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Agar Media That Indicate Acid Production from Sorbitol by Oral Microorganisms

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Two varieties of agar medium (Trypticase [BBL Microbiology Systems]-serum-sorbitol-bromcresol purple agar [TSSB] and Trypticase-blood-sorbitol-$CaCO_3$ agar [TBSCa]) indicating microbial acid production from sorbitol were tested. The media were devised for use in studies on the prevalence of sorbitol-fermenting human oral microorganisms incubated in an anaerobic or microaerophilic atmosphere containing 5 to 6% CO_2. TSSB contains bromcresol purple as the pH indicator and $NaHCO_3$ as the main buffering salt. TBSCa contains $CaCO_3$ as both the buffering salt and the indicator of acid production. The growth yield of pure cultures of oral microorganisms on TBSCa was shown to equal that on blood agar incubated under similar conditions. TSSB inhibited the growth of several bacteria to various extents. The recovery of sorbitol-fermenting microorganisms from oral specimens was the greatest when the specimens were assayed with TBSCa. The poorer results obtained with TSSB were mainly due to the decoloration of the pH indicator in this medium and the presence of greater numbers of sorbitol false-positive colonies.

The identification and enumeration of microorganisms producing acid from various carbohydrates are commonly done with culture media containing pH indicators (3). In such agar media, the color reaction of the indicator around and within the colonies is registered (2, 8–10). In connection with a study on persons who frequently consumed sorbitol-containing foodstuffs and sweets, bacteriological analyses were performed with such media containing sorbitol as the main carbon source (D. Birkhed, S. Edwardsson, and G. Svensäter, Scand. Assoc. Dent. Res., abstr. no. 93, 1980). The samples were mainly incubated in H_2–5% CO_2 or N_2–6% CO_2. However, anaerobic incubation often led to results which were difficult to interpret. The indicator in part or all of the agar was often more or less discolored and bleached, presumably because of the low redox potential existing in an anaerobic atmosphere (7). Furthermore, false-positive reactions of the fermentative properties of the cultured bacteria were not uncommon. It could be demonstrated that this acid reaction was created by the CO_2 in the incubation gas mixture. The pH on the surfaces of some agar media incubated in H_2 with 5 to 6% CO_2 decreased by 0.5 to 1.5 units, as compared with that on similar plates incubated aerobically. To overcome this disadvantage, we tested two new varieties of sorbitol-containing solid medium.

MATERIALS AND METHODS

The base of the first medium has previously been used in our laboratory (Birkhed et al., Scand. Assoc. Dent. Res., abstr. no. 93, 1980). The main modification of this medium is the addition of $NaHCO_3$. The medium (TSSB) has the following composition: Trypticase soy agar (BBL Microbiology Systems), 40 g; yeast extract (Difco Laboratories), 2 g; sorbitol, 10 g; bromcresol purple, 80 mg. The ingredients were dissolved in 870 ml of distilled water by being boiled for 5 min, 1 g of cysteine hydrochloride (Merck & Co., Inc.) was added, and the medium was autoclaved in 500-ml volumes at 121°C for 20 min. The medium was then cooled to 45°C. A 20-ml quantity of inactivated horse serum, 10 ml of sterile 5% hemin, 0.1 ml of sterile filtered 5% menadione, and 100 ml of sterile filtered 1.68% $NaHCO_3$ (freshly prepared) were added to the entire 870 ml of medium and mixed well. The medium was poured into petri dishes and stored at room temperature until used.

The storage time never exceeded 2 weeks. When the plates were stored in air, the pH increased by approximately 1 unit after 24 to 48 h; when the plates were kept in an atmosphere containing 5 to 6% CO_2, the pH was maintained at 7.1 to 7.3. The sorbitol-fermenting properties of the cultured strains were determined by examining the color of the colonies and the medium surrounding them.

