Deoxyribonucleic Acid Base Sequence Studies on Glucan-Producing and Glucan-Negative Strains of *Streptococcus mitior*

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The deoxyribonucleic acid base sequence similarities among 14 strains of *Streptococcus mitior* were assessed by hybridization. Some strains produce glucan. Deoxyribonucleic acid was purified by both a conventional method and by centrifugation in CsCl. Hybridizations were done in solution with S1 nuclease and on membrane filters. The results showed two groups of moderately homologous strains. The glucan-producing strains represented a group which included some glucan-negative strains. Hybridization by the S1 method was less than with the filter method, indicating some sequence divergence within the two groups. There was a slight difference in guanine-plus-cytosine contents of the two groups.

Schottmüller used the name *Streptococcus mitior* *seu viridans* in 1903 for streptococci that were weakly hemolytic and produced small grey to greenish-black colonies (16). This name has priority over the binomial *S. mitis*, which was used in 1906 by Andrewes and Horder to designate streptococci which were rarely pathogenic, grew in short chains, and usually failed to ferment inulin and raffinose (1). *Index Bergeyana* (3) recognizes the binomial *S. mitior* (Schottmüller) as validly published and legitimate but regards *S. mitis* (Andrewes and Horder) illegitimate. It is difficult to know which of these early investigators' descriptions is better for these organisms, and it is likely that both Schottmüller and Andrewes and Horder studied streptococci of more than one species, if one applied current taxonomy. Andrewes and Horder suggested the name *S. mitis* as a "non-committal term fairly expressing its habitually non-virulent character" (1). Sherman et al. (17) discussed the heterogeneity of "*S. mitis*," and this species is still described as a "heterogeneous group" in *Bergey's Manual of Determinative Bacteriology* (8th ed.) (9). Lack of a clear definition of these cocci has limited the usefulness of the term *S. mitis*. The species has often been a receptacle for oral viridans streptococci which were not *S. sanguis*, *S. salivarius*, or *S. mutans*. Colman and Williams gave these organisms a clearer definition and recommended use of the earlier name *S. mitior* (5). Many microbiologists, especially oral microbiologists, now use this name for alpha-hemolytic streptococci that do not split esculin or arginine and do not ferment inulin.

Some "*S. mitior*-like" strains produce a polymer from sucrose which is regarded as a dextran

or glucan (5). This property is shared by *S. sanguis*, and glucan-producing strains otherwise resembling *S. mitior* have often been considered to be *S. sanguis* (see reference 4 for example). Colman and Williams included the "*S. mitior*-like" glucan-producing strains within *S. mitior*, and others have accepted this grouping (12, 15). By using deoxyribonucleic acid (DNA)-DNA base sequence hybridization, we have shown that glucan-producing strains otherwise resembling *S. mitior* are definitely not *S. sanguis* (8). We report here results of DNA base sequence homology experiments designed to show the relationships between the glucan-producing *S. mitior*-like strains and the glucan-negative *S. mitior* strains.

MATERIALS AND METHODS

Bacterial strains. The streptococcal strains used in this study are listed in Table 1. All fit the description of *S. mitior* as set forth by Colman and Williams (5). They do not ferment mannitol, sorbitol, or inulin and do not hydrolyze arginine or esculin. Fermentation of raffinose varies among the strains. They produce H₂O₂. Some strains synthesize glucan from sucrose. When grown on Mitis-salivarius agar (BBL Microbiology Systems, Cockeysville, Md.), which is 5% sucrose, these bacteria generally form very flat, thin, dull colonies, except the glucan-producers, which produce very hard, domed colonies that depress the agar, and are indistinguishable from *S. sanguis* colonies. When grown for DNA extraction, the cultures were Gram-stained and streaked on Mitis-salivarius agar to confirm purity.

Preparation of DNA. Fresh overnight cultures in 100 ml of Todd-Hewitt broth (BBL) were added to 400 ml of fresh, warm (37°C) broth containing an additional 1% (wt/vol) glucose and incubated at 37°C. Bacterial growth was measured turbidimetrically. In

TABLE 1. *Strains used in this study*^a

Strain	Origin
10557 ^b	Isolated from blood; obtained from J. Carlsson (see ref. 4); derived from ATCC strain 10557, formerly considered to be <i>S. sanguis</i> (see ref. 8)
72 × 35 ^b	Culture of strain 10557 obtained from B. Rosan
9811 ^b	Obtained from B. Rosan; derived from <i>S. mitis</i> (<i>mitior</i>) ATCC strain 9811
MPD1, ^b HPA1 ^b	Isolated by J. Carlsson and obtained from D. Bratthall
OP51, HV51, OS51, NS51	Isolated by and obtained from J. Carlsson
OTF1 _{flat} , OTF1 _{beady}	OTF1 isolated by and obtained from J. Carlsson; sub-strains growing flat colonies and beady colonies on Mitis-salivarius agar were established
FW103, FW69, BU174 ^b	Isolated by and obtained from G. Colman (see ref. 5)

^a Strain designations are those used by the cited authors.

