Efficiency of Various Growth Media in Recovering Oral Bacterial Flora from Human Dental Plaque

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MM10 sucrose blood agar (MM10 SB agar), N₂C agar, Schaedler agar (SH agar), and mitis salivarius agar (MS agar) were tested for their ability to recover human dental plaque flora by a continuous anaerobic procedure and by a conventional anaerobic method. MM10 SB agar yielded higher recovery of bacteria from plaque samples as determined by the enumeration of colony-forming units (CFU). The CFU on N₂C agar, SH agar, and MS agar were lower than MM10 SB agar when the continuous anaerobic procedure was used. The superior performance of MM10 SB agar was much more apparent when used for the cultivation of dental plaque flora by the conventional anaerobic method. Under these conditions the counts were consistently higher on MM10 SB agar as compared to the other media tested. However, the differential counts of *Streptococcus sanguis* and *S. mutans* from various plaque samples were in general comparable on all culture media. Deletion of blood from MM10 SB agar did not lower counts. The elimination of dithiothreitol from this medium resulted in a significantly lower recovery of bacteria from the plaque samples when cultured by the conventional anaerobic method. The storage of MM10 SB agar for varying periods of time under aerobic conditions did not seem to affect its performance. These findings suggest that MM10 SB agar is an ideal culture medium for the isolation, nonselective enumeration, and differential counts of bacteria present in normal and disease-associated plaques.

Human dental plaque flora is composed of bacteria which differ in their sensitivity to molecular oxygen. A good portion of these organisms cannot be isolated by conventional anaerobic methods. Consequently, attempts were made in recent years to improve the methods of isolation of these organisms. Aranki et al. (1), working with gingival plaque, showed that higher viable counts of bacteria could be obtained by the anaerobic glove box procedure as compared to the anaerobic jar method. Gordon et al. (10), by using gingival plaque, compared the efficacy of the Hungate roll tube technique (11) with that of the aerobic and anaerobic jar methods and found the roll tube technique most efficient in the isolation of bacteria from oral samples. Although these investigators gave valuable data showing effects of variation in the anaerobic techniques on the recovery of gingival bacteria, little or no data have been published to show the efficacy of various culture media for nonselective isolation of oral bacteria from different types of dental plaque. Gilmour and Poole (9) found a growth stimulatory effect of hemin and other nutrients on the recovery of bacteria from nondisease-associated plaque. However, their studies were limited to samples which were cultured by the anaerobic jar method. In the present investigation, direct comparison of the effects of various media on the total colony counts were made by using nondisease-associated and disease-associated human plaque samples. The performance of a modified medium 10 (MM10 medium) of Caldwell and Bryant (4) was evaluated for its usefulness in the isolation of dental plaque bacteria. The efficiency of MM10 medium was compared with N₂C medium (9), Schaedler agar (SH agar), and mitis salivarius agar (MS agar). The plaque samples were cultured on these media by using the conventional anaerobic method (aerobic plating fol-
lowed by anaerobic incubation) and the anaerobic glove box procedure. Results of the experiments with these media are presented in this paper.

MATERIAL AND METHODS

Plating media. \( N_2 \)C medium was prepared by the method described by Gilmour and Poole (9). This medium contains 1.5 mg of hemin and 1 g of cysteine per liter. MM10 sucrose blood agar (MM10 SB agar) was prepared as described elsewhere (20). Unless otherwise mentioned, MS agar (Difco) was prepared without the addition of potassium tellurite. SH agar (BBL) was also prepared according to the manufacturer's instructions, but agar concentration was increased to 1.5%. Whenever differential counts of \textit{Streptococcus mutans} and \textit{S. sanguis} were desired, \( N_2 \)C and SH agar were supplemented with 5% sucrose.

Transport medium. A dithiothreitol (DTT) poised reduced transport fluid (RTF) was used for collection and dilution of samples. It was prepared as described previously (20). After its preparation the RTF was introduced into the anaerobic glove box (1) and exposed to the anaerobic atmosphere (85% \( N_2 \), 10% \( H_2 \), 5% \( CO_2 \)) for 48 h. The anaerobically kept RTF was referred to as prereduced RTF to differentiate it from the medium which was aerobically stored.

