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Characterization of strains of viridans streptococci by deoxyribonucleic acid hybridization and physiological characteristics

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Characterization of Strains of Viridans Streptococci by Deoxyribonucleic Acid Hybridization and Physiological Characteristics

by

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THESIS

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DEDICATION

This manuscript is dedicated to everyone whose love, friendship or inspiration aided my completion of this work.

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INTRODUCTION

Use of the term streptococcus to describe chain forming cocci dates to 1874 when Billroth noted bacteria in wounds and discharges (Jones, 1978). The first systematic classification of the genus was produced in 1937 by Sherman and included divisions for pyogenic streptococci, enterococci, lactic streptococci, and viridans streptococci. Sherman identified a combination of characteristics which he felt appeared to justify a separate grouping for the viridans streptococci. The characteristics included "1. inability to cause true beta hemolysis of blood 2. the possession of high minimum and relatively high maximum temperatures of growth 3. weak reducing action 4. a limited tolerance to methylene blue, salt and alkali and 5. the inability to produce ammonia from peptone (Sherman, 1937)." The term viridans was initially used to describe the ability of many members of this group to produce alpha hemolysis on blood agar, although some strains have no action on blood. There is little agreement in past literature regarding the ability of these strains to produce beta hemolysis (Colman, 1972; Wilson and Miles, 1975; Facklam, 1977; Pulliam 1980).

Viridans streptococci constitute the predominant streptococcal flora of the mouth and pharynx and also inhabit the gastrointestinal and genital tracts of man (Jones, 1978). These organisms are the most frequent cause

of endocarditis and may be associated with suppurative infections such as abscesses and empyema (Parker and Ball, 1976; Facklam, 1977). Streptococcus mutans, a species within the viridans group, has been associated with coronal dental caries (Edwardsson, 1968; Gibbons and Van Houte, 1975; Van Houte, 1980).

The organisms in the viridans group are generally characterized today as cytochrome negative, homofermentative gram positive cocci, which are usually alpha hemolytic or nonhemolytic on blood agar and seldom resistant to penicillin (Deibel, 1974; Facklam, 1977). Most of these streptococci do not show group or species specificity with the Lancefield serological antigens A-G, although occasional reactions occur with group A,C,E,F or G antisera (Bratthall, 1972; Facklam, 1977). Species differentiation has therefore been based on other serological methods or physiological test results (Facklam, 1977).

In the last fifteen years, detailed investigations of the physiological properties of the viridans streptococci have contributed to a vast array of data. Principal authorities in the field (Carlsson, 1967, 1968; Guggenheim, 1968; Colman and Williams, 1972; Facklam, 1977; Pulliam, 1980) generally tend to agree on the physiological characteristics of Streptococcus mutans and Streptococcus salivarius (Tables 1 and 2). However, confusion still

exists concerning the classification and/or nomenclature of the remaining species.

The physiological characteristics of the strains designated as Streptococcus mitis (Carlsson, 1968; Guggenheim, 1968; Facklam, 1977; Pulliam, 1980) or Streptococcus mitior (Colman and Williams, 1972; Hardie and Bowden, 1976) are generally consistent in various studies (Table 3). Distinguishing characteristics of S. mitis include formation of acid in lactose and sucrose broths and failure to hydrolyze esculin or form acid in mannitol, inulin and raffinose broths (Facklam, 1977).

Problems arise with differentiation of Streptococcus sanguis I and Streptococcus sanguis II strains. Earlier investigators probably designated some of these strains as S. mitis or S. mitior (Colman and Williams, 1972) and several investigators have claimed that S. sanguis II ATCC 10557 should be considered an aberrant member of the species, if it is included in the taxon at all (Lai, Listgarten and Rosan, 1973; Cole and Kolstad, 1974; Coykendall and Specht, 1975). Facklam (1977) prefers to classify most dextran producing S. mitior strains as S. sanguis II based on acid formation in raffinose broth. According to Facklam, distinguishing physiological characteristics of S. sanguis II strains include formation of acid in lactose, raffinose and sucrose broths, and

failure to hydrolyze esculin or form acid in mannitol or inulin broths. S. sanguis I strains are characterized by acid formation in inulin, lactose and sucrose broths and dextran production on five percent sucrose agar (Facklam, 1977). Facklam (1977) found that most S. sanguis I strains are alpha hemolytic and hydrolyze arginine (Table 4 and 5).

Organisms first described as Streptococcus anginosus by Andrewes and Horder (1906) or Streptococcus milleri by Guthof (1956) have been variously characterized as Streptococcus MG (Mirick et al., 1944), minute beta hemolytic streptococci (Long and Bliss, 1934), Streptococcus MG - intermedius (Facklam, 1977) Streptococcus anginosus - constellatus (Facklam, 1977), Streptococcus constellatus (Holdeman and Moore, 1974), and Streptococcus intermedius - MG - anginosus group (Pulliam, 1980). Colman and Williams (1972) felt that S. MG and S. milleri were the same species, and could be identified by their ability to hydrolyze esculin and arginine and their inability to produce acid in mannitol, sorbitol and inulin broths. Poole and Wilson (1976) considered S. MG (nonhemolytic streptococci of group C,F or G) and S. anginosus (minute beta hemolytic streptococci of group F and G) the same species and recommended use of the name S. milleri. These same investigators confirmed earlier reports which indicated that S. anginosus is often characterized by Lancefield group F or G reaction (Liu, 1954; Ottens and Winkler, 1962). Facklam

(1977) agrees with Colman and Williams'(1972) physiological findings, but separates these organisms into two groups. Lactose negative strains are designated S. anginosus - constellatus and lactose positive strains are classed as S. MG - intermedius (Facklam, 1977). Pulliam (1980) puts lactose positive strains together in the S.intermedius-MG-anginosus group and considers S. constellatus to be the only strain (with the exception of Streptococcus morbillorum) unable to produce acid in lactose broth (Table 6).

Streptococcus morbillorum is considered a member of the viridans streptococci in studies by Facklam (1977) and Pulliam (1980), following Holdeman and Moore's (1974) suggestion to re-classify these homofermentative, facultative streptococci in the genus Streptococcus (Table 7).

The difficulties encountered by microbiologists attempting to identify strains of viridans streptococci result from the conflicting opinions regarding the classification of these organisms. Few studies have questioned whether there is any correlation between phenotypic heterogeneity and genetic divergence. Have too many species been created or are there strains representing new taxons which at present have no taxonomic designation? Because most taxonomic work involving the viridans streptococci has been based on phenotypic relationships,

unreliable estimates of the degree of relatedness between species may have been provided. The potential for errors in identification based solely on phenotypic data is evident when one considers that a battery of 300 tests would assay, at most, only between five and twenty percent of the genetic potential of a bacterium (Brenner, 1980). Additional possible sources of error must be recognized when identifying on the basis of phenotype alone. According to Brenner (1980)

"1. Different enzymes (specified by different genes) may catalyze the same reaction.
2. Negative reactions can occur when the metabolic gene is present and functional. They can occur through any of several mechanisms, including the inability of the substrate to enter the cell, and a regulatory or suppressor mutation.
3. A negative reaction can occur when the gene is present, but not functional because of a mutation in a portion of the gene that is necessary for enzyme activity.
4. The correlation between a reaction and the number of genes (or enzymes) necessary to carry out that reaction is not necessarily one to one. Six enzymatic steps may be involved in a given pathway. If one assays for the end product, a positive reaction indicates six similar enzymes, whereas a negative reaction can mean the absence or nonfunction of anywhere from one to six enzymes.
5. Fastidious strains will not cluster with non-fastidious strains of the same species.
6. Plasmids carrying metabolic genes can enable strains to carry out reactions that are rarely, if ever, seen in the absence of the plasmid."

