Glucose-Sucrose-Potassium Tellurite-Bacitracin Agar, an Alternative to Mitis Salivarius-Bacitracin Agar for Enumeration of *Streptococcus mutans*

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An agar medium for selective recovery and enumeration of *Streptococcus mutans* was developed as an alternative to mitis salivarius-bacitracin (MSB) agar. Combinations of dyes, antibiotics, and tellurite were added to a nonselective medium which, because of its sucrose content, allowed easy recognition of *S. mutans* colonies. Candle jar incubation for 2 days, by comparison with anaerobic incubation, reduced background flora but did not diminish *S. mutans* recoveries from clinical samples. Quantitative comparisons were made of the simultaneous recoveries of a number of authentic *S. mutans* serotype representatives and fresh clinical isolates, using various glucose-sucrose-potassium tellurite-bacitracin (GSTB) formulations and mitis salivarius, MSB, and blood agars. Mitis salivarius counts were not detectably different from blood counts, but counts on MSB were distinctly lower. A formulation of the new medium containing 5% glucose, 5% sucrose, 0.001% potassium tellurite, 0.3 U of bacitracin per ml (hence GSTB), and 2% agar gave recoveries nearly equal to those on mitis salivarius agar and much greater than those on MSB. The medium yielded readily recognized *S. mutans* colonies and facilitated detection of intracellular polysaccharide formers upon flooding with I₂ reagent. Freshly isolated serotype *c*, *E*, and *f* colonies could often be distinguished from serotype *d* and *g* colonies, a distinction made reliable by testing for intracellular polysaccharide. A study of 300 salivary samples revealed GSTB to give significantly higher recoveries than MSB. About 72% of all samples were substantially underestimated for *S. mutans* with MSB, and 6.7% of samples were falsely negative for *S. mutans* with MSB. Recovery of background flora on GSTB was as low or lower than on MSB, and both types of agar could be stored for at least 9 weeks without notable change of selectivity. Thus, GSTB agar appears to be simple and reliable to use and requires no anaerobic incubation. Caution is voiced about interpretation of data previously reported which evaluated *S. mutans* on MSB agar.

Much information implicates *Streptococcus mutans* as the prime, albeit not sole, cause of caries of the crowns of teeth of experimental animals and humans (5, 7, 12, 15, 23, 28, 31, 34). Studies of levels of *S. mutans* colonization of human subjects rely on sampling of saliva or dental plaque or both and differential enumeration of *S. mutans* on sucrose-supplemented agar media (2, 14, 16). Because of its greatly localized colonization of tooth surfaces (7), *S. mutans* usually represents a small proportion of pooled plaque and total salivary flora and of their total streptococci, usually evaluated on a streptococcus-selective medium termed mitis salivarius agar (MS; 3). Extensive serial dilution is required to allow differential enumeration of discrete CFU from plaque and salivary samples on agar plates, often resulting in false-negative evaluations for *S. mutans*. Consequently, several putatively selective media (2, 8, 11, 14, 21, 32) have been described which attempt to minimize this need: increasing the sensitivity of *S. mutans* detection, simplifying work, and minimizing cost. The most popular of these is so-called MSB agar (MS agar containing added sucrose and bacitracin [0.2 U/ml]; 14). However, in the course of our longitudinal clinical studies directed at characterizing the possible therapeutic suppression of *S. mutans* in the oral cavity, it became evident that MSB, although excellent for its recognition, severely depressed or totally obliterated the recovery of *S. mutans* from a number of subjects' samples. Indeed, other workers have expressed dissatisfaction with MSB agar (10, 22, 27) on studying pure cultures.

This paper describes the formulation of a simple and easily used alternative medium containing 5% glucose-5% sucrose-0.001% potassium tellurite-0.3 U of bacitracin per ml (GSTB) for partially selective and differential enumeration of *S. mutans*, compares data on the recovery of *S. mutans* from a large number of fresh clinical samples simultaneously cultivated on GSTB and MSB, examines the level of non-*S. mutans* background flora and storability of these agars, and describes the morphologies of *S. mutans* colonies typically recovered on GSTB agar. A preliminary partial report of these data has been given (29).

