A physiological classification of viridans streptococci by use of the API-20STREP system

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Summary. Physiological reactions of viridans streptococci were examined by the API-20STREP system and a selection of conventional tests. Cluster analysis of these results produced a classification similar to a taxonomic scheme based on that of Colman and Williams. The organisms could be divided into the six recognised species—*Streptococcus mutans*, *S. bovis*, *S. mitior*, *S. sanguis*, *S. salivarius* and *S. milleri*. Analysis confirmed that *S. mitior* and *S. sanguis* can be distinguished in the API-20STREP test by hydrolysis of arginine but not by dextran production. Although *S. mutans*, *S. mitior* and *S. sanguis* can be divided into two further subgroups, the taxonomic significance of this is unclear. With this means of classification, most organisms could be identified easily by a small number of tests. API-20STREP is convenient for performing physiological tests on viridans streptococci, but the information provided by the manufacturers in regard to identification and nomenclature is in need of revision.

Introduction

Viridans streptococci comprise a proportion of the normal mouth and bowel flora, have a relatively low virulence for man, and show variable reactions with Lancefield's antisera (Sherman, 1937; Colman, 1976; Hardie and Bowden, 1976; Parker and Ball, 1976; Facklam, 1977). Although they are named "viridans", not all produce α -haemolysis; this property of "greening" on blood agar has been recognised for many years to be affected by the growth medium used (Medical Research Council, 1929). The viridans streptococci of the mouth are associated with caries, dental sepsis, endocarditis, septicaemia and abscess formation.

Because the viridans streptococci give poor and variable reactions in serological and conventional physiological tests and, in many cases, are of relatively low virulence, they have been studied less extensively than the β -haemolytic streptococci. Consequently there is no universally accepted system for the study of the taxonomy of viridans streptococci and in most studies some strains remain unclassified no matter which identification scheme is adopted (Drucker and Melville, 1971; Colman and Williams, 1972; Parker and Ball, 1976;

Hardie and Marsh, 1978). Furthermore, there is disagreement over grouping and nomenclature among different authorities. Facklam (1977) in the USA recognised the following: *Streptococcus salivarius*, *S. mutans*, *S. sanguis* types I and II, *S. mitis*, *S. MG-intermedius*, *S. anginosus-constellatus*, *S. bovis*, and three species that are only rarely found in man—*S. acidominus*, *S. morbillorum* and *S. uberis*. This scheme is used in the Streptococcal Reference Laboratory at the Centers for Disease Control, Atlanta. However, Colman and Williams (1972) in the UK did not accept *S. sanguis* II or *S. mitis* as described by Facklam, but placed organisms resembling these species in a group called *S. mitior*, defined on the basis of cell-wall carbohydrate composition. They identified dextran-producing and non-producing strains of both *S. sanguis* and *S. mitior*, considering these variants taxonomically significant. Colman and Williams classified strains resembling Facklam's *S. MG-intermedius* and *S. anginosus-constellatus* as *S. milleri*, and with Parker and Ball (1976) did not consider that there was sufficient evidence for subdivision of this group. Coykendall *et al.* (1987) concluded from DNA homology studies that *S. milleri*, *S. constellatus* and *S. intermedius* should be described as a single species, *S. anginosus*. Colman and Williams divided *S. bovis* into two groups corresponding to Facklam's "typical" and "variant" strains (Facklam, 1972, 1977). This scheme is used in the Streptococcal

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Reference Laboratory at the Central Public Health Laboratory, Colindale, London, and has been generally adopted in Bergey's Manual of Systematic Bacteriology (Hardie, 1986). The differences between the British and American classification schemes were discussed by Facklam (1984).

Undoubtedly some of the disagreement about taxonomy is related to the variation in the results of tests used for the identification of viridans streptococci. Results of tolerance tests, such as growth in salt, bile and at different temperatures, vary significantly amongst different studies, and results of tests for the production of dextran and levan again depend on the system used.

The API-20STREP system is a commercial test kit for the identification of streptococci; it utilises many of the conventional streptococcal tests in addition to others that include the production of a diverse range of enzymes. The system identifies viridans streptococci on the basis of the Facklam classification, but includes additional biotypes for several species. With the API system, *S. pneumoniae* can be confused with *S. sanguis* unless further tests such as bile solubility or optochin sensitivity are performed. The API-20STREP kit was reviewed favourably by Colman and Ball (1984) for the identification of viridans streptococci, but this system is not the same as the API-20S system described by Nachamkin *et al.* (1982) and Appelbaum *et al.* (1984). The API-20STREP system gave consistent results for the hydrolysis of arginine and aesculin (Price *et al.*, 1986).

To clarify the taxonomy of the viridans streptococci we performed a physiological classification of a collection of these organisms using the API-20STREP system and several other potentially reproducible and discriminatory tests. The API-20STREP system was assessed for its reproducibility and not used for identification of strains. The data were analysed by cluster analysis.

Materials and methods

Organisms

Clinical isolates from St Thomas's Hospital, London, or clinical and reference strains kindly donated by colleagues were examined. Most of the 178 strains were isolated from the mouth (63) or blood (68); 51 isolates were from cases of endocarditis and eight were from animals. There were 12 duplicate strains, usually identical strains held in different culture collections under different codes or strains with the same code number donated from different institutions.

Serological reactions

The Streptex method was used to test for streptococci of Lancefield's groups A, B, C, D, F and G. Group D streptococci react poorly in this test, but nearly always give positive results in the bile-aesculin test. Some strains of *S. mutans* had been serotyped by the donors (table III).

