JOURNAL OF BACTERIOLOGY, Apr. 1971, p. 192-196
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Vol. 106, No. 1
Printed in U.S.A.

Genetic Heterogeneity in *Streptococcus mutans*[1]

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Received for Publication 14 January 1971

The genetic homogeneity among eight cariogenic strains of *Streptococcus mutans* was assessed by deoxyribonucleic acid (DNA)-DNA reassociation experiments. DNA species were extracted from strains GS5, Ingbritt, 10449, FA1, BHT, E49, SL1, and K1R. Labeled DNA (^{14}C-DNA) was extracted from strains 10449, FA1, and SL1. Denatured ^{14}C-DNA fragments were allowed to reassociate, i.e., form hybrid duplexes, with denatured DNA immobilized on membrane filters incubated in 0.45 M NaCl-0.045 M sodium citrate at 67 or 75 C. At 67 C, 10449 ^{14}C-DNA reassociated extensively only with GS5 and Ingbritt DNA. FA1 ^{14}C-DNA hybridized extensively only with BHT DNA, and SL1 ^{14}C-DNA reassociated with K1R and E49 DNA. DNA which hybridized extensively at 67 C also reassociated to a high degree at 75 C. Thermal elution of ^{14}C-FA1-BHT duplexes showed that the hybrid duplexes were thermostable. The results indicate that *S. mutans* is a genetically heterogeneous species. The strains studied can be divided into three (possibly four) genetic groups, and these groups closely parallel antigenic groups.

Streptococcus mutans Clarke (4) is a common "viridans" organism which preferentially colonizes human tooth surfaces (4, 14). Strains of *S. mutans* ferment a wide range of sugars, including sorbitol and mannitol, produce high molecular weight dextran, and induce dental caries in experimental animals (9, 10). Their phenotypic homogeneity was demonstrated by numerical taxonomic methods (3, 8) but they are antigenically heterogeneous (1, 22, 24; and Tow and Shklair, Naval Dental Research Institute Report NDRI 67-06, 1967). Analysis of deoxyribonucleic acid (DNA) base contents of several strains indicated that the species is also genetically heterogeneous (6). To clarify the genetic relationships within this species, the similarity of DNA base sequences among eight strains was estimated by DNA-DNA reassociation (hybridization) techniques.

MATERIALS AND METHODS

Organisms. The strains studied are listed in Table 1. The *S. mutans* strains are all cariogenic for at least one experimental animal. Cariogenic *Streptococcus* sp. 167 (Shklair et al., Abstracts of the 45th General Meeting of the International Association for Dental Research, p. 69, 1967) which does not conform to *S. mutans*, and cariogenic *Streptococcus sanguis* 10556 (Shklair et al., Abstracts of the 48th General Meeting of the International Association for Dental Research, p. 54, 1970) were also included.

All organisms were maintained in Todd-Hewitt broth with 2.5% glucose and $CaCO_3$. Cultures were periodically tested for purity by Gram stain, growth on mitis salivarius and blood-agar media, fermentation of mannitol and sorbitol (*S. mutans*), and the production of ammonia from arginine (*S. mutans* strains FA1 and BHT, and *S. sanguis;* reference 20).

Cell lysis and extraction of DNA. Cells of one-liter, anaerobic cultures in the log phase were treated with penicillin (10 units/ml of culture), harvested, washed three times with water, incubated with 10 mg of lysozyme in 25 ml of 0.15 M tris(hydroxymethyl)aminomethane (Tris, *p*H 6.4), and lysed by sodium lauryl sulfate as previously detailed (6). DNA was isolated from the lysate by phenol and chloroform deproteinizations and precipitation with ethanol. The precipitate was redissolved in SSC/100 (SSC = 0.15 M NaCl-0.015 M sodium citrate), treated with pancreatic ribonuclease (Calbiochem) and further deproteinized with Pronase, phenol, and chloroform. The DNA was reprecipitated with ethanol and dissolved in 1 × SSC (15, 18). This product contained considerable polysaccharide which was eliminated by centrifugation at 80,000 × *g* for 1 hr. The ratio of ultraviolet absorbance of the DNA preparations at 260:230:280 nm approached or exceeded 1.0:0.43:0.52 (18), indicating little carbohydrate or protein contamination. Labeled DNA was isolated from cells grown in phosphate-Trypticase-yeast extract broth (17) modified to contain 25% of the usual amount of yeast extract and supplemented with 1 μCi/ml of ^{14}C-thymidine (Calbiochem or New England Nuclear). Labeled DNA was sheared in a French press at 10,000 psi.