MATERIALS AND METHODS

Microorganisms. Authentic strains of *S. mutans* were maintained either by monthly transfer in fluid thioglycolate medium supplemented with 20% (vol/vol) meat extract (BBL Microbiology Systems) and excess CaCO₃ or by biweekly transfer on blood agar plates. Strains representative of the several serotypes described by Bratthall (1) and Perch et al. (25) (which are now proposed as separate species [4]) were from our own culture collections. Numerous fresh clinical isolates were also studied, and many were identified as to serotype, as described below.

Selective and nonselective agars. Blood agar was made with Trypticase soy agar supplemented with 5% sheep blood (BBL). MS agar (Difco Laboratories) was supplemented, as recommended, with 1% potassium tellurite (Difco). MSB was formulated according to Gold et al. (14). Multiple modifications of a basal medium (GS) previously shown to be excellent for cultivation of *S. mutans* both in broth (17)

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TABLE 1. Formulation of GSTB agar^a

Component	Amt
Soln A	
Trypticase peptone (BBL)	5 g
Yeast extract (Difco)	5 g
K ₂ HPO ₄	5 g
Na ₂ CO ₃	0.05 g
Agar (BBL)	20 g
Salt solution (consisting of 1.15 g of MgSO ₄ · 7H ₂ O, 0.19 g of MnSO ₄ · H ₂ O, 0.068 g of FeSO ₄ · H ₂ O per 10 ml of water)	0.5 ml
Deionized water	800 ml
Boil, cool, and adjust pH to 7.2 with HCl	
Soln B	
Glucose	50 g
Deionized water	q.s. ^b 100 ml
Soln C	
Sucrose	50 g
Deionized water	q.s. 100 ml

^a Solutions A, B, and C are autoclaved separately at 121°C for 20 min, combined, and allowed to cool to 50 to 55°C before adding 1.0 ml of 1% potassium tellurite (Difco) and 300 U of bacitracin (Sigma), both filter sterilized. After thorough stirring at 55°C, plates are poured. GS medium is made identically, except that tellurite and bacitracin are deleted.

^b q.s., Quantum satis (sufficient quantity).

and on agar surfaces (30) were studied. The solid medium had the composition detailed in Table 1. This medium supports nonselective rapid growth of a number of microorganisms upon incubation at 37°C. Potassium tellurite and crystal violet, constituents of MS agar, were added singly and in various combinations, as was bacitracin and polymyxin B (Sigma Chemical Co.). All were added to the 50 to 55°C molten agar medium either from sterile commercial preparations or after being sterilized by filtration through 0.22-μm-pore-diameter membrane disks (Millipore Corp.). After extensive stirring, molten agar was poured into petri dishes (100 by 15 mm), allowed to solidify, and dried overnight at 37°C. Agar media were stored at 4°C until used within 14 days, unless otherwise stipulated. A medium consisting of the above basal formulation and 1.0 ml of 1.0% potassium tellurite and 300 U of bacitracin per liter was found, as will be described below, to give good selectivity for typical *S. mutans* colonies with minimal loss of *S. mutans* CFU numbers in clinical samples. Because it contained glucose, sucrose, tellurite, and bacitracin, it was termed GSTB agar.

Clinical samples and enumeration of *S. mutans*. Salivary and plaque samples from human volunteers with a wide range of *S. mutans* levels, collected by previously described methods (26, 34), were dispersed in VMG II transport medium (24). They were plated such that 25 μl of suspension was deposited, in duplicate and in at least three log₁₀ dilutions (33), on agar plates of GSTB and MSB. GSTB plates were routinely incubated in candle extinction jars, whereas MSB plates were incubated in either 95% N₂-5% CO₂ or 95% H₂-5% CO₂ for 2 days before evaluation.

Confirmation of identities, intracellular polysaccharide synthesis, and serotype identification. Colonies suspected of being those of *S. mutans* were confirmed as such by examination for typical colonial morphology upon growth at 37°C on MS agar for 48 h anaerobically, by their fermentation of sorbitol, mannitol, raffinose, and melibiose, and by their adhesive growth on the walls of culture tubes and fermentation in the presence of sucrose (4, 25). Iodophilic intracellular polysaccharide synthesis (13) was tested on GSTB plates,

as previously detailed (30). For many isolates, serotypic identification was accomplished according to the methods detailed by Bratthall (1), using strains (serotype) AHT (*a*), FA-1 (*b*), KPSK2 (*c*), B13 (*d*), LM7 (*E*), OMZ175 (*f*), OMZ65 (*g*), and SL-1 (*SL*) to raise specific antisera and as controls. Numerous colonies of doubtful *S. mutans* identity were confirmed as such by these colonial, fermentative, and serological criteria.

