A numerical taxonomic study of the “*Streptococcus milleri*” group based upon conventional phenotypic tests and pyrolysis mass spectrometry

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Summary. Clinical strains presumptively identified as *Streptococcus milleri* (60), and blind coded collection strains (21) were characterised in conventional tests and pyrolysis mass spectrometry. Comparison of the clusters found by these two approaches revealed five clearly distinct centres of variation. Three corresponded to the DNA homology groups suggested by Whiley and Hardie (1989) as representing the species *S. anginosus*, *S. intermedius* and *S. constellatus*; a fourth comprised three Lancefield group C β -haemolytic strains; the fifth may represent a biotype of *S. anginosus*. The characteristics of the latter group are described.

Introduction

Organisms of the “*Streptococcus milleri* group” are part of the normal flora of the human oral cavity, upper respiratory, gastrointestinal and female urogenital tracts.^{1–7} They are the most common cause of streptococcal abscesses of the mouth and internal organs, including the brain, liver, lungs and spleen^{8–15} and are also isolated in cases of appendicitis, peritonitis, endocarditis, meningitis, obstetric and neonatal infections, and infections of the skin and soft tissues.^{5, 13, 16, 17} Evidence for the pathogenicity of these streptococci has been reviewed.^{18–20}

The “*S. milleri* group” has contained various taxa over the years, including: *S. anginosus*;²¹ “minute haemolytic streptococci” from the throat;¹ the human faecal isolates of Smith and Sherman;²² and the non-haemolytic *Streptococcus MG*.² The name *S. milleri* was first introduced by Guthof²³ to describe a physiologically distinct group of organisms isolated from oral sites which were predominantly of Lancefield group F.²⁴ Ottens and Winkler⁹ found the group F antigen in both haemolytic and non-haemolytic isolates and showed that these could possess any one of five carbohydrate typing antigens.

On the basis of cell wall analysis, numerical taxonomy and DNA transformation, Colman and Williams²⁵ proposed a revised description of the “*S. milleri* group”, incorporating: *S. milleri*;²³ all group F streptococci; *Streptococcus MG*; certain non-haemolytic streptococci of groups A, C and G; strains possessing an Ottens and Winkler⁹ typing antigen but

no Lancefield group antigen; and the “minute haemolytic streptococci”.¹

However, this revision was not accepted in the USA, where Facklam²⁶ found high similarity between *Streptococcus MG*,² *S. intermedius*,²⁷ *S. constellatus*²⁸ and *S. anginosus*,²¹ and divided the “*S. milleri* group” into non- β -haemolytic lactose fermenters (*S. MG-intermedius*) and non- β -haemolytic non-lactose fermenters (*S. anginosus-constellatus*). Later,²⁹ the American nomenclature was revised as follows: β -haemolytic strains (whether Lancefield group A, C, G, F or ungroupable) were called *S. anginosus*; *S. MG-intermedius* became *S. intermedius*; and *S. anginosus-constellatus* became *S. constellatus*.

Workers have disagreed on the homogeneity of the “*S. milleri* group”. Some have found the group to be homogeneous in phenotypic tests^{11, 30–33} and DNA hybridisation.^{32, 34–36} However, heterogeneity has been reported on the basis of: haemolysis;^{8, 25} whole cell trimethylsilyl sugar profiles;³⁷ fermentation patterns;^{5, 7} long chain fatty acid analysis;^{38, 39} antigenic composition;^{11, 25, 40} multilocus enzyme electrophoresis;⁴¹ sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins;⁴² and G + C content.⁴³

Kilpper-Bälz *et al.*⁴⁴ clearly distinguished the type strains of *S. constellatus* and *S. anginosus* by DNA hybridisation under stringent conditions; *S. intermedius* was also distinct but more similar to *S. constellatus*. Two homology groups were found, one showing high homology with *S. constellatus* and the other with *S. anginosus*. Knight and Shlaes⁴⁵ found evidence of at least three genetically and phenotypically distinguishable groups within 18 representatives of *S. intermedius*.

