Deoxyribonucleic Acid Relatedness of Strains of Yellow-Pigmented, Group D Streptococci

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Deoxyribonucleic acid (DNA) homology was used to examine genetic relatedness among 25 yellow-pigmented strains of group D streptococci and to clarify the possible genetic relatedness of these strains to *Streptococcus faecium* and *Streptococcus faecalis*. In all cases, the DNA of the yellow-pigmented strains hybridized with the DNA of *S. faecium* and *S. faecalis* at a level of less than 25%. Based on median thermal dissociation temperatures (T_m 's) and hybridization tests, the yellow-pigmented strains were divided into two groups (I and II). DNAs of strains in group I had relatively low T_m 's and did not exhibit significant homology with DNA of the herein designated type strain (ATCC 25788) of *S. faecium* subsp. *casseliflavus*. The DNAs of group II strains, on the other hand, exhibited high T_m 's and had a high degree of homology with the DNA of the type strain of the above-mentioned subspecies. Three physiological traits were found to be peculiar to group II organisms: ability to grow in the presence of 6.5% sodium chloride, inability to ferment sorbitol, and inability to decarboxylate tyrosine. It is proposed that the group II strains constitute a separate and distinct species. Because the strains presently placed in this species include the type strain of *S. faecium* subsp. *casseliflavus* Mundt and Graham, the name of this species, according to the rules of the *Bacteriological Code*, is *Streptococcus casseliflavus* (Mundt and Graham) comb. nov.

Mundt and Graham (11) proposed the name *Streptococcus faecium* subsp. *casseliflavus* for group D yellow-pigmented streptococci which were isolated from plants (11, 12). Taken into consideration for the establishment of this subspecies were its pigmentation, physiology, and ecology, which set this subspecies apart from *Streptococcus faecalis* and the other subspecies of *Streptococcus faecium*.

In their original description of *S. faecium* subsp. *casseliflavus*, Mundt and Graham (11) did not designate a type strain. We are here designating as the type strain one of the strains on which the original description was based; this strain is referred to as strain 4 in this paper. The properties of this strain are described in Table 1 of Mundt and Graham (11), and a culture of it has been deposited with the American Type Culture Collection (ATCC) under the number 25788.

Since the proposal of Mundt and Graham (11), Taylor et al. (14) observed that yellow pigmentation in the group D streptococci with which they worked seemed to vary from organism to organism independent of other characteristics, thus rendering pigmentation of questionable value for taxonomic purposes.

Amstein and Hartman (1) used gas chromatography to compare the relative fatty acid composition of various strains of enterococci, includ-

ing *S. faecalis*, *S. faecium*, *S. faecium* subsp. *durans*, and three strains of *S. faecium* subsp. *casseliflavus*. Based on differences between the fatty acid composition of the strains of *S. faecium* subsp. *casseliflavus* and that of the other strains, it was concluded that *S. faecium* subsp. *casseliflavus* deserved varietal (now subspecies), if not species, status.

Roop et al. (13), using deoxyribonucleic acid (DNA)-DNA hybridization, observed that an authentic strain of *S. faecium* subsp. *casseliflavus* exhibited little homology with reference strains of *S. faecium*, *S. faecalis*, and *S. lactis*.

Most of the taxonomic studies of the yellow-pigmented streptococci have included one or a very few of the yellow-pigmented strains. The principal purpose of the work reported here was to use DNA-DNA hybridization to examine genetic relatedness among a large number of these strains and to determine whether these strains are genetically related to *S. faecium* and *S. faecalis*.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. All of the strains except numbers 12, 13, and 27, which were obtained from W. R. Chesbro, were isolated locally from sources specified in Table 1. Strain 4 is the type strain of *S. faecium* subsp. *casseliflavus*. Strain 5, which has cultural and physi-

