Journal of General Microbiology (1989), **135**, 2623–2633. *Printed in Great Britain*

DNA–DNA Hybridization Studies and Phenotypic Characteristics of Strains within the '*Streptococcus milleri* Group'

By ROBERT A. WHILEY* AND JEREMY M. HARDIE

Department of Oral Microbiology, The London Hospital Medical College, London E1 2AD, UK

(*Received 4 April 1989; revised 26 June 1989; accepted 4 July 1989*)

Twenty-five strains resembling '*Streptococcus milleri*' were compared by DNA–DNA hybridization, by whole-cell-derived polypeptide patterns on SDS-PAGE, and by biochemical tests. Four homology groups were revealed by DNA–DNA hybridization. DNA homology groups 1, 2 and 3 were closely related and contained the type strains NCDO 2226 (*Streptococcus constellatus*), NCDO 2227 (*Streptococcus intermedius*) and NCTC 10713 (*Streptococcus anginosus*), respectively. DNA homology group 4 consisted of four strains received as variants of *Streptococcus intermedius* which were found not to be closely related to strains in groups 1–3. The data from SDS-PAGE polypeptide patterns and biochemical tests supported the recognition of three centres of variation within the '*Streptococcus milleri* group' corresponding to DNA homology groups 1–3 and indicated that strains of DNA homology group 4 are members of an as yet undescribed species within the viridans streptococci.

INTRODUCTION

'*Streptococcus milleri*' is the name frequently used for a group of streptococci that form part of the normal flora of the human mouth and upper respiratory, intestinal and urinogenital tracts (Attebury *et al.*, 1972; Mejare & Edwardsson, 1975; Poole & Wilson, 1979; Ruoff & Kunz, 1982). In recent years these bacteria have increasingly been recognized as having clinical importance, particularly by their association with abscesses and other purulent infections (Parker & Ball, 1976; Ball & Parker, 1979; Poole & Wilson, 1979; Shlaes *et al.*, 1981; Gossling, 1988). Despite the accumulated evidence for the pathogenicity of these bacteria (see Gossling, 1988, for a review of the literature) there is still disagreement about the taxonomy and nomenclature of the '*S. milleri* group' (Hardie, 1986).

The name '*Streptococcus milleri*' was first proposed by Guthof (1956) for a group of non-haemolytic streptococci isolated from abscesses of the oral cavity that grew on 40% bile agar and at 45 °C, hydrolysed aesculin and arginine but did not produce acid from mannitol or sorbitol. On the basis of cell wall analysis, numerical taxonomy and DNA transformation studies, Colman & Williams (1972) broadened the description to include those previously reported non-haemolytic streptococci isolated from the human respiratory tract and called '*Streptococcus MG*' (Mirick *et al.*, 1944), the haemolytic and non-haemolytic streptococci possessing the so-called type antigens of Lancefield group F (Ottens & Winkler, 1962) and the minute-colony-forming streptococci of Lancefield groups F and G (Long & Bliss, 1934; Bliss, 1937). Subsequently, Facklam (1977) reported a high degree of similarity between '*Streptococcus MG*' (Mirick *et al.*, 1944), *Streptococcus intermedius* (Holdeman & Moore, 1974), *Streptococcus anginosus* (Andrewes & Horder, 1906; Deibel & Seeley, 1974) and *Streptococcus constellatus* (Holdeman & Moore, 1974). Facklam (1977) chose to divide these non-haemolytic, '*milleri*-like' streptococci into two groups on the basis of lactose fermentation, namely '*Streptococcus MG-intermedius*' (lactose fermenters) and '*Streptococcus anginosus–constellatus*' (lactose non-fermenters). In an attempt to clarify the situation in which different names were used in Europe and the United States, it was suggested that β-haemolytic strains (whether or not they possessed Lancefield group antigens),

be called *Streptococcus anginosus*, while non-β-haemolytic strains be divided into *Streptococcus intermedius* (lactose fermenters) and *Streptococcus constellatus* (lactose non-fermenters) (Facklam, 1984, 1985). Despite the differences in nomenclature, several taxonomic studies have demonstrated a high degree of phenotypic similarity and a close relationship between strains resembling '*S. milleri*' (Mejare, 1975; Lutticken *et al.*, 1978; Labbe *et al.*, 1985; Coykendall *et al.*, 1987; French *et al.*, 1989). However, heterogeneity within the '*Streptococcus milleri* group' has been reported on the basis of several different criteria that include fermentation patterns (Poole & Wilson, 1979; Ruoff & Kunz, 1982; Drucker & Lee, 1981), long-chain fatty acid analysis (Drucker & Lee, 1981; Cookson *et al.*, 1989), DNA base composition determinations (mol% G+C) (Drucker & Lee, 1983), antigenic composition (Colman & Williams, 1972; Lutticken *et al.*, 1978; Yakushiji *et al.*, 1988) and multilocus enzyme electrophoresis (Gilmour *et al.*, 1987).

Evidence from DNA–DNA hybridization studies has been somewhat contradictory. Some authors have concluded that strains resembling '*S. milleri*' should be included within a single homology group (Welborn *et al.*, 1983; Farrow & Collins, 1984; Ezaki *et al.*, 1986; Coykendall *et al.*, 1987) whereas others have demonstrated the presence of more than one DNA homology group (Kilpper-Balz *et al.*, 1984; Knight & Shlaes, 1988). Coykendall *et al.* (1987) proposed that strains variously described as '*S. milleri*', *S. constellatus* and *S. intermedius* be included within a single species, *S. anginosus*. Subsequently, however, strains identified as *S. intermedius* were observed to fall into three DNA homology groups (Knight & Shlaes, 1988). In addition, a preliminary DNA–DNA hybridization experiment in our own laboratory indicated that the type strains of *S. anginosus*, *S. constellatus* and *S. intermedius* belonged to separate homology groups (Whiley, 1987).

In view of the continued confusion surrounding the taxonomy of strains resembling '*S. milleri*', we decided to carry out extensive DNA–DNA hybridization experiments together with phenotypic characterization on a representative collection of clinical and reference strains, in an effort to clarify the taxonomic structure of the '*Streptococcus milleri* group'.