Until recently, genetic information about bacteria has played a small role in bacterial systematics (Jones and Sneath, 1970). Advances in the demonstration of gene transfer, and data from studies on DNA base ratios and

nucleic acid pairings indicate that genetic tests of relatedness among bacteria should provide the best indication of species differentiation (Mandel, 1969). In particular, introduction of DNA reassociation data can serve to clarify classifications of bacteria. Coykendall (1971, 1974) and Coykendall and Specht (1975) used DNA reassociation experiments to observe genetic heterogeneity among strains of S. mutans and S. sanguis which demonstrate phenotypic homogeneity. Brenner (1978) employed DNA hybridization methods in order to identify species of Enterobacteriaceae which could not be readily differentiated by biochemical tests.

This study includes deoxyribonucleic acid reassociation data in addition to biochemical and serological reactions in an effort to clarify the genetic relationships among select species of viridans streptococci.

The physical properties of the DNA molecule make DNA reassociation experiments possible. Under appropriate conditions, such as heating at high temperatures or treating with alkali, the double stranded DNA helix unwinds, resulting in two separate strands. The single strands of DNA can then reassociate with complementary strands. If this reassociation occurs among strands from different strains, a hybrid molecule is formed. Determination of nucleotide sequence homology in the hybrid molecule (the

fraction of complementary nucleotide pairs) allows comparison of the entire genomes of two bacteria. This comparison enables a prediction of the biological relatedness of the two organisms based on their genetic composition rather than their phenotypic characteristics.

It is possible to select a system for homology studies from two general methods currently in use:

1. reassociation with DNA from one source immobilized prior to the addition of radiolabelled DNA and
2. reassociation which occurs with both nucleic acids free in solution.

In the first method, denatured, unlabelled DNA is immobilized, but remains available for binding with complementary fragments of radioactive DNA. Various techniques for immobilization have been used, including attachment of DNA to nitrocellulose columns (Bautz and Hall, 1962), agar-gel columns (Bolton and McCarthy, 1962), and nitrocellulose filters (Denhardt, 1966). An advantage of these immobilizing techniques is the prohibition of self-reassociation of the single stranded, unlabelled DNA. In addition, since relatively large amounts of labelled DNA (as compared to free solution experiments) are required to insure adequate reassociation with immobilized DNA, lower specific activity DNA preparations may be used (10^3 cpm/µg DNA or greater) (Johnson, 1981).

The free solution method results in reassociation reactions which may be measured in several ways. Optical monitoring of the annealing reaction can be achieved without use of radiolabelled DNA by measuring the decrease in absorbance at 260 nm on a recording spectrophotometer (DeLey, Cattoir and Reynaerts, 1970). Alternatively, a liquid scintillation counter may be employed to detect radiolabelled duplexes which form when a small amount of high specific activity denatured DNA is incubated with an excess of unlabelled, denatured DNA. In the latter instance, newly formed duplex DNA must be separated from single stranded fragments by absorption of the double strands to hydroxyapatite or by digestion of single stranded DNA with S_1 nuclease. For free solution reassociations with labelled DNA, the specific activity must be in the range of 5.0×10^4 to 2.5×10^5 cpm/µg DNA (Johnson, 1981).

DNA relatedness is determined by the percentage of reassociation in the heterologous reaction (reaction between labelled and unlabelled DNA from different sources) relative to that of the homologous reaction (labelled and unlabelled DNA from the same source). Relatedness is referred to as the relative binding ratio (RBR) (Steigerwalt et al., 1976). The equation (Brenner et al., 1978) used is:

RBR = % relatedness = [(% heterologous DNA bound/
% homologous DNA bound)x100].

The degree of relatedness is dependent upon the criteria (temperature and ionic strength) used for incubation. Optimal relatedness values are obtained at temperatures 25-30^{o}C below the temperature of denaturation for a given DNA (Marmur and Doty, 1961). Organisms may be referred to as closely or highly related if the percent relatedness is greater than or equal to 65, or distantly related if percentages less than 25 are obtained (Steigerwalt et al., 1976 and Brenner et al., 1978). At increased temperatures (supraoptimal) decreased binding may occur, but the thermal stability of the duplexes formed is greater due to reduced numbers of unpaired bases (Brenner and Falkow, 1971). The increased incubation temperature has little effect on reactions involving DNA from closely related organisms, but in all other reactions, the binding is decreased at the higher temperature as compared to the optimal temperature (Brenner, 1978).

MATERIALS AND METHODS

Organisms. The strains studied are listed in Table 8. Four of the American Type Culture Collection (ATCC) strains were chosen because they have been designated as type strains (Skerman, McGowan and Sneath, 1980). Included in this group are S. constellatus ATCC 27823, S. intermedius ATCC 27335 S. sanguis I ATCC 10556, and S.mitis ATCC 33399 (NCTC 3165). Although designated the type strain for S. mitis, ATCC 33399 was placed in the S. sanguis taxon by Colman and Williams (1972) and Cole et al. (1976). S. mitis ATCC 9895 was included due to an aberrant esculin hydrolysis reaction which casts doubt on its correct taxonomic designation within the viridans group. In addition, this strain has been referred to as Streptococcus MG in the literature. (Horsfall, 1951; Williamson, 1964; Weissman, 1966; Facklam, 1977) S. sanguis II, ATCC 10557, was included because of the controversy surrounding its inclusion in the S. sanguis taxon. Colman and Williams (1972) placed this strain in S. mitior. The group G strain and the two group F strains were chosen to represent beta hemolytic strains with biochemical patterns matching the S. anginosus - constellatus designation described by Facklam (1977). The remaining two strains (S. MG - intermedius SS 899 and S. mitis SS 429) are stock strains obtained from the Center for Disease Control (CDC) which yielded typical biochemical reactions as reported by Facklam (1977).

Frozen stock cultures were maintained at -70°C in trypticase soy broth (TSB) with 15% glycerol. Working cultures were maintained on trypticase soy agar containing 5% sheep blood.

Biochemical Reactions. A battery of biochemical tests was chosen from the physiological tests studied by Facklam (1977) for differentiation of viridans streptococci. Hemolytic action was determined microscopically after anaerobic incubation of sheep blood agar plates (trypticase soy agar with 5% sheep blood) inoculated by the streak-stab method (Facklam, 1980). Aerobic acid production was tested in heart infusion broth base (Difco) with bromocresol purple pH indicator (BBL) and one percent (w/v) final concentration of the following carbohydrates: glucose, inulin, lactose, mannitol, melibiose, raffinose, salicin, sorbitol, sucrose or trehalose (Difco). Esculin hydrolysis was determined in the basal medium containing .03% or 0.1% esculin (K and K Rare Chemicals) and no bromocresol purple indicator. Two drops of one percent ferric ammonium citrate (Baker) solution were added after incubation to detect esculin hydrolysis. Production of dextran was tested on heart infusion agar (Difco) with five percent sucrose (Difco). The ability to hydrolyze starch was tested on heart infusion agar (Difco) with two percent soluble starch (Difco). Reduction and curd production in litmus milk (Difco) were

also tested for each strain. Two drops of an overnight culture grown in 10 ml of Todd Hewitt Broth (THB, Gibco Diagnostics) served as inoculum for the physiological tests. Starch and five percent sucrose plates were incubated in five percent CO_2 at 35°C. All other media were incubated in air at 35°C. Reactions were read at 24 and 48 hours, or up to five days for poorly growing strains.