^b Produces glucan.

the late logarithmic phase, 10 U of penicillin per ml was added and incubation was continued until growth stopped. The cells were harvested by centrifugation after 7.5 g of sodium citrate was added, washed twice with 0.015 M tris(hydroxymethyl)aminomethylmethane (Tris) buffer (pH 8), treated with lysozyme (5 mg in 15 ml of 0.15 M Tris [pH 8]) for 2 h, suspended in 15 ml of saline sodium citrate (SSC; 0.15 M sodium chloride, 0.015 M sodium citrate), and finally lysed by the addition of 0.5 ml of 25% (wt/vol) sodium lauryl sulfate. After heating for 20 min at 60°C, the material was treated with 0.5 ml of 5% (wt/vol) protease (Sigma Chemical Co., St. Louis, Mo.). For radioactive (index) DNA, 1 mCi of [*methyl*-³H]thymidine (New England Nuclear Corp., Boston, Mass.) was added to the culture medium.

The first stage of DNA purification was deproteinization with phenol and chloroform and precipitation with cold ethanol. The DNA was further purified by two methods. Some was centrifuged to equilibrium in CsCl as follows. The precipitated DNA was dissolved in 0.03 M Tris–0.005 M ethylenediaminetetraacetic acid–0.05 M NaCl. A 4-ml volume of the solution was added to 25 ml of a CsCl solution (about 127 g of CsCl dissolved in 100 ml of water), and the density was adjusted to exactly 1.70 g/cm³ by the addition of more CsCl. This mixture was centrifuged at 40,000 rpm for 36 h in a Beckman 60Ti rotor at 15°C. After centrifugation, the DNA was collected by puncturing the bottom of the tube and collecting the viscous fraction. The DNA solution was then dialyzed against 0.42 M NaCl for 2 days at 5°C with five changes of the salt solution.

Other DNA was purified by dissolving the deproteinized nucleic acid in SSC/100 and treating it with ribonuclease A (Sigma) at a concentration of 50 U/ml

of DNA solution for 30 min at 60°C and 30 min at 37°C, followed by incubation for 2 h at 37°C with protease (at 0.05% [wt/vol]) and sodium lauryl sulfate (at 0.5% [wt/vol]). Finally, the mixture was deproteinized again with phenol and chloroform and precipitated in cold ethanol. This DNA was redissolved in dilute saline and dialyzed against 0.42 M NaCl as described above. This DNA is referred to below as "conventional DNA."

The purified DNA was quantitated by the absorption of ultraviolet light at 260 nm, assuming that 10 µg of DNA per ml results in a reading of 0.250. The ratios of absorbance at 230:260:270:280 nm were consistently about 0.44:1.082:0.54. DNA was prepared from most strains by both of the above methods.

When sheared DNA was required, it was disrupted by sonic treatment with a model 185 sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), using a 3-mm tip at the number-two power setting (38 W) for 1 min in 15-s bursts. The DNA was in a 13-mm tube immersed in ice water. This treatment preceded dialysis so that small bits of DNA (below 12,000 molecular weight, or about 25 base pairs) would be eliminated.

Hybridization by the S1 nuclease method. The method described by Barth and Grinter (2) was used. Single-strand-specific S1 endonuclease was used to eliminate unreassociated and incompletely reassociated DNA. A 25-µg portion of sheared DNA was mixed with 0.05 µg of sheared, labeled "index" DNA in a total volume of 1.25 ml of 0.42 M NaCl in siliconized test tubes. These mixtures were denatured in a water-ethylene glycol bath at 103 to 105°C for 20 min (this species of DNA denatures at 90 to 91°C in 0.42 M NaCl). The mixtures were then incubated at 65°C for 6 to 7 h. This short incubation time (about 2 × C_{0t1/2}) was used since it resulted in adequate heteroduplex formation, and the self-reassociation of labeled DNA was minimized. In some experiments, the amount of label used was increased 10-fold, which resulted in some unobjectionable self-reassociation of the index DNA under these conditions.

