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Physiological Characteristics and Deoxyribonucleic Acid Relatedness of *Streptococcus intermedius* Strains

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Fifteen strains of *Streptococcus intermedius* and three mannitol-fermenting *S. intermedius*-like isolates from urine were studied by the S1 nuclease DNA homology assay and two physiological characterization methods. Three distinct homology groups (I, II, and III) were found. The three mannitol-fermenting *S. intermedius*-like strains and 2 of the 15 strains of *S. intermedius* did not fall within these groups. Each of the distinct homology groups could be distinguished by physiological testing. Homology group I strains were bile esculin positive (BE^+), starch negative (Sta^-), non-CO_2 dependent, and melibiose negative (mel^-); homology group II strains were BE^-, Sta^-, CO_2 dependent, and mel^-; homology group III strains were BE^-, Sta^+, non-CO_2 dependent, and mel^+. The two nonhomologous *S. intermedius* strains and three *S. intermedius*-like isolates from were phenotypically distinct from those in homology groups I through III. Characterization by the Rapid Strep system (Analytab Products, Plainview, N.Y.) identified homology groups I and II as either "*S. milleri*" I or II and homology group III strains as *S. sanguis* I or II. One nonhomologous *S. intermedius* strain was identified as *S. sanguis* I, and the other "*S. milleri*" I and the *S. intermedius*-like mannitol-fermenting strains were identified as *S. milleri* III by this method. We conclude that homology groups I, II, and III and the *S. intermedius*-like isolates from urine may represent separate taxa, but we do not make such a proposal on the basis of our limited data.

Over the past 80 years, viridans streptococci resembling the beta-hemolytic strains of Lancefield group F have been assigned at least seven different species names: *Streptococcus anginosus*, "*Streptococcus* MG," "*S. milleri*," *S. intermedius*, *S. constellatus*, "*S.* MG-*intermedius*," and "*S. anginosus-constellatus*" (1, 3, 5, 11, 12, 15, 16, 18, 25, 33, 34). Some researchers have argued that the minute beta-hemolytic streptococci and all these viridans streptococci belong in a single taxon (5, 9, 15, 20, 23, 35). In fact, Facklam (12) has recently proposed that "*S. milleri*" and "*S.* MG-*intermedius*" be brought under a single taxon, *S. intermedius*, but he still separates the minute beta-hemolytic streptococci as *S. anginosus*.

The criteria of Facklam and Carey (13) for the classification of *S. intermedius* indicates variability in the hemolytic, bile esculin, arginine, starch, and raffinose reactions. Poole and Wilson (28) found that 75% of their "*S. milleri*" isolates from vaginal and Bartholin's gland abscesses formed acid from raffinose and melibiose, whereas only 25% of the strains from normal feces and appendiceal abscesses and anal abscesses formed acid from raffinose and melibiose. Most of their "*S. milleri*" strains were nonhemolytic and serologically were Lancefield group F or nongroupable. Ruoff and Kunz (30) found that most isolates from urine of streptococci resembling "*S. milleri*" formed acid from mannitol as well as from raffinose and melibiose and were nonhemolytic.

DNA relatedness studies have been reported for *S. intermedius*, *S. anginosus*, and the minute beta-hemolytic streptococci (9, 19, 20, 35). Welborn et al. (35), using a small number of strains, showed that *S. constellatus*, *S. intermedius*, and a few minute beta-hemolytic streptococci of undetermined species of Lancefield group F were all closely related by DNA homology. Farrow and Collins (15) subsequently found the type strain of *S. anginosus* (NCTC 10713), *S. constellatus*, *S. intermedius*, and "*S. milleri*" closely related by DNA homology. A more recent study (9) suggested that the *S. anginosus* taxon is closely related to the minute beta-hemolytic streptococci of Lancefield groups A, C, F, and G. Several *S. intermedius* strains were examined by Kilpper-Balz et al. (20) and shown to be genetically distinct from *S. anginosus* and to form three homology groups, although the authors thought that the *S. intermedius* strains should be included in a single species. Coykendall and co-workers (7) studied a large number of strains including "anginosus" and beta-hemolytic isolates. Despite homologies that ranged from 41 to 120% for strains identified as "*S. milleri*" biotype I, II, or III by the API Rapid Strep Identification (Analytab Products, Plainview, N.Y.) method, they thought that all such strains should be classified as a single taxon, *S. anginosus*. This work is limited in that all strains were chosen by the API Rapid Strep method for identification.