TABLE 1. *Strains used in this study*

Serial no. of strain	Laboratory strain designation	Identification	Source
1	MUTK 1	<i>S. faecium</i>	Human
2	MUTK 4	<i>S. faecium</i>	Human
3	MUTK 60	<i>S. faecalis</i>	Human
4	MUTK 20	<i>S. faecium</i> subsp. <i>casseliflavus</i>	Plant
5	MUTK 21	<i>S. faecium</i> subsp. <i>casseliflavus</i>	Plant
6	MUTK 6	Yellow streptococcus	Plant
7	MUTK 12	Yellow streptococcus	Plant
8	MUTK 31	Yellow streptococcus	Plant
9	MUTK 33	Yellow streptococcus	Plant
10	MUTK 68	Yellow streptococcus	Plant
11	MUTK 74	Yellow streptococcus	Plant
12	MUTK 559	Yellow streptococcus	Soil
13	MUTK 564	Yellow streptococcus	Soil
14	MUTK 595	Yellow streptococcus	Plant
15	MUTK 730	Yellow streptococcus	Plant
16	MUTK 41	Yellow streptococcus	Plant
17	MUTK 46	Yellow streptococcus	Plant
18	MUTK 61	Yellow streptococcus	Plant
19	MUTK 63	Yellow streptococcus	Plant
20	MUTK 64	Yellow streptococcus	Plant
21	MUTK 66	Yellow streptococcus	Plant
22	MUTK 441	Yellow streptococcus	Plant
23	MUTK 442	Yellow streptococcus	Plant
24	MUTK 443	Yellow streptococcus	Plant
25	MUTK 444	Yellow streptococcus	Plant
26	MUTK 445	Yellow streptococcus	Plant
27	MUTK 562	Yellow streptococcus	Soil
28	MUTK 627	Yellow streptococcus	Plant

ological properties similar to those of strain 4, has approximately 75% DNA homology with strain 4 (see results); therefore, these two strains are used as reference strains in the nucleic acid homology studies reported here.

Cultural and physiological characters. The cultural and physiological characters of the yellow-pigmented strains (Table 2) were determined by methods described previously (11, 12).

Extraction and preparation of DNA. Both unlabeled and labeled DNA was prepared by the modification described by Roop et al. (13) of the procedure of Marmur (9). With some of the yellow-pigmented strains, it was necessary to extract the DNA extensively first with phenol and then with chloroform-isoamyl alcohol (24:1, vol:vol) before attempting to precipitate the DNA with ethanol. The phenol used in the extraction was redistilled and saturated with standard saline citrate (0.15 M NaCl-0.015 M sodium citrate) immediately before use.

All DNA used for reassociation studies was sheared in a Raytheon model DF-101 magnetostriuctive sonicator to an average molecular weight of 3×10^6 (13). Labeled DNA was dialyzed against 0.12 M phosphate buffer, denatured by heating for 10 min at 100°C, and further purified by passage through hydroxyapatite (Bio-Gel MT) at 60°C. This rid the DNA of any material which bound nonspecifically to hydroxyapatite during reassociation reaction assays and eliminated most of the material which, in reassociation assays on hydroxyapatite, would give false-positive reassociation results (3).

Determination of T_m and G+C content of DNA.

The median thermal dissociation temperature (T_m) of each of the DNA samples was determined by the method of Marmur and Doty (10). Samples were dissolved in standard saline citrate at a concentration of 25 μ g of DNA per ml. The samples were heated in an Acta CIII dual-beam spectrophotometer equipped with a jacketed cuvette chamber and a calibrated platinum thermocouple probe which was fixed in a cuvette filled with mineral oil. The temperature was increased automatically at a rate of 0.1°C/min through the range 25 to 100°C by means of a programmable Haake temperature control unit. Optical densities of each sample were recorded at every 0.1°C change in temperature.

The guanine plus cytosine (G+C) content of the DNA was calculated by the equation of De Ley (7): $\text{mol\% G+C} = 2.44 (T_m - 69.4)$.