RESULTS

Fluid thioglycolate medium cultures of several *S. mutans* strains serially diluted in phosphate-buffered saline gave similar CFU counts on blood and MS agars, consistent with the literature (10, 14, 22), as did plating on the basal medium (GS) under study. Salivary and plaque samples cultured on this basal medium were more selective for streptococci if 100 U of polymyxin B per ml (11), 0.001% potassium tellurite, or 0.08 mg of crystal violet per 100 ml (3), or all three, were present. Incubation in CO₂-enriched air (candle extinction jar) substantially reduced the number of non-*S. mutans* CFU recovered but not *S. mutans* CFU, by comparison with anaerobic incubation. However, whereas such incubation and inclusion of 0.001% potassium tellurite did not reduce *S. mutans* CFU numbers detectably, addition of crystal violet and polymyxin B did. Upon this background information, various formulations led to the recognition that the GS medium required supplementation only with 0.001% potassium tellurite, appropriate levels (below) of bacitracin, and incubation in CO₂-enriched air to render it highly selective for, but not inhibitory to, *S. mutans* while retaining its readily recognized colonial morphology.

Optimization of bacitracin levels and study of pure cultures.

To determine the optimal concentration of bacitracin for inclusion in GSTB, pure cultures grown in NIH fluid thioglycolate broth (Difco, 0257-01) were simultaneously plated onto MS, MSB, and GSTB agars, the latter containing bacitracin at 0.1 to 5.0 U/ml. Figure 1 illustrates that for almost all of the 10 reference strains MSB substantially underestimated *S. mutans* numbers by comparison with MS, whereas recoveries on GSTB containing 0.1 or 0.5 U of bacitracin per ml, in almost all cases, better approximated those on MS. Strain SL-1 appeared somewhat inhibited by MS, as well as by MSB, because its CFU numbers on GSTB with 0.1 to 1.0 U of bacitracin per ml were higher than on MS.

Analogous studies were conducted which more closely examined a range of bacitracin concentrations, 0.1 to 0.7 U/ml, by comparison with recoveries on MS and MSB. In general, recoveries with pure cultures were better with GSTB with 0.1 and 0.3 U of bacitracin per ml than with higher bacitracin levels or with MSB (Fig. 2). Like MSB, GSTB formulations entirely inhibited growth of three serotype *a* strains. Two serotype *b* strains, very poorly recovered on MSB, were well recovered on GSTB with 0.1 and 0.3 U of bacitracin per ml. With the strains representing the other serotypes, there was considerable between-strain and between-serotype variability. Also, eight fresh clinical isolates, retrospectively serotyped as *c*, *d/g*, and *E*, gave recoveries like those of the reference strains. Overall, 0.1 and 0.3 U of bacitracin per ml in GSTB gave CFU counts for the average nonserotype *a* strain of 94.9 ± 4.0 and 80.1 ± 4.6%, respectively, compared with 57.5 ± 6.3% for MSB; thus, both of these formulations of GSTB gave recoveries significantly higher than MSB (*P* < 0.01).

Because preliminary clinical studies indicated more complete inhibition of background flora of fresh salivary and

FIG. 1. Comparison of simultaneous recoveries of stock *S. mutans* cultures, representing its various serotypes, on MS, MSB, and formulations of GSTB with concentrations of bacitracin ranging from 0.1 to 5.0 U/ml. Recoveries are expressed as a percentage of those on MS. Symbols: Serotype *c*—GuSt8 (●), Ingbritt (○), NCTC 10449S (■), NCTC 10449S-805 (□); serotype *d*—B13 (△); serotype *E*—B2 (▽); LM7 (◆); serotype *f*—OMZ175 (◇); serotype *g*—OMZ65 (▼); serotype *SL*—SL-1 (▲). The lines connect recovery values for each strain at various medium formulations.

dental plaque cultures by use of the 0.3-U/ml rather than the 0.1-U/ml bacitracin level, the former was routinely chosen for clinical use and simply named GSTB, the formulation of which is detailed in Table 1.

