New Medium for the Isolation of *Streptococcus mutans* and Its Differentiation from Other Oral Streptococci

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A new, improved agar medium for the isolation of *Streptococcus mutans*, the etiological agent of dental caries, was developed. In contrast to mitis-salivarius agar, this medium not only recovers a greater number of *S. mutans* strains from most oral specimens but, because of its mannitol and sorbitol content, it also facilitates the differentiation of *S. mutans* from other oral streptococci, e.g., *S. salivarius*, *S. mitis*, and *S. sanguis*, which do not grow or produce scanty growth only after 10 days of incubation. The medium is easy to prepare because of its simple and unique composition, is characterized by the presence of an acid indicator, and can be utilized under aerobic and anaerobic conditions as well. The medium cannot be used to distinguish among the eight serotypes, *a* to *g* and SL-1, of *S. mutans*. Mannitol-utilizing bacteria such as streptococci (e.g., *S. faecalis*) and other microorganisms (e.g., *Staphylococcus aureus*) are able to grow on this medium and can be distinguished from *S. mutans* by their unique colony morphology.

Streptococcus mutans routinely is isolated together with other oral streptococci on mitis-salivarius (MS) agar. *S. mutans* is differentiated from the other oral streptococci by its ability to ferment mannitol and sorbitol and to adhere to smooth surfaces in the presence of sucrose (17, 19). The recovery of *S. mutans* from MS agar is never quantitative (5) and depends on the origin of MS agar (12, 18). In the following study we utilized the unique ability of *S. mutans* among oral streptococci to ferment mannitol and sorbitol to develop a new isolation and differentiation medium specifically for this *Streptococcus* species.

MATERIALS AND METHODS

Bacterial strains and isolation specimen. For comparative growth studies, 28 defined *Streptococcus* strains were utilized: 13 *S. mutans* strains (Table 1), representing the eight serotypes, *a* to *g* and SL-1, according to Bratthall (1) and Perch et al. (14); and 15 other streptococcal strains (Table 1) typical of viridans (2 *S. sanguis*, 3 *S. salivarius*, and 3 *S. mitis* strains), enterococcus (2 *S. faecalis*, 1 *S. faecalis* subsp. *liquefaciens*, and 1 *S. durans* strain), pyogenic (1 *S. pyogenes* and 1 *S. agalactiae* strain) and lactic groups (1 *S. lactis* strain). For enumeration of unknown microbial strains, a specimen was used that was composed of 3 ml of saliva mixed with 100 mg of plaque material (from a human source) diluted with 9 ml of distilled water.

Media and chemicals. MSFA agar, used for isolation and differentiation of *S. mutans*, was composed of: D-sorbitol (Sigma Chemical Co., St. Louis, Mo.),

10 g; D-mannitol (Sigma), 10 g; yeast extract (Difco Laboratories, Detroit, Mich.), 20 g; sodium azide (Fisher Scientific Co., Fair Lawn, N.J.), 100 mg; basic fuchsin (Allied Chemical Co., Morristown, N.J.), 5 mg; CaCO₃ (precipitated; Merck & Co., Inc., Rahway, N.J.), 10 g; agar (Fisher Scientific Co.), 15 g; and distilled water, 1,000 ml; the pH was adjusted to 7.0. MS agar (Difco) was prepared according to the manufacturer's recommendation. Blood agar was prepared by mixing tryptic soy agar (Difco), prepared according to the manufacturer's recommendation, with 50 ml of sheep blood per 1,000 ml. Trypan blue for growth inhibition studies was obtained from Matheson Coleman & Bell, Norwood, Ohio, and crystal violet was obtained from Difco. The *Streptococcus* strains were maintained in screw-cap test tubes containing 9 ml of a medium containing 2% glucose (dextrose, Difco) and 2% yeast extract (Difco) at pH 6.5, sterilized in an autoclave for 10 min at 20 lb/in² and 126°C. The tubes were supplemented with 10% inoculum and incubated at 28°C. The cultures were transferred every 5 to 6 days.