Whiley⁴⁶ found that type strains of *S. anginosus*, *S. constellatus* and *S. intermedius* belonged to separate DNA homology groups. Whiley and Hardie⁴⁷ examined 25 strains by DNA–DNA hybridisation and phenotypic characteristics, finding three genetic groups containing the type strains of *S. constellatus*, *S. intermedius* and *S. anginosus*, respectively; a fourth homology group was later named *S. parasanguis*.⁴⁸ Subsequently, phenotypic discrimination of the three DNA homology groups was demonstrated by enzyme detection tests with fluorogenic substrates that allowed a collection of 157 strains to be identified.⁴⁹ Emended descriptions recognising *S. anginosus*, *S. constellatus* and *S. intermedius* as distinct species have recently been published as a consequence of this work.⁵⁰ This may end the controversy over taxonomy and nomenclature that has impeded attempts to link pathogenicity and clinical source with membership of subgroups of “*S. milleri*”.

In earlier work with coryneform bacteria,⁵¹ classifications derived from conventional test reaction patterns (CTRPs) and pyrolysis mass spectrometry (PMS) were successfully combined. In the present study, the taxonomy of the “*S. milleri* group” was investigated by a similar combination of approaches. Clinical strains were collected prospectively and a collection of extensively characterised marker strains was obtained for comparison. Previously published DNA–DNA hybridisation data for the collection strains⁴⁶ was used to validate the classification results.

Materials and methods

Strains

Eighty-one strains of “*S. milleri*” were examined. Sixty comprised clinical strains isolated in the routine laboratory at the Royal Hallamshire Hospital between April and August, 1990; three were from the upper respiratory tract, 36 from urogenital sites, and 21 from other clinical sources. Two patients yielded paired isolates (laminectomy wound, blood culture; vaginal swab, blood culture) which were examined as duplicate cultures of the same strain. The remainder were strains previously examined in DNA homology studies⁴⁷ and received with coded designations. Three of the latter strains, NCDO 2226, NCDO 2227 and NCTC 10713, were examined in duplicate, one culture as part of the coded collection, and one as a “known” marker strain. The identities of the coded strains were not revealed until cluster analysis had been completed. All strains were presumptively identified as “*S. milleri*”.⁵² A further four strains were examined: two strains of *S. parasanguis*, each included once in the coded set and once as a marker strain; one blood culture isolate; and one throat isolate. The latter two were identified in API-20 STREP (API-bioMerieux, France) as *S. mitis* and *S. sanguis* respectively. Strains were stored in glycerol 10% broth at -70°C and cultured on blood

agar (Columbia Agar Base, Oxoid, with horse blood, Difco, 5%) (HBA) incubated anaerobically at 37°C for 48 h.

Biochemical tests

Atmospheric requirements were determined by incubation in air, anaerobically, or in CO_2 7% in air at 37°C . Haemolysis, colony size, the uniformity of a slide suspension of 3–5 colonies in saline, and growth at 43°C were recorded after anaerobic incubation for 48 h on HBA. Tolerance of NaCl 6.5%, bile salts 10% and tetracycline 2 mg/L was determined by agar incorporation methods. Acid production from glycerol, adonitol, inositol, salicin, xylose, fucose, rhamnose, glucose, fructose, galactose, mannose, sucrose, maltose, turanose, melezitose, melibiose and cellobiose was determined with 5% substrate in API-20 STREP Medium, dispensed in microtitration trays and incubated at 37°C for 24 h. Further reactions (acetoin, pyrrolidonyl-arylamidase, α -galactosidase, β -glucuronidase, β -galactosidase, alkaline phosphatase, leucine arylamidase and arginine dihydrolase production; hippurate and aesculin hydrolysis, and acid production from ribose, L-arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, starch and glycogen) were determined in the API-20 STREP micro-gallery system. Starch hydrolysis, hyaluronidase production,⁴⁷ H_2O_2 production,⁵³ β -fucosidase and α -amylase production (A/S Rosco rapid identification tests; Rosco Diagnostika, Taastrup, Denmark) and the presence of Lancefield group antigens A, C, G and F⁵⁴ were determined. Fluorogenic substrates were used to test for the presence of the enzymes sialidase, β -D-fucosidase, β -N-acetylgalactosaminidase, β -N-acetylglucosaminidase, β -galactosidase, β -glucosidase, α -galactosidase and α -glucosidase.⁴⁹