METHODS

Bacterial strains and growth conditions. The strains and their sources are given in Table 1. Unless otherwise stated, incubation was at 37 °C in an anaerobic atmosphere of $H_2/CO_2/N_2$ (20:10:70, by vol.). For DNA preparation, strains were cultivated in 2 or 3 litres of Streptococcus Sugar Base broth (SSB), containing 2% (w/v) protease peptone (Oxoid), 0·5% (w/v) yeast extract (Difco), 0·5% (w/v) NaCl, 0·1% (w/v) Na_2HPO_4, pH 7·6, supplemented with 0·5% (w/v) glucose and 0·25% (w/v) $NaHCO_3$. Cells were harvested in the late exponential phase by centrifugation.

Preparation of DNA. DNA was isolated and purified from approximately 3 g (wet weight) of cells by the method of Garvie (1976). Radioactive DNA was labelled with deoxy[1′,2′,5-^{3}H]cytidine triphosphate (Amersham) by nick-translation using a commercial kit (Amersham).

DNA base composition determinations. The thermal denaturation method of Marmur & Doty (1962) was used to determine the guanine (G) plus cytosine (C) content (mol% G+C) of the DNA. Determinations were performed in duplicate in standard saline citrate (SSC: 0·15 M-NaCl plus 0·015 M-trisodium citrate, pH 7·0) as described previously (Beighton *et al.*, 1984). The mol% G+C was calculated after Owen & Hill (1979) using the following equation: mol% G+C = 50·9 +2·44(T_{mX} − T_{mR} where T_{mX} is the melting temperature of DNA from the test strain and T_{mR} is the melting temperature of DNA from *Escherichia coli* strain NCTC 9001.

DNA–DNA hybridizations. These were done using the S1-nuclease method of Crosa *et al.* (1973) as modified by Shah *et al.* (1982). Hybridizations were performed, in triplicate, in 300 µl 0·42 M-NaCl containing sheared, denatured, unlabelled DNA (30 µg) and sheared, denatured, labelled DNA (0·02 µg). Experiments were done under optimum (60 °C) and stringent (75 °C) conditions for 90 h to obtain a C_0t value of 100 (Britten & Kohne, 1968). After incubation, hybridization mixtures were cooled in an ice bath and treated with 540 µl ice-cold S1-nuclease buffer (0·4 mM-$ZnSO_4$, 0·15 M-NaCl, 0·03 M-sodium acetate, pH 4·8) containing 30 µg sheared denatured calf thymus DNA ml^{-1} and 20 units S1-nuclease (Boehringer). The mixtures were incubated at 50 °C for 30 min before the addition of 250 µl ice-cold 25% (w/v) trichloracetic acid. The S1-nuclease-treated duplexes were trapped on glass-fibre filters (Whatman GF/F) and washed with 3 vols ice-cold 5% (w/v) trichloracetic acid. The filters were dried and their radioactivity measured by scintillation counting. Controls included a homologous reassociation as a positive (100%) control and calf thymus DNA as a negative (0%) control. For each test DNA the degree of sequence homology was expressed as a percentage of the homologous control after correction for self-reassociation in the negative control.

Biochemical tests. Acid production from carbohydrates, etc. (arabinose, amygdalin, cellobiose, glucose, glycerol,

Table 1. *Details of Streptococcus strains*

Name/strain code	Lancefield group (as received)	Source (human)
S. anginosus		
NCTC 10713^T (ATCC 12395^T, DSM 20563^T)	G	Throat
S. intermedius		
NCDO 2227^T (ATCC 27335^T, DSM 20573^T)	–	Unknown
CDC: 415-87	–	Brain abscess
CDC: 1007-77	A	Wound
CDC: 2236-81	C	Blood
CDC: 2405-81	–	Blood
S. intermedius variant strains		
CDC: SS-895 (ATCC 15909)	G	Throat
CDC: SS-898 (ATCC 15912)	G	Throat
CDC: 2156-81	–	Blood
CDC: 85-81	F	Blood
S. constellatus		
NCDO 2226^T (ATCC 27823^T, NCTC 11325^T, DSM 20575^T)	–	Purulent pleurisy
'S. milleri'		
NMH 2	–	Brain abscess
NMH 10	A	Perforated ulcer
KR 455	–	Urine
KR 687	F	Axillary abscess
AM 699	–	Dental plaque
PC 4890	C	Dental plaque
UNS 35	–	Brain abscess
G5:3	–	Dental plaque
NCTC 11062	–	Root canal
NCTC 11063	–	Throat
'Streptococcus MG'		
NCTC 8037	–	Respiratory tract
ATCC 9895 (*'S. mitis-MG'*)	F	Throat
'Streptococcus sp. group F'		
NCTC 5389	F	Unknown
NCTC 10714	F	Throat
S. sanguis		
NCTC 7863^T (ATCC 10556^T, DSM 20567^T)	H	Subacute bacterial endocarditis
S. mitis		
NCTC 3165^T (ATCC 33399^T, DSM 20568^T)	–	Pyorrhoea
S. oralis		
LVG1^T (NCTC 11427^T)	–	Mouth
S. salivarius		
NCTC 8618^T (DSM 20560^T)	K	Throat
S. vestibularis		
MM1^T (NCTC 12166^T, ATCC 49124^T)	–	Mouth
S. mutans		
NCTC 10449^T (ATCC 25175^T, DSM 20523^T)	–	Carious dentine

* T, Type strain; ATCC, American Type Culture Collection, Rockville, Maryland, USA; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, UK; CDC, strains from Dr R. Facklam, Center for Disease Control, Atlanta, Georgia, USA; DSM, Deutsche Sammlung von Mikroorganismen, Gottingen, FRG; KR, strains from Dr K. Ruoff, Massachusetts General Hospital, Massachusetts, USA; NMH, strains from Mr M. W. D. Wren, North Middlesex Hospital, London N18, UK; PC, AM, strains from the Department of Oral Microbiology, The London Hospital Medical College, London, UK; G, strain from Dr B. Mejare, School of Dentistry, University of Lund, Malmo, Sweden; UNS, Dr P. Unsworth, Central Public Health Laboratory, Colindale, UK.