Sample collection. The dental plaque samples were collected from three different populations: (i) adults with no clinical evidence of active oral disease, (ii) children and teenagers with clinical symptoms of dental caries, and (iii) mentally retarded patients with acute gingivitis and periodontal disease. The first group was comprised of individuals whose ages ranged between 21 to 40 years. The subjects in the second group were either children or teenage boys between 7 to 16 years. Initial studies with the samples from these patients showed the presence of \textit{S. mutans} in their plaques. The patients in the last group were 11 to 22 years old. Both males and females were represented in all three populations. The interproximal plaques from the first population were removed by means of a sterile unwaxed dental floss (2.5 cm in length) which was passed between the maxillary or mandibular premolar and molar. One floss sample from each normal subject was collected in 10 ml of prereduced RTF in 18- by 150-mm test tubes kept closed with rubber stoppers. The interproximal plaque samples from the caries population were removed in the same manner as in the first group except that four samples representing the four quadrants were collected in 5 ml of prereduced RTF in 13- by 100-mm test tubes provided with rubber stoppers. Occasional samples were also removed from this population by scraping the surfaces of the molar teeth with orthodontic wires (1.0 cm in length) and pooled in the same way as the interproximal samples. The non-disease-associated plaques were cultured immediately. However, there was a 12 to 15 h delay between the collection of carious samples and the culturing process.

Supragingival plaque samples from each of the institutionalized patients were removed by periodontal scalers, weighed on a sterile preweighed piece of aluminum foil, and then added to 10 ml of prereduced RTF in 18- by 150-mm test tubes with rubber stoppers. All samples were cultured within 5 h after collection.

Continuous anaerobic procedure. This method was used to culture the samples under strict anaerobic conditions. The plating media, transport medium, and dilution tubes were prereduced in the anaerobic glove box atmosphere for 48 h prior to their use for culturing plaque samples. Each plaque sample was dispersed for 10 s by the sonic disruption method (20). The tubes containing the plaque were flushed with a stream of oxygen-free gas (85% \( N_2 \), 10% \( H_2 \), 5% \( CO_2 \)) during the sonic disruption of the samples on the bench top to prevent or minimize oxygen contamination from the air. The samples were introduced into the anaerobic glove box where they were diluted in RTF by a serial 10-fold dilution method, plated in 50-\( \mu \)liter amounts by using an Eppendorf pipette on the test media, and spread by sterile L-shaped glass rods. The inoculated plates were incubated at 37°C in the anaerobic glove box for 1 week. After incubation the colony-forming units (CFU) were enumerated.

Conventional anaerobic method. In this method the collection tubes and dilution tubes of RTF were prereduced. However, sonic disruption was performed without benefit of a flush with the oxygen-free gas. The plaque samples were serially diluted and plated on the bench top in the aerobic atmosphere and transferred without delay into the anaerobic glove box atmosphere for incubation purposes. The total CFU were made after 1 week.

Differential colony counts. \textit{S. sanguis} and \textit{S. mutans} colonies were counted with a stereomicroscope. For comparison purposes, known strains of these organisms were grown on the test media under similar conditions. This facilitated the identification of the organisms by colonial morphology. Additional tests were done on representative colonies of \textit{S. mutans} and \textit{S. sanguis} from all test media to confirm the presumptive identification of these organisms. These included Gram stain, single-cell morphology, sucrose and mannitol fermentation, and ability of the organisms to form plaque on wire (18). Intracellular polysaccharide-forming colony counts were made by flooding the plates with 0.2% KI-iodine solution (8). Colonies showing dark brown, black, or dark blue colors were considered positive.

Storage of MM10 SB agar and the recovery of plaque bacteria. Interproximal nondisease-associated plaque samples removed by dental floss were cultured on MM10 SB agar plates which had been stored at refrigeration temperature under aerobic conditions for varying periods of time. The inoculated plates were incubated in the anaerobic glove box atmosphere for 1 week, and then total viable counts were determined by a stereomicroscope. Whenever possible, intracellular polysaccharide-forming colonies and \textit{S. sanguis} colonies were counted.

