Serological variation in oral *Streptococcus milleri*

T. YAKUSHIJI, R. KONAGAWA, MOTOKO ODA and M. INOUE

Department of Preventive Dentistry, Kagoshima University Dental School, 1208-1 Usuki-cho, Kagoshima 890, Japan

Summary. Serological variation in 71 oral isolates and three reference strains of *Streptococcus milleri* was examined. Antisera were raised by immunising rabbits with cells of 10 selected strains, followed by absorption of non-specific antibodies. Double diffusion of the typing sera and the Rantz and Randall extracts of the strains in agar gel demonstrated that 70 strains were divided into 10 serotypes (*a–j*) on the basis of cell-surface carbohydrate antigens. Only four strains were untypable. The typing scheme proposed depends on type antigens other than the Lancefield group antigens A, C, F, G and others, although strains belonging to the serotypes *a*, *c* and *f* strictly corresponded to those of the groups A, C and F respectively. Close correlation between the present serotyping scheme and the previously proposed biotyping scheme for *S. milleri* was demonstrated. Distribution of these strains in dental plaque obtained from young adults was also investigated.

Introduction

The role of *Streptococcus milleri* in clinical infections has been appreciated increasingly (Parker and Ball, 1976; Shlaes *et al.*, 1981; van der Auwera, 1985; Admon *et al.*, 1987; Kambal, 1987). *S. milleri* is a common organism in certain areas of the body and is closely associated with infection in various organs as well as the mouth (Bowden *et al.*, 1975; Edwardson and Mejäre, 1978; Poole and Wilson, 1979).

Several investigators have reported similarities between biochemical and genetic aspects of the organisms (Labbe *et al.*, 1985; Coykendall *et al.*, 1987). However, *S. milleri* strains vary in some of their characteristics, including haemolysis (Colman and Williams, 1972; Ball and Parker, 1979), cellular fatty-acid fingerprint (Drucker and Lee, 1981), cell-wall sugar composition (Aluyi and Drucker, 1983), and DNA base pair ratios (mol% G + C, Drucker and Lee, 1983). Furthermore, like other viridans streptococci, *S. milleri* strains are serologically heterogeneous; they often carry Lancefield group antigens A, C, F and G, or Ottens type antigens I, II, III, IV and V, or both (Colman and Williams, 1972; Lütticken *et al.*, 1978); however, a significant number of *S. milleri* strains are ungroupable and untypable.

Serological classification of these clinically im-

portant streptococci would be of great value not only for taxonomic studies but also for ecological and epidemiological studies. The provision of a simple, rapid and reliable method to determine sources and transmission of systemic infections would be valuable. In an earlier report we isolated 71 strains of *S. milleri* from the dental plaque of 15 young adults and demonstrated a variety of physiological profiles in these strains (Yakushiji *et al.*, in press). The aim of the present study was to determine serological variation in oral strains of *S. milleri*.

Materials and methods

S. milleri strains

A total of 71 clinical isolates and three reference strains of *S. milleri* was used. Physiological characteristics of the strains have been described previously (Yakushiji *et al.*, in press). They were grown in Brain Heart Infusion Broth (BHI) (Difco) anaerobically at 37°C for 18h.

Protocol for immunisation

Ten strains were selected as immunogens to prepare typing antisera (table I). Cells of the strains were collected by centrifugation at 6000 *g* for 20 min at 4°C from the BHI cultures, washed three times in distilled water, and lyophilised. They were then resuspended to a concentration of 0.5 mg (dry weight)/ml in saline containing merthiolate 0.025%.

Female rabbits weighing around 3 kg were given

intravenous injections of the whole-cell suspensions on alternate days (3 days a week) for 3 weeks; 0.5 ml, 1.0 ml and 2.0 ml were injected on the first, second and third weeks, respectively. Blood was collected about 10 days after the last injection.