Reassociation reaction mixtures. The buffer for the reaction mixtures was 0.24 M phosphate buffer. Each reaction mixture consisted of 0.33 μ g of labeled DNA and 167 μ g of unlabeled DNA in a total volume of 1 ml. Each mixture was placed in a sealed vial, dissociated by heating at 100°C for 10 min, and incubated for 17 h (to a C_{ot} [5] of 100). To correct for self-reassociation of labeled DNA, reaction mixtures were also prepared with 0.33 μ g of labeled DNA in 1.0 ml of 0.24 M phosphate buffer with no unlabeled DNA present. At the end of the incubation period, the reaction mixtures were cooled with ice and frozen at -20°C until subjected to assay.

Hydroxyapatite assays of reassociation reac-

TABLE 2. *Physiological characteristics of strains of yellow-pigmented group D streptococci*^a

Property	Group I strains										Group II strains															
	6	7	8	9	10	11	12	13	14	15	4	5	16	17	18	19	20	21	22	23	24	25	26	27	28	
Growth in broth	+	+	+	+	+	+	0	0	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
At 10°C	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
At 45°C	+	+	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
At pH 9.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
With 6.5% NaCl	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Ability to survive 60°C for 30 min	+	+	+	-	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
Growth on agar medium																										
With 5% bile salt	+	-	-	+	+	+	+	+	w	+	+	+	+	+	+	+	w	+	w	+	+	w	w	+	+	+
With tellurite	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-	-
Reduction of methylene blue	+	-	+	+	-	0	w	+	0	0	+	-	+	+	0	+	+	+	0	0	0	0	0	0	+	0
Acid curd in milk	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Tiny pink colonies on tetrazolium medium	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	0	-	-
Fermentation of:																										
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	-	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Melezitose	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	0	0	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+
Dulcitol	+	-	-	-	-	-	0	0	+	+	0	0	+	-	-	+	-	+	-	-	-	+	+	-	0	-
Deamination of arginine	+	-	-	-	+	+	+	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+
Decarboxylation of tyrosine	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Production of heme peroxidase	+	-	+	+	+	+	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+

^a +, positive reaction; -, negative reaction; 0, information not available; w, weak positive reaction.

tions. Assays were carried out by the hydroxyapatite batch procedure of Brenner et al. (3). In step 1, a hydroxyapatite suspension (0.28 g [dry weight] per ml of water) was boiled to reduce nonspecific binding of DNA (2). The hydroxyapatite solution was diluted with 0.001 M phosphate buffer so that 10 ml contained three times as much hydroxyapatite as was needed to bind the DNA in each reaction vial (approximately 60 mg/ml). In step 2, samples (10 ml) of the hydroxyapatite were added to 40-ml polypropylene tubes, placed in a centrifuge maintained at 70°C, and centrifuged at $1,000 \times g$ for 4 min. In step 3, the supernatant fluid was discarded, and the hydroxyapatite was washed three times with 15-ml portions of 0.12 M phosphate buffer containing 0.4% sodium dodecyl sulfate and 0.005 M ethylenediaminetetraacetic acid. The tubes containing the hydroxyapatite sediment were placed in water baths at 60, 70, or 75°C, the temperature of the water bath depending on the temperature at which the reassociations were carried out. In step 4, reaction mixtures were thawed, diluted with 1.0 ml of distilled water and 13 ml of preheated 0.12 M phosphate buffer containing 0.4% sodium dodecyl sulfate and 0.005 M ethylenediaminetetraacetic acid, and mixed with the hydroxyapatite. The tubes were placed in the water bath for 20 min and were agitated frequently. The temperature of both the water bath and the tubes always remained above 55°C (3). In step 5, the tubes were centrifuged, and the solutions were poured into scintillation vials. The hydroxyapatite was washed with 15-ml portions of the buffer until no counts were detected in the buffer. In step 6, to determine the amount of double-stranded DNA still bound to hydroxyapatite after exhaustive washing with buffer, the washed hydroxyapatite was dissolved in 5 N HCl and the solution was poured into scintillation vials for counting.