Growth and enumeration of bacterial strains. The agar plates were inoculated with each of the 28 *Streptococcus* strains by streaking 0.1 ml of a suspension onto the plate, cutting into the agar surface at the last 0.5 inch (ca. 1.27 cm) of the streak. The plates were incubated aerobically at 36°C for up to 2 weeks. Growth was observed at intervals of 4 days. For anaerobic growth conditions, the plates were incubated in an anaerobic jar (BBL, Cockeysville, Md.) in the presence of a gas-generating envelope (GasPak no. 70304, BBL) and an anaerobic indicator strip (GasPak no. 70404, BBL) for 36°C for up to 2 weeks. For enumeration of the unknown *Streptococcus* strains, a dilution series of the aqueous saliva-

TABLE 1. *The 28 Streptococcus species studied*

Strain	Serotype	Origin	Source
S. mutans			
NCTC 10449	c	Human	H. D. Slade, Chicago, Ill.
AHT	a	Human	
B-13	d	Human	
B-14	e	Human	
FA-1	b	Rat	
SL-1	SL-1/d/g	Human	
OMZ-176	d	Human	B. Guggenheim, Zürich, Switzerland
OMZ-65	g	Human	
OMZ-175	f	Human	
BHT	b	Human	D. D. Zinner, Miami, Fla.
HS-6	a	Hamster	R. J. Fitzgerald, Miami, Fla.
P-4	e	Human	S. Edwardsson, Malmö, Sweden
B-2	e	Human	
S. mitis ATCC 15909			American Type Culture Collection, Rockville, Md.
S. mitis ATCC 15910			
S. salivarius ATCC 9759			
S. salivarius ATCC 13419			
S. sanguis ATCC 10556			
S. sanguis ATCC 10557			
S. faecalis ATCC 19433			
S. faecalis subsp. *liquefaciens*			Midwest Culture Service, Terre Haute, Ind.
S. pyogenes			
S. durans			
S. agalactiae			
S. lactis			
S. salivarius			
S. mitis			
S. faecalis			

plaque material mixture was prepared, and 0.2-ml portions were evenly distributed, with the aid of a bent glass rod, on top of the agar plates, which were incubated at 36°C for up to 2 weeks. Then the colonies were counted with the aid of a colony counter (model C-110; New Brunswick Scientific Co., New Brunswick, N.J.).

Growth inhibitions studies. Stock solutions of sodium azide, basic fuchsin, crystal violet, and trypan blue were prepared and sterilized in an autoclave; the solutions were then diluted in sterile water to yield the desired inhibitor concentrations. For each test, 1.0 ml of the appropriate inhibitor dilution and 0.5 ml of sterile water were added to 5.0 ml of a 2% glucose-2% yeast extract medium at pH 6.5 in screw-cap test tubes. The final concentrations of sodium azide, basic fuchsin, crystal violet, and trypan blue in the medium were 20 to 1,000, 1 to 500, 1 to 500, and 1 to 500 μg/ml, respectively. Each tube was then inoculated with 0.5 ml of a 48-h culture of the appropriate *Streptococcus* strain to yield a total volume of 7.0 ml. Inoculated tubes with 1.5 ml of sterile water added to the glucose-yeast extract medium served as controls. The test tubes were incubated in a gyratory water bath shaker (model G76, New Brunswick Scientific Co.) at 36°C. After 24 h the optical density, as a measure of growth, was determined at 546 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. For each inhibitor dilution, a blank tube was prepared to aid adjustment of the spectrophotometer's transmission.

RESULTS

Growth inhibition studies. Most *Streptococcus* species are relatively resistant to sodium azide treatment (Fig. 1); some strains are still able to survive the presence of 1,000 μg/ml. At a concentration of 200 μg/ml, on the average, a 50% growth inhibition was obtained with all tested strains.

A significant growth inhibition of *S. mutans*, but not of *S. faecalis*, by basic fuchsin was obtained at a concentration of 10 μg/ml (Fig. 2); concentrations lower than 5 μg/ml had no or little effect on streptococcal growth.