In each experiment, DNA from *E. coli* and/or calf thymus (Sigma; sheared and dialyzed, as the other DNA) was included. All hybridizations were performed in duplicate. After 6 h, the hybridization mixtures were treated with S1 nuclease as follows. To each mixture was added 1.8 ml of "reaction mixture" (50 mM sodium acetate–1.67 mM ZnSO₄ [pH 4.8]), 25 µg of sheared, denatured calf thymus DNA, and 20 U of S1 nuclease. The mixtures were then returned to 65°C for 20 min. The mixtures were then placed in ice water, and 3.2 ml of ice-cold trichloroacetic acid (10% [wt/vol]) was added to stop the reaction and precipitate the DNA.

Three pairs of control mixtures were included. All were mixtures of 25 µg of calf thymus DNA and 0.05 µg of index DNA. One pair was not denatured and did not receive S1 nuclease. When precipitated, this DNA represented the maximum labeled DNA recoverable. Another pair was not denatured but received S1 nuclease. This tested the effect of the enzyme on double-stranded DNA. The remaining pair of controls was denatured and treated with S1 nuclease immediately after cooling to 65°C, to test the efficiency of the enzyme against single-stranded DNA. Radioactive

counts from this control were considered "background."

The precipitated DNA was collected on glass-fiber filters (Whatman GF/C). The filters were prerinsed with 4 ml of 5% trichloroacetic acid (wt/vol) containing 0.2 mM thymine. The DNA was filtered, and then the filters were rinsed four times with 4 ml of 5% acid with thymine, the first 4 ml being used to rinse the reaction tube that had contained the DNA. After a final rinse with 2.5 ml of acetone, the filters were dried, and entrapped labeled DNA (hybrids) was counted by liquid scintillation using a commercial scintillant (Aquasol, New England Nuclear).

The degree of relative hybridization, or "homology," was calculated by subtracting the "background" from all reassociation counts and comparing heterologous reassociation (hybrid) values to the homologous control (labeled index DNA reassociated with unlabeled DNA of the same strain).

Hybridization on filters. For comparison, some tests of DNA homology were undertaken by the filter method of Denhardt (10). Solutions of 50 μ g of denatured, unsheared DNA in 6 $\times$ SSC were applied to 25-mm filters (HAWP, Millipore Corp., Bedford, Mass.), dried, and incubated as described by Denhardt (10), with 1 or 0.5 μ g of sheared, denatured index DNA. After incubation overnight at 67°C, the filters were rinsed in 3 $\times$ SSC, dried, and counted as described above.

With DNA prepared by two methods and hybridizations done by two methods, four types of experiments were possible. Most of the DNA was used in the S1 nuclease experiments. The DNA from only a few strains was used in the filter experiments.

Determination of DNA base content. The base content (moles percent guanine plus cytosine [G+C]) of each strain's DNA was determined three to five times by thermal denaturation, using the formula: $\text{mol\% G+C} = (T_m - 69.3)/0.41$ (14).

RESULTS

Effectiveness of the S1 method. Using 20 U of S1 nuclease per reaction mixture, it was possible to eliminate 90% of the single-stranded DNA in 20 min. The amount of trichloroacetic acid-precipitable DNA was very similar between duplicate hybridization mixtures. Using a 500:1 ratio of unlabeled DNA to index DNA, the amount of labeled, precipitated DNA from the control mixtures containing *E. coli* or calf thymus DNA was seldom much higher than that of background. When the ratio was reduced to 50:1, these mixtures yielded amounts of precipitable DNA of 5 to 7% of the homologous control. This probably represents the formation of homoduplexes of labeled DNA at this proportionally higher concentration, which resulted in a slight inflation of the relative hybridization values of the *S. mitior* strains.

Hybridizations. The degree of DNA-DNA hybridizations between DNA from different strains was never high when the S1 nuclease method was used (Table 2). The results indicate

the existence of two groups of strains, arbitrarily called 1 and 2. DNA from strains in group 1 hybridized between 60 and 80% with the index strains 10557 and OP51 (DNA of strain 72 $\times$ 35 hybridized almost completely with 10557 DNA, but they are actually the same strain [see Table 1], and this was expected). DNAs from the strains of group 2 hybridized about 30 to 35% with the group 1 index DNAs.

When DNAs of strains in group 2 were used as index DNAs, the reciprocal results were seen. Within this group, hybridizations were 60 to 75% with index strains FW103 and NS51, although strain FW69 DNA always hybridized less. The group 1 DNAs hybridized only 30 to 40% with the group 2 index strain DNAs in two experiments, and 40 to 50% in one experiment. The DNA purification method (CsCl versus conventional method) did not seem to have any effect on the results.

When the filter hybridization method was used, the degree of hybridization was always significantly higher, but paralleled the results of the S1 method hybridizations.