Effect of DTT elimination on the recovery of plaque bacteria. MM10 SB agar plates with and without DTT were prepared and used for the comparison of total, \textit{S. mutans} and \textit{S. sanguis} viable counts of
interproximal and occlusal plaque samples collected from dental caries patients. These experiments were done by the conventional anaerobic method.

RESULTS

Media comparison by continuous anaerobic procedure. The results of the series of experiments which were conducted with nondisease-associated plaque samples are shown in Table 1. The total CFU on MM10 SB agar were higher than SH agar counts. The counts on N4C agar were comparable to the counts on MM10 SB agar and blood-deficient MM10 medium.

In our next studies, various plaque samples were cultured on the various media. N4C agar and SH agar were supplemented with 5% sucrose to facilitate the differential counts of S. mutans, S. sanguis, and intracellular polysaccharide-forming bacteria. The results of this experiment are given in Table 2. The total counts of all five samples were considerably higher on MM10 SB agar than the other media. The recoveries from N4C agar were higher than on SH and MS agar. S. mutans counts on MM10 SB agar were approximately 1.5 times higher than the counts on N4C and SH agar. However, the counts of this organism on MS agar were similar to those obtained on MM10 SB agar. S. sanguis counts on MM10 SB agar were twice as high as on the other media. The intracellular polysaccharide-forming colony counts were comparable on all media tested.

The periodontal plaque collected from the mentally retarded subjects was cultured on MM10 SB agar with and without blood and on SH agar. Since considerable amounts of plaque were present on the tooth surfaces of these individuals, it was possible to weigh the plaque and report the data in terms of CFU per milligram wet weight basis (Table 3). The total counts on MM10 SB agar were higher than MM10 medium from which blood had been omitted and on SH agar.

In another experiment, the periodontal plaque samples were cultured on N4C agar, MM10 SB agar, and MM10 agar without blood. N4C agar consistently yielded lower counts than the latter media (Table 3). Since there was a 4-day delay between the collection of the samples and bacterial analysis, the results could not be compared with those obtained from the earlier experiment.

Media comparison by conventional anaerobic method. The plaque samples were collected from the three different populations in the same manner as in the first studies. Nondisease-associated plaque samples were plated on MM10 SB agar and SH agar in one experiment, and in another experiment, the recovery of bacteria on MM10 SB agar, N4C agar, and MS agar was compared (Table 4). The counts on MM10 SB agar were 1.5 times higher than those of SH agar. Similarly, MM10 SB agar gave rise to counts 2.5 times higher than N4C agar and 12.6 times higher than MS agar. Also, the counts of individual samples on MM10 SB agar were higher than all other media tested under identical conditions.

Plaque samples were collected from caries-active patients who were previously shown to have S. mutans in their oral cavities. The colonies of this organism, S. sanguis, and iodo-philic colonies, as well as total counts were determined and compared on all media (Table 5). In the initial experiment, five samples were cultured on MM10 SB agar, MS agar, N4C agar, and SH agar supplemented with 5% sucrose. The data showed highest total counts on MM10 SB agar either on individual samples or mean viable counts basis. MS agar yielded one-third of the total counts of MM10 SB agar. Recoveries on N4C agar and SH agar were approximately one-half of those of MM10 SB agar. The S. mutans counts were comparable on MM10 SB and MS media, with both media being superior to N4C and SH media in this regard. S. sanguis counts were significantly higher on MM10 SB agar (P < 0.01, t test); intracellular polysaccharide (ICP) counts on N4C agar were lower than on MM10 SB agar and SH agar.