Conventional characterisation tests

These were based on the methods of Cowan (1974) and those recommended by the Streptococcal Reference Laboratory (Waitkins *et al.*, 1980). Tests for catalase production and urease activity followed Cowan's method 1, and arginine and aesculin hydrolysis were assessed by a combination of Cowan's agar methods. Fermentation of ribose, L-arabinose, mannitol, sorbitol, lactose, trehalose, inulin and raffinose was determined by culture in serum water sugars (Cowan, 1974). Starch hydrolysis was tested by the method of Waitkins *et al.* (1980). To detect the production of H₂O₂, organisms were incubated for 24 h in Todd Hewitt broth (Southern Group Laboratories, no. 0510H), the culture was centrifuged at 3000 rpm for 10 min, and the supernate decanted; a Merckoquant Peroxide Test strip (Merck, Darmstadt, FDR) was dipped into the deposit and, after 5 s, the colour reaction was compared with the manufacturer's colour chart. For the production of dextran and levan, cultures in glucose and sucrose broths (Bailey and Oxford, 1958, 1959) were incubated for 5 days and then centrifuged at 3000 rpm for 10 min; 0.5 ml of the glucose supernate was placed in one universal container and 0.5 ml of sucrose supernate in each of two others, and 4.5 ml of 10% sodium acetate was added to all three containers; 2.5 volumes of absolute alcohol was added to the one containing glucose (control) and to one of the sucrose samples, and 1.2 volumes to the other. All the universal containers were mixed well and incubated at room temperature. The formation of precipitates in the sucrose solutions within 3 h indicated the production of polysaccharides; the glucose solution (control) remained clear. A precipitate in both sucrose bottles indicated dextran production; precipitation in only the one containing 2.5 volumes of alcohol indicated levan production.

The API-20STREP system

This commercial kit tests for the following characters: possession of seven enzymes (pyrrolidonylarylamidase, α -galactosidase, β -galactosidase, β -glucuronidase, alkaline phosphatase, leucine aminopeptidase and arginine dihydrolase), production of acetoin (the Voges-Proskauer, VP, test), hydrolysis of hippurate and aesculin, and fermentation of ribose, L-arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, starch and glycogen. The test for the possession of arginine dihydrolase was regarded as equivalent to a test for arginine hydrolysis. Organisms were grown for 24 h on blood agar in CO₂ 10% in air. The inoculum was a heavy suspension in distilled water, to a turbidity between MacFarland

Tubes 5 and 6, and the galleries were inoculated according to the manufacturer's instructions. After incubation for 4 h at 37°C in air, the reagents were added and colour changes were read by comparison with the API chart in daylight. The reactions for aesculin and arginine hydrolysis, and all the fermentation tests were read after 24 h, as recommended.

Cluster analysis

This was performed with the Clustan suite of programs (Clustan 1c, May 1975) (Wishart, 1975) running on mainframe computers at the University of London's computer centre. Analyses were performed with the Simple Matching Coefficient (Clustan coefficient 4) (Sokal and Sneath, 1973), and the squared Euclidean distance (Clustan coefficient 2) (Sokal and Sneath, 1973). The similarity matrices were then analysed by three clustering methods: single linkage (Nearest neighbour) (Sneath, 1957), Gower's Median (Gower, 1967) and Ward's Error Sum of Squares (Ward, 1963). After initial clustering had been obtained with each of these methods the classification was tested within Clustan by a relocation technique (Wishart, 1975).

Results

Comparison between conventional and API-20STREP test results

Identical results were obtained in conventional and API-20STREP tests for the fermentation of ribose, arabinose, mannitol, sorbitol and trehalose. However, there were some discrepancies between the two methods in tests for hydrolysis of arginine and aesculin, production of acetoin, and fermentation of raffinose, inulin and lactose (table I). Except for inulin fermentation the API system gave a

Table I. Comparison between physiological test results produced by API-20STREP (API) and conventional (Con) methods

Test	API-positive Con-negative	Con-positive API-negative
<i>Hydrolysis of:</i>		
arginine	12 (a)	0
aesculin	13 (b)	0
<i>Production of:</i>		
acetoin (VP)	10 (c)	0
<i>Fermentation of:</i>		
raffinose	6 (d)	0
inulin	0	19 (e)
lactose	3 (f)	0

(a) 12 *S. sanguis*; (b) 10 *S. sanguis*, 3 *S. milleri*; (c) 8 *S. salivarius*, 2 *S. milleri*; (d) 5 *S. salivarius*, 1 *S. mitior*; (e) 6 *S. mutans*, 5 *S. mitior* and 8 *S. sanguis*; (f) 2 *S. mitior*, 1 *S. sanguis*.

greater number of positive results for the above tests than the conventional methods. We have demonstrated previously that the API-20STREP results for arginine and aesculin hydrolysis are taxonomically more consistent for the speciation of organisms resembling *S. sanguis* and *S. mitior*, (Price *et al.*, 1986) and table I shows that the system is also more satisfactory for testing acetoin production in strains of *S. salivarius* and *S. milleri*; these are typically positive in this test. Differences in carbohydrate fermentation results appeared to be less significant taxonomically. Therefore, the API-20STREP results were adopted and are presented in this paper.

Tests uniformly positive or negative

All 178 viridans streptococci were gram-positive cocci in chains; they did not produce catalase, hydrolyse hippurate or ferment arabinose; they gave negative results in tests for pyrrolidonylaryl-amidase activity and positive for leucine aminopeptidase. Only one strain (STH28, a probable *S. bovis* II) showed β -glucuronidase activity. As these tests were not useful for taxonomy of viridans streptococci, they were not used in the analyses.