Thermal elution studies. To assess the thermal stability of the hybrids trapped on hydroxyapatite, the assay procedure described above was carried out through step 5, and the DNA was progressively eluted at increasing temperature. Specifically, fractions were eluted with the same buffer used in step 4 but at temperatures above the original incubation temperature. Successive elutions were carried out at intervals of 2.5°C to a final value of 100°C. The fractions eluted at each temperature were assayed for radioactivity. For purposes of graphic representation (Fig. 1 to 4), the total amount of radioactivity eluted at or below a given temperature was determined and normalized to the total radioactivity of the sample, yielding cumulative percent DNA eluted.

Assay of radioactivity. The radioactivity of the samples was assayed in a Packard Tri-Carb scintillation counter by counting the Cerenkov radiation generated by the ^{32}P in the DNA (6).

RESULTS

T_m and G+C content of the DNA. T_m 's and G+C contents of DNAs from all strains are shown in Table 3. *Escherichia coli* K-12 was used as a standard for the T_m determination. The T_m ($90.2 \pm 0.2^\circ\text{C}$) determined for the strain used compared favorably with those found in

TABLE 3. T_m , G + C content, and percent hybridization of the DNAs of group I and group II strains

Strain	T_m^a (°C)	G+C content (mol%)	% Hybridization ^b with strain:	
			4	5
Group I				
6	85.2	38.6	16	4
7	83.5	34.7	19	9
8	85.1	38.3	7	8
9	84.8	37.6	41	22
10	85.8	39.4	7	7
11	84.8	37.6	26	22
12	84.9	37.8	9	6
13	85.0	38.1	12	9
14	86.0	40.5	9	13
15	84.4	36.6	32	22
Group II				
4	86.6	42.0	100	77
5	86.4	41.5	75	100
16	86.7	42.2	90	88
17	86.8	42.5	77	99
18	86.8	42.5	86	99
19	86.4	41.5	87	77
20	86.3	41.5	96	82
21	86.2	41.0	79	94
22	86.6	42.0	95	83
23	86.6	42.0	97	80
24	86.7	42.2	95	83
25	86.6	42.0	95	85
26	86.7	42.2	96	86
27	87.8	44.9	75	77
28	86.0	40.5	91	89

^a T_m , Temperature at which thermally induced hyperchromicity reached one half its maximum value.

^b At 60°C with reference strains of *S. faecium* subsp. *casseliflavus*.

the literature (10, 13). The T_m 's of the streptococci ranged from 83.6 to 87.8°C with an average of $85.9 \pm 0.98^\circ\text{C}$. The G+C content ranged from 34.7 to 44.9 with an average of 40.4 ± 2.4 mol%. Strain 4, the type strain of *S. faecium* subsp. *casseliflavus*, was found to have a T_m of 86.6°C.

Strain 27, reported as atypical of either *S. faecium* or *S. faecalis* (14), was found to have a T_m of 87.8°C. Strain 13, reported (14) as being similar to strains of *S. faecium* subsp. *casseliflavus*, had a T_m of 85.0°C. Strain 12 was reported as *S. faecium*-like (14) and had a T_m of 86.6°C.

DNA-DNA reassociation at 60°C. Several of the yellow-pigmented strains tested showed a high degree of DNA homology with reference strains 4 and 5. All of these strains exhibited high T_m 's and G+C contents as compared with those strains not exhibiting high homology with strains 4 and 5 (Table 3).

The DNA-DNA reassociation data and the T_m 's of the DNAs allowed division of the yellow-

pigmented strains into two groups (Table 3). Group I exhibited T_m 's equal to or less than 86.0°C, DNA base (G+C) compositions equal to or less than 40.5 mol%, and low reassociation (4 to 41%) with strains 4 and 5. Group II includes strains with T_m 's equal to or greater than 86.0°C, G+C contents equal to or greater than 40.5 mol%, and high reassociation with strains 4 and 5. The group with the lower T_m 's had a mean T_m of $85.0 \pm 0.7^\circ\text{C}$, which corresponds to a mean G+C content of 38.1 mol%. The group with the higher values had a mean T_m of $86.6 \pm 0.4^\circ\text{C}$, corresponding to a mean G+C content of 42.0 mol%.