At a concentration of 10 μg/ml, crystal violet inhibited most of the tested *Streptococcus* strains (Fig. 3); even 1 μg of this compound per
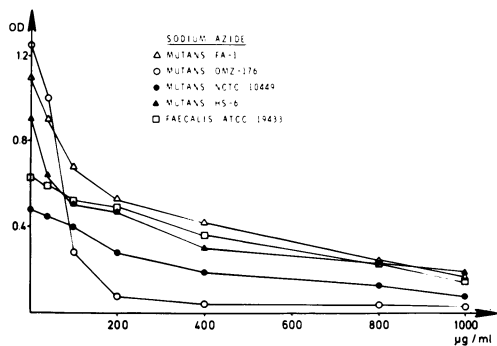
FIG. 1. *Growth inhibition (optical density [OD] at 546 nm) of glucose-grown Streptococcus strains in the presence of sodium azide.*
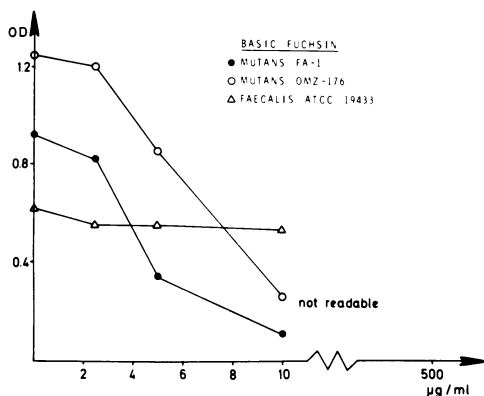

FIG. 2. *Growth inhibition (optical density [OD] at 546 nm) of glucose-grown Streptococcus strains in the presence of basic fuchsin.*
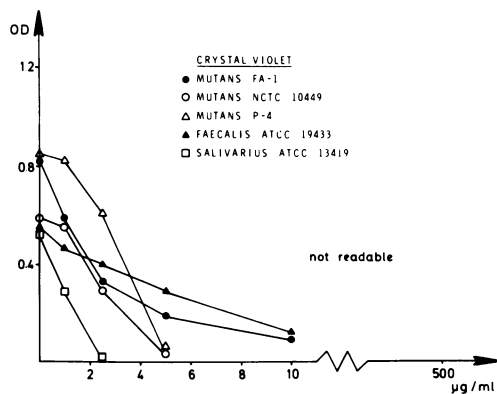

FIG. 3. *Growth inhibition (optical density [OD] at 546 nm) of glucose-grown Streptococcus strains in the presence of crystal violet.*

ml inhibited the growth of all tested strains up to 25%.

Growth of the *Streptococcus* strains in the presence of trypan blue concentrations higher

than 50 μg/ml could not be determined under the experimental conditions. Three of the tested *S. mutans* strains, P-4, HS-6, and NCTC 10449, were almost completely growth inhibited by a concentration of 50 μg/ml (Fig. 4); therefore, a trypan blue concentration of 75 μg/ml, as used in MS agar preparations, is definitely not desirable for isolation and growth of many *S. mutans* strains. A significant reduction in streptococcal growth, up to 35%, was obtained at a trypan blue concentration of 10 μg/ml (Fig. 4).

Growth on MSFA agar and MS agar. Most of the studied *S. mutans* strains grew much better on MSFA agar than on MS agar (Table 2). The other *Streptococcus* species that grew well on MS agar, e.g., *S. salivarius, S. mitis,* and *S. sanguis,* did not grow or produced only scanty growth on MSFA agar. On MSFA agar most streptococci colonies were pink to red, some having a purple to blue tint. The colonies were often pinpoint to small and were shiny or had a silky to frosted-glass appearance; some appeared to be raspberry-like. *S. faecalis* grew in large mat colonies, often with a center indentation. Good growth on MSFA agar developed usually after 1 week of incubation, but, in the cases of *S. salivarius, S. mitis,* and *S. sanguis,* scanty growth appeared after an incubation period of 10 days to 2 weeks.