Classification of viridans streptococci

Classifications can be constructed intuitively by giving special weight to certain test results that are considered traditionally to have more importance than others. This is in contrast to numerical or Adansonian taxonomy in which all tests are given equal weight and the taxonomic divisions are made mathematically. We analysed the physiological results by both intuitive and numerical methods.

Intuitive classification. We have previously shown that organisms resembling *S. sanguis* that are arginine-negative and aesculin-positive in the API-20STREP system also have a low cell-wall rhamnose content, and thus can be classified as *S. mitior*; similar organisms that are arginine-positive and aesculin-negative have high rhamnose contents and are *S. sanguis* (Price *et al.*, 1986). With this modification of the Colman and Williams classification, the collection of strains could be divided intuitively into six species: *S. mutans*, *S. salivarius*, *S. milleri*, *S. sanguis*, *S. mitior* and *S. bovis*. Dextran-positive and dextran-negative variants of both *S. sanguis* and *S. mitior* were recognised.

Of the 57 strains of *S. mutans*, 26 resembled Perch's biotype 1 (Perch *et al.*, 1974); seven of these were serotype a, seven serotype c, three serotype e, four serotype f and the remainder were not typed.

Five strains hydrolysed arginine and were classified, therefore, as Perch biotype 2; four of these were serotype b, and the other serotype g. Seven arginine-negative strains were serotype d or g. None fermented raffinose (unlike all but one of the other 31 *S. mutans* strains) and were therefore regarded as Perch biotype 3. In contrast with the expected reactions for biotype 3, all but one of these strains hydrolysed aesculin, and none produced H₂O₂. None possessed α -galactosidase, although this enzyme was present in 30 of the 31 *S. mutans* strains of other biotypes.

Only three of the 15 strains thought to be *S. bovis* resembled *S. bovis* II; they hydrolysed aesculin but not arginine, and only one hydrolysed starch. All produced acetoin but not dextran, and none

fermented mannitol or sorbitol. All three were identified as *S. bovis* II by API-20STREP.

Five organisms could not easily be classified. These were strain STH102, an α -haemolytic organism resembling *S. sanguis* but H₂O₂-negative; strains STH11 and STH20, two highly unreactive α -haemolytic strains; and the reference strains NCTC 7868 and HF/F90A (equivalent to *S. sanguis* ATCC 12369), which are similar to *S. sanguis* but are β -haemolytic and H₂O₂-negative. Table II shows the results for the intuitive classification if the reference strains are classified with *S. sanguis*; the three other unidentified strains and the three *S. bovis* II strains are excluded.

Cluster analysis. All analyses produced identical results with the technique of relocation, and for the

Table II. Intuitive classification of 155 strains of viridans streptococci

Test	Percentage of strains* that gave positive results						BO1 (14)
	MUT (38)	SAL (17)	MIL (25)	SAN (32)	MD + (12)	MD - (17)	
<i>Haemolysis:</i>							
α -	3	12	52	88	100	100	29
β -	0	6	16	6	0	0	0
Non-	97	82	32	6	0	0	71
<i>Hydrolysis of:</i>							
Arginine	13	0	100	100	0	0	0
Aesculin	97	100	88	100	33	41	100
Starch	0	0	0	12	0	0	100
Urea	0	71	0	0	0	0	0
<i>Production of:</i>							
Acetoin (VP test)	100	100	100	0	0	0	100
H ₂ O ₂	0	0	16	94	100	100	0
Dextran	97	0	0	84	100	0	44
Levan	0	94	0	0	0	0	0
<i>Fermentation of:</i>							
Mannitol	100	0	8	0	0	0	93
Sorbitol	92	0	0	0	8	0	0
Raffinose	79	94	12	38	92	53	100
Inulin	87	41	0	56	0	0	100
Lactose	97	94	80	94	100	100	100
Trehalose	95	71	100	97	33	18	93
Ribose	3	0	0	3	0	29	0
Starch	0	59	40	44	75	47	100
Glycogen	0	0	4	0	0	0	100
<i>Enzyme activity:</i>							
α -galactosidase	76	12	23	38	92	47	100
β -galactosidase	0	94	0	0	0	12	0
alkaline-phosphatase	0	12	100	53	92	47	0

MUT = *S. mutans*; SAL = *S. salivarius*; MIL = *S. milleri*; SAN = *S. sanguis*; MD + = *S. mitior*, dextran-positive; MD - = *S. mitior*, dextran-negative; BO1 = *S. bovis* I.

* These results exclude duplicates, three strains of probable *S. bovis* II, and three unclassifiable organisms, but group the atypical reference strains NCTC 7868 and HF/F90A (ATCC 12369) with *S. sanguis*.

single linkage method there was no difference between the clustering produced by the Simple Matching Coefficient (Clustan coefficient 4) and the squared Euclidean distance metric (Clustan coefficient 2). Gower's (Median) clustering method produced large numbers of small clusters, and was unhelpful. The single linkage method with the Simple Matching Coefficient produced a cluster analysis that placed organisms resembling *S. sanguis*, *S. mitior* and *S. milleri* in one large group, and *S. salivarius*, *S. mutans* and *S. bovis* in another. Moreover, with this method many isolates were placed into small, often single-strain clusters, that joined the larger groups independently. In contrast, Ward's method with Clustan coefficient 2 produced clusters that corresponded very closely with intuitive groupings. Therefore, detailed results are presented only for this method of cluster analysis.