Strains 14 and 28 had T_m 's of 86.0°C and were placed in groups I and II, respectively, on the basis of the ability of their DNA to reassociate with that of strains 4 and 5.

Intermediate levels of DNA-DNA reassociation with strains 4 and 5 were obtained with strains 9, 11, and 15. All of the strains which had high and intermediate levels of homology with strains 4 and 5, and some which showed poor levels of binding with strains 4 and 5, were examined further in reassociation experiments with incubation temperatures of 70 and 75°C.

DNAs in homologous reactions consistently exhibited 80 to 90% reassociation (Table 4). Results were corrected for nonspecific binding (3 to 4%) and were normalized to the homologous reaction. Unlabeled DNA from strains 1 and 2 of *S. faecium* hybridized with labeled DNA from *S. faecalis* strain 3 at a level of 95% or greater. Unlabeled DNA from strain 3 bound 90% of labeled DNA from strain 2. DNA from all of the yellow-pigmented strains showed low (less than 25%) homology with DNA from strains of *S. faecium* and *S. faecalis*.

Thermal elution studies. To test the stability of duplexes formed during the reassociation studies at 60°C incubation, thermal stability studies were done on duplexes which had been incubated at 70°C and on those which had been incubated at 75°C, and a thermal elution midpoint ($T_{m(e)}$) was determined for each duplex (Tables 5 and 6). $T_{m(e)}$ is the temperature at which 50% of the DNA bound to the hydroxyapatite is dissociated. The higher temperatures provide more stringent conditions than does 60°C and prevent the reassociation of all but the highly complementary duplexes. For each reaction mixture, whether incubated at 70 or at 75°C, the thermal binding index was calculated. The thermal binding index is the ratio of binding at 70 or 75°C to that at 60°C. The thermal binding index approaches 1.0 in closely related organisms (4). In general, the following relationships were shown. Those strains which exhibited greater than 50% DNA-DNA reassociation with strains

TABLE 4. Reassociation of DNA from reference strains of *S. faecium*, *S. faecium* subsp. *casseliflavus*, and *S. faecalis* with DNA from group I and group II strains

Source of unlabeled DNA	% Hybridization ^a of unlabeled DNA with labeled DNA from:			
	<i>S. faecium</i> strain 2	<i>S. faecium</i> subsp. <i>casseliflavus</i>		<i>S. faecalis</i> strain 3
		strain 4	strain 5	
<i>S. faecium</i> strains				
1	97	14	17	95
2	100	15	11	96
<i>S. faecalis</i> strain 3	90	17	12	100
<i>S. faecium</i> subsp. <i>casseliflavus</i> strains				
4	8	100	77	7
5	13	75	100	10
Yellow-pigmented strains				
Group I				
6	14	16	4	8
7	5	19	9	4
8	9	7	8	4
9	4	41	22	2
10	12	7	7	8
11	1	26	22	11
12	13	9	6	21
13	10	12	9	4
14	4	9	13	3
15	16	32	22	12
Group II				
16	9	90	88	8
17	8	77	99	13
18	1	86	99	11
19	5	87	77	3
20	10	96	82	11
21	21	79	94	21
22	7	95	83	5
23	8	97	80	8
24	9	95	83	6
25	9	95	85	5
26	17	96	86	11
27	9	75	77	2
28	20	91	89	12

^a Expressed as percentage of homologous reaction values.

4 and 5 at 60°C also showed greater than 50% binding at 70°C, as well as at 75°C, when tested at this temperature. Those strains which showed less than 50% binding with strains 4 and 5 at 60°C showed even less binding at 70 and 75°C.