inulin, lactose, mannitol, melibiose, raffinose, salicin, sorbitol and trehalose) was tested for in microtitre trays by the method of Beighton *et al.* (1981). The ability to produce acetoin (VP test) was determined as described by Cowan (1974). Aesculin hydrolysis and the production of ammonia from arginine were tested according to Bisset & Davis (1960); the production of ammonia from arginine was detected by the addition of Nessler's reagent. Starch hydrolysis was tested on brain heart infusion medium (Difco) supplemented with 0·2% (w/v) soluble starch and

1·2% (w/v) agar (Oxoid no. 1). After 5 d incubation, plates were flooded with Lugol's iodine; the hydrolysis of starch was indicated by the appearance of clear zones around the colonies. Hydrogen peroxide production was tested for by the method of Whittenbury (1964) as described previously (Whiley & Hardie, 1988), with strains incubated in 10% CO_2-in-air at 37 °C for 4 d. Hydrogen peroxide production was detected when the surrounding medium became dark brown or black.

API 20 STREP. All test strains were examined using the API 20 STREP rapid identification kit according to the manufacturer's instructions.

Haemolysis. Strains were streaked onto blood agar plates containing blood agar base (40 g l^{-1}; Oxoid) plus 5% (v/v) defibrinated horse blood (Gibco) and incubated for 48 h anaerobically before the type of haemolysis produced (α, β or γ) was recorded.

Hyaluronidase production. This was tested for by the rapid plate method of Smith & Willet (1968). To a basal medium consisting of brain heart infusion and 1% (w/v) agar (Oxoid no. 1) were added filter-sterilized, aqueous solutions of hyaluronic acid (4 mg ml^{-1}; Sigma, grade III-S) and 5% (v/v) bovine serum albumin fraction V (Sigma) to final concentrations of 400 μg ml^{-1} and 1% (w/v) respectively. Plates were poured (3–4 mm depth) and strains were inoculated onto the agar surface in discrete spots. Incubation was carried out for 48 h under anaerobic conditions. After incubation the plates were flooded with 2 M-acetic acid for 10 min. Strains producing hyaluronidase were identified by the appearance of a clear zone around the growth.

Comparison of strains by SDS-PAGE. Whole-cell-derived polypeptide patterns were obtained by SDS-PAGE, done in both 7% and 12% (w/v) polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970). Approximately 60 μg of sample protein from each strain, prepared as described previously (Whiley *et al.*, 1982), was loaded into each well. After electrophoresis was completed the polypeptide patterns were visualized by staining with Coomassie Brilliant Blue-R (Sigma).

RESULTS

DNA studies

The mol% G+C contents of the test strains and the results of the DNA–DNA hybridization experiments are shown in Table 2. The mol% G+C range of 36·7–39·5 for homology groups 1–3 is in good agreement with values obtained in previous studies on strains resembling '*S. milleri*' (Kilpper-Balz *et al.*, 1984; Ezaki *et al.*, 1986; Coykendall *et al.*, 1987). However, the mol% G+C range within homology group 4 (41·5–42·7) falls outside that of most published data on this group of bacteria, although values as high as 43% were obtained by Drucker & Lee (1983).

The 25 strains resembling '*S. milleri*' fell into four DNA homology groups (1–4), with members within a group exhibiting a high degree of base sequence homology under both optimum and stringent hybridization conditions. A higher degree of intergroup relatedness was observed between groups 1, 2 and 3 than was seen between any of these groups and homology group 4. In most cases the latter group showed <30% homology with the other groups under optimum conditions. Homology group 1 (mol% G+C 36·7–38·5) contained reference strains of *S. constellatus* (NCDO 2226), Lancefield group F (NCTC 5389 and NCTC 10714) and '*S. milleri*' (NCTC 11063), together with '*S. milleri*' strain AM 699, isolated from dental plaque. Homology group 2 (mol% G+C 37·2–38·5) included the reference strain of *S. intermedius* (NCDO 2227) together with three strains isolated from brain abscesses: '*S. milleri*' NMH 2 and UNS 35, and *S. intermedius* 415–87. Homology group 3 (mol% G+C 38·1–39·5) contained the type strain of *S. anginosus* (NCTC 10713), reference strains of '*Streptococcus MG*' (NCTC 8037, ATCC 9895) and '*S. milleri*' (NCTC 11062) together with strains designated as '*S. milleri*' and *S. intermedius* that had been isolated from dental plaque, wound swabs, blood, an axillary abscess and urine. Homology group 4 (mol% G+C 41·5–42·7) consisted of the four variant strains of *S. intermedius* obtained from Dr R. Facklam of the CDC, Atlanta, Georgia, USA, that had been isolated from blood cultures and human throats.

Biochemical characteristics

The biochemical test results for the strains are shown in Table 3. All 25 test strains produced acid from glucose but none was able to ferment arabinose, glycerol or inulin. Virtually all strains failed to ferment mannitol or sorbitol. Most strains produced acetoin, hydrolysed arginine and fermented salicin and trehalose but failed to ferment melibiose or raffinose. Strains comprising DNA homology group 1 were characterized by not fermenting lactose or amygdalin, in not producing hydrogen peroxide and in being mainly β-haemolytic. Most strains in homology

Table 2. *DNA homologies of strains resembling 'S. milleri' using optimum (60 °C) and stringent (75 °C) hybridization conditions*