Serological Reactions. All strains were tested for group specific antigens with the Streptex rapid latex test kit (Wellcome). The extracts were prepared from twenty four hour cultures grown on trypticase soy agar with 5% sheep blood and the procedure was performed as directed in the package insert.

Growth and Harvest of Cells. A pure culture of the organism was obtained on trypticase soy agar with 5% sheep blood. THB (30 ml) was inoculated from a 24 hour blood agar plate and incubated overnight at 35°C. After gram staining and subculturing to check for purity, the overnight culture was centrifuged and the cell button was resuspended in fresh THB (10 ml). The resuspended cells were added to one liter of THB with one percent (w/v) phosphate buffer (Na_2HPO_4) and one percent (w/v) filter sterilized glucose. The pH was adjusted to 8.0 prior to autoclaving, and the final pH after addition of glucose was 7.5. Incubation for 18 hours at 37°C was usually carried out in a shaking water bath. Three

strains (Group G, S. sanguis I ATCC 10556 and S. sanguis II 10557), were grown overnight in a Microferm® Fermentor (New Brunswick Corp.) with the pH control set at 6.5. For growth of labelled cells, the 10 ml inoculum was added to 500 ml of the above medium containing 1 mCi [^{3}H] methyl thymidine (ICN Pharmaceuticals). (On occasion, it was also necessary to add 1 mCi [^{3}H] methyl thymidine to the 30 ml inoculum culture.) After overnight incubation, gram stains and subcultures were obtained so that physiologic reactions could be tested to confirm purity. Penicillin was added (10 units/ml of culture) and incubation was continued for twenty minutes (Coykendall, 1970). Sodium citrate (15 grams per liter) was then added to inhibit deoxyribonuclease (Perry and Slade, 1962) and the cells were harvested by centrifugation and washed three times in 25 ml distilled water (Coykendall, 1970).

Cell Lysis and Extraction of DNA. The washed cells were resuspended in 25 ml of 0.15 M tris (hydroxymethyl)amino-methane buffer (pH 8.0) containing ten milligrams of lysozyme (Coykendall, 1970). Incubation at 37°C for two to three hours followed. The cells were then centrifuged and resuspended in 25 ml of 0.1 M NaCl - 0.1 M Na citrate. Lysis was achieved by the addition of two milliliters of a 25% sodium lauryl sulfate solution and subsequent heating at 60°C for ten to thirty minutes. Following lysis, 1.25 ml of five percent pronase was added and the lysate was incubated

overnight at 37°C. DNA was isolated from the lysate by phenol and chloroform deproteinizations and precipitation with ethanol and isopropanol as follows (Coykendall, 1970; Marmur, 1961):

1. Add an equal volume of 25% (w/v) phenol in standard saline citrate buffer (SSC) to the lysate. (SSC = 0.15 M NaCl; 0.015 M trisodium citrate)

2. Gently swirl in an ice water bath for 30 minutes.

3. Centrifuge at 4080 x gravity for twenty minutes at 4°C.

4. Save the aqueous phase.

5. Add an equal volume of chloroform: isoamyl alcohol (24:1) to the aqueous phase and shake gently for 15 minutes.

6. Centrifuge at 4080 x gravity for 20 minutes at 4°C.

7. Repeat steps 4-6.

8. Remove the aqueous phase to a clean flask and precipitate the DNA by the addition of two volumes of cold ethanol. Spool the DNA onto a glass rod or centrifuge to sediment broken strands.

9. Dissolve the DNA in a minimum amount of SSC/100.

10. Incubate the dissolved nucleic acids at 37°C for 30 minutes with 50 µg/ml ribonuclease I (Marmur, 1961) and 30 µg/ml ribonuclease T_1 (Saito and Miura, 1963).

11. Repeat steps 1-8.

12. Dissolve the DNA in 9.0 ml SSC/100 and add 1.0 ml 3.0 M sodium acetate/0.001 M EDTA (pH=7.0).

13. Add 0.54 volumes of isopropyl alcohol dropwise while stirring rapidly.

14. Collect precipitated DNA on a glass stirring rod and rinse free of acetate and salt in increasing concentrations (70 - 95%) of ethyl alcohol.

15. Dissolve the DNA in a minimum amount of SSC/100 and check purity and yield.

Yield estimates were obtained by the following method. Following heat denaturation at 102°C for fifteen minutes, the absorbance of the single stranded DNA at 260 nm (Beckman Model 25 Spectrophotometer) was compared to a standard curve of calf thymus DNA. The ratio of extinctions at 260:230:280 nm was obtained to determine the purity of the DNA preparation. A ratio which approached or exceeded 1:0.43:0.55 indicated little protein or carbohydrate contamination (Marmur, 1961). Pure DNA preparations of sufficient yield were stored in one milliliter aliquots at -20°C until required for hybridization experiments.

In addition to determination of purity and yield, specific activity measurements were required for labelled DNA. One milliliter of a 1 µg/ml sample of the labelled DNA was counted for one minute on a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 3320) to determine the counts per minute per microgram.

DNA Hybridization. DNA was hybridized on 22mm BA 85 membrane filters (Schleicher and Schuell) according to the dimethyl sulfoxide (DMSO) method. (Legault - Démare et al., 1967; DeLey and Tijtgat, 1970). Unlabelled DNA was denatured by heating at 102°C for 15 minutes (concentration of

the stock DNA was adjusted to 140 - 160 µg/ml in SSC/100 prior to denaturation). Following denaturation, the DNA was chilled rapidly by placing the tube on ice, and the exact concentration was determined by the absorbance at 260 nm. It was then possible to determine the volume of DNA required for addition of 60 µg of DNA to each filter. The filters were soaked in water overnight and in cold 6xSSC for 30 minutes, before being placed on a Millipore filter apparatus and pre-rinsed with 5 ml of ice cold 6xSSC. (Coykendall, Kaczmarek and Slots, 1980). The DNA was then added (with 5 ml ice cold 6xSSC) and the filter was rinsed with an additional 5 ml ice cold 6xSSC (A.L. Coykendall - personal communication). The filters were dried at room temperature, and then overnight at 60°C before storage in a dessicator at room temperature (Johnson, 1981). Blank filters, (lacking DNA) were prepared using the same procedure.