For each heterologous reaction, $\Delta T_{m(e)}$ was calculated (Table 5). $\Delta T_{m(e)}$ is the difference between the $T_{m(e)}$ of the heterologous and homologous reactions. It has been estimated that the presence of each 1.0% mismatched bases lowers the thermal stability about 1°C (2).

The data were plotted, and the resulting thermal elution curves are shown in Fig. 1 to 4. Homologous reactions are shown in Fig. 1 and 2,

TABLE 5. *Reassociation of DNAs from reference strains with DNAs from other strains of S. faecium subsp. casseliflavus*

Reaction mix- tures ^a	% DNA-DNA reassociation ^b with incubation at:		
	60°C	70°C	75°C
4/4	85 ± 1.7	82 ± 2.8	83 ± 1.3
4/5	66 ± 3.6	61 ± 2.0	63 ± 2.7
4/9	36	14	3
4/16	68	58	— ^c
4/17	68	56	60
4/18	68	64	60
4/19	73	66	—
4/20	82	69	—
4/21	67	58	63
4/11	24	11	—
4/25	72	64	75
4/12	9	9	—
4/27	75	72	—
4/15	29	16	—
5/5	87 ± 2.1	85 ± 1.4	85 ± 2.9
5/4	67 ± 2.0	58 ± 3.3	56 ± 3.2
5/9	22	9	—
5/17	79	79	79
5/18	83	80	—
5/19	61	60	60
5/20	72	64	62
5/21	74	60	72
5/11	21	9	—
5/25	56	51	60
5/12	7	4	—
5/27	69	65	61
5/15	21	9	—

^a The labeled strain is represented by the number to the left of the slash mark, and the unlabeled strain is represented by the number to the right of the slash.

^b All values represent actual nonnormalized experimental values.

^c—, No experimental determination.

and representative heterologous reactions are shown in Fig. 3 and 4. The homologous reactions exhibited sigmoid curves. The heterologous reactions exhibiting low reassociation, and some of those exhibiting higher (50%) reassociation at 60°C showed a significant increase in counts eluted as lower melting duplexes and perhaps less specific pairing of bases.

When homologous reassociations were carried out at 70 and 75°C, the curves were virtually the same as those at 60°C (Fig. 1 and 2). The main differences appeared in the 0 to 10% section of the curve where the curve represents a relatively small number of counts. When heterologous reassociations were carried out at 70 or 75°C with strains which had given low reassociation at 60°C, a large fraction of the duplexes eluted at low temperatures, showing that there had been some nonspecific binding at 60°C (Fig. 4). Heterologous reassociations at 70 and 75°C with

those strains which displayed intermediate homology at 60°C gave varying results. Most of the heterologous reassociations exhibiting high binding at 60°C had elution patterns similar to those of homologous reactions and showed high reassociation at 70 and 75°C (Fig. 3).

Characteristic physiological properties of groups I and II. Eight of the 10 strains in group I decarboxylated tyrosine, whereas all 14 strains tested in group II failed to decarboxylate tyrosine. Eight of the strains in group I fermented sorbitol, whereas 6 of the 15 in group II fermented the alcohol. Three of the strains in group I grew in broth with 6.5% NaCl, whereas 13 of 15 strains in group II did so.

DISCUSSION

The existence of a group of streptococci distinct from *S. faecium* and *S. faecalis* is shown by DNA-DNA reassociation. The organisms in this group (group II) consistently showed high (50%) homology with the two reference strains of this group, strains 4 and 5, but showed little homology with strains of *S. faecium* and *S. faecalis*.

The purpose of this work was to examine the genetic relatedness among yellow-pigmented strains of group D streptococci. We also wished to determine whether any correlation existed between the physiological characteristics and the DNA homologies of the yellow-pigmented strains; therefore, strains which differed somewhat in their characteristics were included in this study. Because of the variation in physiological characteristics among the strains, one might expect a continuum in the degree of reassociation. Rather, we found division of the strains into two classes: those showing greater than 50% reassociation with the reference strains and those showing less than 50% reassociation with the reference strains, the majority of the latter showing less than 20% reassociation with strains 4 and 5. Those eight strains which showed poor homology with the reference strains, like those which showed good homology, were variable in their physiological characteristics.