Subsequent experiments were done on plaque samples collected from a larger population of caries-active subjects. Pooled interproximal and occlusal plaque samples were collected separately in either prereduced RTF or freshly

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Table 1. Comparison of total viable counts of bacteria from nondisease-associated plaque samples cultured on various growth media by the continuous anaerobic procedure

| No. of samples | Total viable counts per floss sample* |
|---------------|-------------------------------------|
|               | MM10 SB agar | N4C agar | MM10 S+ plain agar | SH agar |
| 10            | 236 (81–508) | 265 (62–460) | 195 (63–399) |
| 10            | 276 (81–508) | 151 (51–383) | 147 (42–321) |
| 17            |                | 138 (55–289) |

|               | Based on mean CFU of six plates per sample for each medium. To be multiplied by 2 × 106. |
|---------------|-------------------------------------|
| * MM10 S+ indicates MM10 medium with sucrose and without blood. |
| * Numbers in parentheses indicate count range. |

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TABLE 2. Comparison of the recovery of bacteria (CFU) from various plaque samples cultured on various media by the continuous anaerobic procedurea

| Medium                          | Total viable countsa | Differential countsa |
|---------------------------------|----------------------|----------------------|
|                                 |                      | S. mutans | S. sanguis | ICPc |
| MM10 SB agar                    | 204 (68-270)d        | 7 (0.3-23) | 2 (0-5)    | 38 (12-71) |
| N2C agar + 5% sucrose           | 162 (26-207)         | 5 (0.2-19) | 1 (0-2)    | 35 (1-65)  |
| SH agar + 5% sucrose            | 146 (65-211)         | 4 (0.2-14) | 1 (0.2-2)  | 39 (13-82) |
| MS agar (no tellurite)          | 149 (27-188)         | 7 (0-18)   | 1 (0-6)    | Not done   |

*Five interproximal plaque samples.
* Based on mean CFU of six plates per sample for each medium. To be multiplied by 2 x 10a.
* Intracellular polysaccharide CFU.
* Numbers in parentheses indicate count range.

TABLE 3. Comparison of the recovery of bacteria from periodontal plaque samples cultured on various growth media

| Culturing method                  | No. of samples | Total viable counts x 107 per mg (wet weight) | MM10 SB agar | MM10 S agar | SH agar | N2C agar |
|-----------------------------------|----------------|----------------------------------------------|--------------|-------------|---------|---------|
| Continuous anaerobiosisa          | 6              | 5 (2-10)b                                    | 4 (1-7)      | 3 (2-5)     | 4 (1-7) |
|                                   | 5c             | 5 (2-8)                                      | 6 (2-9)      |             |         |
| Conventional anaerobic methoda    | 7              | 5 (1-11)                                     | 3 (1-7)      | 2 (1-5)     |         |

* Total CFU represent mean counts of six plates per sample for each medium.
* Numbers in parentheses indicate count range.
* Samples were stored for 4 days at refrigeration temperature.
* Total CFU represent mean counts of two plates per sample for each medium.

TABLE 4. Effect of growth media on the recovery of bacteria from nondisease-associated plaque samples cultured by the conventional anaerobic method

| No. of samples | Medium                        | Total viable countsa |
|----------------|-------------------------------|----------------------|
| 7              | MM10 SB agar                  | 48 (8-99)d           |
|                 | SH agar                       | 26 (8-62)            |
| 5              | MM10 SB agar                  | 19 (4-94)            |
|                 | N2C agar                      | 7 (2-23)             |
|                 | MS agar (no tellurite)        | 2 (1-7)              |

* Based on mean CFU of four plates per sample for each medium. To be multiplied by 10a.
* Numbers in parentheses indicate count range.

MS agar supplemented with potassium tellurite (MST) were considerably lower than the other two media and comprised approximately one-third of the N2C counts and one-sixth of MM10 SB agar counts, respectively.

When differential counts were compared in this experiment, S. mutans, S. sanguis, and ICP counts from interproximal plaque samples were approximately two to three times higher on MM10 SB agar than on N2C agar. However, the differential counts of these organisms from occlusal plaque samples were comparable on the two media, with the exception of ICP counts which were higher on MM10 SB agar. The S. mutans and S. sanguis counts on MST agar were three to four times lower than on either N2C or MM10 SB agar. This is in marked contrast to S. mutans counts obtained when tellurite is omitted from this medium (Table 5).