Colonial morphologies. After 2 days of growth, *S. mutans* was easily identified on GSTB, as it was on MSB (Fig. 3A and B). The colonies, viewed with incident light, ranged from 0.02 to 0.7 mm in diameter. Fresh clinical isolates of *c*, *E*, and *f* cells had typically rough frosted morphologies (Fig. 3A), whereas fresh clinical isolates of *d* and *g* cells had typically smoother, though frosted, morphologies (Fig. 3B). Serotype *c*, *E*, and *f* colonies were generally circular to irregular in form. They were pulvinate to umbonate in elevation, and their margins ranged from entire to undulate. Generally, fresh isolates of serotype *d/g* strains were circular in form, pulvinate to umbonate in elevation, and entire in margin. All types often exhibited "bubble" formation on the colony's apex and, with further incubation, "puddle" formation around colonies. They also tended to become increasingly rough to lobate in margin with time so that identification of colonies as *S. mutans* was more difficult with more than 3 days of incubation. Stock cultures sometimes were more or less rougher than fresh isolates of the same serotype. Differences in coloration ranging from beige to brown and dark grey or even green for *c*, *E*, and *f* were notable on GSTB, but these hues were not a reliable guide to serotype. The serotype *d/g* cells were usually slate grey. No serotype *a* cells grew on any of these agars and, whereas serotype *b* stock strains and the type strain of serotype *SL* grew well on GSTB, no serotype *b* or *SL* strains were isolated in the course of these studies; thus, the typical morphology of their fresh isolates could not be demonstrated.

Whereas the formation of intracellular iodophilic polysaccharide could be tested on both MSB and GSTB, it was very easily detected on the clear GSTB plates. Serotype *d/g* cells were always negative; serotype *c*, *E* and *f* fresh isolates were always positive, albeit to varying degrees, consistent with the literature (13).

Quantitative recoveries from clinical samples. In a series of clinical studies, 300 salivary cultures were simultaneously

FIG. 2. Comparison of simultaneous recoveries of stock *S. mutans* cultures representing its various serotypes and fresh isolates on MS, MSB, and formulations of GSTB with concentrations of bacitracin ranging from 0.1 to 0.7 U/ml. Recoveries are expressed as a percentage of those on MS. Data are for two serotype *a* (●), two serotype *b* (■), two serotype *c* (□), six serotype *d/g/SL* (▲), three serotype *E* (△), and one serotype *f* (▽) strains and 10 fresh clinical isolates (○).

FIG. 3. Colonial morphologies of (A) serotype *c*, *E*, and *f* strains and (B) serotype *d* and *g* strains on GSTB (left) and MSB (right) agars after incubation as stipulated in the text.

enumerated for *S. mutans* on MSB and GSTB. Figure 4A is a scattergram of absolute frequency as a function of the ratio of GSTB/MSB CFU recoveries. The vast majority of GSTB/MSB recovery ratios was >1 ; many were much higher. If one disregards those ratios of $>150/1$, so as to foster statistical analyses based on normally distributed data, the remaining 278 samples had a mean GSTB/MSB ratio of 2.87 ± 0.40 standard error of the mean. A paired *t*-test comparison established that absolute GSTB values were greater than absolute MSB values, $P < 0.0001$ ($n = 278$). Considering all samples, the median ratio was 1.667.

Figure 4B presents the same data in histogram format and illustrates frequency of probable error estimates with MSB. Because there was an estimated maximum 20% error in plating and counting duplicate samples for *S. mutans* on the two media, ratios of 0.81 to 1.20 were conservatively viewed as giving concordant data on the two agars. These represented only 20.3% of all 300 samples. Samples for which the GSTB/MSB ratio was <0.81 constituted 7.7% of the total, with the greatest observed discrepancy being 14-fold lower (GSTB/MSB ratio, 0.071). GSTB values were greater than those of MSB (ratio, 1.20 to infinity) for about 72% of all