Unlabelled DNA from:	Mol% G+C	Percentage DNA homology* using ^{3}H-labelled DNA from: NCDO 2226^T 60 °C	NCDO 2226^T 75 °C	NCDO 2227^T 60 °C	NCDO 2227^T 75 °C	NCTC 10713^T 60 °C	NCTC 10713^T 75 °C	85-81 60 °C	85-81 75 °C
Homology group 1									
NCDO 2226^T	36·7	**100**	**100**	70	40	57	24	24	NT
AM 699	38·5	**122**	**78**	NT	NT	56	22	24	NT
NCTC 10714	38·5	**72**	**102**	69	35	44	NT	NT	NT
NCTC 11063	38·5	**101**	**98**	58	19	44	NT	NT	NT
NCTC 5389	37·8	**78**	**68**	63	25	37	NT	25	NT
Homology group 2									
NCDO 2227^T	37·2	29	NT	**100**	**100**	44	NT	21	NT
NMH 2	38·5	44	NT	**106**	**61**	45	NT	10	NT
UNS 35	37·5	33	NT	**102**	**57**	29	NT	NT	NT
415-87	37·5	39	NT	**103**	**60**	35	NT	21	NT
Homology group 3									
NCTC 10713^T	38·1	32	NT	42	20	**100**	**100**	16	NT
2405-81	39·2	44	NT	28	NT	**92**	NT	NT	NT
NMH 10	38·5	66	39	40	9	**81**	**50**	30	NT
G5:3	38·4	53	32	23	NT	**79**	**69**	NT	NT
2236-81	39·0	46	NT	43	NT	**76**	**59**	19	NT
1007-77	39·0	62	12	46	NT	**78**	**50**	NT	NT
PC 4890	39·5	67	24	48	11	**75**	**53**	NT	NT
NCTC 11062	37·8	50	37	45	NT	**74**	NT	NT	NT
KR 687	38·8	37	NT	42	5	**72**	**55**	15	NT
NCTC 8037	38·7	55	26	51	26	**69**	**60**	NT	NT
KR 455	39·0	24	NT	36	NT	**62**	**61**	NT	NT
ATCC 9895	38·2	57	15	47	25	**54**	**51**	20	NT
Homology group 4									
85-81	42·7	1	NT	10	NT	6	NT	**100**	**100**
SS-895	41·5	15	NT	14	NT	12	NT	**93**	**62**
2156-81	42·0	12	NT	28	2	17	NT	**110**	**60**
SS-898	42·0	2	NT	10	NT	11	NT	**72**	NT
S. sanguis NCTC 7863^T	45·0	9	NT	10	NT	18	NT	33	NT
S. mitis NCTC 3165^T	38·8	13	NT	13	NT	13	NT	19	NT
S. oralis LVG1^T (NCTC 11427^T)	40·5	10	NT	11	NT	20	NT	15	NT
S. salivarius NCTC 8618^T	41·6	5	NT	5	NT	7	NT	16	NT
S. vestibularis MM1^T (NCTC 12166^T)	39·0	17	NT	17	NT	14	NT	23	NT
S. mutans NCTC 10449^T	37·0	0	NT	2	NT	5	NT	18	NT

* Only hybridizations showing ≥ 50% base sequence homology under optimum conditions were repeated under stringent conditions. Intra-homology group values are shown in **bold** print. NT, Not tested.

group 1, with the exception of strain AM 699, were assigned to '*S. milleri*' biotype I in the API 20 STREP rapid identification system. Members of homology group 1 were either serologically ungroupable or belonged to Lancefield group F and were further characterized by the production of hyaluronidase.

Strains of homology group 2 differed from those of group 1 by fermenting lactose and usually cellobiose and in being weakly haemolytic (α) or non-haemolytic (γ). All strains were assigned to '*S. milleri*' biotype II in the API 20 STREP rapid identification system. As with homology group 1, hyaluronidase was produced by all members of this group. No Lancefield serological groups were represented in homology group 2.

Table 3. *Phenotypic characteristics of strains resembling 'S. milleri'*

All strains produced acid from glucose. No strain produced acid from arabinose, inulin or glycerol. Abbreviations: amygdalin (amygd); cellobiose (cello); mannitol (mann); melibiose (melib); raffinose (raff); salicin (sal); sorbitol (sorb); trehalose (treh); arginine (arg); aesculin (aesc); hyaluronidase (hyal). NT, Not tested. +/−, Weak positive result.

	Acid produced from:									Hydrolysis of:			Production of:			Haemo-lysis	Lance-field gp.	API*
	Amygd	Cello	Lac	Mann	Melib	Raff	Sal	Sorb	Treh	Arg	Aesc	Starch	Acetoin	H_2O_2	Hyal			
Homology group 1																		
NCDO 2226^T	−	−	−	−	−	−	+	−	+	+	+	−	+	−	+	γ	−	I
AM 699	−	−	−	−	−	−	+	−	+	+	+	−	+	−	NT	β	−	II
NCTC 10714	−	+	−	−	−	−	+	−	+	+	−	−	+	−	+	β	F	I
NCTC 11063	−	−	−	−	−	−	+	−	−	+	+	−	+	−	+	α	−	I
NCTC 5389	−	−	−	−	−	−	−	−	+	+	+/−	−	+/−	−	+	β	F	I
Homology group 2																		
NCDO 2227^T	+	+	+	−	−	−	+	−	+	+	+	−	+	−	+	γ	−	II
NMH 2	+	+	+	−	−	−	+	−	+	+	+	−	+	−	+	γ	−	II
UNS 35	−	−	+	−	−	−	−	−	+	+	−	+	+	−	+	α	−	II
415-87	−	+	+	−	−	−	+	−	+	+	+	−	+	+	+	α	−	II
Homology group 3																		
NCTC 10713^T	+	+	+	−	+	+	+	−	+/−	+	+	+/−	+	+	−	β	G	II
2405-81	+	+	+	−	−	−	+	−	+	+	+	−	+	+	−	α	−	II
NMH 10	+	+	+	−	−	−	+	−	−	+	+	+	+	+	−	γ	A	II
G5:3	+	+	+	−	−	−	+	−	+	+	+	−	+	+	−	α	−	II
2236-81	+	+	+	−	−	−	+	−	+	+	+	+	+	+	−	α	C	II
1007-77	+	−	+	−	−	−	+	−	+	+	+	−	+	+	−	α	A	II
PC 4890	+	+	+	−	−	−	+	−	+	+	+	+	+	−	−	α	C	II
NCTC 11062	+	−	+	−	−	−	+	−	+	+	+	+	+	+	−	α	−	II
KR 687	+	+	+	+	+	+	+/−	−	+	+	+	−	+	−	−	γ	F	III
NCTC 8037	+	+	+	−	−	−	+/−	−	+	+	+	−	+	+	−	α	−	II
KR 455	+	+	+	+	+	+	+/−	−	+	−	+	−	+	−	−	γ	−	III
ATCC 9895	+	+	+	−	−	−	+	−	+	+	+	+	+	+	−	α	F	II
Homology group 4																		
85-81	−	+	+	−	+	+	+	+	−	+	−	+	−	+	−	α	F	S1/2
SS-895	−	+	+	−	+	+	+	−	+	+	+/−	+	−	−	−	α	G	SII
2156-81	−	−	+	−	+	+	+	−	+	+	+/−	+/−	−	+	−	α	−	SII
SS-898	+	+	+	−	+	+	+	−	+	+	+	+	−	+	−	α	G	SII