Ward's method can be applied to dissimilarity distance (metric) coefficients only, and dissimilarities are expressed in distance units rather than percentages. The pyramidal dendrogram produced by this method is shown in the figure. Two large clusters, containing 64 strains and 114 strains respectively, are separated by a distance of about 15 units. The smaller cluster contains the *S. mutans*

(group I) and *S. bovis* (group II) strains, and the larger cluster divides into two subclusters separated by 7.3 units, one containing strains resembling *S. milleri* (group VI) and *S. salivarius* (group V), and the other containing those corresponding to *S. sanguis* (group IV) and *S. mitior* (group III). These general groupings are similar to the intuitive classification based on the physiological reactions.

Group I (table III) contains all 47 strains identified intuitively as *S. mutans*. It divides into two subgroups separated by a distance of about 1.5 units. Subgroup IB (34 strains) contains 28 strains identified intuitively as *S. mutans* biotype 1 (including the reference strains NCTC 10449 and 10923 and their duplicates), and all six biotype-2 strains. Subgroup IA (13 strains) contains the remainder of the *S. mutans* isolates—all the biotype-3 strains (including the reference strain NCTC 10922) and all four α -galactosidase-negative biotype-1 strains (including the reference strain NCTC 10919). All the strains that make up subgroup IA can be distinguished from those of subgroup IB by their failure to produce α -galactosidase.

Group II (17 strains) (table III), contains all isolates identified intuitively as *S. bovis*, with the exception of the atypical *S. bovis* II strain STH28

Figure. Cluster analysis of the results of 23 physiological tests for 178 strains of viridans streptococci; the dendrogram was produced by Ward's method with the squared Euclidean distance metric (Clustan coefficient 2).

Table III. Biochemical and enzymatic reactions of strains in cluster I from conventional and API-20STREP tests

Strain				Reactions						
STH code	Original code	Ser	H	AESU	APDL	MSARILT	Hi	RSG	PABBPL	APICODE
<i>Group IA (S. mutans)</i>										
151	OIHI	d	G	- + - -	+ - - -	+ - - - - + +	-	- - -	- - - - - +	5040510
159	OMZ/65	g	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040710
142	TH62	g	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040710
118	TH62	g	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040710
133	K1	g	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040710
145	P4	e	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- - - - - +	5040770
125	KPSK 2	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- - - - - +	5040770
152	NCTC 10922	d	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040730
149	NCTC 10919	a	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040730
117	279	g	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040730
072	Z/AHT	a	G	- + - -	+ - + -	+ + - - - + +	-	- - -	- - - - - +	5040730
122	B13	d	A	- - - -	+ - + -	+ - - - - + +	-	- - -	- - - - - +	1040530
071	B13	d	A	- - - -	+ - + -	+ - - - - + +	-	- - -	- - - - - +	1040530
<i>Group IB (S. mutans)</i>										
147	OMZ/51	b	G	+ + - -	+ - + -	+ + - + - + +	-	- - -	- + - - - +	5241750
116	Z/BHT	b	G	+ + - -	+ - + -	+ + - + - + +	-	- - -	- + - - - +	5241750
157	OMZ/71	b	G	+ + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5241770
156	FA1	b	G	+ + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5241770
077	FA1	b	G	+ + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5241770
153	TH21	g	G	+ + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5241770
144	OMZ/70	c	G	- + - -	+ - + -	+ + - + + + -	-	- - -	- + - - - +	5240760
143	OMZ/61	a	G	- + - -	+ - + -	+ + - + + + -	-	- - -	- + - - - +	5240750
109	OMZ/61	a	G	- + - -	+ - + -	+ + - + + + -	-	- - -	- + - - - +	5240760
154	INGBRITT	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
140	KHAN	?	G	- + - -	+ - + -	+ - - + + + +	-	- - -	- + - - - +	5240570
121	161	f	G	- + - -	+ - + -	+ + - + + + +	-	+ - -	- + - - - +	5242770
184	BC: 1817	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
090	BC: 1817	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
158	SE11	f	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
155	FW293	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
154	INGBRITT	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
150	NCTC 10923	e	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
067	LM7 = 10923	e	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
148	NCTC 10449	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
008	NCTC 10449	c	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5241770
141	MCGREGOR	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
134	E49	a	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
132	3720	a	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
127	3720	a	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
131	151	f	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
126	4177	f	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
089	BC: 1813	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
083	SVIR 544	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
045	STREP 206	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
036	SVIR 728	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
026	R79/1374	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
017	R79/2200	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770
009	ROBERTSON	?	G	- + - -	+ - + -	+ + - + + + +	-	- - -	- + - - - +	5240770

STH code = code number of strain in the St Thomas's Hospital collection.

Original code = code number supplied by donor.

Ser = serotype.

H = haemolysis: A = alpha, B = beta, G = gamma (non-haemolytic).

AESU = hydrolysis of arginine, aesculin, starch, urea.

APDL = production of acetoin (VP), H₂O₂, dextran, levan.

MSARILT = fermentation of mannitol, sorbitol, arabinose, raffinose, inulin, lactose, trehalose.

Hi = hydrolysis of hippurate.

RSG = fermentation of ribose, starch, glycogen.

PABBPL = enzyme activity for pyrrolidonylarylamidase, α -galactosidase, β -glucuronidase, β -galactosidase, alkaline phosphatase, leucine aminopeptidase.