The filters were placed in glass liquid scintillation vials containing 1.0 ml of 30% (vol/vol) dimethyl sulfoxide in 2xSSC (2SSC/DMSO) for thirty minutes (Coykendall et al., 1980). During this time period, ^{3}H labelled index DNA was prepared. Radioactive DNA was sheared by sonic treatment for one minute (15 sec x 4) on continuous cycle using a micro tip with output set at 2 (Branson Sonic Power Company Sonifier 350). The sheared, radiolabelled DNA was then denatured for 15 minutes at 102°C and the concentration was confirmed by checking the absorbance at 260 nm. The

sheared, denatured, [^{3}H] DNA was diluted to 50 µg/ml in cold 2xSSC, and 10 µl (0.5 µg) was added to each scintillation vial. Each reaction was run in triplicate. Negative controls (filters bearing calf thymus DNA) and homologous controls (filters bearing DNA from the same strain which served as the labelled index DNA) were included with each experiment. In addition, blank filters served as a control for nonspecific binding of radioactive DNA to the filters. Reassociation of the labelled DNA with the unlabelled, filter immobilized DNA was allowed to proceed for 16 hours at 48.4^{o}C in a water bath. The filters were then rinsed at the incubation temperature by passing each filter through six beakers (100 ml each) of 2xSSC. The rinsed filter was placed in a clean glass liquid scintillation vial, to which 10 ml Aquasol (New England Nuclear) was added. Vials were stored at room temperature for approximately thirty hours (to allow time for the filters to dissolve) before quantitating the hybridized [^{3}H] DNA by liquid scintillation.

RESULTS

Physiological Characteristics

The results of the biochemical and serological tests are presented in Table 9. Each strain was tested with the battery of tests a minimum of three times. The esculin hydrolysis results with .03% esculin were confirmed with the 0.1% esculin formulation used at the Center for Disease Control in Atlanta, Georgia (Dowell and Hawkins, 1977).

The ability to hydrolyze esculin was an aberrant characteristic of S. mitis ATCC 33399 and S. mitis ATCC 9895 based upon the identification scheme proposed by Facklam (1977). The production of acid from inulin by S. mitis, ATCC 33399, was also atypical according to Facklam's (1977) scheme. S. mitis SS 429 was the only typical S. mitis strain tested, as it conformed to all the biochemical characteristics for S. mitis as described by Facklam (1977). This strain reacted weakly with group C antisera. None of the other S. mitis strains reacted with group A,B,C,D, F or G antisera.

The two group F isolates (minute beta hemolytic colonies on 5% sheep blood agar) were identical in their carbohydrate reactions to S. constellatus ATCC 27823 and the Group G strain. Except for some variation in the ability to

hydrolyze starch, these four isolates reacted identically in the remaining biochemical tests. Some variation was also evident in the serological grouping as S. constellatus ATCC 27823 did not react with group A,B,C,D,F or G antisera. Also noted was the inability of S. constellatus ATCC 27823 to hemolyze sheep red blood cells, while the other three isolates produced beta hemolysis.

S. MG-intermedius SS 899 was biochemically identical to S. intermedius ATCC 27335 although variation occurred with the serological grouping. S. intermedius ATCC 27335 reacted with group G antisera and S. MG-intermedius SS 899 reacted weakly with group F antisera. Both strains were non-hemolytic on trypticase soy agar plates with 5% sheep blood.

The reactions for S. sanguis I ATCC 10556 and S. sanguis II ATCC 10557 agreed with those reported by Facklam (1977). S. sanguis II ATCC 10557 showed some variability in the production of acid from melibiose and salicin upon repeat testing, and S. sanguis I ATCC 10556 showed variable esculin hydrolysis results in .03% esculin. Variability in these results did not alter the identification of the organisms when using Facklam's (1977) scheme.

Hybridizations. The unlabelled DNA in the homologous reactions bound 36% to 86% of the total labelled DNA ($\bar{x}$ = 51.2, n = 10). Blank filters bound 2.4% to 7.0% of the

labelled DNA ($\bar{x}$ = 3.4, n = 10). The relative binding ratio (RBR) in heterologous reactions (expressed as percent of homologous binding) remained consistent for most strains even if variation occurred in the percent of homologous binding in subsequent experiments. The mean standard deviation of the RBR obtained for a specific pairing in repeat experiments was 9.6. Examination of reverse reactions (A[^{3}H] + B and A + B[^{3}H]) revealed good correlation ($\pm$ 10%) for most pairings, although a tendency toward lower relative binding ratios in heterologous reactions involving unlabelled DNA from S. intermedius ATCC 27335 was noted. The reason for this phenomenon was not clear.

The results of the hybridization experiments are shown in Table 10 and reflect the average relative binding ratios obtained in several experiments. High relative binding ratios (72% to 93%) were obtained with labelled DNA from S. constellatus ATCC 27823 (type strain) and unlabelled DNA from the following stains: S. intermedius ATCC 27335 (type strain), S. MG-intermedius SS 899, Group F SFGH 879, Group F MGH 8153, and S. mitis ATCC 9895. This grouping was confirmed with results from two separate experiments utilizing labelled DNA from S. intermedius ATCC 27335, (type strain), and S. mitis ATCC 9895. Most of the pairings gave relative binding ratios above 70% although three of the values were slightly lower. Unlabelled DNA from S. MG-intermedius

SS-899, hybridized at a very high level (RBR = 104%) with labelled DNA from S. mitis ATCC 9895.

Hybridization experiments using unlabelled DNA from the six strains described above and labelled DNA from S. mitis ATCC 33399 (type strain), and S. sanguis II ATCC 10557 did not yield any relative binding ratios above 45%, although several pairings with S. mitis ATCC 33399 approached this figure.

Unlabelled DNA from the Group G strain hybridized at very low levels (RBR less than 25%) with each labelled index DNA tested.

Low to moderate DNA relatedness values (RBR 13%-47%) were obtained when unlabelled DNA from S. sanguis I ATCC 10556 (type strain) or S. sanguis II ATCC 10557 was incubated with each labelled index DNA.

Unlabelled DNA from S. mitis ATCC 33399 (type strain), yielded relative binding ratios below 48% with each labelled index DNA. The highest RBR (47%) was with DNA from S. sanguis II ATCC 10557. S. mitis SS 429 gave comparable results to S. mitis ATCC 33399 in experiments with labelled index DNA.

The unlabelled DNA from S. mitis ATCC 9895, yielded low relative binding ratios (36% and 20%) with labelled DNA from S. mitis ATCC 33399 (type strain) and S. sanguis II ATCC 10557, respectively. The extent of hybrid formation was greatest with labelled DNA from S. constellatus ATCC 27823 (type strain) and S. intermedius ATCC 27335 (type strain) as indicated earlier.

DISCUSSION

Choice of Procedures. Biochemical tests were selected from a battery of tests recommended by Richard Facklam (Center for Disease Control - Bacteriology Division) for the identification of viridans streptococci. These tests have served as the reference method for the recent development of several identification systems for viridans streptococci.

Procedures for the isolation and purification of labelled and unlabelled DNA were carefully chosen since the majority of the technical problems encountered in hybridization experiments occur in these areas. For some of the more fastidious strains, such as S. intermedius ATCC 27335 and S. constellatus ATCC 27823 it was difficult to obtain sufficient cell volumes to yield adequate amounts of DNA. Several different liquid media were employed, but none gave significantly larger yields for S. intermedius than incubation in THB (1% Glucose; 1% Na_2HPO_4). Cell yields of S. constellatus were increased by supplementing the THB (1% Glucose; 1% Na_2HPO_4) with 0.1% Tween 80 or 0.1% CVA (Co-factors, vitamins and amino acids, Gibco Diagnostics).

The specific activities of labelled DNA, (1,200-6,500 cpm/µg DNA), obtained from cells grown in THB (500 ml) with addition of 1 mCi methyl ^3H thymidine were lower than desired for initial plans to employ the free solution

hybridization technique (Johnson, 1981). Attempts to increase specific activities with substitution of 50 µCi ^{14}C thymidine were not successful. Because the membrane filter hybridization method requires lower specific activity DNA preparations (10^3 cpm/µg or greater) (Johnson, 1981), a decision was made to follow that technique rather than a free solution procedure.