Pyrolysis mass spectrometry

Four replicate spectra were obtained for each culture. Methods and mathematical data processing were as described previously.⁵¹

Taxonomic analysis

Classification and identification analyses were as described previously.⁵¹ Briefly, similarity coefficients were the simple matching coefficient for CTRP data and squared Euclidian distance for PMS data; hierarchical clustering was by the unweighted pair group method with arithmetic averages (UPGMA). Cross tabulations of cluster membership in these classifications were examined; clusters were re-ordered to reflect the similarity matrices, and to optimise matching along a diagonal of correlated cluster membership. This tabulation suggested a unified classification (table I) reflecting the inter-strain similarities found in both PMS and CTRPs. The identities of coded strains were not revealed until the unified

Fig. 1. Dendrogram based on CTRPs. The similarity measure is the simple matching coefficient.

classification was finalised, to minimise the possibility of bias.

Results

Three strains of "*S. milleri*" were examined as both coded cultures and "known" marker strains, and two clinical strains as paired concurrent isolates. In each case the similarity in PMS was so great as to suggest that the duplicated cultures were indistinguishable. The duplicate cultures of *S. parasanguis* showed similar behaviour. Several pairs of clinical strains also appeared indistinguishable, although there was no documented connection between the patients from whom they originated. The non-*milleri* streptococci were clearly distinct from the main cluster of "*S.*

Fig. 2. Dendrogram based on PMS data. The dissimilarity measure is squared Euclidean distance, which is approximately equal to the probability on a χ^2 distribution with 19 degrees of freedom that strains were indistinguishable.

milleri" strains in PMS, with a squared Euclidean distance > 1000. These are not mentioned further.

As in the study of coryneform bacteria,⁵¹ classifications based on CTRPs (fig. 1) and PMS (fig. 2) showed extensive correlation of group membership, revealed by cross-tabulation (table I). As before, this strategy suggested a unified classification, combining the order revealed in the classifications from PMS and CTRP data. The five unified classification blocks are shown in table II, as are PMS cluster, CTRP cluster and block membership for individual strains. CTRPs are shown in table III.

Block 1 comprised fifteen strains including NCDO 2226 (the type strain of *S. constellatus*), NCTC 5389 and NCTC 10714 ("*Streptococcus* sp. group F"), NCTC 11063 and AM699 ("*S. milleri*"). All of these strains are known to be members of DNA homology

Table I. A cross tabulation of group membership in the CTRP and PMS classifications showing the five blocks of the unified classification

CTRP cluster	PMS cluster														Block
	H	I	E2	F	G2	C2	G1	C1	E1	B	A1	D	A2	A3	
7 3 4a-h 5a	1														1
		1	2	2	3	4									
						1									
1a-f 2a						2*	2	2	5				1*		2
									1						
5b-f										3	6				3
6a-b												3			4
2b-f													6	35	5

* Unassigned strains; CTRP, conventional test reaction patterns; PMS, pyrolysis mass spectrometry.

Table II. Origin, API-20 STREP biotype, DNA homology group (HG) and cluster membership for individual strains

Designation	API biotype	DNA HG	Cluster		Number of strains
			CTRP	PMS	
Block 1 (<i>S. constellatus</i>)					
NCDO 2226	I	1	4a	I	1
AM699	I	1	4d	E2	1
NCTC 5389	II	1	4g	E2	1
NCTC 10714	I	1	4c	F	1
NCTC 11063	I	1	4b	C2	1
	I		7	H	1
	I		4b	F	1
	I		4f	G2	1
	I		3	G2	1
	I		4e	G2	2
	I		4b	C2	1
	I		4e	C2	1
	I		5a	C2	1
	I		4h	C2	1
Block 2 (<i>S. anginosus</i>)					
PC4890	II	3	1a	G1	1
NMH10	II	3	1d	C1	1
CDC 1007-77	II	3	1a	C1	1
NCTC 11062	II	3	1a	E1	1
CDC 2405-81	II	3	1f	E1	1
NCTC 10713	II	3	2a	E1	1
ATCC 9895	II	3	1c	E1	1
G5:3	II	3	1c	E1	1
NCTC 8037	II	3	1c	E1	1
	II		1b	G1	1
Block 3 (<i>S. intermedius</i>)					
NCDO 2227	II	2	5d	B	1
NMH2	II	2	5d	B	1
CDC 415-87	II	2	5f	B	1
UNS35	II	2	5b	A1	1
	II		5e	A1	1
	II		5b	A1	1
	II		5c	A1	3
Block 4 (undesigned)					
	II		6	D	3
Block 5 (mannitol fermenting <i>S. anginosus</i>)					
KR687	III	3	2b	A2	1
KR455	III	3	2c	A2	1
	III		2c	A2	3
	II		2f	A2	1
	II		2f	A3	1
	III		2d	A3	1
	III		2e	A3	4
	III		2c	A3	29