Preparation of typing antisera

To prepare type-specific antisera, crude antisera were absorbed with the whole cells of cross-reacting strains. One ml of antiserum was added to 50 mg (dry weight) of cells in 1 ml of distilled water and incubated at 37°C for 1 h, then at 4°C overnight with occasional shaking. Finally the cells were removed by centrifugation at 3000 rpm for 10 min at 4°C. The absorption procedures were repeated if necessary under the same condition until all non-specific antibodies were removed. When the antibody titre was markedly lowered by absorption, antiserum was concentrated by use of Collodion Bags (Sartorius GmbH, Göttingen) or an Amicon membrane (PM10, Amicon Corporation, Massachusetts).

Preparation of antigens

Cell-surface carbohydrate antigens were extracted from the lyophilised cells according to the method of Rantz and Randall (1955) (RR extract). Cells were resuspended in saline at a concentration of 20 mg/ml and heated at 120°C for 20 min; the supernate was then separated by centrifugation.

Immunodiffusion

Double diffusion in agar gel was performed by the routine method in Noble Agar (Difco) 1% gel in 0.01 M sodium phosphate buffer containing 0.15 M NaCl and Na₂N₃ 0.1%; the pH was 7.2. Appropriate amounts of the RR extracts and immune sera (c. 25 µl) were placed in wells and incubated overnight at room temperature.

Reagents

All the chemicals used were purchased from commercial sources. Lancefield group antigens A–G and antisera to these (the Bacto-Streptococcus Antigen/Antisera set) were from Difco.

Biotypes, haemolysis and colonial forms of the strains used in this study have been reported previously (Yakushiji *et al.*, 1988).

Results

Serotyping of oral isolates of *S. milleri*

Immune sera were obtained by immunising rabbits with whole cells of three reference and seven oral strains (table I). Double diffusion in gel demonstrated that six of the antisera reacted with

Table I. *S. milleri* strains used as immunising agents or absorbents

		Antisera		Serotype designation
	raised with cells of strain (biotype)		absorbed with cells of strain (biotype/serotype)	
FW73	(Ia)	—	—	<i>a</i>
10708 ¹	(Ia)	K214-2K	(Ia/—*)	<i>b</i>
K51Y ²	(Ia)	K217K	(Ia/d)	<i>c</i>
K215-1K	(Ia)	—	—	<i>d</i>
K226K	(Ia)	—	—	<i>e</i>
9895	(Ia)	—	—	<i>f</i>
K1K ³	(IIc)	K201K	(IIb/j)	<i>g</i>
K18K	(IIc)	—	—	<i>h</i>
K39K ⁴	(Ia)	K16-1K	(IIc/h)	<i>i</i>
K201K	(IIb)	—	—	<i>j</i>

FW73, *S. milleri* FW73, 10708; *S. milleri* NCTC 10708; 9895, *S. MG* ATCC 9895. —, none or *untypable. The four crude antisera (1–4) also cross-reacted with the following strains. 1: K218K (Ia/—); 2: K215-1K (Ia/d), K216K (Ia/d); 3: K213K (IIc/—); 4: K235K (Ia/h), K236K (Ia/h), K16-2K (IIb/h), K18K (IIc/h), K232-2K (IIc/h), K233aK (IIc/h), K233K (IId/h).

the RR antigens of different *S. milleri* strains. In contrast, the remaining four antisera, anti-10708, anti-K51Y, anti-K1K and anti-K39K, cross-reacted with RR extracts of several strains (see below). Absorption of these sera with whole cells (table I) removed non-specific antibodies (fig. 1). The 10 specific antisera thus obtained were used as standardised typing sera (fig. 2). Serotypes are listed in table I.

RR antigen extracts of 70 strains each reacted with only one of the 10 specific typing antisera. Precipitin lines formed with RR extracts of the strains clustered in one type completely fused with the line formed between the typing antiserum and its homologous antigen extract (fig. 3).

Thus, the majority of the *S. milleri* strains examined here were divisible into 10 serotypes, tentatively designated *a–j* (table I). Only four strains were untypable. Distribution of the *S. milleri* strains among these serotypes are summarised in table II. Serotypes *f* and *i* constituted major clusters, serotypes *c*, *g* and *h* were intermediate and the other serotypes were less prominent.