The treatment of cultures with penicillin prior to cell harvest and subsequent lysis procedures utilizing lysozyme and detergent were essentially those of Coykendall (1970) due to the special problems encountered with streptococci. While gram negative organisms are readily lysed by sodium lauryl sulfate alone (Marmur, 1961), streptococci usually require treatment with lysozyme prior to addition of the detergent if maximum lysis is to be obtained. The addition of penicillin prior to harvest of the cells improved lysis, (thereby increasing DNA yields), according to Coykendall and Specht (1975). The isolation of DNA from the lysate required a combination of phenol (Coykendall, 1970) and chloroform - isoamyl alcohol (Marmur, 1961) procedures to result in sufficiently pure DNA preparations. Not all strains required the isopropanol extraction step (Marmur, 1961), and it was omitted when possible to reduce unnecessary loss of DNA.

The decision to store purified DNA preparations at -20°C was based upon the study by Crombach (1973) which indicated that storage at that temperature for up to one year did not significantly affect results of hybridization experiments.

Two different membrane filter hydridization methods were considered, the Denhardt (1966) method and the DMSO technique (Leqault-Démare et al., 1967; Deley and Tijtgat, 1970). The disadvantage of the Denhardt method proved to be the 5-6 hour preincubation step where the filters containing immobilized, unlabelled DNA were immersed in 3xSSC with 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine albumin. The purpose of the preincubation step prior to the addition of labelled DNA was to prevent nonspecific binding of the labelled, single stranded DNA to the filters, while still allowing the specific binding of single stranded DNA fragments to complementary strands previously immobilized on the filter. It was necessary to remove the caps of the reaction vials for the addition of labelled DNA, and then re-cap the vials for an additional 16 hour incubation period at 67°C.

The DMSO procedure has several advantages over the Denhardt method. First, no preincubation is necessary, as nonspecific attachment of labelled single stranded DNA to the filter is prevented by hybridization in the presence of DMSO (Legault-Démare et al., 1967). In addition, a lower

temperature is required for hybridization, since the melting point of DNA in 2SSC-DMSO is 19°C lower than in 2SSC alone (De Ley and Tijtgat, 1970). Legault-Démare et al. (1967) reported an optimum temperature of 40-50°C in 2SSC/DMSO. De Ley and Tijtgat (1970) indicated that the highest temperature required for the DMSO technique is 66°C and that the procedure can be recommended for the entire guanine + cytosine (% G+C) range. Their studies on the effect of temperature on the release of filter fixed DNA revealed potential problems with the higher temperature and preincubation period required by the Denhardt method.

The DMSO procedure was chosen for this work due to the previously mentioned advantages over the Denhardt method. The BA 85 membrane filters (Schleicher and Schuell) used are synonomous with earlier references to B-6 filters (Schleicher and Schuell) (Denhardt, 1966; Coykendall, 1971). Filters measuring 22 mm were required to avoid crimping of the edges in the reaction vials. The decision to add 60 µg of unlabelled DNA to the filters, resulting in approximately 25 µg/cm^2, was based on calculations presented by Johnson (1981). (The effective filtering surface was 2.5 cm^2). The procedure for immobilizing the unlabelled DNA on the filters and the preparation of labelled DNA and subsequent addition of 0.5 µg to the reaction mix (1.0 ml 2SSC-DMSO) followed Coykendall's recommendation. (A.L. Coykendall-personal communication). The final ratio of unlabelled to

labelled DNA in the experiments was 120:1. This met the requirement for an excess number of available reassociation sites for the labelled fragments (Brenner and Falkow, 1971). Some experiments were run in parallel with reaction vials containing 1.0 µg labelled DNA rather than 0.5 µg. This slight variation in the concentration of labelled DNA did not alter the relative binding ratios. Johnson and Ordal (1968) observed the same phenomenon using the Denhardt technique.

The optimal renaturation rate temperature (T_{OR}) for the hybridization experiments in 2SSC-DMSO was determined from the equation presented by De Ley and Tijtgat (1970). [T_{OR} = 0.51 x % G+C + 28.0] An average DNA base composition of 40% G+C was used in the calculation of the optimal renaturation rate temperature. Several factors influenced the decision to use an average percent G+C value. Varying base contents have been reported in the literature for viridans streptococci. Bergey's Manual of Determinative Bacteriology (1974) reports G+C values ranging from 38%-42% for several species of viridans streptococci. Coykendall and his associates have reported values ranging from 36%-46% for strains of S. mutans and S. sanguis (Coykendall and Specht, 1975; Coykendall and Lizotte, 1978). Past observations have shown that the base composition of a given genus has an average standard deviation of 4.06% G+C (De Ley, 1969), although different techniques of base composition

determination may yield slightly different values for the same organism. If the lower limit (36%) of the base contents reported for viridans streptococci had been used for calculations, the optimum temperature for renaturation would have been 46.4°C. Calculations utilizing the upper limit reported (46% G+C) result in an optimum renaturation temperature of 51.5°C. The temperature chosen, (48.4°C) is approximately halfway between these two values, and is appropriate for these preliminary studies, based on the observation by DeLey and Tijtgat (1970) that "for organisms which are very closely related the temperature used is rather unimportant." They also indicated that the optimum temperature for reassociation is affected more by the method used than by slight differences in the percent G+C of the organisms tested.

In addition to controlling temperature, ionic strength and time for the hybridization experiments, the volume of water in the water bath was an important factor to monitor. According to Johnson, (1981) condensation inside the vial may alter ionic strengths and nucleic acid concentrations. In an effort to minimize the possibility of a condensation effect on these experiments, the water level was kept above the shoulder of the reaction vials throughout the incubation period.

Interpretation of Results. The DNA hybridization results indicated a close relationship (RBR $\geq$ 65%) (Steigerwalt, 1976) among the following strains: 1. S. constellatus ATCC 27823 (type strain) 2. S. intermedius ATCC 27335 (type strain) 3. S. mitis ATCC 9895 4. S. MG - intermedius SS 899 5. Group F SFGH 879 and 6. Group F MGH 8153. The inclusion of S. mitis ATCC 9895 in this group is not surprising in view of past references in the literature which referred to this strain as S. MG. (Horsfall, 1951; Williamson, 1964; Weissman 1966, Facklam, 1977) The physiologic test results for these six organisms fell into two main groups. Lactose fermenting, non-hemolytic strains included S. mitis, S. intermedius, and S. MG - intermedius. These strains yielded identical results in the biochemical reactions tested, although serological reactions varied. The second group did not ferment lactose and included S. constellatus and both Group F isolates. The lactose negative strains showed variation in hemolytic ability and starch hydrolysis reactions in addition to variable serological results. The observation that the nonhemolytic Group F strain (SS 899) produced acid from lactose while the beta hemolytic group F strains were lactose negative agreed with findings described by Ottens and Winkler (1962). These investigators used cross- absorption tests to show that the group antigen of group F streptococci is identical, regardless of the hemolytic ability of the organism.

The grouping of these six strains based on physiologic tests alone follows Facklam's (1977) description of the lactose negative S. anginosus - constellatus group and the lactose positive S. MG. - intermedius group, although beta hemolytic strains were not included in his study.