group 1.⁴⁷ Typically, colonies were small (<0.5 mm), β -haemolytic, adherent, and gave a granular saline suspension. Lancefield antigens (F) and CO₂ requirement were detected in only a few strains. Most strains in this block were identified as *S. milleri* biotype I in API-20 STREP, and produced hyaluronidase and α -glucosidase but not H₂O₂. The block was diverse, comprising strains from CTRP clusters 3, 4, 5a and an outlier in cluster 7, which were members of PMS clusters C2, E2, F, G2 and the outlying clusters H and I. The outlier in CTRP cluster 7 showed an unusual CTRP (turannose and mannose positive) and was also an outlier in PMS (cluster H).

Block 2 comprised 10 strains, including: NCTC 10713 (the type strain of *S. anginosus*); PC4890, NMH 10, NCTC 11062 and G5:3 ("*S. milleri*"); CDC 1007-

77 and CDC 2405-81 (*S. intermedius*); ATCC 9895 and NCTC 8037 (*Streptococcus MG*). These strains are known to be members of DNA homology group 3, with two exceptions (see block 5). Strains ATCC 9895, NCTC 8037 and G5:3 were closely similar in PMS and CTRP reactions. Typically, colonies were large, α - or non-haemolytic, non-adherent, and gave a granular saline suspension. Lancefield antigen was detected in most strains, but varied (A, C, G or F); none required CO₂. All strains were identified as *S. milleri* biotype II in API-20 STREP, and produced H₂O₂ and β -glucosidase but not hyaluronidase. This block comprised strains from CTRP clusters 1 and 2a, which were members of PMS clusters G1, C1 and E1.

Block 3 comprised nine strains, including NCDO 2227 (the type strain of *S. intermedius*), CDC 415-87

Table III. Block percentage positivity in conventional tests

Tests	Percent of results positive in block*				
	1 (15)	2 (10)	3 (9)	4 (3)	5 (41)
α -Haemolysis	0	80	11	0	83
β -Haemolysis	60	10	0	100	2
Colonies adherent	40	10	0	0	0
Granular suspension	67	90	100	100	12
Colonies >0.5 mm diameter	20	90	44	0	100
CO ₂ requirement	13	0	89	33	0
Tetracycline resistance	47	10	22	33	98
Lancefield group:					
A	0	20	0	0	0
C	0	10	0	100	0
G	0	10	0	0	5
F	20	40	0	0	39
Hydrolysis of:					
hippurate	0	0	0	0	12
aesculin	0	100	22	0	100
starch	47	90	44	0	100
Production of:					
α -galactosidase (API)	0	10	0	0	95
H ₂ O ₂	0	100	0	0	0
hyaluronidase	80	0	100	100	0
Acid from:					
ribose	0	0	0	0	12
lactose	40	100	100	100	83
mannitol	7	0	0	0	98
trehalose	87	100	100	33	100
raffinose	0	10	0	0	95
amygdalin	13	60	89	100	100
glycogen	0	0	11	0	2
galactose	67	100	67	66	100
melibiose	7	10	0	0	98
fructose	87	100	78	100	100
cellobiose	7	70	56	0	100
salicin	60	100	33	100	100
API-20 STREP biotype:					
I	93	0	0	0	0
II	7	100	100	100	5
III	0	0	0	0	95
Fluorogenic enzyme tests:					
sialidase	0	0	100	0	0
α -galactosidase	7	0	0	50	53
β -galactosidase	27	10	100	100	63
α -glucosidase	100	0	100	100	97
β -glucosidase	13	80	50	100	100
β -D-fucosidase	0	0	100	0	0
β -N-acetylgalactosaminidase	0	0	100	0	0
β -N-acetylglucosaminidase	0	0	100	0	0