The second medium (TBSCa) is a modification of a medium described earlier for the recovery of acidogenic and aciduric bacteria from samples from the oral cavity (4, 5, 11). The composition is as follows: Trypticase soy agar (BBL), 40 g; yeast extract (Difco), 2 g; sorbitol, 10 g; $CaCO_3$, 10 g; KH_2PO_4, 912 mg; K_2HPO_4, 228 mg; KNO_3, 250 mg; NaCl, 17.1 mg; $MgSO_4 \cdot 7H_2O$, 7 mg; $(NH_4)_2SO_4$, 17.1 mg. The ingredients were boiled in 875 ml of distilled water as described above. Cysteine hydrochloride (1 g) was added, and the medium was autoclaved in 500-ml amounts at 121°C for 30 min. After the medium was cooled to 45°C, 14 ml of inactivated horse serum, 20 ml of laked human blood, and 10 ml of sterile 5% hemin were added to the entire volume. To avoid sedimentation of the insoluble $CaCO_3$, the medium was kept in a dispenser bottle at 45°C under continuous stirring. A layer of 8 ml of the medium was poured into petri dishes and allowed to set rapidly on a precooled plate, and 8 ml of the same medium was poured on top.

The plates were stored as described for TSSB. The pH of TBSCa is approximately 7.2. Storage of the agar plates in air and at room temperature and incubation of them in a 5 to 6% CO_2-containing atmosphere at 37°C resulted in pH changes of less than 0.2 units. The low solubility of $CaCO_3$ resulted in the presence of particles of less than 25 μm in diameter in the medium, easily visible with a stereomicroscope (magnification, ×30 to 40). In the presence of acids, the $CaCO_3$ particles reacted, forming water-soluble calcium salts. Microbial sorbitol fermentation was indicated by the formation of particle-free zones around the colonies owing to the

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reaction of the $CaCO_3$ particles with the acids produced from sorbitol (Fig. 1).

Blood agar (6) and MM10-sorbitol agar (MM10-S), which has been used to indicate the presence of sorbitol-fermenting bacteria in the oral cavity (10), were also included for comparative purposes. MM10-S contains bromcresol purple as the pH indicator.

The growth of various oral microorganisms was evaluated by studying 64 pure cultures of bacteria and yeasts from the reference strain stock at our department. They belonged to the genera *Actinomyces*, *Bacteroides*, *Candida*, *Capnocytophaga*, *Eubacterium*, *Fusobacterium*, *Haemophilus*, *Lactobacillus*, *Neisseria*, *Propionibacterium*, *Rothia*, *Streptococcus*, *Torulopsis*, *Veillonella*, and *Wollinella*. A total of 16 strains within the genera *Actinomyces*, *Lactobacillus*, *Rothia*, and *Streptococcus* fermented sorbitol. Inocula of the strains prepared from 48-h cultures grown on blood agar were suspended in 0.1 M phosphate buffer (pH 6.8) to correspond to a McFarland no. 1 standard. This suspension was diluted in the buffer solution, and samples of appropriate dilutions were inoculated onto blood agar, MM10-S, TSSB, and TBSCa. The plates were incubated at 37°C in H_2–6% CO_2 for 7 days. For each strain, the numbers of CFU on the three sorbitol-containing media were compared with those on blood agar.

The degradation of sorbitol to acids by the tested bacteria was confirmed by culturing the organisms in prereduced peptone-yeast broth (6) and phenol red broth (lot B92; Difco) in the presence and absence of sorbitol at 37°C for 7 days. A difference in the pH decrease of 1 unit or more between the sorbitol-containing and base broths was considered to indicate a positive fermentation reaction.

Seven plaque and three saliva samples from seven subjects were also used to study the recovery of sorbitol-fermenting organisms by using the three sorbitol-containing agar media and by following the procedures described by Birkhed et al. (1). A total of 150 sorbitol-fermenting and -non-fermenting strains (50 strains from every medium) were isolated, and their fermentation characteristics were confirmed as described above.