All of the glucan-producing strains joined group 1.

G+C contents. The DNA G+C contents of the strains are listed in Table 3. There is a slight, but significant ($P < 0.01$), difference between the mean base contents of the two groups: 42.14 mol % for group 1 and 40.44 mol % for group 2.

DISCUSSION

We consider bacterial strains whose DNAs are 80% homologous (in the membrane filter technique) to form "genospecies," i.e., organisms that have similar genetic molecules. (We would like to have genospecies and species be synonymous.) In the case of these *S. mitior* strains, it is clear that DNA base sequences of members of group 1 are significantly different from those of members of group 2. Indeed, the small but significant difference in the mean base contents of the two groups predicts some sequence dissimilarity. Strains within the groups were usually over 80% homologous in the few filter experiments that were done and in one previous experiment with glucan-producing strains (8), but the lower hybridization with the S1 nuclease method indicates a degree of heterogeneity within the groups also.

We believe that the strains within each group may have areas in their genomes which are not species-specific. The fact that strain 72 $\times$ 35 hybridized to nearly 100% with strain 10557 DNA in S1 experiments indicates that the lack of higher hybridization values among the other strains within each group was not purely technical, and that there are indeed areas in the

TABLE 2. Degree of DNA homology between strains^a

Unlabeled DNA	Index (labeled) DNA									
	10557			OP51			FW103		NS51	
	CsS1 ^b	CoS1	CoF	CsS1	CoS1	CsF	CsS1	CsF	CsS1	CoS1
Group 1										
10557 ^c	100	100	100	70.1	76.9	—	39.9	—	32.8	48.0
72 × 35 ^c	96.5	97.3	90.6	69.4	70.7	87.5	40.3	61.8	30.4	44.1
9811 ^c	63.9	— ^d	—	64.4	73.5	75.9	36.1	64.6	31.1	43.8
MPD1 ^c	73.1	60.2	89.4	65.6	63.7	88.6	37.0	60.9	30.2	48.7
HPA1 ^c	63.2	—	91.3	65.2	79.6	—	38.6	—	34.4	43.2
BU174 ^c	65.0	—	—	60.0	75.3	—	—	—	—	45.8
OP51	65.4	64.4	—	100	100	100	40.7	60.0	34.1	48.4
OTF1 _{flat}	—	—	—	—	74.0	—	—	—	—	41.3
OTF1 _{beady}	64.6	—	—	68.0	80.6	83.5	37.5	—	31.7	41.3
Group 2										
HV51	32.3	—	—	29.8	56.3	57.9	64.8	75.0	67.9	73.6
OS51	32.4	21.9	—	35.4	50.4	—	60.0	—	58.7	67.9
NS51	35.1	—	—	37.9	27.5	—	67.4	—	100	100
FW103	30.8	—	—	36.8	37.0	57.4	100	100	68.5	72.1
FW69	33.9	—	—	33.2	16.2	—	49.2	—	53.0	62.8
<i>Escherichia coli</i>	5.0	6.9	4.5	0	5.5	6.7	2.0	6.4	0	6.3
Calf thymus	1.8	—	—	0	—	—	3.6	—	0	—

^a Expressed as percent hybrid duplex formation compared to homologous duplex formation.^b Abbreviations denote the type of experiment; CsCl-purified DNA, using the S1 nuclease method (CsC1); CsCl DNA, using the filter method (CsF); conventionally purified DNA, using the S1 method (CoS1); conventional DNA, using the filter method (CoF).^c Strain that produces glucan.^d Hybridization not done.

TABLE 3. DNA base contents of the strains

Strain	Mol% G+C	±SD ^a	Raffinose fermentation ^b
Group 1			
10557	42.2	0.71	+
72 × 35	42.6	0.52	+
9811	42.2	0.41	—
MPD1	42.5	0	+
HPA1	42.1	0.42	+
BU174	42.4	0.36	+
OP51	42.2	1.01	+
OTF1 _{flat}	41.8	0.36	—
OTF1 _{beady}	41.3	0.89	—
Group 2			
HV51	40.8	0.91	+
OS51	40.2	1.04	—
NS51	41.0	1.14	+
FW103	40.3	0.26	—
FW69	39.9	0.35	—

^a SD, Standard deviation.^b +, Acid produced; —, no acid produced.

genomes of these strains which are not homologous. Thus, these *S. mitior* genomes may have areas where their base sequences are similar but not identical, so that these sequences can form weak, infidelical heteroduplexes which contain many mismatched base pairs. These duplexes

can appear as hybrids on membrane filters, but cannot endure treatment with S1 nuclease.