* API '*S. milleri*' biotypes: I (aesc −, α-galactosidase (α-GAL) −, mann −, lac −, raff −); II (aesc +, α-GAL −, mann −, lac +, raff −); III (aesc +, α-GAL +, mann +, lac +, raff +). API *S. sanguis* biotypes: S1/2 [acetoin (VP) −, aesc + or −, alkaline phosphatase (PAL) −, arginine dihydrolase (ADH) +]; SII (VP −, aesc −, PAL +, ADH + or −).

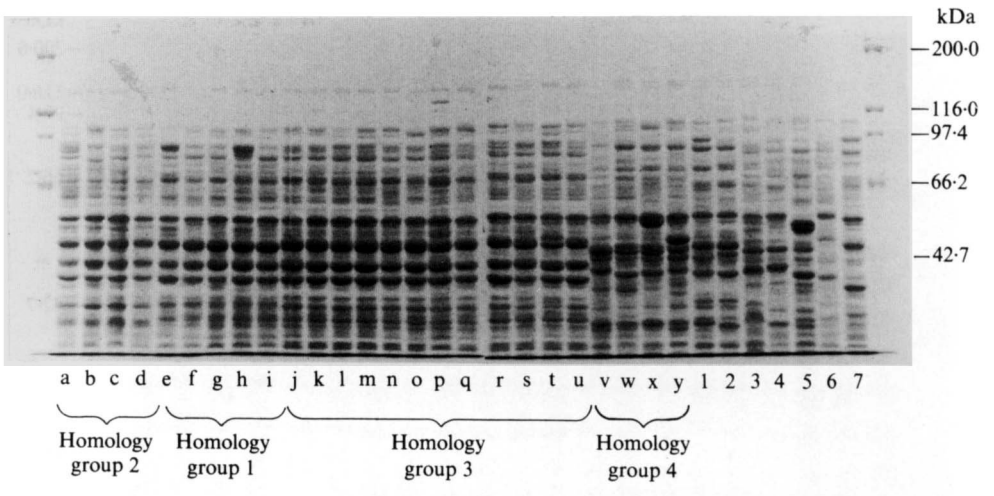

Fig. 1. SDS-PAGE (7% acrylamide) of whole-cell-derived polypeptide patterns stained with Coomassie Brilliant Blue. Homology group 2: lane a, NCDO 2227; b, UNS 35; c, NMH 2; d, 415-87. Homology group 1: lane e, NCDO 2226; f, NCTC 10714; g, NCTC 11063; h, NCTC 5389; i, AM 699. Homology group 3: lane j, NCTC 10713; k, PC 4890; l, 1007-77; m, NMH 10; n, NCTC 11062; o, 2236-81; p, KR 687; q, 2405-81; r, NCTC 8037; s, KR 455; t, G5:3; u, ATCC 9895. Homology group 4: lane v, 85-81; w, SS-895; x, SS-898; y, 2156-81. Lane 1, *S. sanguis* (NCTC 7863); 2, *S. mitis* (NCTC 3165); 3, *S. oralis* (LVG1); 4, *S. salivarius* (NCTC 8618); 5, *S. vestibularis* (MM1); 6, *S. mutans* (NCTC 10449); 7, *S. sobrinus* SL-1 (ATCC 33478).

Strains of homology group 3 were characterized by their ability to ferment amygdalin and lactose and by the frequent fermentation of cellobiose and production of hydrogen peroxide. Strains in this group were also weakly or non-haemolytic (α, γ), with the exception of NCTC 10713 (β), and serologically heterogeneous, with strains of Lancefield groups A, C and F as well as serologically ungroupable strains present. None of the 12 strains in homology group 3 produced hyaluronidase and most were assigned to '*S. milleri*' biotype II in the API 20 STREP identification system, apart from two strains (KR 455 and KR 687) which fell into biotype III.

Homology group 4 strains differed from the other groups by being unable to produce acetoin. All four strains in this group fermented lactose and melibiose; three fermented cellobiose and raffinose, and three produced hydrogen peroxide. No strains of homology group 4 produced hyaluronidase and all were α-haemolytic. These strains were serologically heterogeneous, with strains possessing Lancefield group antigens F and G as well as serologically ungroupable strains present. In the API 20 STREP identification system the strains in group 4 were assigned to *S. sanguis* biotype II (SS-895, SS-898, 2156-81) or to *S. sanguis* biotype 1/2 (strain 85-81) although, with the exception of strain 2156-81, the level of confidence for these identifications was less than 56%.

SDS-PAGE polypeptide patterns

The polypeptide profiles of the test strains together with those from selected type strains of other viridans streptococcal species are shown in Fig. 1. Strikingly similar polypeptide patterns were shared by strains of homology groups 1, 2 and 3. These were characterized by four dominant bands of approximate molecular masses 53·3, 45·3, 41·3 and 38·0 kDa. Considerable inter-strain heterogeneity was observed amongst the bands in the range 58·3–106·0 kDa, even between strains belonging to the same DNA homology group (Figs 1 and 2). However, the band patterns of polypeptides in the lower size range of 25·5–34·0 kDa were less heterogeneous and fell into three main pattern types corresponding to the DNA homology groups (Fig. 2).