Note: The results are in a different order from the arrangement on the API-20STREP gallery, but the API profile number is correct.

which clusters in group VI with strains resembling *S. milleri*. Group II is reasonably homogeneous, but a small subgroup containing only three strains splits off the main cluster at a dissimilarity distance of about 0.5 units; two of these strains (STH13 and STH174) were identified intuitively as *S. bovis* II, and the third was the atypical mannitol-negative, trehalose-negative reference strain of *S. bovis* I, NCTC 8177.

Group III (30 strains) (table IV), contains all the isolates identified intuitively as *S. mitior*, together with the arginine-positive strains STH139 and the previously unidentified strain STH102. All 30 strains were α -haemolytic; all except strains STH139 and STH102 were arginine-negative; and all except strain STH102 produced H_2O_2 . Group III divides into two subgroups separated by a distance of about 1.8 units. Subgroup IIIA (16 strains) contains only raffinose-positive, α -galactosidase-positive and alkaline-phosphatase-positive isolates. It includes all 11 strains previously identified as dextran-positive *S. mitior* (including the reference strain NCTC 7864) and five other strains—the dextran-negative *S. mitior* strains STH21, STH27 and STH58; the unidentified strain STH102 (α -haemolytic, aesculin-positive, arginine-positive, H_2O_2 -negative and dextran-negative); and the dextran-negative, arginine-positive, aesculin-positive strain STH139 which had been identified intuitively as *S. sanguis*. Subgroup IIIB (14 strains) contains all strains intuitively identified as dextran-negative *S. mitior* (including the reference strain NCTC 10712), except for strains STH21, STH27 and STH58 which clustered with subgroup IIIA. The cluster analysis subdivided IIIB into raffinose-positive and raffinose-negative varieties, separated by a distance of about 0.8 units, but this did not appear to be taxonomically significant. It is not possible to create a dichotomous key to allocate *S. mitior* strains uniquely to subgroup IIIA or IIIB. Strains within IIIB tend to be less reactive than those in IIIA, but no reactions are mutually exclusive.

Group IV (36 strains) (table V), contains all organisms intuitively identified as *S. sanguis* with the exception of the atypical reference strain NCTC 7868 which clusters with group VI (*S. milleri*), and the dextran-negative strain STH139 which clusters with strains resembling *S. mitior*. All members of group IV produced H_2O_2 and all except strain STH135 hydrolysed arginine. Two subgroups, IVA (19 strains) and IVB (17 strains), were separated by a distance of about 1.8 units; they may be distinguished by the fermentation of sorbitol—all but one strain of IVA were positive and all of IVB

were negative. Both subgroups contained dextran-negative and dextran-positive varieties, but this characteristic does not appear to be taxonomically significant.

Group V (17 strains) (table VI) is homogenous and contains all the *S. salivarius* isolates except the atypical strain STH130 (LINH 33142) which clusters with *S. milleri* strains in group VI. Urease production has been proposed as a diagnostic characteristic for *S. salivarius*. In this study no other species produced this enzyme, but only 14 out of 18 *S. salivarius* strains (including strain STH130) gave positive results.

Group VI (31 strains) (table VI) comprises organisms resembling *S. milleri* and six other strains: two atypical *S. sanguis* reference strains, NCTC 7868 and STH113 (HF/F90A) (equivalent to ATCC 12369), both β -haemolytic, arginine-positive, aesculin-positive, dextran-positive and H_2O_2 -negative; strain STH130 (LINH 33142), an atypical urease-negative, raffinose-negative, inulin-negative, lactose-negative, β -galactosidase-negative strain of *S. salivarius*; strain STH28, an atypical arginine-negative and alkaline phosphatase-negative strain identified as *S. bovis* by API-20STREP; and two H_2O_2 -negative strains, STH11 and STH20, of uncertain identity. Only 10 strains in Group VI reacted with Lancefield's antisera; seven were group F, two group C and one group G. Haemolysis on blood agar was variable and did not appear to contribute to any natural divisions within this group.

Discussion

Our results with this collection of viridans streptococci demonstrated that most of the organisms could be classified intuitively by the results of the tests in the API-20STREP kit into the six species defined by Colman and Williams (1972), but there were insufficient strains resembling *S. bovis* II for comment to be made on this subgroup. The most difficult isolates to speciate are organisms resembling *S. sanguis*/*S. mitior*. We have shown previously that, for this group, a positive result in the API test for the possession of arginine dihydrolase (a test for arginine hydrolysis) correlates well with a high cell-wall rhamnose content, and is characteristic of *S. sanguis* (Price *et al.*, 1986). In contrast, *S. mitior* is API arginine-negative and has a low rhamnose content. Strains of both *S. sanguis* and *S. mitior* can hydrolyse aesculin. With this convention, organisms resembling *S. sanguis* and *S. mitior* can be easily classified. They are also characterised by the production of H_2O_2 during

Table IV. Biochemical and enzymatic reactions of strains in clusters II and III from conventional and API-20STREP tests