Periodontal plaque samples were cultured within 5 h of collection. The results of this experiment are presented in Table 3. MM10 SB agar counts were approximately twice as high as on N2C agar and SH agar. Also, the total CFU recovered from the samples on SH and MM10 prepared RTF and cultured 12 to 15 h after collection. Almost twice as many CFU were recovered from either interproximal or occlusal plaque on MM10 SB agar as compared to N2C agar supplemented with 5% sucrose (Table 5).

When compared with N2C on an individual sample basis, MM10 SB agar yielded higher recoveries in 78 out of 81 samples. Total CFU on
SB agar were comparable to those of the continuous anaerobic method.

**DTT elimination and recovery of plaque flora on MM10 SB agar.** Interproximal plaque and occlusal plaque samples collected from caries-active patients were plated on MM10 SB agar with and without 0.02% DTT. The samples were cultured by the conventional anaerobic method. The results presented in Table 6 show that there was about a 50% decrease in the total viable counts on the MM10 medium without DTT, suggesting a beneficial role of DTT on the recovery of plaque flora. *S. mutans* counts did not seem to be affected by DTT. However, the *S. sanguis* counts on MM10 SB agar containing DTT were two to three times higher than on DTT-deficient MM10 SB agar.

**Evaluation of counts on aerobically stored MM10 SB agar.** Nondisease-associated dental plaque samples were cultured on MM10 SB agar plates stored for 2, 8, and 14 days at refrigeration temperature and incubated under anaerobic conditions. The results presented in Table 7 show that there were no adverse effects of aerobic storage of MM10 SB agar on the recovery of plaque bacteria. When *S. sanguis*...
yields highest counts from both types of plaque samples.

Most laboratories are not equipped to culture the plaque samples under continuous anaerobic procedures. Therefore, the various media were compared by using bench manipulation of the samples followed by anaerobic incubation. The superior performance of MM10 SB agar in recovering bacteria from dental plaque was best demonstrated when the plaque samples were cultured by the conventional anaerobic method. In all instances the total counts were significantly higher on MM10 SB agar than on the other media tested (Tables 3–5). Also, the recovery of bacteria on MM10 SB agar anaerobically stored for 14 days was comparable to that obtained on 2-day-old medium. These observations suggested the possible role of DTT in maintaining a lower oxidation reduction potential in MM10 SB agar under aerobic atmosphere and its subsequent effect on the recovery of bacteria from all types of plaque samples. Since $N_2C$ agar had cysteine as a reducing agent, a compound of relatively low poising capacity (19) under aerobic atmosphere, this medium would be more easily oxidized than MM10 SB agar. Thus, $N_2C$, SH, and MS media (the latter having no reducing agents) could, under aerobic storage, be more oxidized than MM10 SB agar, a factor which could explain the poor performances of these media. Subsequent studies on the protective role of DTT in preserving the viability of bacteria under aerobic conditions showed that the bacterial recoveries on MM10 SB agar containing DTT were significantly higher ($P = 0.01$, Sign test [3]) than on DTT-deficient MM10 SB agar.

If the media are compared when cultured under continuous anaerobiosis on a plaque type basis, the difference in the recoveries of bacteria from nondisease-associated plaque samples were statistically not significant. This could be attributed to the better performance of all media under strict anaerobic conditions. As far as periodontal plaque and carious plaque were concerned, the recoveries from these samples were always higher on MM10 SB agar, either under strict anaerobic conditions or by conventional anaerobic method, and are statistically highly significant ($P = 0.01$, Sign test [3]). The counts of *S. mutans* and *S. sanguis* were comparable on all media, with the exception of MST agar. This is noteworthy, as MS agar with tellurite is the usual medium for the isolation and enumeration of these organisms (5, 12–14). Since the total counts on MM10 SB agar were higher, the differential counts would appear lower than the other media on a percentage basis.
basis and would lead to erroneous conclusions with respect to these organisms in the plaque samples.