The pigment of *S. faecium* subsp. *casseliflavus* has been identified as a carotenoid (14), possibly a flavonol or a flavanone (11). In our experiments, we merely observed the pigment in isolated colonies on sucrose agar, in the pellet of cells centrifuged after 48 h of growth in tryptic soy broth, or in pellets of cells from which DNA was to be isolated. Strains 5, 13, 15, 16, 22 to 26, and 28 were observed to have a faint or fine yellow pigment. Strains 4, 6, 7, 10, 12, 14, 17 to 21, and 27 had more intense yellow color. Strains

TABLE 6. *Relative binding of DNA-DNA reassociation reactions at 60, 70, and 75°C*

Reaction mixture ^a	60°C			70°C			75°C				
	% Binding ^b	$T_{m(e)}$ ^c	$\Delta T_{m(e)}$ ^c	% Binding	$T_{m(e)}$	$\Delta T_{m(e)}$	TBI ^d 70°C/60°C	% Binding	$T_{m(e)}$	$\Delta T_{m(e)}$	TBI 75°C/ 60°C
4/4	100	87.3	— ^e	100	87.3	—	—	100	88.6	—	—
4/5	77	82.2	5.1	74	84.3	3.0	0.96	75	86.7	1.9	0.97
4/9	41	—	—	18	87.2	0.1	0.44	—	—	—	—
4/16	79	83.0	4.3	70	85.5	1.8	0.89	—	—	—	—
4/17	79	82.0	5.3	68	84.5	2.8	0.86	71	86.5	2.1	0.90
4/18	80	83.5	3.8	78	84.4	2.9	0.98	71	86.8	1.8	0.89
4/19	86	85.6	1.7	80	86.7	0.6	0.93	—	—	—	—
4/20	96	85.8	1.5	84	86.5	0.8	0.88	—	—	—	—
4/21	78	81.9	5.4	70	84.0	3.3	0.90	75	86.0	1.6	0.96
4/11	26	82.8	4.5	11	84.3	3.0	0.42	—	—	—	—
4/25	84	85.6	1.7	77	85.4	1.9	0.92	90	88.2	0.4	1.07
4/12	08	84.8	2.5	09	85.8	1.5	1.12	—	—	—	—
4/27	88	87.3	0.0	87	87.2	0.1	0.99	—	—	—	—
4/15	32	83.2	4.1	18	85.9	1.4	0.56	—	—	—	—
5/5	100	87.8	—	100	88.0	—	—	100	88.7	—	—
5/4	76	83.8	4.0	68	85.5	2.5	0.89	65	86.3	2.4	0.86
5/9	23	83.9	3.9	09	85.8	2.2	0.39	—	—	—	—
5/16	80	79.5	8.3	78	84.9	3.1	0.98	—	—	—	—
5/17	80	84.7	3.1	93	88.2	0.2	1.03	95	88.5	0.2	1.06
5/18	95	87.2	0.6	94	87.9	0.1	0.99	—	—	—	—
5/19	69	82.5	5.3	70	85.0	3.0	1.01	70	86.4	2.3	1.01
5/20	82	83.3	4.5	75	84.7	3.3	0.91	72	87.1	1.6	0.88
5/21	85	85.9	1.9	70	83.6	4.4	0.82	84	87.5	1.2	0.99
5/11	22	82.7	5.1	08	87.0	1.0	0.36	—	—	—	—
5/25	63	84.4	3.4	59	84.6	3.4	0.94	70	86.6	2.1	1.11
5/12	06	84.7	3.1	03	84.8	3.2	0.50	—	—	—	—
5/27	79	84.0	2.8	76	84.5	3.5	0.96	71	86.6	2.1	0.90
5/15	22	78.3	9.5	09	84.7	3.3	0.41	—	—	—	—

^a The labeled strain number is to the left of the slash mark, and the unlabeled strain is to the right.