Because of continuing confusion in this area, we studied *S. intermedius* strains of various biotypes, including the mannitol-positive, raffinose- and melibiose-fermenting isolates from urine described by Ruoff and Kunz (30) and others (8). We also studied a group of strains which resembled *S. intermedius*, fermented raffinose and melibiose, but were mannitol negative. In addition, we studied the ability of the API Rapid Strep Identification method to separate these strains.

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TABLE 1. Bacterial strains used in this study

Homology group	Strain	Source, comments[a]
I	*S. intermedius* F-1482	Veterans Administration Medical Center, Cleveland, Ohio; human; blood
	S. intermedius F-2076	Veterans Administration Medical Center, Cleveland, Ohio; human; blood
	S. intermedius F-845	Veterans Administration Medical Center, Cleveland, Ohio; human; blood
	S. intermedius F-1962	Veterans Administration Medical Center, Cleveland, Ohio, human; gall bladder
	S. intermedius ATCC 9895	ATCC; F. Horsfall (*Streptococcus* MG); human; sputum
II	*S. Intermedius* ATCC 27335^T	ATCC; W. E. C. Moore VPI 3372 A; A. Prevot 1877; neotype; anaerobic
	S. intermedius F-855	Veterans Administration Medical Center, Cleveland, Ohio; human; wound or body fluid
	S. intermedius JD16	New England Deaconess Hospital, Boston, Mass.; human
III	*S. intermedius* ATCC 43268	Veterans Administration Medical Center, Cleveland, Ohio; human; Jackson-Pratt drain site
	S. intermedius MG145	Massachusetts General Hospital, Boston, Mass.; human; blood
	S. intermedius MG413	Massachusetts General Hospital, Boston, Mass.; human; urine
	S. intermedius CDC-DS-2270-81	CDC (R. Facklam); human; blood
	S. intermedius CDC-DS-85-81	CDC (R. Facklam); human; blood
Mannitol-fermenting *S. intermedius*-like isolates from urine	*Streptococcus* sp. MG455	Massachusetts General Hospital, Boston, Mass.; human; urine
	Streptococcus sp. MG461	Massachusetts General Hospital, Boston, Mass.; human; urine
	Streptococcus sp. MG481	Massachusetts General Hospital, Boston, Mass.; human; urine
Ungrouped streptococci phenotypically resembling group II	*Streptococcus* sp. MG576	Massachusetts General Hospital, Boston, Mass.; human; peritoneal abscess
	Streptococcus sp. MG578	Massachusetts General Hospital, Boston, Mass.; human; pelvic fluid
Others	*S. sanguis* I ATCC 10556^T	ATCC; human endocarditis
	S. sanguis II ATCC 10557	ATCC; human endocarditis

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control, Atlanta, Ga.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Both phenotypic tests and DNA hybridizations were performed.

Phenotypic tests. The phenotypic traits used to differentiate the organisms are shown in Table 3. The tests for hemolysis, bile esculin (40% bile tolerance and production of esculetin), 6.5% NaCl tolerance (salt tolerance), and inulin were performed by conventional methods (10). The Minitek (BBL Microbiology Systems, Cockeysville, Md.) disk method was used to detect acid production from lactose, D-mannitol, sorbitol, sucrose, raffinose, and melibiose as described by Setterstrom et al. (32). Arginine dihydrolase (Moeller method) and esculin broth hydrolysis tests were performed by conventional methods (13). A modification of the Barritt method for the Voges-Proskauer test was used (17). The inoculated methyl red–Voges-Proskauer medium was incubated for 48 h at 35°C instead of 24 h and allowed to stand for 30 min after the reagents were added to detect acetoin production. The hydrolysis of starch was determined by flooding the surface of a Mueller-Hinton agar plate with Gram iodine 48 h after inoculation and incubation (27). A zone of hydrolysis appeared colorless, and a dark blue-to-purple color indicated that the starch had not been hydrolyzed.