Strain			Reactions						
STH code	Original code	H	AESU	APDL	MSARILT	Hi	RSG	PABBPL	APICODE
<i>Group II (S. bovis)</i>									
174	2.77/192.76	A	- + - -	+ - - -	- - - + - + +	-	- + +	- + - - - +	5240453
062	NCTC 8177	A	- + + -	+ - + -	- - - + + + -	-	- + +	- + - - - +	5240463
013	R79/3146	A	- + + -	+ - - -	- - - + - + -	-	- + +	- + - - - +	5240443
074	R79/2631	G	- + + -	+ - - -	+ - - + + + +	-	- + +	- + - - - +	5240573
175	2.77/185a.7	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
163	DAVIS 33995	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
138	M	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
093	R79/2850	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
086	R79/2715	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
003	R79/2715	A	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
085	STREP 259	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
078	R79/2445	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
075	SVIR 971	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
052	SVIR 375	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
042	STREP 295	G	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
022	R79/3113	A	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
004	BC: 1782	A	- + + -	+ - + -	+ - - + + + +	-	- + +	- + - - - +	5240573
<i>Group IIIA (S. mitior)</i>									
112	S5732	A	- - - -	- + + -	- - - + - + +	-	- + -	- + - - - +	0260451
068	SVIR 616	A	- - - -	- + + -	- - - + - + -	-	- - -	- + - - - +	0260440
172	6.78/198a.7	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
171	1.80/187.79	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
123	OPA1	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
115	H240	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
103	B88	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
034	NCTC 7864	A	- - - -	- + + -	- - - + - + -	-	- + -	- + - - - +	0260441
104	B159	A	- + - -	- + + -	- - - + - + -	-	- + -	- + - - - +	4260441
107	H362	A	- + - -	- + + -	- - - + - + +	-	- + -	- + - - - +	4260451
061	STREP 527	A	- + - -	- + + -	- - - + - + +	-	- - -	- + - - - +	4260450
027	MAYS 1892	A	- - - -	- + - -	- - - + - + +	-	- - -	- + - - - +	0260450
139	T	A	+ + - -	- + - -	- - - + - + +	-	- + -	- + - - - +	4261451
102	LH045/80BC	A	+ + - -	- - - -	- - - + - + +	-	- + -	- + - - - +	4261451
058	BC: 1780	A	- + - -	- + - -	- - - + - + +	-	- + -	- + - - - +	4260451
021	R79/2188	A	- + - -	- + - -	- - - + - + +	-	- + -	- + - - - +	4260451
<i>Group IIIB (S. mitior)</i>									
100	LH134/80BC	A	- + - -	- + - -	- - - + - + -	-	+ - -	- + - - - +	4242440
098	LH833/80BC	A	- + - -	- + - -	- - - + - + -	-	+ - -	- + - - - +	4242440
073	SVIR 722	A	- + - -	- + - -	- - - + - + -	-	+ + -	- + - - - +	4242441
182	NCTC 10712	A	- - - -	- + - -	- - - + - + -	-	- + -	- + - - - +	0240441
041	R79/3328	A	- - - -	- + - -	- - - + - + -	-	- + -	- + - - - +	0240441
101	LH023/81BC	A	- - - -	- + - -	- - - - - + -	-	- + -	- - - - - +	0060401
087	BURY GEN	A	- - - -	- + - -	- - - - - + -	-	- + -	- - - - - +	0060401
051	STREP 351	A	- - - -	- + - -	- - - - - + -	-	+ - -	- - - - - +	0042400
038	SVIR 975	A	- + - -	- + - -	- - - - - + -	-	+ - -	- - - - - +	4042400
161	WELBEL' VED	A	- - - -	- + - -	- - - - - + -	-	- + -	- - - - - +	0040401
092	BC: 1800	A	- - - -	- + - -	- - - - - + -	-	- - -	- - - - - +	0040400
031	R75/1027	A	- - - -	- + - -	- - - - - + -	-	- - -	- - - - - +	0040400
094	LH537/80BC	A	- + - -	- + - -	- - - - - + -	-	- - -	- - - + - +	4050400
012	R79/3386	A	- - - -	- + - -	- - - - - + -	-	- - -	- - - + - +	0050400

See footnote to table III.

Table V. Biochemical and enzymatic reactions of strains in cluster IV from conventional and API-20STREP tests

Strain			Reactions						
STH code	Original code	H	AESU	APDL	MSARILT	Hi	RSG	PABBPL	APICODE
<i>Group IVA (S. sanguis)</i>									
167	M371	A	++--	-+--	---+--+	-	---	-+---+	4241050
160	WELLB'VED 1	A	++--	-+--	---+--+	-	++-	-+---+	4263451
169	M408	A	++--	-+--	---+--+	-	++-	-+---+	4241451
137	L	A	++--	-+--	---+--+	-	++-	-+---+	4241451
120	A43	A	+++	-+--	---++++	-	++-	-+---+	4241471
106	OMZ/9	G	+++	-+--	---++++	-	++-	-+---+	4241471
110	M19	A	++--	-+--	-+---++	-	++-	-+---+	0241671
097	LH867/80BC	A	++--	-+--	---+--+	-	++-	-+---+	4241071
168	P695	A	++--	-+--	---+--+	-	++-	-+---+	4241471
136	K	A	++--	-+--	---+--+	-	++-	-+---+	4241471
056	M517	A	++--	-+--	---+--+	-	++-	-+---+	4241471
166	KENDALL	G	++--	-+--	---+--+	-	++-	-+---+	4041401
177	NCTC 7863	A	++--	-+--	---+--+	-	++-	-+---+	4041411
162	BRENCHLEY	A	+++	-+--	---+--+	-	++-	-+---+	4041411
091	BC: 1821	A	+++	-+--	---+--+	-	++-	-+---+	4041430
088	BC: 1805	A	++--	-+--	---+--+	-	++-	-+---+	0041411
054	T427	A	++--	-+--	---+--+	-	++-	-+---+	0041431
173	6.77/74a.77	A	++--	-+--	---+--+	-	++-	-+---+	0041431
015	STH23	A	++--	-+--	---+--+	-	++-	-+---+	0041431
<i>Group IVB (S. sanguis)</i>									
055	BC: 1736	A	++--	-+--	---++++	-	---	---++	4061430
180	NCTC 10231	A	++--	-+--	---++++	-	---	---++	4061430
128	H44	A	++--	-+--	---++++	-	---	---++	4061430
066	R79/3247	A	++--	-+--	---++++	-	---	---++	4061430
037	BC: 1746	A	++--	-+--	---++++	-	---	---++	4061430
018	BC: 1770	A	++--	-+--	---++++	-	---	---++	4061430
016	L/SANG	A	++--	-+--	---++++	-	---	---++	4061430
135	J	A	-+--	-+--	---++	-	---	---++	4040410
170	NP506	A	++--	-+--	---++	-	---	---++	4041410
096	LH524/80BC	A	++--	-+--	---++	-	---	---++	4041410
178	NCTC 7865	A	++--	-+--	---++	-	---	---++	4061410
044	NCTC 7865	A	++--	-+--	---++	-	---	---++	4061410
164	SINGH	A	++--	-+--	---++	-	---	---++	4061410
099	GPF1	A	++--	-+--	---++	-	---	---++	4061410
046	CARLSSON 903	A	++--	-+--	---++	-	---	---++	4061410
024	H730	A	++--	-+--	---++	-	---	---++	4061410
007	R79/3344	A	++--	-+--	---++	-	---	---++	4061410

