by the three isolates of "*S. milleri*" studied also is in accord with DNA hybridization data from two separate laboratories (16, 44).

Within the sanguis complex, there is an apparent difference in the relative degree of genotypic relatedness among *S. sanguis* genotypes 1 and 2 and *S. oralis* (*mitior*) indicated by multilocus enzyme electrophoresis and DNA hybridization. Enzyme electrophoresis showed that the three groups were approximately equidistant from one another (Fig. 2B), whereas DNA hybridization demonstrated RBR values of 40 to 60% between *S. sanguis* genotypes 1 and 2 but only 20 to 40% between each of those two genotypes and *S. oralis* (*mitior*) (18). Interpretation of the results obtained by the two methods is difficult, however, because there is genetic diversity among isolates of each group and different isolates were examined in the two studies, except for ATCC strains 10558, 10556, and 10557, representing *S. sanguis* genotypes 1 and 2 and *S. oralis* (*mitior*), respectively; none of these strains was used as a reference in the DNA hybridization experiments. Between the isolate pairs 10556 and 10558, 10557 and 10556, and 10557 and 10558, the values were 0.256, 0.242, and 0.196, respectively, for $1 - S_G$ and 50%, 30 to 40%, and 30 to 40%, respectively, for percent DNA hybridized (14). (Note that the ranges of RBR values for these comparisons approximate those obtained for the replicate experiments shown in Fig. 4.) Neither technique indicated great variation in degree of relatedness between pairs.

Position of *S. ferus*. Our finding that *S. ferus* is genotypically more similar to species of the sanguis complex than to those of the mutans complex is at variance with evidence obtained from a limited study of hybridization of labeled 23S rRNA from *S. sobrinus* B13 with DNA from one isolate of *S. ferus* and one isolate of the sanguis complex (54). However, the difference between the two $\Delta T_{m(e)}$ values was small and of questionable significance. Our conclusion is supported by evidence that isolates of both *S. ferus* and species of the sanguis complex are less aciduric and acidogenic than those of the mutans complex and are noncariogenic or less cariogenic in animal models (29, 40).

The common ancestor of *S. mutans*, *S. rattus*, *S. sobrinus*, and *S. cricetus* can be envisioned as branching off from the main ancestral line of the oral streptococci with the loss of the G6P and 6PG loci. We postulate that after branching off from the ancestor common to it and to the sanguis complex, *S. ferus* independently lost the same two loci and that the M1P locus subsequently was lost in the line leading to the present-day species of the sanguis complex. According to this scenario, the resemblance of *S. ferus* to species of the mutans complex is a consequence of convergent evolution rather than close phylogenetic relationship.

Structure of the *S. mutans* complex. Our analysis has confirmed Coykendall's (14) separation of "*S. mutans*" into five species, *S. mutans*, *S. rattus*, *S. sobrinus*, *S. cricetus*, and *S. ferus*. Oral microbiologists and dental researchers

have been slow to accept this new classification and to understand its implications for oral biology. We endorse the recommendation of Gibbons (30) that isolates of the mutans complex be referred to by the correct specific epithets, because use of the name "mutans" all-inclusively is both incorrect and misleading. Continued disregard for the extensive interspecific genetic diversity of the oral streptococci can only retard progress in the study of pathogenesis in this diverse group of bacteria.

ACKNOWLEDGMENTS

We thank D. A. Caugant for helpful discussions and A. L. Coykendall, A. D. Eisenberg, W. F. Liljemark, R. E. Marquis, B. Perch, I. L. Shklair, and P. H. A. Sneath for providing strains. W. H. Bowen donated specific antibodies, and K. M. Schilling supplied instruction in serotyping. We acknowledge the technical assistance provided by C. M. Sommers and L. M. Tremblay; P. E. Pattison edited and typed the manuscript.

This research was supported by Public Health Service grant DE-06031 from the National Institute of Dental Research to R.K.S.