Strains of homology group 4 gave polypeptide patterns that were clearly unrelated to those of DNA homology groups 1–3 (Fig. 1). With the exception of a dominant 53·3 kDa band, which was present in virtually all streptococci examined here, there was little or no similarity between

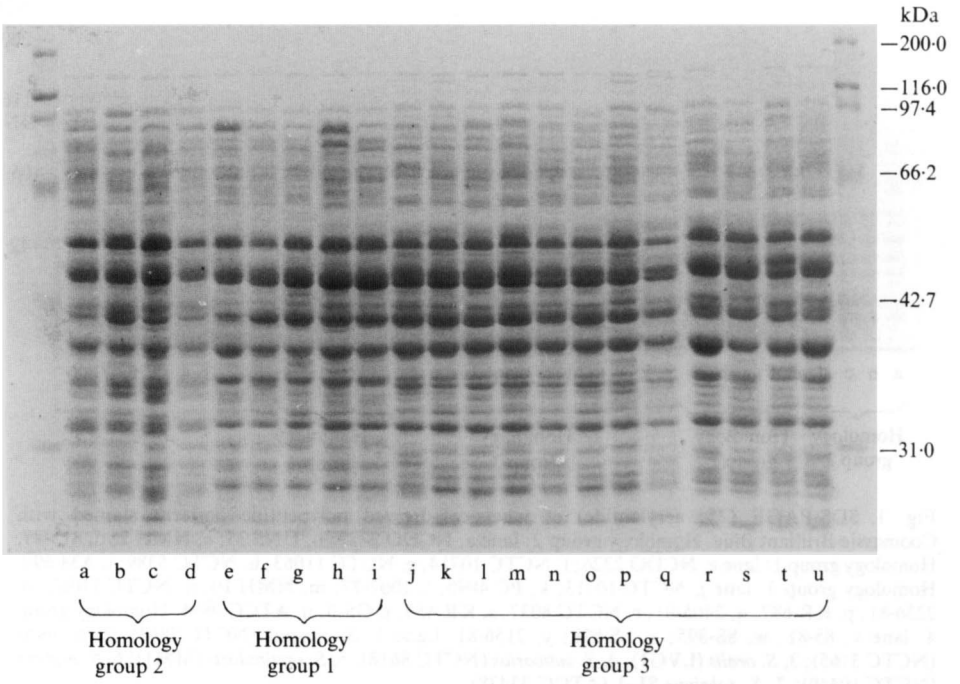

Fig. 2. SDS-PAGE (12% acrylamide) of whole-cell-derived polypeptide patterns stained with Coomassie Brilliant Blue. Homology group 2: lane a, NCDO 2227; b, UNS 35; c, NMH 2; d, 415–87. Homology group 1: lane e, NCDO 2226; f, NCTC 10714; g, NCTC 11063; h, NCTC 5389; i, AM 699. Homology group 3: lane j, NCTC 10713; k, PC 4890; l, 1007-77; m, NMH 10; n, NCTC 11062; o, 2236-81; p, KR 687; q, 2405-81; r, NCTC 8037; s, KR 455; t, G5:3; u, ATCC 9895.

the patterns of the strains in homology group 4 and those of homology groups 1–3. Considerable heterogeneity within group 4 was also noted throughout the 29–106 kDa range resolved on the 7% gel (Fig. 1), despite the obvious close relationship between these strains as shown by DNA–DNA hybridization.

DISCUSSION

The data obtained from the DNA studies and SDS-PAGE gels demonstrate that strains resembling '*S. milleri*' consist of three distinct, albeit closely related, groups. The inclusion of the type strains of *S. constellatus*, *S. intermedius* and *S. anginosus* in separate DNA homology groups confirms and extends the results of Kilpper-Balz *et al.* (1984), who obtained DNA homology groups corresponding to *S. anginosus* and *S. constellatus*, with the type strain of *S. intermedius* (DSM 20573) remaining ungrouped. The formation of a fourth, more distantly related, genetic group by the variant strains of *S. intermedius* is in accord with the data obtained by Knight & Shlaes (1988). Unfortunately these authors did not include reference strains of *S. constellatus* or *S. anginosus* in their study so it is not possible to equate any of the homology groups found by them with homology groups 1 and 3 of this study.

The conflicting data in the literature concerning the inclusion of strains resembling '*S. milleri*' in a single homology group by some authors (Welborn *et al.*, 1983; Farrow & Collins, 1984; Ezaki *et al.*, 1986; Coykendall *et al.*, 1987) and the demonstration of several distinct DNA homology groups by others (Kilpper-Balz *et al.*, 1984; Knight & Shlaes, 1988) has most probably been due to the respective stringencies of the different methods employed. Such discrepancies have been recognized before (Bouvet & Grimont, 1986; Coykendall & Munzenmaier, 1978, 1979) and have been acknowledged as a likely cause for disagreement in previous studies on the '*S. milleri* group' (Coykendall *et al.*, 1987; Knight & Shlaes, 1988). In addition, the fact that in some cases DNA–DNA hybridization studies have only been done on strains preselected on the

basis of haemolysis type and lactose fermentation (Ezaki *et al.*, 1986; Knight & Shlaes, 1988) has made it difficult to draw conclusions regarding the '*S. milleri* group' as a whole.

As concluded by previous authors (Kilpper-Balz *et al.*, 1984; Knight & Shlaes, 1988) there remains a need for reliable phenotypic tests or markers with which to differentiate the three homology groups within the '*S. milleri* group'. Of the tests used in the present study, acid production from amygdalin and lactose, and the production of hydrogen peroxide and hyaluronidase seem the most useful. However, the assignment of freshly isolated strains to one or other of the genetic groups on the basis of these tests alone may well prove equivocal, as was found to be the case by Kilpper-Balz *et al.* (1984), where both $lac^+/hyal^+$ and $lac^+/hyal^-$ strains were found to belong to the *S. constellatus* DNA homology group. The observation by these authors that the type strain of *S. intermedius* did not produce hyaluronidase was not confirmed in the present study, where hyaluronidase production was found to be a characteristic of all strains in the *S. intermedius* homology group. Subsequently, we have also hybridized other versions of the *S. intermedius* type strain [i.e. DSM 20573 and CDC strain SS1090 (=ATCC 27823)] with NCDO 2227. These gave 84% and 112% DNA base sequence homology, respectively, with NCDO 2227 (data not shown) and were also able to produce hyaluronidase.