Strains of serotypes *d* and *h* produced cross-reacting bands with the crude antisera against cells of strains K51Y (serotype *c*) and K39K (*i*) respectively (table I). The serotype *j* strain produced a precipitin line cross-reacting with the crude anti-K1K (*g*) serum. Also, one untypable strain cross-reacted with the anti-K1K (*g*) serum and the other two untypable strains (both of which contained the

Fig. 1. Preparation of type-specific antiserum. Anti-K51Y serum (well a) yielded cross-reacting precipitin lines with RR extracts of strains K217K (well 1) and K51Y (well 2). After absorption with the strain K217K cells, the resultant antiserum (anti-c serum; well b) did not react with the antigen extract of the absorbent strain (well 1). Precipitin lines of the anti-K51Y and anti-c sera with the K51Y antigen fused.

Fig. 2. Specificity of a typing serum. Anti-c serum (wells a) reacted only with the homologous RR antigen (well 11) among the antigen extracts of the 10 vaccine strains and one untypable strain (wells 1–11). Well 1, FW73 (a); 2, 10708 (b); 3, K215-1K (d); 4, K226K (e); 5, 9895 (f); 6, K1K (g); 7, K18K (h); 8, K39K (i); 9, K201K (j); 10, K214-2K (untypable); 11, K51Y (c).

Fig. 3. Clustering of *S. milleri* strains by a typing serum. Anti-c serum (wells a) reacted with the RR extracts of all the strains clustered in serotype c (wells 1–6), yielding precipitin lines fusing with that produced between the typing serum and its homologous RR extract (well 1). Well 1, K51Y; 2, K59YS; 3, K59YL; 4, K103K; 5, K104K; 6, K106K.

Table II. Distribution of oral *S. milleri* strains among serotypes and Lancefield groups

Serotype	Number of strains belonging to serotype			Number of strains with Lancefield group antigen				
	reference (3)	clinical (71)	total (74)	A (1)	C (6)	F (24)	G (2)	UG (41)
a	1	0	1	1	0	0	0	0
b	1	3	4	0	0	0	0	4
c	0	6	6	0	6	0	0	0
d	0	3	3	0	0	0	0	3
e	0	2	2	0	0	0	0	2
f	1	23	24	0	0	24	0	0
g	0	6	6	0	0	0	0	6
h	0	8	8	0	0	0	0	8
i	0	15	15	0	0	0	0	15
j	0	1	1	0	0	0	0	1
UT	0	4	4	0	0	0	2	2

UG = ungroupable; UT = untypable.

Lancefield group G antigen) with the anti-10708 (*b*) serum.

Relation of serotypes and Lancefield groups

Reactivity of antigen extracts with Lancefield grouping sera indicated that the serotype *a* strain (1 strain), the type *c* strains (6 strains), the type *f* strains (24 strains), and the untypable strains (2 strains) carried the Lancefield group antigens A, C, F, and G respectively (table II). As shown in fig. 4, the commercial anti-group C serum yielded a fusing precipitin line with the RR antigen extract of strain K51Y (serotype *c*) and the commercial group C antigen preparation. However, the anti-*c* (K51Y) serum did not react with the group C antigen although the K51Y (*c*) antigen cross-reacted with the type *c* serum and the anti-C serum. Similar results were demonstrated between serotype *a* and Lancefield group A and between serotype *f* and group F.

Relations between serotypes and biological characteristics

Table III shows that all the 16 strains classified as serotypes *a*–*e* belonged to the biotype Ia, and the 24 serotype *f* strains to the biotypes Ia or Ib. Most strains of serotypes *g*–*j* were members of the biotype II, mostly subtypes IIc and IID.