All strains grew at 43°C and on NaCl 6.5%, produced alkaline phosphatase, leucine arylamidase, acetoin and arginine dihydrolase. All but one produced acid from turanose and mannose; all but two produced acid from sucrose, maltose and glucose. No strain produced pyrrolidonyl-arylamidase, α -amylase, α -fucosidase or β -glucuronidase. None produced acid from sorbitol, inulin, arabinose, rhamnose, adonitol, xylose, melezitose or fucose. Only one strain showed enhanced growth under anaerobic conditions, and only two produced acid from glycerol. Two strains were bile tolerant.

* Block 1, *S. constellatus*; block 2, *S. anginosus*; block 3, *S. intermedius*; block 4, undesignated; block 5, *S. anginosus* (mannitol fermenting); () = number of strains in block.

(*S. intermedius*), NMH2 and UNS35 ("*S. milleri*"), all of which are known to be members of DNA homology group 2. Strains NCDO 2227 and NMH 2 were closely similar in PMS and CTRP patterns. Typically, colonies were non-haemolytic, non-adherent, gave a granular saline suspension, but were variable in size. Lancefield antigen was not detected, and most required

CO₂. All strains were identified as *S. milleri* biotype II in API-20 STREP, and produced hyaluronidase, β -D-fucosidase, β -N-acetylgalactosaminidase, sialidase, β -N-acetylglucosaminidase, β -galactosidase and α -glucosidase but not H₂O₂. This tight block comprised strains from CTRP clusters 5b to 5f, which were members of PMS clusters B and A1.

Block 4 comprised three strains which gave CTRPs similar to those of block 3. However, they produced small, β -haemolytic colonies, possessed Lancefield group C antigen, and were similar to block 5 strains in the fluorescent substrate enzyme tests (FETs). This tight block comprised strains from CTRP cluster 6, which were members of PMS cluster D. None of these strains was from the reference collection.

Block 5 comprised 41 strains, including KR687 and KR455 ("*S. milleri*"), i.e., the two remaining known members of DNA homology group 3. Typically, colonies were large, α -haemolytic, non-adherent and gave a smooth saline suspension. Many possessed Lancefield group F antigen, and none required CO₂. Almost all strains were identified as *S. milleri* biotype III in API-20 STREP, produced acid from a wide range of sugars, and produced β -glucosidase, β -galactosidase, α -galactosidase and α -glucosidase but not hyaluronidase or H₂O₂. This was the most homogeneous block of strains in both CTRPs (clusters 2b-f) and PMS (clusters A2 and A3).

Three strains had uncertain affiliations. The coded strain CDC 2236-81 and clinical strain C1084 appeared similar to block 1 in PMS, but to block 2 in CTRPs. CDC 2236-81 is known to be a member of DNA homology group 3. Clinical strain M2792 appeared similar to block 2 in CTRPs, but to block 5 in PMS. All three were similar to block 2 in FETs and were identified as *S. milleri* biotype II in API-20 STREP.

As in the study of coryneforms,⁵¹ corroboration of the unified classification was sought by comparison of identification results with actual group membership. Strains were divided into two approximately equal sets; the first (teaching) set provided descriptions of the five blocks, and strains of the second (challenge) set were identified by comparison with these descriptions; the sets were then reversed, so that all strains were identified from a challenge set. Inevitably, blind identification of the three strains in block 4 gave poor agreement (0% CTRP, 66% PMS); the teaching sets comprised at most two strains, a number clearly inadequate to provide a description of block characteristics. With this exception, conventional identification in the unified classification gave accurate and unequivocal results (98–100% agreement, table IV); PMS identification gave acceptable results (70–100% agreement).

Discussion

These findings show that the "*S. milleri* group" contains several distinct taxa that can be differentiated

Table IV. Results of blind identification

Block no.	Total number of strains	Number of strains showing firm agreement* with block membership for identifications based on	
		CTRPs	PMS
1	15	15 (0)	12 (3)
2	10	10 (0)	7 (1)
3	9	9 (0)	9 (0)
4	3	0 (2)	2 (1)
5	41	40 (1)	41 (0)

* The number of strains yielding equivocal identification results in which the first or second choice identification agreed with the actual block membership are given in parenthesis.