Total viable counts of bacteria on various growth media. A dilution series of the saliva-plaque material specimen was prepared, and portions of the specimen dilutions were plated on the three different media: blood agar, MS agar, and MSFA agar. Results in Table 3 represent readings from plates that received inoculum from the 10^{-4} dilution. Blood agar produced the highest viable count of bacteria (=100%), followed by MS agar, which recovered 84% of
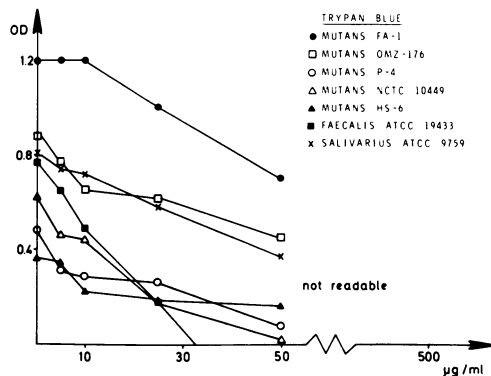

FIG. 4. *Growth inhibition (optical density [OD] at 546 nm) of glucose-grown Streptococcus strains in the presence of trypan blue.*

TABLE 2. Growth of 28 Streptococcus strains on the new isolation medium (MSFA agar) and on MS agar

Strain	Growth[a] on MSFA agar	Colony characteristics	Growth[a] on MS agar
S. mutans			
OMZ-175	+ +	Pink to red, frosted glass	+
OMZ-176	+ +	Pink, frosted glass	+ +
OMZ-65	+ +	Pink, raspberry-like	+
SL-1	+ + +	Pink, smooth	+
HS-6	+ +	Pink to red, frosted glass	+
B-13	+ +	Pink, silky	(+)
B-14	+ + +	Red, silky	+
BHT	+ +	Pink, mat	+ +
AHT	+ + +	Pink to red, frosted glass	+ +
NCTC 10449	+ + +	Blue-purple, silky	+ +
P-4	+ +	Red, silky	+
FA-1	+ +	Pink, silky	+
B-2	+ +	Red, silky	+
S. mitis ATCC 15909	(+)	Pink-red, mat	+
S. mitis ATCC 15910	(+)	Pink-red, silky	+ + +
S. mitis	+	Pink-red, mat	+ +
S. salivarius ATCC 9759	−		+ + + +
S. salivarius ATCC 13419	(+)	Pink-blue, smooth	+ + + + +
S. salivarius	+	Pink-blue, smooth	+ +
S. sanguis ATCC 10556	−		+ +
S. sanguis ATCC 10557	(+)	Pink-blue, mat	+
S. faecalis ATCC 19433	+ + +	Pink-blue, indented	+ +
S. faecalis	+ +	Light pink, mat	+ +
S. faecalis subsp. liquefaciens	+ + +	Pink, mat	+ + +
S. pyogenes	(+)	Light pink, silky	−
S. durans	+	Pink, mat	+ +
S. agalactiae	+	Pink-red, mat	+
S. lactis	+ + +	Pink-blue, silky	+ + +

[a] Growth after 10 days of aerobic incubation at 36°C; colonies usually appeared circular and convex. Symbols: −, no growth; (+), scanty growth (haze of growth); +, light growth (pinpoint colonies); + +, moderate growth (colony size ca. 1 mm); + + +, very good growth (colony size ca. 2 mm); + + + +, excellent growth (colony size ca. 3 mm); + + + + +, luxuriant growth (colony size > 3 mm).

TABLE 3. Comparison of total viable counts of bacteria from a saliva-plaque material sample (diluted 10^{-4}) on various growth media

Agar medium	No. of colonies/ml	Recovery (%)
Blood	155	100
MS	130	84
MSFA	120	77

the viable cells. Many colonies on MS resembled S. salivarius according to their large size and smooth convex appearance. On MSFA agar the recovery of colonies was 77% as compared with that on blood agar, or 92% as compared with that on MS agar. It was difficult to isolate S. salivarius, S. mitis, and S. sanguis colonies from MSFA agar because of scanty growth.