M10 medium has been used by several investigators as a nonselective medium for the determination of total counts of anaerobic bacteria present in a variety of samples (2, 4, 7, 17). In all instances it was found to be satisfactory. In our studies we found MM10 SB agar to be a highly valuable medium for the nonselective isolation of bacteria present in the normal and disease-associated plaques. It also serves as a useful medium for the differential counts of S. mutans and S. sanguis (16). It is an ideal medium for culturing plaque samples by either strict anaerobic conditions or by conventional anaerobic methods. As it contains DTT, a compound that is resistant to oxidation under aerobic conditions (6), it facilitates improved recovery of bacteria from all types of plaques by preserving their viability during the culturing procedure. This medium was used in our laboratory in the past to study the predominant cultivable flora from periodontal and carious plaque (15, 16). On the basis of the results obtained from such studies, it can be stated that it is an all-purpose medium that allows growth of a variety of gram-positive members of the oral flora such as streptococci, actinomycetes, lactobacilli, diphtheroids, clostridia, bacteroides, fusobacteria, and veillonella.

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LITERATURE CITED

1. Aranki, A., S. A. Syed, E. B. Kenney, and R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Appl. Microbiol. 17:568-576.
2. Barnes, E. M., and G. C. Burton. 1970. The effect of hibernation on the cecal flora of the thirteen lined ground squirrel (Citelus tridecemlin-eatus). J. Appl. Bacteriol. 33:505-514.
3. Beyer, W. H., ed. 1966. Handbook of tables for probability and statistics, p. 397-398. The Chemical Rubber Co., Cleveland, Ohio.
4. Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794-801.
5. Carlsson, J. 1967. Presence of various types of non-hemolytic streptococci in dental plaque and in other sites of the oral cavity in man. Odontol. Revy 18:55-74.
6. Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3:480-482.
7. Eller, C., M. R. Crabill, and M. P. Bryant. 1971. Anaerobic roll tube media for nonselective enumeration and isolation of bacteria in human feces. Appl. Microbiol. 22:522-529.
8. Gibbons, R. J., and S. S. Sooranausk. 1962. Intracellular polysaccharide storage by organisms in dental plaques. Its relation to dental caries and microbial ecology of the oral cavity. Arch. Oral Biol. 7:73-80.
9. Gilmour, M. N., and A. E. Poole. 1970. Growth stimulation of the mixed microbial flora of human dental plaques by hemin. Arch. Oral Biol. 15:1343-1353.
10. Gordon, D. F., M. Stutman, and W. J. Loesche. 1971. Improved isolation of anaerobic bacteria from gingival crevice area of man. Appl. Microbiol. 21:1046-1050.
11. Hungate, R. E. 1950. The anaerobic mesophilic cellulosic bacteria. Bacteriol. Rev. 14:1-49.
12. Jordan, H. V., H. R. Engleander, and S. Lim. 1969. Potentially cariogenic streptococci in selected population groups in the western hemisphere. J. Amer. Dent. Ass. 78:1331-1335.
13. Kraiss, B. 1954. The proportional distribution of Streptococcus salivarius and other streptococci in various parts of mouth. Odontol. Revy 5:203-211.
14. Littleton, N. W., S. Kakehashi, and R. J. Fitzgerald. 1970. Recovery of specific "caries-inducing" streptococci from carious lesions in the teeth of children. Arch. Oral Biol. 15:461-464.
15. Loesche, W. J., R. N. Hockett, and S. A. Syed. 1972. The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch. Oral Biol. 17:1311-1325.
16. Loesche, W. J., and S. A. Syed. 1973. The predominant cultivable flora of carious plaque and carious dentine. Caries Res. 7:201-216.
17. Mah, R. A., and C. Sussman. 1967. Microbiology of anaerobic sludge fermentation. I. Enumeration of non-methanogenic bacteria. Appl. Microbiol. 16:338-361.
18. McCabe, R. M., P. H. Keyes, and A. Howell, Jr. 1967. An in-vitro method for assessing the plaque forming ability of oral bacteria. Arch. Oral Biol. 12:1653-1666.
19. Reed, G. B., and J. H. Orr. 1943. Cultivation of anaerobes and oxidation reduction potentials. J. Bacteriol. 45:309-320.
20. Syed, S. A., and W. J. Loesche. 1972. Survival of human dental plaque flora in various transport media. Appl. Microbiol. 24:638-644.