^b All percentages are normalized to homologous reactions.

^c $T_{m(e)}$ is the temperature at which half of the DNA bound to hydroxyapatite is eluted. $\Delta T_{m(e)}$ denotes the difference between $T_{m(e)}$ for the appropriate homologous DNA reaction under consideration (2).

^d TBI 70°C/60°C and TBI 75°C/60°C denote the thermal binding index, defined as the ratio of binding at 70°C to that at 60°C and the ratio of binding at 75°C to that at 60°C, respectively (2).

^e—, No experimental determination.

8 and 9 had an extremely bright yellow pigment. There seems to be no group correlation of intensity of pigmentation and DNA homology.

Based on their physiological characteristics, the strains with which we worked would ordinarily be classified either as atypical strains of *S. faecalis* or *S. faecium* or as strains intermediate between these two species, since they have some characteristics of both and some characteristics not shared by these two species. Moreover, none of the yellow-pigmented strains hybridized at a level of more than 21% with strains of either *S. faecium* or *S. faecalis*. Thus, according to the criteria proposed by Johnson (8), they are at most only distantly related to these species.

The DNA of the pigmented streptococci of group II hybridized at a level of at least 75% with the DNA of strains 4 and 5. Thus, this

group appears to constitute a distinct taxon. The strains in group I, for the most part, have a DNA complement which hybridized at a level of about 10% with strains 4 and 5. These strains appear to be only distantly related to the taxon centering around strains 4 and 5.

Reassociation experiments appear to be a useful tool in determining which physiological characteristics should be weighted in a scheme for identifying bacteria. Based on the results of these experiments, it appears that there are certain physiological characteristics which should carry more weight than others in the identification of the pigmented streptococci. The characteristics to be emphasized are tyrosine decarboxylation, sorbitol fermentation, and growth in 6.5% NaCl.

On the basis of fatty acid content, Amstein and Hartman (1) found sufficient differences

FIG. 1. Thermal elution from hydroxyapatite of duplexes of ^{32}P -labeled and unlabeled DNA from strain 4.

FIG. 3. Thermal elution from hydroxyapatite of duplexes of ^{32}P -labeled DNA from strain 5 and unlabeled DNA from strain 20.

FIG. 2. Thermal elution from hydroxyapatite of duplexes of ^{32}P -labeled and unlabeled DNA from strain 5.

FIG. 4. Thermal elution from hydroxyapatite of duplexes of ^{32}P -labeled DNA from strain 5 and unlabeled DNA from strain 15.

between strains of *S. faecium* subsp. *casseliflavus* and other strains of *S. faecium* to conclude that *S. faecium* subsp. *casseliflavus* "deserves varietal, if not species, status." The possibility of assigning species status to *S. faecium* subsp. *casseliflavus* was further suggested by our finding (13) that the DNA of an authentic strain of *S. faecium* subsp. *casseliflavus* has negligible sequence homology with the DNA of an authentic strain of *S. faecium* and by the physiological differences that we have just reviewed. We deferred proposing a change in the taxon, however, pending an examination of the DNA hybridization behavior of additional yellow-pigmented strains. As reported here, we have now tested 25 different yellow-pigmented group D streptococci and have found none which exhibits significant DNA sequence homology with *S. faecium*. Therefore, we now feel justified in proposing that *S. faecium* subsp. *casseliflavus* be elevated to the rank of species and be designated *Streptococcus casseliflavus* (Mundt and Graham) comb. nov. Our earlier suggestion (13) that the yellow-pigmented group D streptococci are not a homogeneous group is further borne out by the results reported here, and we conclude that the strains we have placed in group I should be excluded from the proposed species. The status of the group I strains cannot be established without further work.

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REPRINT REQUESTS

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