Growth of other microorganisms besides Streptococcus species on MSFA agar. Besides the Streptococcus species already mentioned, several other mannitol-utilizing microorganisms were able to grow on MSFA agar. Lactobacillus casei produced good growth in pink-blue mat colonies; L. acidophilus produced very

scanty growth. Under semianaerobic conditions, Actinomyces israelii grew well in spreading, tan, mat-scaly colonies; Actinomyces viscosus produced pink to red mat colonies under aerobic conditions. Staphylococcus aureus exhibited fair growth in mucoid, pink-blue, silky colonies on MSFA agar, and also Escherichia coli ATCC 25922, which grew in pink-red mat colonies. Furthermore, Neisseria sicca exhibited scanty growth in pink-red mat colonies with raspberry-like centers. Occasionally, diphtheroid colonies from saliva specimens could be recovered on MSFA agar.

DISCUSSION

S. mutans, the etiological agent of dental caries, was first discovered and described in 1924 (3). Since that time several investigators have isolated, from carious teeth of humans and animals, numerous S. mutans strains by using various complex growth media (7, 10, 20) including MS medium (11). MS agar is now commonly used to isolate S. mutans as well as other Streptococcus species from natural

sources. MS agar was modified to be more specific for the isolation of *S. mutans* by the addition of either sulfonamide (2), polymyxin (6), or bacitracin (9). By comparison of *S. mutans* growth on various selective media, different rates of recovery were observed (5). Different MS agar preparations from different manufacturers (BBL; Difco) recovered different numbers of *S. mutans* strains from the same specimen (12), and some *S. mutans* strains were even inhibited by these different MS agar preparations (18). This growth inhibition of MS agar preparations to certain strains of *S. mutans* was probably caused by the high (75 µg/ml) trypan blue content of the medium. We found that this large amount of trypan blue in MS agar definitely exerted a growth inhibitory effect on most *S. mutans* strains.

Modified MS agar preparations are also not very useful in the differentiation of *S. mutans* from other oral streptococci, e.g., *S. salivarius*, *S. sanguis*, and *S. mitis*; this differentiation is usually achieved after isolation of several *Streptococcus* spp. from MS agar and utilizes the ability of *S. mutans* to ferment mannitol and sorbitol and to adhere to smooth surfaces in the presence of sucrose (17, 19). Recently, it was found that some strains of *S. mutans* are not able to ferment sorbitol (4). *S. salivarius*, *S. sanguis*, and *S. mitis* ferment neither mannitol nor sorbitol.

On this basis we developed an isolation and differentiation medium (MSFA agar) for *S. mutans*, which contains the carbohydrates mannitol and sorbitol as carbon sources and yeast extract mainly as a nitrogen source. The addition of sodium azide and basic fuchsin provides some advantages over other isolation media (8); the former suppresses the growth of gram-negative microorganisms, including *Proteus*, and the latter reduces the development of undesirable gram-positive organisms. In addition, basic fuchsin has the advantage of producing colored colonies. Since *S. mutans* and other *Streptococcus* species are high acid producers (13, 15), some precipitated calcium carbonate was added to the medium to neutralize produced acids, which, in turn, enhanced the microbial growth on the agar surface. Due to the calcium carbonate content of the medium, acid-producing colonies appeared to be surrounded by a clear zone, a convenient visual check. Therefore, the new MSFA agar in comparison with MS medium is more useful for the isolation of *S. mutans* strains and its differentiation from other oral streptococci. In addition, the new medium is easy to prepare because of its simple and unique composition, is chemically stable, and has a long shelf life. The medium can be utilized under both aerobic and anaerobic conditions.

MSFA agar allows growth of most mannitol-utilizing microorganisms, e.g., *S. faecalis*, *S. faecalis* subsp. *liquefaciens*, *Staphylococcus aureus*, and *Actinomyces* spp. The new medium cannot be utilized for biochemical determination of the eight serological groups, *a* to *g* and SL-1, of *S. mutans* according to Bratthall (1) and Perch et al. (14); previously a biochemical scheme was developed for the separation of *S. mutans* into five biotypes that correlated with the serotypes *a* to *e*, based on the utilization of mannitol with and without bacitracin and other biochemical tests (16).

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