Hybridizations. Hybridizations were carried out by the membrane filter technique of Denhardt (7). Fifty-microgram samples of unlabeled heat-denatured DNA

[1] From the Bureau of Medicine and Surgery, Department of the Navy, Research Task MR005.05.01-0015A3JJ.

TABLE 1. *Cariogenic streptococci used in the study*

Strain	Origin	Guanine plus cytosine[a]
S. mutans derived from NCTC 10449	Human mouth	37.9 ± 0.79
S. mutans GS5	Human mouth	37.7 ± 0.67
S. mutans Ingbritt	Human mouth	37.1 ± 1.15
S. mutans FA1	Rat tooth	42.2 ± 0.96
S. mutans BHT	Human mouth	43.4 ± 0.35
S. mutans E49	Hamster tooth	43.7 ± 0.47
S. mutans SL1	Human mouth	45.1 ± 0.60
S. mutans K1R	Human mouth	45.2 ± 0.77
Streptococcus sp. 167	Human mouth	43.2 ± 0.25
S. sanguis 10556	Case of subacute bacterial endocarditis	45.5 ± 0.66

[a] Results expressed as per cent; data obtained from Coykendall (6).

in cold 6 × SSC (0.90 M NaCl-0.09 M sodium citrate) were applied to 25-mm Bactiflex B-6 filters (Schleicher & Schuell, Inc.). The filters were then incubated 5 to 6 hr in 3 × SSC (0.45 M NaCl-0.045 M sodium citrate) containing 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin (7). A 0.5-μg amount of sheared, denatured, labeled DNA was then added, and the mixture was incubated for 16 hr at 67 C, after which the filters were rinsed six times in 6 × SSC at 67 C and dried. Labeled DNA bound to the immobilized DNA was quantitated by liquid scintillation. The extent of heterologous duplex formation is expressed as the percentage of DNA bound (hybridized) in heterologous reassociations compared to the homologous control (i.e., labeled and immobilized DNA from the same strain). Filters containing *Escherichia coli* DNA served as negative controls. In some experiments, the incubation temperature was raised to 75 C.

Thermal elution. The thermal stability of some DNA-DNA duplexes formed by hybridization at 67 C was tested by thermal elution (5). For this procedure, the amounts of immobilized DNA and labeled DNA fragments in the reaction mixture were doubled, and bound ^{14}C-DNA was measured in a gas-flow beta radiation counter (Wide-Beta, Beckman Instrument Co.). Filters were eluted with 2.1-ml samples of increasingly warm SSC/30 containing 5×10^{-4} M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer (*p*H 7). The temperature of the 3 × SSC was increased by about 2.5 C per sample. The eluate was added directly to 15 ml of dioxane-based scintillant (Aquafluor, New England Nuclear) and counted to estimate eluted ^{14}C-DNA. The temperature at which one-half of the ^{14}C-DNA was eluted was designated E_m.

RESULTS AND DISCUSSION

Hybridizations. In the homologous reaction, the immobilized DNA bound 42 to 70% of the labeled DNA ($\bar{x}$ = 57%, n = 9). The extent of binding between heterologous DNA (expressed here as per cent of the homologous binding) was consistent for a given pair of strains even if the level of homologous binding varied in replicate experiments. The results of hybridization experiments carried out at 67 C are presented in Table 2. *S. mutans* strains GS5 and Ingbritt reassociated 85 and 92% with labeled DNA from strain 10449 (10449 ^{14}C-DNA). Strains FA1 and BHT DNA reassociated about 40% with this labeled DNA. The DNA from the remaining strains bound very little 10449 ^{14}C-DNA.

Labeled FA1 DNA reassociated extensively only with BHT DNA. This FA1 ^{14}C-DNA bound about 50% to 10449, GS5, and Ingbritt DNA, and less than 30% to the remaining strains.

A strong relationship between K1R and SL1 was evidenced by extensive reassociation between SL1 ^{14}C-DNA and K1R DNA (89%). Considerable SL1 ^{14}C-DNA bound to E49 DNA (55%) but other strains reassociated only slightly with SL1 ^{14}C-DNA.