An absolute requirement for 5 to 10% CO_2 for growth on tryptic soy agar base supplemented with 5% sheep blood (GIBCO Diagnostics, Madison, Wis.) after incubation for 48 h at 35 to 37°C was used to indicate CO_2 dependence. Production of *N*-acetyl-β-D-glucosaminidase (NABDG) was determined by adding 0.025 ml of a 100-mg/ml solution of 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma Chemical Co., St. Louis, Mo.) prepared in 0.02 M $NaKHPO_4$ buffer (pH 6.4) containing 1 mM $MgCl_2$–0.1 mM EDTA–3 mM NaN_3 (pH 6.4) to 0.025 ml of an 18- to 24-hold tryptic soy broth culture, mixing, and allowing the mixture to stand at room temperature for 1 h. Detection of NABDG was made by adding 0.15 ml of 0.50 M Na_2CO_3, mixing, and observing for fluorescence with a long-wave UV-366 nm lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.). The Streptex (latex test) for streptococcal grouping (Wellcome Diagnostics, Research Traingle Park, N.C.) was used to detect Lancefield group antigens.

API Rapid STREP Identification method. API Rapid Strep Identification kits were inoculated, incubated, and read as described in brochures provided by Analytab Products. All identifications included in the results were made after 24 h of incubation.

DNA hybridization tests. Using previously described methods (21, 22) we performed deoxyribonucleic acid (DNA) hybridization tests with all strains listed in Table 1 under optimal conditions (60°C) and stringent conditions (75°C) by using [^{3}H]thymidine-labeled DNA from the following strains: *S. intermedius* F-1482, *S. intermedius* ATCC 27335^T, and *S. intermedius* ATCC 43268.

RESULTS

Guanine-plus-cytosine content and DNA relatedness. The guanine-plus-cytosine contents of *S. intermedius* F-1482, *S. intermedius* 27335^T, and *S. intermedius* ATCC 43268 were 38, 37, and 41 moles percent, respectively. These values were determined by J. L. Johnson (Virginia Polytechnical Institute, Blacksburg) from thermal melting temperatures.

Labeled DNA from *S. intermedius* F-1482, *S. intermedius* 27335^T, and *S. intermedius* ATCC 43268 was used for homology studies (Tables 1 and 2). Table 2 indicates that the DNAs from *S. intermedius* F-1482, *S. intermedius* ATCC 27335^T, and *S. intermedius* ATCC 43268 represent three homology groups (I, II, and III), respectively. DNAs from all strains within these homology groups having greater than 50% relatedness under optimal conditions were subsequently tested under stringent conditions. Strains having a

TABLE 2. DNA homology groups of *S. intermedius* phenotypes

Source of unlabeled DNA	% Homology with ^{3}H-labeled DNA from[a]:					
	S. intermedius F-1482 (homology group I)		*S. intermedius* ATCC 27355^T (homology group II)		*S. intermedius* ATCC 43268 (homology group III)	
	60°C	75°C	60°C	75°C	60°C	75°C
S. intermedius (homology group I)						
F-1482	100	100	41	ND[b]	12	ND
F-2076	70	93	45	ND	17	ND
F-845	65	73	35	ND	14	ND
F-1962	80	70	58	10	30	ND
ATCC 9895	51	50	35	ND	0	ND
S. intermedius (homology group II)						
ATCC 27335^T	41	ND	100	100	9	ND
F-855	45	ND	78	58	15	ND
JD16	58	6	94	80	17	ND
S. intermedius (homology group III)						
F-2218	7	ND	5	ND	100	100
MG145	17	ND	12	ND	87	53
MG413	39	ND	45	ND	72	52
CDC-DS-2770-81	10	ND	1	ND	79	93
CDC-DS-85-81	12	ND	9	ND	88	96
Mannitol-fermenting *S. intermedius*-like isolates from urine						
MG455	55	34	58	25	8	0
MG461	55	26	72	22	17	0
MG481	0	0	16	0	9	0
Streptococci phenotypically resembling group II						
MG576	51	7	60	0	0	ND
MG578	45	ND	49	23	0	ND

[a] Data are expressed as averages of values from at least two experiments. Both optimal (60°C) and stringent (75°C) conditions were used for those organisms having homology levels greater than 50%.
[b] ND, Not determined.