Most (20 out of 24) of the serotype *f*, group F strains and the untypable (3 of 4) or group G (2 of 2) strains produced rough forms on Carlsson's MC agar plates or changed the appearance of their colonies on blood, MS and MC media (table IV). Among others, only one or two of each of the

Table III. Relation between serotypes and biotypes

Serotype	Number of strains	Number of strains of biotype					
		Ia (32)	Ib (15)	IIa (4)	IIb (4)	IIc (10)	IId (9)
<i>a</i>	1	1	0	0	0	0	0
<i>b</i>	4	4	0	0	0	0	0
<i>c</i>	6	6	0	0	0	0	0
<i>d</i>	3	3	0	0	0	0	0
<i>e</i>	2	2	0	0	0	0	0
<i>f</i>	24	10	14	0	0	0	0
<i>g</i>	6	0	0	1	1	3	1
<i>h</i>	8	2	0	0	1	4	1
<i>i</i>	15	1	1	3	1	2	7
<i>j</i>	1	0	0	0	1	0	0
UT	4	3	0	0	0	1	0

UT = untypable.

ungroupable *b*, *g*, *h* and *i* strains produced rough colonies or a change in colonial appearance.

Only a few strains of the serotypes *a* and *f*, the ungroupable serotypes *b* and *d*, and the untypable group G were α -haemolytic.

Distribution of serotypes in plaque samples

Table V summarises the frequencies of various serotypes in plaque samples. The serotype *f* strains which were the most widely distributed, were isolated from 6 of the 15 plaque samples. Serotype *i* and serotypes *g* and *h*, *b* and *c*, and *d*, *e* and *j* were isolated from four, three, two, and one plaque samples, respectively. No serotype *a* strain was detected in any of the samples examined.

Serological variations amongst strains from any one plaque sample were not so marked. One or two serotypes were usually found in each sample. Three serotypes were detected in only two samples.

Discussion

Considerable serological variations amongst *S. milleri* strains have been known: the Lancefield group antigens and the Ottens type antigens (Ottens and Winkler, 1962) are distributed among the streptococcal cluster (Colman and Williams, 1972; Lütticken *et al.*, 1978). The present findings indicate that there are far more serological varieties of *S. milleri* than are identified by grouping and typing systems reported hitherto. Oral strains of *S. milleri* were divided into 10 or more types based on variations in their cell-surface carbohydrate antigens.

Fig. 4. Independence of a typing serum from Lancefield group antigens. RR extracts of the type *c* vaccine strain K51Y (well 2) cross-reacted with anti-*c* serum (well b) and anti-group C serum (well a). Anti-*c* serum did not react with group C antigen (well 1). Precipitin lines of group C and type *c* antigens with group C serum fused.

Table IV. Relation of serotypes/serogroups and colonial morphology

Lancefield group	Serotype	Total number of strains (47)	Number of strains producing	
			rough colonies on MC agar (25)	colonial form change (S $\rightleftharpoons$ R) (26)
F	<i>f</i>	24	19	20
G	UT	2	2	2
UG	<i>b</i>	4	1	0
UG	<i>g</i>	6	0	1
UG	<i>h</i>	8	1	2
UG	<i>i</i>	1	1	0
UG	UT	2	1	1

UG = ungroupable; UT = untypable.

The serological variations appeared to correspond well to the biological variations of *S. milleri* demonstrated previously (Yakushiji *et al.*, in press); strains belonging to serotypes *a-f* were confined to biotype I whereas most strains of other serotypes were of biotype II (table III).

A significant number of *S. milleri* strains have been shown to carry Lancefield group antigens A, C, F or G (Colman and Williams, 1972; Ball and Parker, 1979; Yakushiji *et al.*, in press). The observation that the serotypes *a*, *c* and *f* strains and the group A, C, and F strains strictly correspond to

each other suggests the possibility that the specific antibodies contained in the anti-*a*, -*c* and -*f* antisera are the anti-A, -C and -F antibodies, respectively. Indeed, RR extracts of the vaccine strains FW73 (*a*), K51Y (*c*) or ATCC9895 (*f*) cross-reacted with the corresponding typing serum and group-specific reference serum, but the typing serum did not react with the paired reference group antigen (fig. 4). These observations indicate that the specific antibodies in these typing antisera were not derived from the group antigens. Thus, all the 10 typing antisera, including the above three, are type