Streptococcus sp. strain 167 and *S. sanguis* are phenotypically quite different from *S. mutans*, and their lack of relatedness to *S. mutans* was expected.

The DNA from strains which reassociated extensively at 67 C were also incubated at the more stringent 75 C. Absolute binding decreased to about 40% at this temperature, probably due to leaching of immobilized DNA, but relative binding remained consistent. Under these conditions, the binding of 10449 DNA to Ingbritt and GS5 DNA decreased somewhat, but FA1-BHT and SL1-K1R reassociations were equal to the 67 C results. There was a notable decrease in E49-SL1 binding (Table 3).

Elutions. The thermal stability of FA1-^{14}C-FA1, BHT-^{14}C-FA1, and 10449-^{14}C-FA1 DNA duplexes produced on membrane filters at 67 C is represented in Fig. 1. The BHT-^{14}C-FA1 du-

TABLE 2. *Extent of DNA-DNA reassociation at 67 C in 3 × SSC*[a]

Immobilized strands	Radioactive fragments			
	10449	FA1	SL1	*S. sanguis*
10449	100	48	18	10
Ingbritt	85	48	24	11
GS5	92	54	19	13
FA1	42	100	17	21
BHT	38	98	19	20
167	10	22	14	30
E49	15	29	55	21
SL1	22	26	100	18
K1R	16	23	89	18
S. sanguis		17	25	100

[a] Expressed as percentage of labeled DNA bound to immobilized strands, compared to homologous binding.

plexes were as stable as FA1-^{14}C-FA1 duplexes (actually, slightly more stable). The E_m (58.6 and 59.1 C) approached the temperature at which native FA1 DNA would denature in SSC/30 (62 C). Thus, the release of the labeled DNA was probably due to helix-coil transitions which would signify genuine base pairing. On the other hand, the instability of 10449-^{14}C-FA1 duplexes indicated that these strands are not united by long sequences of paired bases. The thermal instability of duplexes formed between strains which hybridize less than 60% under less stringent conditions has been noted previously (2).

TABLE 3. *Extent of DNA-DNA reassociations at 75 C in 3 × SSC*[a]

Immobilized strands	Radioactive fragments		
	10449	FA1	SL1
GS5	69		
Ingbritt	78		
BHT	—[b]	93	
K1R			86
E49			35

[a] Expressed as per cent of the homologous control. (Control reactions are not included in the table.)

[b] Labeled DNA was incubated only with those DNA which formed extensive duplexes at 67 C.

The experiments reported here show that *S. mutans* is a genetically heterogeneous species. Seven of the strains can be assigned to three groups. Group I includes the strains whose DNA contain 37 to 38% guanine plus cytosine (Ingbritt, GS5, and 10449). Strains FA1 and BHT comprise group II. (These two strains can also be distinguished from the others by their production of ammonia from arginine.) Group III is represented by strains K1R and SL1. The ability of the DNA from the strains within any one group to hybridize extensively at both 67 and 75 C is interpreted to indicate a high degree of complementary base pairing as a result of high overall base sequence similarity. This was confirmed in the group II strains by thermal elution. These results are consistent with previous reports that a high degree of reassociation will occur under stringent conditions if reassociation was over 80% at less stringent conditions, and that these duplexes will be stable when subjected to thermal elution (2, 16). The decreased binding of E49 to SL1 at 75 C indicates that although these two strains have some genetic similarity they are not identical. Strain E49 may represent a fourth group.