DNA relatedness of ≥50% under stringent conditions were considered homologous. DNAs from two other strains (MG576 and MG578) did not fall within these three homology groups. DNAs from the three mannitol-fermenting isolates from urine (MG455, MG461, and MG481) did not have significant homology to labeled strains of homology group I, II, or III under stringent conditions (Table 2). Because of some phenotypic resemblance of the homology group III strains with "*S. mitior*" as described by Coykendall and Specht (6) and with *S. oralis* (19) we also tested DNA extracted from *S. sanguis* I ATCC 10556^T and *S. sanguis* II ATCC 10557 against DNA from our labeled strains. ATCC 10557 resembles *S. oralis* of Kilpper-Balz et al. (19) and Bridges and Sneath (4) and "*S. mitior*" of Coykendall and Specht (6). Neither ATCC 10556^T nor ATCC 10557 was homologous (13 and 22%, respectively) with labeled DNA from strain ATCC 43268 (homology group III) under optimal conditions.

Physiological characterization of strains. The following properties were common to the *S. intermedius* and *S. intermedius*-like strains tested (Table 3): alpha- or gamma-hemolysis, lack of growth in 6.5% NaCl, production of acid from lactose and sucrose but not from inulin, and production of arginine dihydrolase.

All streptococcal strains included in Table 3 were identifiable as *S. intermedius* by the criteria of Facklam and Carey (13) except for the three mannitol-positive isolates from urine. All homology group I strains were distinguishable from homology group II and III strains by a positive bile esculin test and production of NABDG, whereas all homology group III strains were distinguishable from homology groups I and II by production of acid in both raffinose and melibiose and hydrolysis of starch. Homology group II strains were dependent on CO_2 for growth, in contrast to homology group I and III strains, which did not require CO_2 for growth. Two strains of streptococci phenotypically resembled homology group II except for hydrolysis of starch and lack of production of NABDG. These two strains were nonhomologous under both optimal and stringent conditions to the three labeled strains used in this study (Tables 2 and 3). Homology group II strain JD16 differed from the other homology group II strains (ATCC 27335^T and F-855) by forming acid in raffinose and failing to produce acetoin in methyl red–Voges-Proskauer medium. Homology group III contained two unusual strains (CDC-DS-2270-81 and CDC-DS-85-81) which formed acid from sorbitol. The mannitol-fermenting isolates from urine were phenotypically distinguishable from all *S. intermedius* strains used in this study (Table 3).

Both *S. sanguis* I ATCC 10556^T and *S. sanguis* II ATCC 10557 were characterized by our methods. Both strains were typical by the criteria of Facklam (data not shown). In 1975, Coykendall and Specht (6) reported *S. sanguis* II ATCC 10557 to be "*S. mitior*" on the basis of DNA relatedness and physiological parameters.

Although most strains carried the Lancefield group F antigen, the Lancefield group reaction was variable and did not provide significant differential characteristics among the strains used in this study.