LITERATURE CITED

1. **Baptist, J. N., M. Mandel, and R. L. Gherna.** 1978. Comparative zone electrophoresis of enzymes in the genus *Bacillus*. Int. J. Syst. Bacteriol. **28**:229–244.
2. **Boyar, R. M., and G. H. Bowden.** 1985. The microflora associated with the progression of incipient carious lesions in teeth of children living in a water-fluoridated area. Caries Res. **19**:298–306.
3. **Bridge, P. D., and P. H. A. Sneath.** 1983. Numerical taxonomy of *Streptococcus*. J. Gen. Microbiol. **129**:565–597.
4. **Bridges, R. B.** 1977. Ribose biosynthesis in *Streptococcus mutans*. Arch. Oral Biol. **22**:139–145.
5. **Brown, A. T., and C. E. Patterson.** 1972. Heterogeneity of *Streptococcus mutans* strains based on their mannitol-1-phosphate dehydrogenases: criterion for rapid classification. Infect. Immun. **6**:422–424.
6. **Caufield, P. W., N. K. Childers, D. N. Allen, J. Hansen, K. Ratanapridakul, D. Crabb, and G. Cutter.** 1986. Plasmids in *Streptococcus mutans*: usefulness as epidemiological markers and association with mutacins, p. 217–223. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing Co., Inc., New York.
7. **Caugant, D. A., L. O. Frøholm, K. Bøvre, E. Holten, C. E. Frasch, L. F. Mocca, W. D. Zollinger, and R. K. Selander.** 1986. Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc. Natl. Acad. Sci. USA **83**:4927–4931.
8. **Chassy, B. M.** 1976. A gentle method for the lysis of oral streptococci. Biochem. Biophys. Res. Commun. **68**:603–608.
9. **Clewell, D. B., and C. Gawron-Burke.** 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu. Rev. Microbiol. **40**:635–659.
10. **Coakley, W. T., A. J. Bater, and D. Lloyd.** 1977. Disruption of microorganisms. Adv. Microb. Physiol. **16**:279–341.
11. **Colman, G., and R. E. O. Williams.** 1972. Taxonomy of some human viridans streptococci, p. 281–299. *In* L. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal disease. Academic Press, Inc., New York.
12. **Coykendall, A. L.** 1971. Genetic heterogeneity in *Streptococcus mutans*. J. Bacteriol. **106**:192–196.
13. **Coykendall, A. L.** 1974. Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. J. Gen. Microbiol. **83**:327–338.
14. **Coykendall, A. L.** 1976. On the evolution of *Streptococcus mutans* and dental caries, p. 703–712. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings: Microbial Aspects of Dental Caries (special supplement to Microbiology Abstracts), vol. 3. Information Retrieval, Inc., Washington, D.C.
15. **Coykendall, A. L., D. Bratthall, K. O'Connor, and R. A. Dvarskas.** 1976. Serological and genetic examination of some nontypical *Streptococcus mutans* strains. Infect. Immun. **14**:667–670.
16. **Coykendall, A. L., and K. B. Gustafson.** 1986. Taxonomy of *Streptococcus mutans*, p. 21–28. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing Co., Inc., New York.
17. **Coykendall, A. L., and A. J. Munzenmaier.** 1978. Deoxyribonucleic acid base sequence studies on glucan-producing and glucan-negative strains of *Streptococcus mitior*. Int. J. Syst. Bacteriol. **28**:511–515.