The genetic data, however, supports the findings of Colman and Williams (1972) who grouped serologically heterogeneous strains with varying abilities to hemolyze red blood cells and ferment lactose into S. milleri. Lancefield groups A,C,F and G were present among the strains examined by Colman and Williams (1972). These investigators found that it was more difficult to separate the minute beta hemolytic streptococci of groups F and G from S. milleri, and they proposed that these strains be considered "varieties" of S. milleri. The hybridization results obtained in this study also indicated that minute beta hemolytic Group F strains should be included in the S. milleri group. Colman and Williams (1972) demonstrated that minute beta hemolytic strains which reacted with Lancefield antisera could be differentiated physiologically from pyogenes-like (i.e. large colony types) strains of the same serological group. This study demonstrated that a pyogenes-like Group G strain has a low genetic relatedness to S. milleri strains, some of which possess the group G antigen, even though the reactions for the physiologic tests chosen were identical.

Collectively, the data from this study implies that certain investigators have divided the S. milleri group of organisms into too many species. In addition, erroneous identification of strains in some culture collections (i.e., S. mitis ATCC 9895) may have occurred in the past. Further hybridization studies utilizing a greater number of S. milleri-like strains and higher incubation temperatures are needed to confirm the optimal temperature reassociation percentages obtained in this study which indicate that these strains are members of the same species.

Interpretation of the data for the S. sanguis and S. mitis strains is more difficult and reflects the problems encountered by earlier investigators who have studied these organisms. Physiologically, except for lack of glucan production (if this is viewed as a stable characteristic), S. mitis ATCC 33399 (type strain) would be classified as S. sanguis I due to acid production in inulin and hydrolysis of esculin. Colman and Williams (1972) and Cole et al. (1976) classified ATCC 33399 (NCTC 3165) in S. sanguis on the basis of physiological and serological findings. The hybridization experiments performed in this study did not show a high relatedness between S. mitis ATCC 33399 and S. sanguis I ATCC 10556 (type strain) or S. sanguis II ATCC 10557. These findings support the classification of Cole et al. (1976), who put these three strains into three separate

groups on the basis of their physiological and serological test results in addition to their antigenic composition as defined by Rosan (1973).

The hybridization results from this study show a low RBR (13%) for the heterologous reaction involving S. sanguis I ATCC 10556 and S. sanguis II ATCC 10557. This confirms Coykendall and Specht's (1975) data from DNA reassociation studies involving ATCC 10556 and ATCC 10557. They found that these two strains fell into two distinct genetic groups, group 3 (S. sanguis subsp. carlsonnii) and group 2 respectively, and concluded that members of group 2 should not be considered members of S. sanguis. The other genetic group (group 1) described by Coykendall and Specht (1975), includes members which are typically S. sanguis. Further studies may show that S. mitis ATCC 33399 is a member of group 1, (S. sanguis subsp. sanguis), thus explaining the only moderate relatedness (47%) to ATCC 10556 (S. sanguis subsp. carlsonnii), obtained in this study.

The physiological characteristics of S. mitis SS 429 are typical of S. mitis strains. This strain did not hybridize significantly with any of the index strains tested. The relative binding ratios obtained with unlabelled SS 429 DNA indicated only moderate relatedness to S. sanguis II ATCC 10557 and the S. milleri group. The moderate relatedness (41%) shown in the heterologous reaction with unlabelled DNA

from SS 429 and labelled DNA from ATCC 33399 indicates that these two strains probably do not belong in the same species. Further hybridization studies involving many strains which have been previously classified as S. sanguis or S. mitis will be required to firmly establish the taxonomic boundaries of these organisms.

CONCLUSION

This study represents an effort to clarify the classification of some of the viridans streptococci by means of DNA hybridization experiments in addition to the more commonly employed physiologic and serologic tests. The results of the hybridization experiments indicated that six of the strains tested (S. intermedius ATCC 27335 [type strain], S. constellatus ATCC 27823 [type strain], S. mitis ATCC 9895, S. MG - intermedius SS 899, Group F 879 and Group F 8153) are closely related to each other genetically even though variable results were obtained in physiologic tests. The relative binding ratios for these strains ranged from 60% - 99%. The majority of the pairings resulted in an RBR >85%, indicating that these strains should be considered members of the same species (S. milleri). The data obtained for the strains of S. sanguis and S. mitis tested were not sufficient to recommend any definite taxonomic changes since the majority of the relatedness values obtained in the hybridization experiments were in the moderate range (25%-50%). The possible incorrect identification of S. mitis ATCC 33399 (type strain) was implied by 1. Biochemical results which were more typical of S. sanguis I, and 2. Low genetic relatedness in hybridization experiments to a strain (SS 429) which had biochemical reactions typical of S. mitis. The need for further studies involving members of S. milleri, S. sanguis and S. mitis was sugggested. The

remaining species of viridans streptococci mentioned in the introduction of this paper should also be studied from a genetic viewpoint for a thorough characterization of this entire group of organisms.

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TABLE 1. REACTIONS OF *S. MUTANS*
(% of strains tested)

INVESTIGATOR	CARLSSON (1968)	COLMAN (1972)	FACKLAM (1977)	PULLIAM (1980)
NO. OF STRAINS	9 (Group II)	12	152 (Clinical)	7
HEMOLYSIS				
ALPHA	0	33	59	14
BETA	33	0	11	0
NONE	67	67	29	86
GROWTH				
40% BILE	NT[1]	50	50	NT
10% BILE	NT	100	71	NT
45°C	11	17	42	NT
4% NaCl BROTH	100	100	44	NT
ACID PRODUCTION				
GLYCEROL (aerobic)	0	8	0	NT
INULIN				
AUTOCLAVE	100	58	99	86
FILTER STERILIZED	89	NT	NT	NT
LACTOSE	100	100	99	100
MANNITOL	89	100	100	100
MELIBIOSE	89	NT	38	NT
RAFFINOSE	100	33	85	71
SALICIN	100	100	92	NT
SORBITOL	100	100	98	71
SUCROSE	NT	100	100	100
TREHALOSE	100	100	100	NT
HYDROLYSIS				
ESCULIN	100	75	90	100
STARCH	0	0	3	NT
ARGININE	11	8	1	NT
DEXTRAN FORMATION	NT	100	100	100

[1]Not tested

TABLE 2. REACTIONS OF *S. SALIVARIUS*
(% of strains tested)

INVESTIGATOR	CARLSSON (1968)	COLMAN (1972)	FACKLAM (1977)	PULLIAM (1980)
NO. OF STRAINS	13 (GROUP III)	10	81 (Clinical)	4
HEMOLYSIS				
ALPHA	0	0	10	0
BETA	0	0	0	0
NONE	100	100	90	100
GROWTH				
40% BILE	[1]NT	50	28	NT
10% BILE	NT	90	59	NT
45°C	0	10	69	NT
4% NaCl BROTH	38	0	21	NT
ACID PRODUCTION				
GLYCEROL (aerobic)	0	0	0	NT
INULIN				
AUTOCLAVE	100	50	100	100
FILTER STERILIZED	38	NT	NT	NT
LACTOSE	85	80	89	100
MANNITOL	0	0	0	0
MELIBIOSE	0	NT	19	NT
RAFFINOSE	85	70	95	100
SALICIN	100	100	88	NT
SORBITOL	0	0	0	0
SUCROSE	NT	100	100	100
TREHALOSE	85	100	68	NT
HYDROLYSIS				
ESCULIN	100	100	91	100
STARCH	0	0	7	NT
ARGININE	0	0	0	NT
DEXTRAN FORMATION	NT	0	11	0