API Rapid Strep identification. The API Rapid Strep

TABLE 3. Phenotypic test results[a]

Strain	Hemolysis	CO_2 dependence[b,c]	Hydrolysis of starch	Bile esculin	Voges-Proskauer	NABDG[d]	Acid production from:				Lancefield group[e]
							Mannitol	Sorbitol	Raffinose	Melibiose	
S. intermedius (homology group I)											
F-1482	Alpha	−	−	+	+	−	−	−	−	−	F
F-2076	Alpha	−	−	+	+	−	−	−	−	−	F
F-845	Alpha	−	−	+	+	−	−	−	−	−	F
F-1962	Alpha	−	−	+	+	−	−	−	−	−	F
ATCC 9895	Alpha	−	−	+	+	−	−	−	−	−	F
S. intermedius (homology group II)											
ATCC 27335^T	Gamma	+	−	−	+	+	−	−	−	−	G
F-855	Alpha	+	−	−	+	+	−	−	−	−	F
JD16	Alpha	+	−	−	−	+	−	−	+	−	Ungroupable
S. intermedius (homology group III)											
ATCC 43268	Alpha	−	+	−	−	+	−	−	+	+	F
MG145	Alpha	−	+	−	−	+	−	−	+	+	Ungroupable
MG413	Alpha	−	+	−	−	+	−	−	+	+	F
CDC-DS-2270-81	Alpha	−	+	−	−	+	−	+	+	+	F
CDC-DS-85-81	Alpha	−	+	−	−	+	−	+	+	+	F
Mannitol-fermenting *S. intermedius*-like isolates from urine											
MG455	Gamma	−	−	+	+	−	+	−	+	+	Ungroupable
MG461	Gamma	−	−	+	+	−	+	−	+	+	Ungroupable
MG481	Gamma	−	−	+	+	−	+	−	+	+	F
Streptococci phenotypically resembling group II											
MG576	Alpha	−	+	−	+	−	−	−	−	−	Ungroupable
MG578	Alpha	−	+	−	+	−	−	−	−	−	Ungroupable

[a] All tests were performed as previously described unless specified otherwise (17,18).
[b] Tested on tryptic soy base supplemented with 5% sheep blood (GIBCO).
[c] +, Growth only in 5 to 10% CO_2 after 48 h; −, growth in ambient air after 48 h.
[d] Production of NABDG in tryptic soy broth (GIBCO) in 24 h.
[e] Determined by the Streptex (latex test).

method identified all homology group I strains as "*S. milleri*" II. Two of three strains in homology group II were identified as "*S. milleri*" I and the other strain (F-855) as "*S. milleri*" II. Three of five strains of homology group III were identified as *S. sanguis* II, whereas the two strains forming acid in sorbitol (CDC-DS-2270-81 and CDC-DS-85-81) were identified as *S. sanguis* 1/2. The two strains phenotypically resembling homology group II (MG576 and MG578) were identified as *S. sanguis* I and "*S. milleri*" I, respectively. *S. sanguis* I ATCC 10556^T and *S. sanguis* II ATCC 10557 were identified as *S. sanguis* I/1 and *S. sanguis* II, respectively.

DISCUSSION

A number of previous studies have presented data supporting the inclusion of beta-hemolytic group F and strains resembling "*S. milleri*" of Guthof (16) in a single taxon (5, 9, 15, 20, 23, 35). Cross absorption studies demonstrated that the group F antigen on the cell walls of viridans strains was identical to that of the beta-hemolytic strains (26, 36). However, subtyping showed the presence of different carbohydrate antigens in nonhemolytic lactose-positive strains compared with those in beta-hemolytic lactose-negative strains (26). Despite this difference, numerical taxonomy classified beta-hemolytic strains with the viridans group "*S. milleri*" (5, 24). In addition, Lutticken et al. (23) described identical protein antigens in both hemolytic and nonhemolytic strains. Welborn et al. (35) demonstrated a close genetic relationship among a few *S. intermedius* and *S. constellatus* strains and some minute beta-hemolytic streptococci of undetermined species of Lancefield group F. More recently, larger studies have shown a genetic relationship among *S. anginosus*, *S. constellatus*, "*S. milleri*," *S. intermedius*, and minute beta-hemolytic streptococci of Lancefield groups A, C, F, and G (9, 15). However, in studying a variety of biotypes of hemolytic and nonhemolytic streptococci resembling *S. intermedius*, Kilpper-Balz et al. (20) also noted the existence of three homology groups.