Additional experiments support the idea that these strains have base sequence heterogeneities detectable by S1 nuclease treatment and that the lack of complete homology was not a technical artifact. DNA was extracted by the conventional method from four strains of *S. sobrinus* (*mutans*) (7). These strains are known to have great DNA base sequence homology (6). Using the membrane filter method, these DNAs showed 96 to 98% homology. When these same DNAs were tested by the S1 method, their homology did not decrease.

Thus, these *S. mitior* strains show a degree of genetic heterogeneity consistent with the often-noted phenotypic heterogeneity, even though these particular strains form a reasonably homogeneous group based on their reactions to arginine, esculin, and inulin. It seems likely that considerable base sequence divergence has occurred in the DNA of this "species." Heterogeneities found among fructose diphosphate aldolases of *S. mitis* (*mitior*) strains (13) also indicate evolutionary divergence.

Facklam has suggested a taxonomic scheme for viridans streptococci which includes a "Type I" *S. sanguis*, a "Type II" *S. sanguis*, and *S. mitis* (11). *S. sanguis* II and *S. mitis* are similar;

they seldom split arginine and never split esculin or ferment inulin. They are differentiated by raffinose; *S. sanguis* II ferments this sugar whereas *S. mitis* does not. We consider Facklam's *S. sanguis* II and *S. mitis* strains to be *S. mitior* strains, especially since we find raffinose fermentation of no value in identifying either *S. mitior* (Table 3) or *S. sanguis*.

Our results show that *S. mitior* strains which produce glucan are genetically indistinguishable from others that do not, and therefore this trait has little taxonomic significance. However, the production of these polymers may have ecological and pathological importance. The glucan producers are more often found on tooth surfaces (4), and Parker and Ball suggest that dextran production enhances the probability that such a streptococcus will cause endocarditis (15).

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REPRINT REQUESTS

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LITERATURE CITED

- Andrewes, F. W., and T. J. Horder. 1906 A study of the streptococci pathogenic for man. *Lancet* ii:708-713.
- Barth, P. T., and N. J. Grinter. 1975. Assay of deoxyribonucleic acid homology using a single-strand-specific nuclease at 75 C. *J. Bacteriol.* 121:434-441.
- Buchanan, R. E., J. G. Holt, and E. F. Lessel, Jr. (ed). 1966. Index Bergeyana, p. 1084-1085. The Williams and Wilkins Co., Baltimore.
- Carlsson, J. 1968. A numerical taxonomic study of human oral streptococci. *Odontol. Revy* 19:137-160.
- Colman, G., and R. E. O. Williams. 1972. Taxonomy of some human viridans streptococci, p. 282-299. In L. W. Wannamaker and J. M. Matsen (ed.), *Streptococci and streptococcal diseases*. Academic Press Inc., New York.
- Coykendall, A. L. 1974. Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. *J. Gen. Microbiol.* 83:327-338.
- Coykendall, A. L. 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on their molecular composition. *Int. J. Syst. Bacteriol.* 27:26-30.
- Coykendall, A. L., and P. A. Specht. 1975. DNA base sequence homologies among strains of *Streptococcus sanguis*. *J. Gen. Microbiol.* 91:92-98.
- Deibel, R. H., and H. W. Seeley, Jr. 1974. Genus I. *Streptococcus* Rosenbach 1884, 22, p. 490-509. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams and Wilkins Co., Baltimore.
- Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.
- Facklam, R. R. 1977. Physiological differentiation of viridans streptococci. *J. Clin. Microbiol.* 5:184-201.
- Hardie, J. M., and G. H. Bowden. 1974. The normal microbial flora of the mouth, p. 47-83. In F. A. Skinner and J. G. Carr (ed.), *The normal microbial flora of man*. Society for Applied Microbiology Symposium Series no. 3. Academic Press Inc., London.
- London, J., N. M. Chace, and K. Kline. 1975. Aldolase of lactic acid bacteria: immunological relationships among aldolases of streptococci and gram-positive non-sporeforming anaerobes. *Int. J. Syst. Bacteriol.* 25:114-123.
- Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5:109-118.
- Parker, M. T., and L. C. Ball. 1976. Streptococci and aerococci associated with systemic infection in man. *J. Med. Microbiol.* 9:275-302.
- Schottmüller, H. 1903. Die Artunterscheidung der für den Menschen pathogenen Streptokokken durch Blutagar. *Muench. Med. Wochenschr.* 50:849-853.
- Sherman, J. M., C. F. Niven, Jr., and K. L. Smiley. 1943. *Streptococcus salivarius* and other non-hemolytic streptococci of the human throat. *J. Bacteriol.* 45:249-263.