See footnote to table III.

growth, but this test is not part of the API-20STREP system. Dextran-positive and dextran-negative varieties of both these species were identified by a sensitive test for polysaccharide production.

It has been suggested that the possession of urease (not tested by the API-20STREP kit) is a characteristic of *S. salivarius* (Feltham, 1979); 14 of the 18 strains of *S. salivarius* tested hydrolysed urea, but the reference strains NCTC 8606 and LINH 33142 were urease negative. Organisms of other species gave uniformly negative results in this test.

Thus urea hydrolysis is a useful additional test for the identification of *S. salivarius*, especially if the production of levan is not tested, but it is not definitive.

The *S. mutans* biotypes of Perch *et al.* (1974) were closely related but not identical to serotype. Biotype 3 was not well-defined, and on these API-20STREP results it would be more useful to characterise it as arginine-positive, raffinose-negative and α -galactosidase-negative. In this study, arginine-positive strains of *S. mutans* were usually serotype b, and raffinose-negative, α -galactosidase-

Table VI. Biochemical and enzymatic reactions of strains in clusters V and VI from conventional and API-20STREP tests

Strain				Reactions						
STH code	Original code	Group*	H	AESU	APDL	MSARILT	Hi	RSG	PABBPL	APICODE
<i>Group V (S. salivarius)</i>										
063	STREP 136		G	- + - -	+ - - +	- - - + + + -	-	- + -	- - - + - +	5050461
053	R79/2894		A	- + - -	+ - - +	- - - + - + -	-	- + -	- - - + - +	5050441
165	BC: 1967		G	- + - +	+ - - +	- - - + - + +	-	- + -	- - - + - +	5050451
111	6.77/16.77		G	- + - +	+ - - +	- - - + - + -	-	- + -	- - - + - +	5050441
048	B128		G	- + - +	+ - - +	- - - + - + -	-	- + -	- - - + - +	5050441
057	STREP 347		A	- + - +	+ - - +	- - - + + + +	-	- + -	- - - + - +	5050471
105	H167		G	- + - +	+ - - +	- - - + + + +	-	- + -	- - - + - +	5050471
047	R79/3114		G	- + - +	+ - - +	- - - + + + +	-	- + -	- - - + - +	5050470
181	NCTC 8606		G	- + - -	+ - - +	- - - + - + +	-	- - -	- + - + + +	5270450
043	55xlc		G	- + - +	+ - - +	- - - + + + +	-	- - -	- - - + - +	5050470
023	M36		G	- + - +	+ - - +	- - - + + + +	-	- - -	- - - + - +	5050470
014	66xlb		G	- + - +	+ - - +	- - - + + + +	-	- - -	- - - + - +	5050470
108	B242		G	- + - +	+ - - +	- - - + - + +	-	- - -	- - - + - +	5050450
050	STREP 290		G	- + - +	+ - - +	- - - + - + +	-	- - -	- - - + - +	5050450
006	R79/3385		G	- + - -	+ - - +	- - - + - + +	-	- - -	- - - + - +	5050450
183	QC552		B	- + - +	+ - - +	- - - + - + -	-	- + -	- + - + + +	5270445
005	QC552		B	- + - +	+ - - +	- - - + - + -	-	- + -	- + - + + +	5270445
<i>Group VI (S. milleri)</i>										
179	NCTC 7868	—	B	+ + - -	- - + -	- - - + + + +	-	- - -	- - - - + +	4061434
113	HF/F90A	—	B	+ + - -	- - + -	- - - + + + +	-	- - -	- + - - + +	4261474
070	COOK 142	—	G	+ + - -	+ - - -	+ - - + - + +	-	- + -	- + - - + +	5261551
065	LASKOWSKI	F	G	+ + - -	+ - - -	+ - - + - + +	-	- + -	- + - - + +	5261551
010	SVIR 891	—	B	+ + - -	+ + - -	- - - + - + +	-	- + -	- + - - + +	5261455
130	LINH 33142	—	G	- + - -	+ - - +	- - - - - + -	-	- + -	- - - - - +	5040011
028	STREP 535	—	G	- + - -	+ - - -	- - - - - + +	-	- - -	- - + + - +	5450410
011	ANDERSON 95	—	A	- - - -	+ - - -	- - - - - + -	-	- - -	+ - - - - +	1140000
084	MCELROY	C	B	+ - - -	+ - - -	- - - - - + +	-	- + -	- - - - + +	1061415
059	R79/307	—	B	+ - - -	+ - - -	- - - - - + -	-	- - -	- - - - + +	1061014
030	SVIR 543	F	B	+ + - -	+ - - -	- - - - - + -	-	- - -	- - - - + +	5061014
060	SVIR 412	—	G	+ + - -	+ - - -	- - - - - + +	-	- + -	- - - - + +	5061411
049	933	—	G	+ + - -	+ - - -	- - - - - + +	-	- + -	- - - - + +	5061411
029	SVIR 931	—	G	+ + - -	+ - - -	- - - - - + +	-	- + -	- - - - + +	5061411
082	SHAW 898	—	G	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
081	COX 471	—	G	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
025	WELLS 6782	—	G	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
080	HUNT 284	F	A	+ - - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	1061410
129	G5: 3	F	A	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
124	G6: 1A	—	A	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
119	G4: 2A	F	A	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
114	G3: 1	C	A	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
069	STREP 566	—	A	+ + - -	+ - - -	- - - - - + +	-	- - -	- - - - + +	5061410
176	2.81/269	G	A	+ + - -	+ + - -	- - - - - + +	-	- + -	- - - - + +	5061411
064	WALSH 1974	F	A	+ + - -	+ - - -	- - - - - + +	-	- + -	- - - - + +	5061411
040	R79/3327	—	A	+ + - -	+ + - -	- - - - - + +	-	- - -	- - - - + +	5061410
039	R79/3340	—	A	+ + - -	+ + - -	- - - - - + +	-	- - -	- - - - + +	5061410
020	850356		A	+ - - -	- - - -	- - - - - + -	-	- - -	- - - - + +	0061010
019	STREP 536	F	A	+ + - -	+ - - -	- - - - - + -	-	- + +	- - - - + +	5061013
079	STREP 562	—	A	+ + - -	+ - - -	- - - - - + -	-	- - -	- - - - + +	5061010
002	STREP 411	—	A	+ + - -	+ - - -	- - - - - + -	-	- - -	- - - - + +	5061010