The homogeneity of each block in the unified classification was tested by identifying half the strains on the basis of block descriptions derived from the properties of the remaining half, then repeating this after reversing the 'blind identification' and 'teaching' sets. Identification results based on CTRPs showed firm agreement with actual block membership for 95% of strains, and those based on PMS showed agreement for 93% of strains, indicating good homogeneity of biochemical and compositional characters within the blocks. The poor agreement rates for block 4 strains probably reflect the small number of strains, with consequent uncertainty in the description of this block, rather than strain heterogeneity.

in CTRPs and PMS. They confirm the existence of the three species suggested by DNA homology⁴⁷ but add two further groups, one comprising mostly saccharolytic strains, the other comprising Lancefield group C β -haemolytic strains producing minute colonies. Each of the five groups appeared homogeneous and distinct.

In common with Whiley *et al.*,⁴⁹ we noted an association between the production of hyaluronidase and β -haemolysis activity among *S. constellatus* (block 1) strains but not among *S. intermedius* (block 3) strains that produced hyaluronidase but were not β -haemolytic.

Phenotypic tests that differentiated between the blocks particularly well were: production of α -galactosidase in API-20 STREP, hyaluronidase, H₂O₂ and acid from mannitol, raffinose or melibiose, together with hydrolysis of aesculin. These tests were technically undemanding and gave clear-cut results. A similar discriminatory scheme was suggested by Whiley and Hardie.⁴⁷ Alternatively, a battery of six of the FETs described by Whiley *et al.*⁴⁹ could be used. These were: production of sialidase (or β -D fucosidase or β -N-acetylgalactosaminidase), β -N-acetylglucosaminidase, β -galactosidase, α -galactosidase, β -glucosidase and α -glucosidase. Discriminatory characters are listed in table V.

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Table V. Discriminatory characters for the unified classification blocks

Tests	Results obtained with strains of block*				
	1	2	3	4	5
H ₂ O ₂	-	+	-	-	-
Aesculin	-	+	d	-	+
Hyaluronidase	d	-	+	+	-
β -Haemolysis	d	-	-	+	-
Lancefield group C	-	-	-	+	-
Mannitol†	-	-	-	-	+
α -Galactosidase (API)	-	-	-	-	+
Fluorogenic enzyme:					
sialidase‡	-	-	+	-	-
α -galactosidase	-	-	-	d	d
β -galactosidase	d	-	+	+	d
α -glucosidase	+	-	+	+	+
β -glucosidase	-	d	d	+	+

* Results are shown as -, + or d, corresponding to <15%, >85% or 15-85% positivity. Block 1, *S. constellatus*; block 2, *S. anginosus*; block 3, *S. intermedius*; block 4, undesignated; block 5, mannitol fermenting *S. anginosus*.

† Raffinose and melibiose yield similar results.

‡ β -D-fucosidase, β -N-acetylgalactosaminidase and β -N-acetylglucosaminidase gave similar results.

The controversy over the taxonomy and nomenclature of the "*S. milleri* group" has been largely resolved by evidence from newer taxonomic techniques. However, some effort should now be directed towards ensuring that the range of species and subspecies of the group has been adequately documented. We could find no convincing match between the CTRPs of the blocks and the classification of Facklam.²⁹ However, blocks 1-3 corresponded to the DNA homology groups which Whiley and Hardie⁴⁷ termed *S. constellatus*, *S. anginosus* and *S. intermedius*, respectively. The evidence presented here suggests that there is a previously undescribed centre of variation, the group C β -haemolytic strains of block 4, and gives clear evidence that the mannitol fermenting strains of *S. milleri* form a compact group or phenon, closely similar in conventional tests and PMS. The latter biotype has been reported previously.^{5,7,8,43} Two of the block 5 strains (KR687 and KR455) have shown high DNA homology with the type strain of *S. anginosus*⁴⁷ and further DNA hybridisation work is required to confirm this relationship. Also, similar work on a larger collection of strains showing the phenotypic characters of strains in block 4 is required to elucidate their taxonomic position.

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