18. **Coykendall, A. L., and P. A. Specht.** 1975. DNA base sequence homologies among strains of *Streptococcus sanguis*. J. Gen. Microbiol. **91**:92–98.
19. **Coykendall, A. L., P. A. Specht, and H. H. Samol.** 1974. *Streptococcus mutans* in a wild, sucrose-eating rat population. Infect. Immun. **10**:216–219.
20. **Crawford, I., and C. Russell.** 1983. Streptococci isolated from the bloodstream and gingival crevice of man. J. Med. Microbiol. **16**:263–269.
21. **De Ley, J., and R. Tijtgat.** 1970. Evaluation of membrane filter methods for DNA-DNA hybridization. Antonie van Leeuwenhoek J. Microbiol. Serol. **36**:461–474.
22. **De Ley, J., R. Tijtgat, J. De Smedt, and M. Michiels.** 1973. Thermal stability of DNA-DNA hybrids within the genus *Agrobacterium*. J. Gen. Microbiol. **78**:241–252.
23. **Ezaki, T., R. Facklam, N. Takeuchi, and E. Yabuuchi.** 1986. Genetic relatedness between the type strain of *Streptococcus anginosus* and minute-colony-forming beta-hemolytic streptococci carrying different Lancefield grouping antigens. Int. J. Syst. Bacteriol. **36**:345–347.
24. **Facklam, R. R.** 1974. Characteristics of *Streptococcus mutans* isolated from human dental plaque and blood. Int. J. Syst. Bacteriol. **24**:313–319.
25. **Facklam, R. R.** 1977. Physiological differentiation of viridans streptococci. J. Clin. Microbiol. **5**:184–201.
26. **Facklam, R. R.** 1980. Streptococci and aerococci, p. 100–102. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
27. **Flavell, R. A., E. J. Birfelder, J. P. M. Sanders, and P. Borst.** 1974. DNA-DNA hybridization on nitrocellulose filters. 1. General considerations and non-ideal kinetics. Eur. J. Biochem. **47**:535–543.
28. **Flavell, R. A., P. Borst, and E. J. Birfelder.** 1974. DNA-DNA hybridization on nitrocellulose filters. Concatenation effects. Eur. J. Biochem. **47**:545–548.
29. **Freedman, M. L., A. L. Coykendall, and E. M. O'Neill.** 1982. *Streptococcus ferus* and dental caries, p. 127–129. *In* S. E. Holm and P. Christensen (ed.), Basic concepts of streptococci and streptococcal diseases. Reedbooks, Ltd., Surrey, U.K.
30. **Gibbons, R. J.** 1986. Concluding remarks and areas for future research, p. 433–445. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing Co., Inc., New York.
31. **Gibbons, R. J., L. Cohen, and D. I. Hay.** 1986. Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect. Immun. **52**:555–561.
32. **Gibbons, R. J., and J. V. Qureshi.** 1980. Virulence-related physiological changes and antigenic variation in populations of *Streptococcus mutans* colonizing gnotobiotic rats. Infect. Immun. **29**:1082–1091.
33. **Gower, J. C.** 1971. A general coefficient of similarity and some of its properties. Biometrics **27**:857–874.
34. **Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin.** 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr. Microbiol. **4**:325–330.
35. **Groves, W. E., J. Calder, and W. J. Rutter.** 1966. Fructose diphosphate aldolase. II. *Clostridium perfringens*. Methods