[1]Not tested

TABLE 3. REACTIONS OF *S. MITIS*

(% of strains tested)

INVESTIGATOR	CARLSSON (1968)	CARLSSON (1968)	COLMAN (1972)	FACKLAM (1977)	PULLIAM (1980)
NO. OF STRAINS	19 (GROUP VA)	12 (GROUP VB)	46	177 (Clinical)	29
HEMOLYSIS					
ALPHA	84	100	78	92	59
BETA	16	0	22	0	0
NONE	0	0	0	8	41
GROWTH					
40% BILE	NT[1]	NT	2	14	NT
10% BILE	NT	NT	13	28	NT
45°C	0	17	0	40	NT
4% NaCl BROTH	0	8	2	14	NT
ACID FROM					
GLYCEROL AEROBIC	0	0	0	0	NT
INULIN					
AUTOCLAVE	32	8	7	0	0
FILTER STERILIZED	21	8	NT	NT	NT
LACTOSE	100	100	74	100	100
MANNITOL	NT	NT	0	0	0
MELIBIOSE	58	67	NT	1	NT
RAFFINOSE	58	67	59	0	0
SALICIN	42	83	13	28	NT
SORBITOL	0	0	0	0	0
SUCROSE	NT	NT	85	100	100
TREHALOSE	42	42	22	25	NT
HYDROLYSIS					
ESCULIN	0	33	4	0	0
STARCH	0	0	13	33	NT
ARGININE	11	100	7	16	NT
DEXTRAN FORMATION	NT	NT	11	13	NT

[1]Not tested

TABLE 4. REACTIONS OF *S. SANGUIS* I
(% of strains tested)

INVESTIGATOR	CARLSSON (1968)	COLMAN (1972)	FACKLAM (1977)	PULLIAM (1980)
NO. OF STRAINS	25 (GROUP IB)	35	202 (Clinical)	22
HEMOLYSIS				
ALPHA	100	69	94	68
BETA	0	31	0	0
NONE	0	0	6	32
GROWTH				
40% BILE	NT[1]	37	42	NT
10% BILE	NT	71	73	NT
45°C	16	0	63	NT
4% NaCl BROTH	88	3	28	NT
ACID PRODUCTION				
GLYCEROL (aerobic)	0	0	0	NT
INULIN				
AUTOCLAVE	96	49	100	100
FILTER STERILIZED	96	NT	NT	NT
LACTOSE	100	86	94	100
MANNITOL	0	0	0	0
MELIBIOSE	44	NT	38	NT
RAFFINOSE	48	40	45	36
SALICIN	100	91	91	NT
SORBITOL	8	0	12	0
SUCROSE	NT	100	99	100
TREHALOSE	100	80	95	NT
HYDROLYSIS				
ESCULIN	72	57	77	86
STARCH	0	31	56	NT
ARGININE	100	91	64	NT
DEXTRAN FORMATION	NT	57	84	NT

[1]Not tested

TABLE 5. REACTIONS OF *S. SANGUIS* II
(% of strains tested)

INVESTIGATOR	CARLSSON (1968)	FACKLAM (1977)	PULLIAM (1980)
NO. OF STRAINS	15	231 (Clinical)	30
HEMOLYSIS			
ALPHA	100	95	75
BETA	0	0	0
NONE	0	5	25
GROWTH			
40% BILE	NT[1]	16	NT
10% BILE	NT	34	NT
45°C	33	60	NT
4% NaCl BROTH	0	18	NT
ACID FROM			
GLYCEROL (AEROBIC)	0	0	NT
INULIN			
AUTOCLAVE	7	0	0
FILTER STERILIZED	7	NT	NT
LACTOSE	100	100	100
MANNITOL	0	0	0
MELIBIOSE	87	82	NT
RAFFINOSE	87	100	100
SALICIN	20	28	NT
SORBITOL	0	0	0
SUCROSE	NT	100	100
TREHALOSE	40	38	NT
HYDROLYSIS			
ESCULIN	0	0	0
STARCH	0	30	NT
ARGININE	0	21	NT
DEXTRAN FORMATION	NT	49	NT

[1]Not tested

TABLE 6. REACTIONS OF *S. MILLERI* GROUP
(*S. milleri*, *S.* MG-intermedius, *S. anginosus*-*constellatus*, *S.intermedius*-MG-*anginosus* and *S. constellatus*)
(% of strains tested)

INVESTIGATOR	COLMAN (1968)	FACKLAM (1977)	FACKLAM (1977)	PULLIAM (1980)	PULLIAM (1980)
NO. OF STRAINS	42 (*milleri*)	231 (MG-*int*) (Clinical)	55 (*ang*-*con*) (Clinical)	51 (*int*-MG-*ang*)	18 (*con*)
HEMOLYSIS					
ALPHA	7	45	40	18	17
BETA	0	0	0	14	50
NONE	93	55	60	68	33
GROWTH					
40% BILE	48	52	38	NT	NT
10% BILE	76	68	49	NT	NT
45°C	2	48	22	NT	NT
4% NaCl broth	10	34	18	NT	NT
ACID FROM					
GLYCEROL	0	0	0	NT	NT
INULIN	7	0	0	0	0
LACTOSE	95	100	0	100	0
MANNITOL	2	0	0	0	0
MELIBIOSE	NT	18	6	NT	NT
RAFFINOSE	7	18	9	20	0
SALICIN	100	84	60	NT	NT
SORBITOL	2	0	0	0	0
SUCROSE	98	100	100	100	100
TREHALOSE	90	73	64	NT	NT
HYDROLYSIS					
ESCULIN	86	100	73	98	61
STARCH	2	23	20	NT	NT
ARGININE	98	26	24	NT	NT
DEXTRAN FORMATION	0	12	0	NT	NT

[1]Not tested

TABLE 7. REACTIONS OF *S. MORBILLORUM*
(% of strains tested)

INVESTIGATOR	FACKLAM (1977)	PULLIAM (1981)
NO. OF STRAINS	46 (Clinical)	3
HEMOLYSIS		
ALPHA	50	0
BETA	0	0
NONE	50	100
GROWTH		
40% BILE	0	NT
10% BILE	0	NT
45°C	13	NT
4% NaCl	1	NT
ACID FROM		
GLYCEROL	0	NT
INULIN	0	0
LACTOSE	0	0
MANNITOL	0	0
MELIBIOSE	0	NT
RAFFINOSE	0	0
SALICIN	4	NT
SORBITOL	0	0
SUCROSE	62	100
TREHALOSE	11	NT
HYDROLYSIS		
ESCULIN	0	0
STARCH	9	NT
ARGININE	0	NT
DEXTRAN FORMATION	0	0

[1]Not tested

TABLE 8. STRAINS USED IN THE STUDY

SENDER'S IDENTIFICATION	SOURCE	SPECIMEN NO.	SPECIMEN CODE
S. constellatus[1]	ATCC	27823	a
S. intermedius[1]	ATCC	27335	b
S. mitis	ATCC	9895	c
S. mitis[1]	ATCC	33399	d
S. sanguis I[1]	ATCC	10556	e
S. sanguis II	ATCC	10557	f
Group F	MGH[2]	8153	g
Group F	SFGH[3]	879	h
Group G	SFGH	1235	i
S. mitis	CDC	SS 429	j
S. MG-intermedius	CDC	SS 899	k