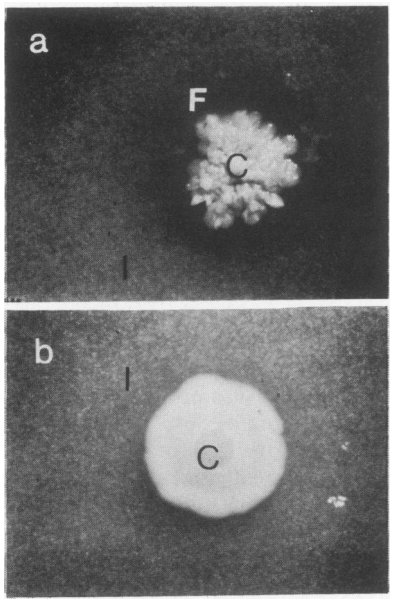

FIG. 1. Indication of acid production on TBSCa. (a) Sorbitol-positive bacterium; C, colony; F, particle-free zone around the colony; I, insoluble $CaCO_3$. (b) Sorbitol-negative bacterium; C, colony; I, insoluble $CaCO_3$ present up to the edge of the colony.

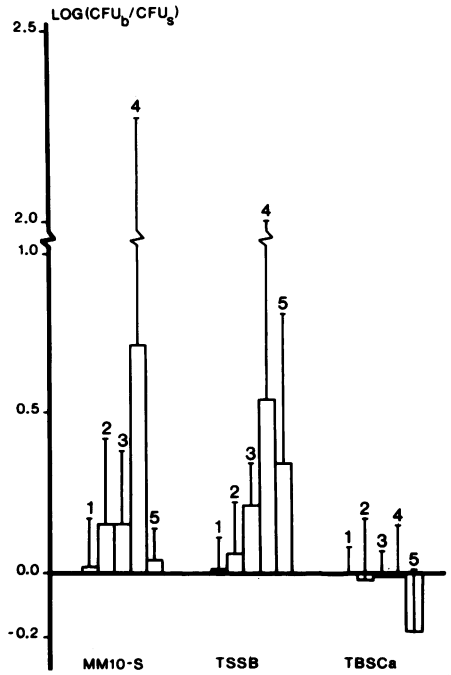

FIG. 2. Relative growth-supporting ability (RGSA) of MM10-S, TSSB, and TBSCa evaluated with pure cultures of oral microorganisms. The RGSA is expressed as the logarithm of the ratio of the CFU of the tested organism on blood agar (CFU_b) to the CFU of the same organism on sorbitol-containing solid medium (CFU_s). The tested organism will grow to the same or greater extent on the examined medium than on blood agar if the RGSA is ≤ 0. The reverse is true if the RGSA is >0. The mean RGSA values and the standard deviations are presented for the following groups of microorganisms: 1, gram-positive cocci (27 strains); 2, gram-positive rods (15 strains); 3, gram-negative cocci (5 strains); 4, gram-negative rods (14 strains); and 5, yeasts (3 strains).

RESULTS

The recovery of the tested microorganisms on MM10-S, TSSB, and TBSCa is shown in Fig. 2. The growth yield was lower on MM10-S and TSSB than on blood agar, especially for the gram-negative rods. All five microbial groups tested showed approximately equally good growth on blood agar and TBSCa. None of the strains tested showed a difference of more than 3-fold in the number of CFU on TBSCa as compared with that on blood agar, whereas a 10-fold difference or more was observed for five strains on MM10-S and for three strains on TSSB.

The capacity of the three media to indicate acid production from sorbitol by the tested strains showed that out of a total of 16 sorbitol-fermenting strains, 14 could be characterized as being sorbitol positive when cultured on all three solid media. The tested *Bacteroides* and *Veillonella* strains decolorized the TSSB and MM10-S plates to various degrees, resulting in difficulties in determining the fermentation reaction. In addition, four strains of oral streptococci and one lactobacillus strain formed yellow colonies on MM10-S, although they were unable to ferment sorbitol in the confirmatory test.