The low level of DNA homology observed between strains comprising homology group 4 and all other streptococci examined indicates that these bacteria represent an as yet unrecognized species. Further studies with additional strains will be necessary to determine the true taxonomic status of these streptococci.

Although these data demonstrate a considerable degree of genetic heterogeneity within the '*S. milleri* group', the relatively low numbers of strains that have, so far, confidently been assigned to one or other of the three homology groups, together with a lack of reliable phenotypic tests, precludes us from making formal taxonomic proposals at present.

REFERENCES

ANDREWES, F. W. & HORDER, T. J. (1906). A study of the streptococci pathogenic for man. *Lancet* **ii**, 703–713.

ATTEBURY, H. R., SUTTER, V. L. & FINEGOLD, S. M. (1972). Effect of a partially chemically defined diet on human fecal flora. *American Journal of Clinical Nutrition* **25**, 1391–1398.

BALL, L. C. & PARKER, M. T. (1979). The cultural and biochemical characters of *Streptococcus milleri* strains isolated from human sources. *Journal of Hygiene* **82**, 63–78.

BEIGHTON, D., RUSSELL, R. R. B. & HAYDAY, H. (1981). The isolation and characterisation of *Streptococcus mutans* serotype h from dental plaque of monkeys (*Macaca fascicularis*). *Journal of General Microbiology* **124**, 271–279.

BEIGHTON, D., HAYDAY, H., RUSSELL, R. R. B. & WHILEY, R. A. (1984). *Streptococcus macacae* sp. nov. from dental plaque of monkeys (*Macaca fascicularis*). *International Journal of Systematic Bacteriology* **34**, 332–335.

BISSET, P. J. M. & DAVIS, G. H. G. (1960). *The Microbiol Flora of the Mouth*. London: Heywood.

BLISS, E. A. (1937). Studies on minute hemolytic streptococci: serological differentiation. *Journal of Bacteriology* **33**, 625–642.

BOUVET, P. J. M. & GRIMONT, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov. and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *International Journal of Systematic Bacteriology* **36**, 228–240.

BRITTEN, R. J. & KOHNE, D. E. (1968). Repeated sequences in DNA. *Science* **161**, 529–540.

COLMAN, G. & WILLIAMS, R. E. O. (1972). Taxonomy of some human viridans streptococci. In *Streptococci and Streptococcal Diseases*, pp. 282–299. Edited by L. W. Wannamaker & J. M. Matsen. New York: Academic Press.

COOKSON, B., TALSANIA, H., CHINN, S. & PHILLIPS, I. (1989). A qualitative and quantitative study of the cellular fatty acids of '*Streptococcus milleri*' with capillary gas chromatography. *Journal of General Microbiology* **135**, 831–838.

COWAN, S. T. (1974). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge: Cambridge University Press.

COYKENDALL, A. L. & MUNZENMAIER, A. J. (1978). Deoxyribonucleic acid base sequence studies on glucan-producing and glucan-negative strains of *Streptococcus mitior*. *International Journal of Systematic Bacteriology* **28**, 511–515.

COYKENDALL, A. L. & MUNZENMAIER, A. J. (1979). Deoxyribonucleic acid hybridisation among strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. *International Journal of Systematic Bacteriology* **29**, 234–240.

COYKENDALL, A. L., WESBECHER, P. M. & GUSTAFSON, K. B. (1987). '*Streptococcus milleri*', *Streptococcus constellatus*, and *Streptococcus intermedius* are later synonyms of *Streptococcus anginosus*. *International Journal of Systematic Bacteriology* **37**, 222–228.

CROSA, J. H., BRENNER, D. J. & FALKOW, S. (1973). Use of a single-strand nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *Journal of Bacteriology* **115**, 904–911.

DEIBEL, R. M. & SEELEY, H. W., JR (1974). Streptococcaceae. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 490–509. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.

DRUCKER, D. B. & LEE, S. M. (1981). Fatty acid fingerprints of '*Streptococcus milleri*', *Streptococcus mitis*, and related species. *International Journal of Systematic Bacteriology* **31**, 219–225.

DRUCKER, D. B. & LEE, S. M. (1983). Possible heterogeneity of *Streptococcus milleri* determined by DNA mol% (guanine plus cytosine) measurement and physiological characterization. *Microbios* **38**, 151–157.

EZAKI, T., FACKLAM, R., TAKEUCHI, N. & YABUUCHI, E. (1986). Genetic relatedness between the type strain of *Streptococcus anginosus* and minute-colony-forming beta-hemolytic streptococci carrying different Lancefield grouping antigens. *International Journal of Systematic Bacteriology* **36**, 345–347.

FACKLAM, R. R. (1977). Physiological differentiation of viridans streptococci. *Journal of Clinical Microbiology* **5**, 184–201.

FACKLAM, R. R. (1984). The major differences in the American and British Streptococcus taxonomy schemes with special reference to *Streptococcus milleri*. *European Journal of Clinical Microbiology* **3**, 91–93.

FACKLAM, R. R. (1984). *Streptococcus milleri*: Reply to D. M. Yajko and W. K. Hadley. *European Journal of Clinical Microbiology* **4**, 356.

FARROW, J. A. E. & COLLINS, M. D. (1984). Taxonomic studies on streptococci of serological groups C, G and L and possibly related taxa. *Systematic and Applied Microbiology* **5**, 483–493.

FRENCH, G. L., TALSANIA, H., CHARLTON, J. R. H. & PHILLIPS, I. (1989). A physiological classification of viridans streptococci by use of the API-20 STREP system. *Journal of Medical Microbiology* **28**, 275–286.