[1] Type strain (Skerman, McGowan and Sneath, 1980)

[2] Massachusetts General Hospital, Dr. L. Kunz

[3] San Francisco General Hospital, Dr. W. K. Hadley

TABLE 9. BIOCHEMICAL AND SEROLOGICAL REACTIONS OF STRAINS USED IN THE STUDY[1]

Strain	Hemolysis	Glucose	Inulin	Lactose	Mannitol	Melibiose	Raffinose	Salicin	Sorbitol	Sucrose	Trehalose	Litmus Milk	Esculin Hydrolysis (0.3%)	Esculin Hydrolysis (0.1%)	Starch Hydrolysis	Arginine Hydrolysis	5% Sucrose	Serological Reaction
S. constellatus[2a]	γ	+	-	-	-	-	-	+	-	+	+	A/NR	-	-	-	+	NA	NR
S. intermedius[2b]	γ	+	-	+	-	-	-	+	-	+	+	A/C	+	+	-	+	NA	G
S. sanguis I[2e]	α	+	+	+	-	-	-	$+_w$	-	+	+	A/C	V	+	+	+	AD	NR
S. mitis[2d]	γ	+	+	+	-	-	-	+	-	+	+	A/NR	+	+	+	+	NA	NR
S. mitis[c]	γ	+	-	+	-	-	-	+	-	+	+	A/C	+	+	w	+	NA	F
S. sanguis II[f]	α	+	-	+	-	V	+	V	-	+	-	A/C	-	-	+	-	AD	NR
Group F[h]	β	+	-	-	-	-	-	+	-	+	+	A/NR	-	-	+	+	NA	F
Group F[g]	β	+	-	-	-	-	-	+	-	+	+	A/NR	-	-	+	+	NA	F
Group G[i]	β	+	-	-	-	-	-	+	-	+	+	A/NR	-	-	-	+	NA	G
S. mitis[j]	α	+	-	+	-	-	-	+	-	+	-	A/C	-	-	-	-	NA	C_w
S. MG-intermedius[k]	γ	+	-	+	-	-	-	+	-	+	+	A/C	+	+	-	+	NA	F_w

[1]Abbreviations: w=weak reaction; v=variable reaction; A=Acid production; C=Curd; NA=Nonadherent; AD=Adherent; NR=No Reaction

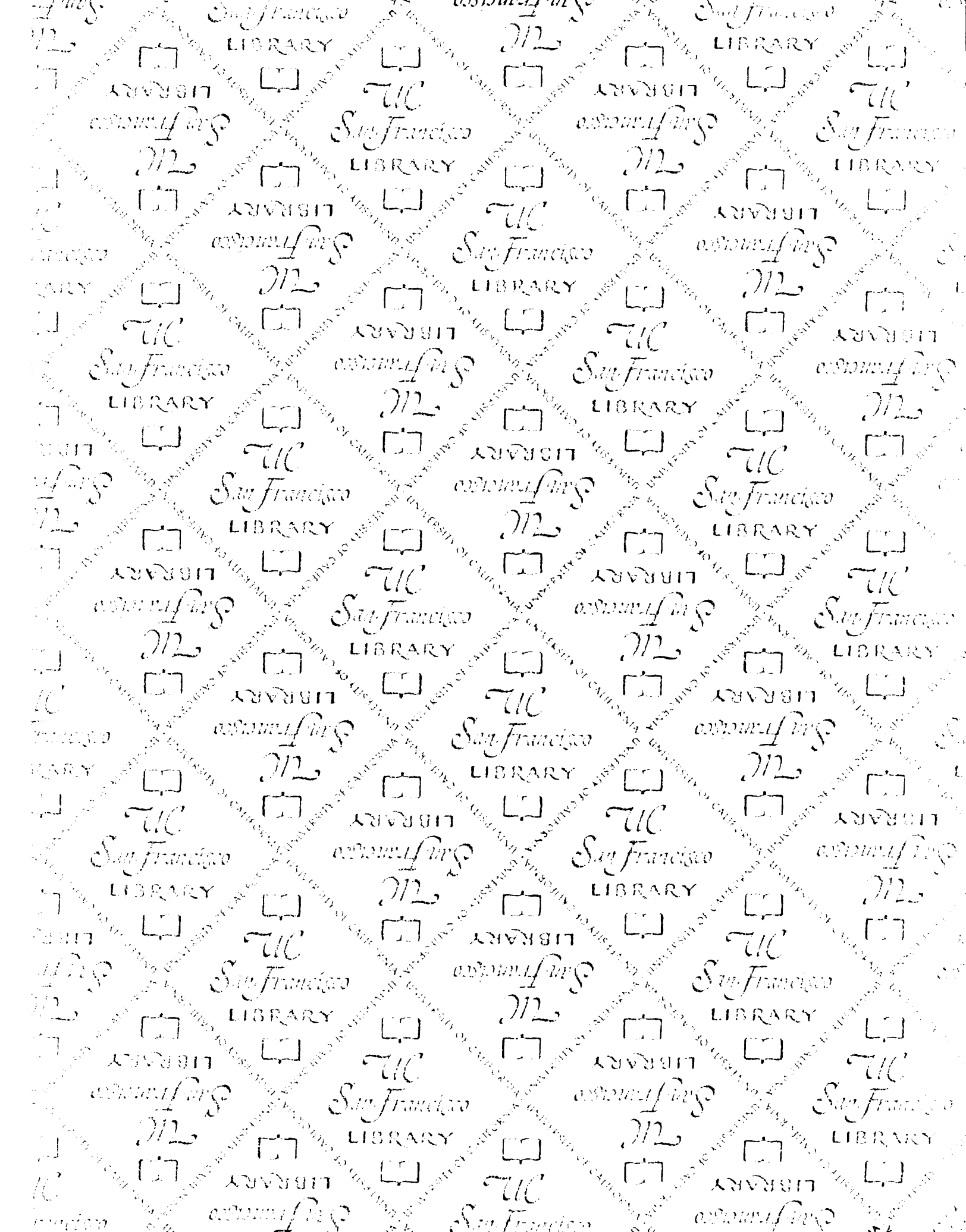
[2]Type strain

a-k Refer to Table 8, p. 55

TABLE 10. RELATIVE BINDING RATIOS

	ATCC[1] 33399[1] [^{3}H]	ATCC 10557 [^{3}H]	ATCC[1] 27823[1] [^{3}H]	ATCC[1] 27335[1] [^{3}H]	ATCC 9895 [^{3}H]
S.constellatus[1] ATCC 27823	37	21	100	89	75
S.intermedius[1] ATCC 27335	20	20	76	100	64
S. MG - int CDC SS 899	45	20	91	99	104
Group F SFGH 879	37	16	93	93	59
Group F MGH 8153	40	14	72	91	62
S.mitis ATCC 9895	36	20	81	90	100
S.mitis CDC SS 429	41	39	27	40	22
S.mitis[1] ATCC 33399	100	47	35	37	27
S.sanguis I[1] ATCC 10556	47	13	18	37	15
S.sanguis II ATCC 10557	40	100	22	36	16
Group G SFGH 1235	23	10	21	21	10
Negative Control (Calf Thymus DNA)	1.7	.02	1.2	1.6	3.2

[1]Type strain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