FIG. 4. Scattergram of the ratio of simultaneous recovery values of *S. mutans* on GSTB and MSB from 300 fresh salivary samples (A) and histogram (B) of data in (A). (A) Note that ratios of <1 are plotted on a normalized inverse ratio scale of the same dimension as those of >1; thus, 0.25 is the same distance below unity as 4 is above unity, and 0.125 is the same distance from unity as 8, etc. (B) Illustrates the frequency of the probable estimation error for samples cultured on MSB compared with GSTB and the absolute number of samples falling into each of the stratifications.

samples, GSTB values 3-fold to infinitely greater than MSB were about 25% of all samples, those values 10-fold to infinitely greater than MSB were 12.7% of all samples, and those where MSB gave a total false-negative (GSTB/MSB ratio, infinity) represented 6.7% of all samples.

Background flora and storability of media. In the course of these studies it was clear that saliva and plaque samples from subjects had variable numbers of background organisms. On GSTB they were most commonly *Candida* sp. and enterococci and on MSB they were most commonly enterococci, but other streptococci were also found on both media. With neither agar was there a serious problem of misidentifying background flora as *S. mutans* once there was a short training period to identify the range of *S. mutans* colonial morphologies. Nonetheless, colonial forms and questionable CFU types were routinely checked for their *S. mutans* or non-*S. mutans* identities, using the criteria listed above.

A study of selectivity loss and, thus, storability of these two media was conducted, using three subjects' saliva samples collected over a 9-week period. Single batches of agar plates were made on the same day and stored at 4°C in sealed plastic sleeves. The three subjects were selected because of their regular availability and the large range of *S.*

mutans levels they typically had in their salivas. Figure 5 illustrates that neither medium, when stored in the cold, notably lost its selectivity with time. However, for the three subjects GSTB was more selective, allowing less growth of background flora than MSB. As seen with both media, there was considerable day-to-day fluctuation of absolute numbers (not shown) of *S. mutans* in the subject's salivas, as described by others (9, 18-20, 34). It probably reflects, at least in part, changes of dietary intake and oral hygiene.

DISCUSSION

Although several media with some degree of selectivity have been described for *S. mutans*, they either fail to differentiate *S. mutans* from other polyol- and sucrose-fermenting microorganisms or they are difficult to use because of high levels of background flora or uncertainty of interpretation (21, 27) or both. Some seriously fail to recover the prevalent serotype *d/g* strains (2, 22), or, as seen here for MSB, they substantially underestimate the presence of *S. mutans* in both pure and mixed clinical culture samples. The present report appears to be the first to attempt both extensive quantitative recovery evaluations with clinical cultures, as well as reference strains, and longitudinal evalu-

FIG. 5. Percentage of total CFU on MSB (○) or GSTB (●) agars which are *S. mutans* repeatedly measured for three subjects (A, B, and C) over 9 weeks, using the same batches of agar plates.

ations of the storability and selectivity of the media under study.

GSTB agar is neither totally selective nor totally free from loss of *S. mutans* numbers. Nonetheless, the current data support the conclusions that: (i) GSTB recoveries are nearer numerically to MS recoveries than are MSB recoveries for pure cultures of diverse *S. mutans* strains; (ii) GSTB is easily and inexpensively formulated; (iii) GSTB is adequately and ideally incubated in CO₂-supplemented air, rendering it useful in field studies with either candle extinction jars or plastic bags inflated with exhaled air and obviating the need for anaerobic incubation; (iv) GSTB is easily interpreted for identification and enumeration of *S. mutans*; (v) GSTB is used with ease for differential identification of intracellular polysaccharide synthesis-positive and -negative *S. mutans* which are of serotypes *c*, *E*, *f*, and *d/g/SL*, respectively; (vi) GSTB yields significantly higher recoveries of *S. mutans*

than the widely used MSB agar and avoids the false-negative values of MSB agar; (vii) GSTB gives as low or lower recovery of background flora than MSB agar; and (viii) GSTB, as well as MSB, can be stored at 4°C without detectable loss of relative selectivity for at least 9 weeks.

In view of the perceived inadequacy of MSB agar for detection and quantitation of *S. mutans* in clinical samples, it is necessary to interpret with caution previously reported *S. mutans* prevalence and incidence data (18, 19, 34) and data on attempts to therapeutically suppress it (9, 20).

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