See footnote to table III.
*Lancefield group.

negative strains were usually serotypes d or g. These results support the views of Hardie and others (Hardie and Bowden, 1976; Hardie and Marsh, 1978; Hamada and Slade, 1980) that the various serotypes of *S. mutans* are not well defined and are inconsistently related to biotype.

Our API-20STREP results show that many of the reference strains of viridans streptococci are atypical in their reactions—in particular strains B13 (*S. mutans*), NCTC 8177 (*S. bovis*), NCTC 8606 and LINH 33142 (*S. salivarius*), NCTC 7868 and ATCC 12369 (*S. sanguis*).

Different methods of cluster analysis produced different taxonomic results. Ward's method combined with the Squared Euclidian Distance metric produced a satisfactory cluster result that corresponded closely with the intuitive classification. The same six species were clearly separated, each about 5 units from its nearest neighbour. There were no major subdivisions of *S. salivarius*, *S. milleri* or *S. bovis*, but subgroups of *S. mutans*, *S. sanguis* and *S. mitior* were distinguished, each pair separated by about 2 units. Additional smaller subclusters separated from larger groups by less than one unit did not appear to be significant. The subdivision of *S. mutans* did not correspond to the Perch biotypes or to serotype. *S. mutans* strains of subgroup IA could be distinguished from IB only by the possession of α -galactosidase, and this division does not seem very useful.

S. sanguis and *S. mitior* strains clustered separately, closely approximating to the intuitive classification. The cluster analysis divided *S. sanguis* by ribose fermentation, but table V suggests that the group might equally well be separated by inulin or raffinose fermentation, or by the possession of α -galactosidase or alkaline phosphatase. Thus the subdivision of *S. sanguis* did not seem to be useful. The cluster subdivision of *S. mitior* was not based on mutually exclusive reactions, and was also unhelpful. The production of dextran did not contribute to any significant division of either *S.*

sanguis or *S. mitior*, and this analysis did not support the existence of a raffinose-positive *S. mitis* group (Facklam, 1977).

These results suggest that α -haemolytic H_2O_2 -positive, acetoin-negative viridans streptococci should be classified into *S. sanguis* and *S. mitior* on the basis of arginine hydrolysis, and that further subdivisions are as yet unjustified. Further evidence for this scheme is provided by the DNA studies of Coykendall and his colleagues which also support the taxa *S. sanguis* and *S. mitior*, but not *S. mitis* or speciation by dextran production (Coykendall and Specht, 1975; Coykendall and Munzenmaier, 1978).

There is a need for international agreement on the taxonomy and nomenclature of viridans streptococci. The present study supports the Colman and Williams classification of *S. mutans*, *S. bovis*, *S. salivarius*, *S. milleri*, *S. sanguis* and *S. mitior*, except that dextran production does not appear to be a taxonomically significant character. With this scheme, the viridans streptococci can be speciated or identified fairly easily with the API-20STREP system if small subgroups are ignored and if *S. sanguis* and *S. mitior* are distinguished by arginine hydrolysis. The tests in the API-20STREP system may be more reliable than conventional test methods, and are probably reproducible. Additional tests for the production of H_2O_2 and the hydrolysis of urea are useful but tests for polysaccharide production are not. The API-20STREP identification codes do not correspond to the simple classification described here, and it would be useful for these to be revised.

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