GARVIE, E. I. (1976). Hybridization between the deoxyribonucleic acid of some strains of heterofermentative lactic acid bacteria. *International Journal of Systematic Bacteriology* **26**, 116–122.

GILMOUR, M. N., WHITTAM, T. S., KILIAN, M. & SELANDER, R. K. (1987). Genetic relationships among the oral streptococci. *Journal of Bacteriology* **169**, 5247–5257.

GOSSLING, J. (1988). Occurrence and pathogenicity of the *Streptococcus milleri* group. *Reviews of Infectious Diseases* **10**, 257–285.

GUTHOF, O. (1956). Über pathogene, vergrunende "Streptokokken": Streptokokkenbefunde bei dentogenen Abszessen und Infiltraten im Bereich der Mundhohle. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. I*, **166**, 553–564.

HARDIE, J. M. (1986). Oral streptococci. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1054–1063. Edited by P. H. A. Sneath. Baltimore: Williams & Wilkins.

HOLDEMAN, L. V. & MOORE, W. E. C. (1974). New genus, *Coprococcus*, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. *International Journal of Systematic Bacteriology* **24**, 260–277.

KILPPER-BALZ, R., WILLIAMS, B. L., LUTTIKEN, R. & SCHLEIFER, K. H. (1984). Relatedness of '*Streptococcus milleri*' with *Streptococcus anginosus* and *Streptococcus constellatus*. *Systematic and Applied Microbiology* **5**, 494–500.

KNIGHT, R. G. & SHLAES, D. M. (1988). Physiological characteristics and deoxyribonucleic acid relatedness of *Streptococcus intermedius* strains. *International Journal of Systematic Bacteriology* **38**, 19–24.

LABBE, M., VAN DER AUWERA, P., GLUPCZYNSKI, Y., CROCKAERT, F. & YOURASSOWSKY, E. (1985). Fatty acid composition of *Streptococcus milleri*. *European Journal of Clinical Microbiology* **4**, 391–393.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227** 680–685.

LONG, P. H. & BLISS, E. A. (1934). Studies on minute hemolytic streptococci; isolation and cultural characteristics of minute beta-hemolytic streptococci. *Journal of Experimental Medicine* **60**, 619–631.

LUTTICKEN, R., WENDORFF, U., LUTTICKEN, D., JOHNSON, E. A. & WANNAMAKER, L. W. (1978). Studies on streptococci resembling *Streptococcus milleri* and on an associated surface-protein antigen. *Journal of Medical Microbiology* **11**, 419–431.

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* **5**, 109–118.

MEJARE, B. (1975). Characteristics of *Streptococcus milleri* and *Streptococcus mitior* from infected root canals. *Odontologisk Revy* **26**, 291–308.

MEJARE, B. & EDWARDSSON, S. (1975). *Streptococcus milleri* (Guthof): an indigenous organism of the human oral cavity. *Archives of Oral Biology* **20**, 757–762.

MIRICK, G. S., THOMAS, L., CURNEN, E. C. & HORSFALL, F. L. (1944). Studies on a nonhemolytic streptococcus isolated from the respiratory tract of human beings. I. Biological characteristics of *Streptococcus MG*. *Journal of Experimental Medicine* **80**, 391–406.

OTTENS, H. & WINKLER, K. C. (1962). Indifferent and haemolytic streptococci possessing group-antigen F. *Journal of General Microbiology* **28**, 181–191.

OWEN, R. J. & HILL, L. R. (1979). The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists*, 2nd edn. (Society for Applied Bacteriology, Technical Series no. 14), pp. 277–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.

PARKER, M. T. & BALL, L. C. (1976). Streptococci and aerococci associated with systemic infection in man. *Journal of Medical Microbiology* **9**, 275–302.

POOLE, M. T. & WILSON, G. (1979). Occurrence and cultural features of *Streptococcus milleri* in various body sites. *Journal of Clinical Pathology* **32**, 764–768.

RUOFF, K. L. & KUNZ, L. T. (1982). Identification of viridans streptococci isolated from clinical specimens. *Journal of Clinical Microbiology* **15**, 920–925.

SHAH, H. N., VAN STEENBERGEN, T. J. M., HARDIE, J. M. & DE GRAAFF, J. (1982). DNA base composition, DNA–DNA reassociation and isoelectrofocussing of proteins of strains designated *Bacteroides oralis*. *FEMS Microbiology Letters* **13**, 125–130.

SHLAES, D. M., LERNER, P. I., WOLINSKY, E. &

GOPALAKRISHNA, K. V. (1981). Infections due to Lancefield Group F and related streptococci. (*S. milleri, S. anginosus*). *Medicine* **60**, 197–207.

SMITH, R. F. & WILLETT, N. P. (1968). Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. *Applied Microbiology* **16**, 1434–1436.

WELBORN, P. R., HADLEY, W. K., NEWBORN, E. & YAJKO, D. M. (1983). Characterization of strains of viridans streptococci by deoxyribonucleic acid hybridisation and physiological tests. *International Journal of Systematic Bacteriology* **33**, 293–299.

WHILEY, R. A. (1987). *A taxonomic study of oral streptococci.* PhD thesis, University of London.

WHILEY, R. A. & HARDIE, J. M. (1988). *Streptococcus vestibularis* sp. nov. from the human oral cavity. *International Journal of Systematic Bacteriology* **38**, 335–339.

WHILEY, R. A., HARDIE, J. M. & JACKMAN, P. J. H. (1982). SDS-polyacrylamide gel electrophoresis of oral streptococci. In *Basic Concepts of Streptococci and Streptococcal Diseases*, (Proceedings of the VIIIth International Symposium on Streptococci and Streptococcal Disease, Reedbooks, Chertsey, Surrey, UK) pp. 61–62. Edited by S. E. Holm & P. Christensen.

WHITTENBURY, R. (1964). Hydrogen peroxide formation and catalase activity in lactic acid bacteria. *Journal of General Microbiology* **35**, 13–26.

YAKUSHIJI, T., KONAGAWA, R., ODA, M. & INOUE, M. (1988). Serological variation in oral *Streptococcus milleri*. *Journal of Medical Microbiology* **27**, 145–151.