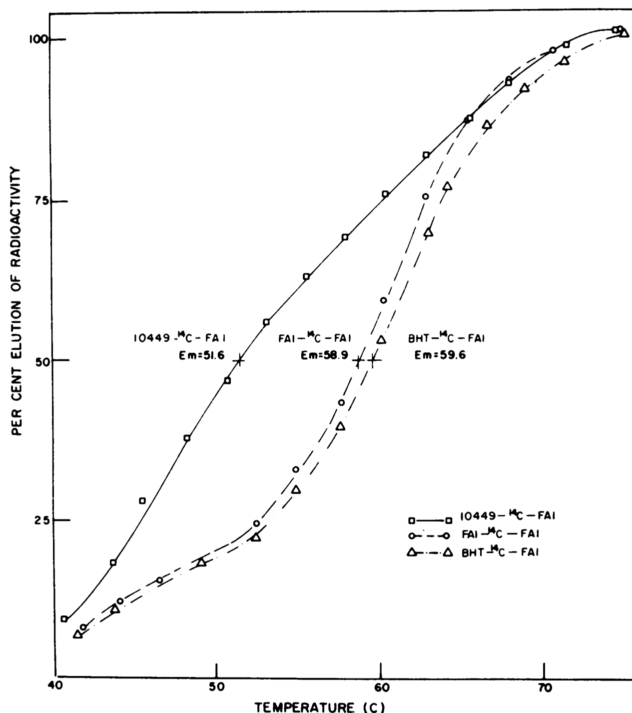

FIG. 1. *Thermal elution patterns of hybrid duplexes formed at 67 C in 3 × SSC by using FA1 ^{14}C-DNA and immobilized BHT, 10449, and FA1 (the homologous control) DNA. Symbols: △, BHT; □, 10449; ○, FA1.*

Although genetic heterogeneity among these strains of *S. mutans* was predicted from their dissimilar DNA base contents, the extent of heterogeneity, especially among strains with similar base contents, was surprising in view of their phenotypic similarity. Similar genetic heterogeneity has been observed in metabolically similar strains of *Chlamydia trachomatis* (21). Also, some strains of *Lactobacillus* species have been found to have dissimilar DNA base contents which imply some genetic diversity (13, 19). Recently a group of organisms nearly indistinguishable from *Lactobacillus leichmannii* were found to have guanine plus cytosine contents sufficiently different from *L. leichmannii* to support the proposal of a new species (12). The guanine-cytosine contents of several strains of several species of *Streptococcus* were determined by Møller-Madsen (*personal communication*), and the wide range of values found within some species was similar to the differences noted in the *Lactobacillus* species. Thus, the common phenotypic characteristics of strains within some species of *Lactobacillaceae* may not account for sufficient common base sequences to offset significant differences in the genome as a whole.

As the result of an extensive serological study of 70 strains of *S. mutans* (including some which did not ferment sorbitol), Bratthall (1) divided the species into five groups. On the basis of the six strains common to both Bratthall's study and the work reported here, the serological groups are identical to the genetic groups. Bratthall's group *c* includes *S. mutans* strains 10449, GS5, and Ingbritt (the "low G-C" strains). Strains FA1 and BHT were the only members of group *b*. Bratthall's group *a* includes strains E49, 3720, and AHT. [Strains E49 and 3720 were both derived from strain HS6, one of the original cariogenic hamster strains (11), and AHT is a human isolate which is serologically identical to HS6 (24).] Although Tow and Shklair found SL1 serologically related to HS6, the relationship is weak on the basis of DNA-DNA reassociations. Unfortunately, K1R and SL1 were not included in Bratthall's collection. The genetic and antigenic divisions of the eight strains are summarized in Table 4. A more formal taxonomic separation of the least related strains may be useful in the future.

The genetic (and serological) differences in *S. mutans* may be related to differences in pathogenicity or ecology. The FA1-BHT organisms can be found in mouths of infants, whereas the HS types become established later and are found associated with carious lesions in adults (23). Also, some strains are cariogenic for some experimental animals and not others. For example, FA1 and BHT are cariogenic for rats (10) but noncariogenic (10, 24) or only moderately cariogenic (22) for hamsters. Conversely, hamster strain HS6 and the serologically identical AHT (24) are not cariogenic for rats (10).

TABLE 4. *Summary of genetic and antigenic groups within S. mutans*

Genetic group	Strains	Antigenic group[a]
I	10449, GS5, Ingbritt	*c*
II	BHT, FA1	*b*
III	K1R, SL1	Not done
IV[b]	E49	*a*

[a] Data obtained from Bratthall (1).
[b] Assignment of E49 to a fourth group is conjectural and based on lack of duplex formation with SL1 ^{14}C-DNA.

ACKNOWLEDGMENTS

I am especially grateful to S. Schramek and E. Weiss for their invaluable advice and encouragement throughout this work. I also thank M. E. Beath, M. Reich, N. A. Schlamm, N. N. Wilson, and C. Wolin for their advice, assistance, and encouragement.

The major part of this work was done in partial fulfillment of requirements for the Master of Science degree at The George Washington University, Washington, D.C.

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