Approximately 58% of the isolated strains grown on

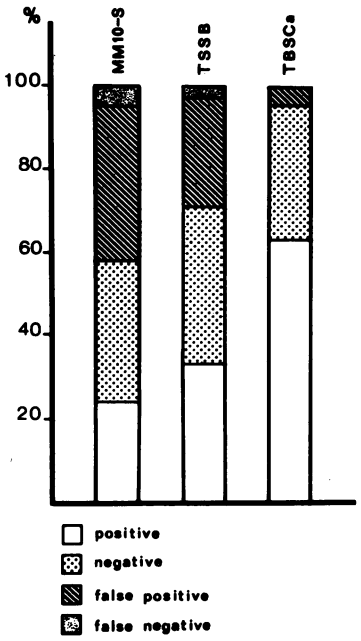

FIG. 3. Comparison of the sorbitol-indicating ability of the three sorbitol-containing agar media MM10-S, TSSB, and TBSCa. A total of 50 colonies were isolated from each plate, that amount being equivalent to 100% in the figure.

MM10-S and recultured in the confirmation test broths showed the same fermentation pattern (Fig. 3). Corresponding values for TSSB and TBSCa were 71 and 95%, respectively. In addition, the buffering capacity of TSSB and MM10-S was found to be less than that of TBSCa. When the number of colonies formed by sorbitol-fermenting organisms exceeded approximately 15 per plate on TSSB and MM10-S, the entire plate or a large part of it showed an acid color reaction.

DISCUSSION

In TSSB, $NaHCO_3$ was added to increase the buffering capacity of the medium; in TBSCa $CaCO_3$ was incorporated both to increase the buffering capacity and to act as the indicator of sorbitol fermentation. When an incubation atmosphere containing 5 to 6% CO_2 was used, the best results were obtained with TBSCa. The ability to indicate sorbitol fermentation can be considered good. The $CaCO_3$ particles and the particle-free zones around sorbitol-positive colonies on TBSCa can easily be observed with a dissection microscope (magnification, ×30 to 40). The formation of the particle-free areas is, however, dependent on the incubation time. Difficulties in determining the fermentative properties of some bacteria may arise when the incubation time is shorter than 48 to 72 h.

Since the indication of carbohydrate fermentation is not dependent on the color of TBSCa, the addition of blood to the agar is possible, resulting in growth yields comparable to those on blood agar for both gram-positive and gram-negative oral microorganisms. The morphological characteristics of the colonies of different strains cultured on TBSCa resemble those on blood agar, whereas the pH indicator-containing media usually show a greater incidence of pleomorphic colonies of the same bacteria.

The recommended double-layer preparation of the TBSCa plates was made to yield a homogenous dispersion of the $CaCO_3$ particles in the medium. The use of laked blood is preferred. Whole blood cells may interfere with the finding of $CaCO_3$ particle-free zones. When determining sorbitol fermentation, it is important to observe the dissolution of the salt particles around the colonies and not only the formation of zones. Some bacteria may cause color changes of the agar medium, mainly through interactions with blood components. This phenomenon was especially observed when samples of oral specimens were cultured on TBSCa in N_2–5% CO_2.

The salt particles were completely dissolved around pH 5.5, which is 1 to 2 pH units lower than that of ordinary agar media. Thus, a clear-cut distinction between fermenting and nonfermenting bacteria should be possible. Recently, we used TBSCa to recover sorbitol-fermenting bacteria from dental plaque material of persons consuming sorbitol frequently. Up to now, more than 200 isolates have been tested. The agreement between the fermentation patterns of the microorganisms on TBSCa and in peptone-yeast broth is as high as that reported in this paper. In the present study, sorbitol was incorporated as the main carbon source. It should also be possible to use TBSCa for the indication of bacterial acid production from other carbohydrates.

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