Enzymol. 9:486–499.
36. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**:331–384.
37. **Hamilton, I. R., and G. C. Y. Lo.** 1978. Co-induction of β-galactosidase and the lactose-P-enolpyruvate phosphotransferase system in *Streptococcus salivarius* and *Streptococcus mutans*. J. Bacteriol. **136**:900–908.
38. **Hardie, J. M.** 1986. Oral streptococci, p. 1054–1063. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
39. **Hardie, J. M., R. A. Whiley, and M. J. Sakin.** 1982. A numerical taxonomic study of oral streptococci, p. 59–60. *In* S. E. Holm and P. Christensen (ed.), Basic concepts of streptococci and streptococcal diseases. Reedbooks, Ltd., Surrey, U.K.
40. **Harper, D. S., and W. J. Loesche.** 1984. Growth and acid tolerance of human dental plaque bacteria. Arch. Oral Biol. **29**: 843–848.
41. **Johnson, J. L., and E. J. Ordal.** 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. J. Bacteriol. **95**:893–900.
42. **Kilian, M., B. Nyvad, and L. Mikkelsen.** 1986. Taxonomic and ecological aspects of some oral streptococci, p. 391–400. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing Co., Inc., New York.
43. **Kilpper-Balz, R., P. Wenzig, and K. H. Schleifer.** 1985. Molecular relationships and classification of some viridans streptococci as *Streptococcus oralis* and emended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int. J. Syst. Bacteriol. **35**:482–488.
44. **Kilpper-Balz, R., B. L. Williams, R. Lutticken, and K. H. Schleifer.** 1984. Relatedness of '*Streptococcus milleri*' with *Streptococcus anginosus* and *Streptococcus constellatus*. Syst. Appl. Microbiol. **5**:494–500.
45. **Kimura, M.** 1983. The neutral theory of molecular evolution. Cambridge University Press, Cambridge.
46. **Lawrence, J. V., and S. Maier.** 1977. Correction for the inherent error in optical density readings. Appl. Environ. Microbiol. **33**:482–484.
47. **London, J., N. M. Chace, and K. Kline.** 1975. Aldolase of lactic acid bacteria: immunological relationships among aldolases of streptococci and gram-positive nonsporeforming anaerobes. Int. J. Syst. Bacteriol. **25**:114–123.
48. **MacIntyre, R. J., and G. E. Collier.** 1986. Protein evolution in the genus *Drosophila*, p. 39–146. *In* M. Ashburner, H. L. Carson, and J. N. Thompson, Jr. (ed.), The genetics and biology of *Drosophila*, vol. 3e. Academic Press, Inc., New York.
49. **Musser, J. M., S. J. Barenkamp, D. M. Granoff, and R. K. Selander.** 1986. Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. Infect. Immun. **52**:183–191.
50. **Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander.** 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166**:230–237.
51. **Parker, M. T., and L. C. Ball.** 1976. Streptococci and aerococci associated with systemic infection in man. J. Med. Microbiol. **9**:275–302.
52. **Richardson, R. H., M. E. Richardson, and P. E. Smouse.** 1975. Evolution of electrophoretic mobility in the *Drosophila mulleri* complex, p. 533–545. *In* C. L. Markert (ed.), Isozymes, vol. IV: Genetics and evolution. Academic Press, Inc., New York.
53. **Russell, R. R. B.** 1979. Comparison of oral *Streptococcus mutans* AHT with strains of serotypes *a* and *g* by biochemical and electrophoretic methods. Arch. Oral Biol. **24**:617–619.
54. **Schleifer, K. H., R. Kilpper-Balz, J. Kraus, and F. Gehring.** 1984. Relatedness and classification of *Streptococcus mutans* and "mutans-like" streptococci. J. Dent. Res. **63**:1047–1050.
55. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51**:873–884.
56. **Selander, R. K., D. A. Caugant, and T. S. Whittam.** 1987. Genetic structure and variation in natural populations of *Escherichia coli*, p. 1625–1648. *In* J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
57. **Selander, R. K., R. M. McKinney, T. S. Whittam, W. F. Bibb, D. J. Brenner, F. S. Nolte, and P. E. Pattison.** 1985. Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. **163**:1021–1037.
58. **Sneath, P. H. A.** 1983. Distortions of taxonomic structure from incomplete data on a restricted set of reference strains. J. Gen. Microbiol. **129**:1045–1073.
59. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy. W.H. Freeman Co., San Francisco.
60. **Tanzer, J. M., A. T. Brown, M. F. McInerney, and F. N. Woodiel.** 1977. Comparative study of invertases of *Streptococcus mutans*. Infect. Immun. **16**:318–327.
61. **Theilade, E., J. Theilade, and L. Mikkelsen.** 1982. Microbiological studies on early dento-gingival plaque on teeth and Mylar strips in humans. J. Periodont. Res. **17**:12–25.
62. **Tsumori, H., A. Shimura, and H. Mukasa.** 1983. Comparative study of *Streptococcus mutans* extracellular glycosyltransferases by isoelectric focusing. J. Gen. Microbiol. **129**:3261–3269.
63. **Ullman, J. S., and B. J. McCarthy.** 1973. The relationship between mismatched base pairs and the thermal stability of DNA duplexes. II. Effects of deamination of cytosmine. Biochim. Biophys. Acta **294**:416–424.
64. **Van Houte, J., and J. Russo.** 1986. Variable colonization by oral streptococci in molar fissures of monoinfected gnotobiotic rats. Infect. Immun. **52**:620–622.
65. **Welborn, P. P., W. K. Hadley, E. Newbrun, and D. M. Yajko.** 1983. Characterization of strains of viridans streptococci by deoxyribonucleic acid hybridization and physiological tests. Int. J. Syst. Bacteriol. **33**:293–299.
66. **Westergren, G., and C. G. Emilson.** 1977. Transformation of streptococci to streptomycin resistance by oral streptococcal DNA. Arch. Oral Biol. **22**:533–537.
67. **Westergren, G., and C.-G. Emilson.** 1983. Prevalence of transformable *Streptococcus mutans* in human dental plaque. Infect. Immun. **41**:1386–1388.