The "*S. milleri*" of wide fermentation pattern as described by Poole and Wilson (28) and, later, by others (8, 30) forms a separate problem. Not all investigators describing these strains tested for fermentation of mannitol. Thus, it is not clear whether all the strains described as genitourinary isolates in the studies of others were identical to the mannitol-fermenting strains of Ruoff et al. (29, 30).

Within the 18 strains of *S. intermedius* we used in this study, we discovered three homology groups (Table 2). Each homology group had a unique phenotype, and two nonhomologous strains phenotypically resembled, but could be distinguished from, homology group II (Tables 2 and 3). The five strains of streptococci in homology group I were all identical in their phenotypic characteristics (Table 3). This group was able to grow in 40% bile, was unable to produce NABDG, and did not require CO_2 for growth. In contrast, the three homology group II strains studied were CO_2 dependent, unable to tolerate 40% bile, and produced NABDG. Homology group III differed phenotypically from

homology group I by being intolerant to 40% bile and from homology group II by not being CO_2 dependent. Homology group III differed from both groups I and II by producing acid in both raffinose and melibiose and hydrolyzing starch. Strains falling into DNA homology group I phenotypically resemble the original strains of "*S. milleri*" described by Guthof (16). Our homology group II strains most closely resemble *S. intermedius* as proposed by Holdeman and Moore (18).

Drucker and Lee (8) reported guanine-plus-cytosine contents (40.3 to 41.6 moles percent) for four vaginal isolates of "*S. milleri*," three of which formed acid from mannitol and melibiose and two of which formed acid from mannitol, raffinose, and melibiose. Poole and Wilson (28) found that 75% of their "*S. milleri*" isolates from vaginal and Bartholin's gland abscesses formed acid from raffinose and melibiose, whereas only 25% of the strains from normal feces and appendiceal abscesses and anal abscesses formed acid from raffinose and melibiose. The ability to produce acid from mannitol was not tested, but most of their "*S. milleri*" strains were nonhemolytic and serologically Lancefield group F or nongroupable. Ruoff and Kunz (30) found that 54 isolates from urine of streptococci resembling "*S. milleri*" were nonhemolytic; formed acid from mannitol, raffinose, and melibiose; and produced acetoin. Three of these strains included in our study (Table 1) had identical biochemical profiles (Table 3) and were not homologous with homology group I, II or III (Table 2).

The API Rapid Strep Identification method has been extensively evaluated by others (2, 14, 31). In this study, 24-h readings indicated that all homology group I and II strains were "*S. milleri*" I or II. Thus, this method does not separate these homology groups by phenotypic characteristics. Three of five strains of homology group III coded as *S. sanguis* II, whereas the two strains forming acid in sorbitol coded as *S. sanguis* I/2. The mannitol-fermenting *S. intermedius*-like isolates from urine (Tables 1, 2, and 3) coded as "*S. milleri*" III. This distinction is based on the production of acid from raffinose and mannitol. *S. sanguis* I ATCC 10556^T and *S. sanguis* II ATCC 10557 were identified as *S. sanguis* I/1 and *S. sanguis* II, respectively. Both of these were nonhomologous to labeled strains in homology groups I through III. Thus, it is clear that homology group III strains do not fall in the *S. sanguis* I or II taxa and that the API Rapid Strep Identification system does not currently separate our homology group III from *S. sanguis*.

Our work differs from that of Coykendall et al. (7) in that we included only those strains that would be classified as *S. intermedius* by physiological tests recommended by Facklam and Carey (13). We were not, therefore, limited to biotypes identified by the API Rapid Strep Identification system. In addition, the method we used for DNA-DNA hybridization is probably more stringent than that used by Coykendall et al. (7) and is similar to the method used by Kilpper-Balz et al. (20). This may partly explain the similarity of our results to those of the latter workers, who identified three homology groups among their collection of reference strains.

In conclusion, our work presents evidence that the *S. intermedius* taxon consists of three or more genetically and phenotypically distinguishable groups which may represent separate taxa. We chose not to make formal proposals on the basis of our limited data.

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