Table V. Distribution of oral *S. milleri* serotypes in 15 plaque samples

Plaque sample	Number of strains in serotype											Total (71)	Number of serotypes /sample
	<i>a</i> (0)	<i>b</i> (3)	<i>c</i> (6)	<i>d</i> (3)	<i>e</i> (2)	<i>f</i> (23)	<i>g</i> (6)	<i>h</i> (8)	<i>i</i> (15)	<i>j</i> (1)	UT (4)		
IK	0	1	0	0	0	0	0	0	0	0	0	1	1
KK	0	0	3	0	0	1	0	0	0	0	0	4	2
MM	0	0	0	0	0	0	0	0	0	0	1	1	1
MK	0	0	0	0	0	3	0	0	0	0	0	3	1
HK	0	0	0	0	0	1	0	3	4	0	0	8	3
YI	0	0	0	0	0	4	0	2	0	0	0	6	2
IH	0	2	3	0	0	0	0	0	0	0	0	5	2
NT	0	0	0	3	0	0	1	0	0	0	3	7	3
MT	0	0	0	0	2	0	0	3	0	0	0	5	2
TA	0	0	0	0	0	1	0	0	7	0	0	8	2
YK	0	0	0	0	0	13	0	0	0	0	0	13	1
YA	0	0	0	0	0	0	2	0	0	0	0	2	1
YY	0	0	0	0	0	0	3	0	0	0	0	3	1
AY	0	0	0	0	0	0	0	0	3	0	0	3	1
TF	0	0	0	0	0	0	0	0	1	1	0	2	2
Number of positive samples													
All	0	2	2	1	1	6	3	3	4	1	2		

UT = untypable.

specific. The serological classification of *S. milleri* proposed here is totally independent of the Lancefield group antigens, A, C, F and G.

S. milleri possess Ottens type antigens I–V in various combinations along with the Lancefield group antigens C, F and G (Ottens and Winkler, 1962; Colman and Williams, 1972; Lütticken *et al.*, 1978), suggesting the possibility that some of the typing antisera are specific to one or the other of the Ottens type antigens.

One of the most readily proved associations is that the anti-*f* serum obtained by immunisation with *S. MG ATCC9895* contains antibodies specific to the Ottens type III antigen. The vaccine strain ATCC9895 has been shown to belong to Lancefield group F, Ottens type III, category F, III (Willers *et al.*, 1964), but the usual preparations of the rabbit anti-ATCC9895 serum are reported to contain anti-type III antibodies alone and not anti-group F antibodies (Willers *et al.*, 1964). This may also be so for our own anti-*f* serum. Any relationship of specific typing sera other than the anti-*f* serum to the Ottens type antigens, I, II, IV and V is not known at present; clarification of the chemical properties of these type-specific antigens and their identities with the Ottens type antigens require further study.

The crude anti-10708 serum did not react with the group G antigen but cross-reacted with RR extract of the untypable group G strains. Furthermore, absorption of the crude antiserum with cells of the untypable group G strain K214-2K provided the specific anti-*b* serum (table I). These results suggest that strain NCTC10708 carries two type antigens; one is serotype *b*-specific and the other is the same as that of strain K214-2K. It should be noted that there is evidence for strain NCTC 10708 being a member of Lancefield group F. However, there have been discrepant descriptions with respect to the antigenic and physiological properties of this strain (Drucker and Green, 1978; Drucker and Lee, 1983). Reasons for these inconsistent findings are obscure but the strain may be prone to alter in antigenicity.

The proposed serotyping scheme for oral *S. milleri* is not yet established, but the typing sera could be useful not only to initiate classification of oral *S. milleri* but also to clarify the route of transmission of this clinically important streptococcus.

We thank Dr T. Koga (NIH, Tokyo) for helpful advice and discussion and also Miss Kayoko Yuge for typing the manuscript.

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