An improved medium for isolation of *Streptococcus mutans*

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Summary. A medium based upon tryptone, yeast extract, cystine (TYC) agar and incorporating bacitracin and sucrose has been evaluated for selective isolation of *Streptococcus mutans*. The effect of varying the concentrations of sucrose and bacitracin on the recovery of two standard strains was investigated. Growth of *S. mutans* NCTC 10449 was significantly inhibited by increasing concentrations of sucrose but was not affected by bacitracin; the reverse was seen with *S. sobrinus* strain 6715. The best compromise between recovery of the streptococci and growth of other organisms was obtained with a final sucrose concentration of 20% and bacitracin 0.2 units/ml. In comparison with three other selective media, this medium gave the highest recovery rate of standard strains, indicating that it is superior to mitis-salivarius bacitracin (MSB) agar for the recovery of *S. mutans* from saliva.

Introduction

There is considerable evidence incriminating *Streptococcus mutans* as the most important organism in the initiation of dental caries (McGhee and Michalek, 1981). The attention focussed on *S. mutans* in studies on the epidemiology and aetiology of caries, and the fact that this organism constitutes a relatively small proportion of dental plaque and salivary flora, has led to the development of many selective media for its isolation. These include a medium similar to mitis-salivarius (MS) agar containing a sulphonamide (Carlsson, 1967); mitis-salivarius-40% sucrose (MS40S) agar (Ikeda and Sandham, 1972a); mitis-salivarius-sucrose-bacitracin (MSB) agar (Gold *et al.*, 1973); mannitol-sorbitol-fuchsin-azide (MSFA) agar (Linke, 1977); and glucose-sucrose-tellurite-bacitracin (GSTB) agar (Tanzer *et al.*, 1983).

The shortcomings of these media prompted the formulation of a medium based on tryptone, yeast extract, cystine (TYC) agar (de Stoppelaar, 1971) with the addition of sucrose and bacitracin (Van Palenstein Helderman *et al.*, 1983; Wade *et al.*, 1983). The effects of varying the concentration of sucrose and bacitracin on the recovery of two standard strains of *S. mutans* and on the numbers of contaminating organisms have been investigated and this new medium (TYCSB) compared to MSB, MSFA and GSTB.

Materials and methods

Bacterial strains

The standard strains employed in the study were *S. mutans* NCTC 10449 (serotype c) and *S. sobrinus* strain 6715 (*S. mutans* serotype d/g).

Saliva samples

Whole unstimulated saliva was collected from 20 randomly selected adult dentate subjects.

Effect of different concentrations of sucrose and bacitracin in TYC agar on recovery of *S. mutans*

Sucrose was added to TYC agar before autoclaving to give final concentrations of 10, 20 and 30% w/v. When the agar had cooled to 50°C, bacitracin was added to give concentrations of 0.1, 0.2 and 0.3 units/ml.

Suspensions of *S. mutans* NCTC 10449 and *S. sobrinus* strain 6715 were made from 48-h anaerobic plates in phosphate-buffered saline (PBS) and disaggregated by vortex mixing for 30 s and passage five times through a 25-gauge needle (Adams *et al.*, 1982). Ten-fold dilutions were then prepared in PBS and 0.1 ml of the 10³, 10⁴ and 10⁵ dilutions spread in triplicate on blood agar, TYC agar and the TYC agar containing different combinations of sucrose and bacitracin. The plates were incubated anaerobically for 48 h at 37°C. Plates with colony counts between 30 and 300 were selected. The result for each combination was expressed as a percentage of the

count on blood agar and the effects of sucrose and bacitracin assessed statistically by analysis of variance.

Effect of different concentrations of sucrose and bacitracin on the numbers of contaminants growing on supplemented TYC plates inoculated with saliva

Concentrations of sucrose and bacitracin added to TYC agar were used as described above. Five saliva samples were disaggregated by passage through a 25-gauge needle and a 10^2 dilution in PBS prepared; 0.1 ml of each sample was plated on to each of the combinations in duplicate. The plates were incubated anaerobically for 48 h at 37 °C. After incubation the plates were examined and the number of organisms other than *S. mutans* assessed. The density of growth was scored semi-quantitatively: "0" indicated no contaminating organisms and "3" a "lawn" of contaminants.

The recovery of standard strains of S. mutans on TYC agar containing sucrose 20% and bacitracin 0.2 units/ml compared to that on other selective media

The recovery of *S. mutans* on TYC agar containing bacitracin 0.2 units/ml and supplemented with sucrose at a final concentration of 20% was compared with that on three other selective media—MSB (Gold *et al.*, 1973), MSFA (Linke, 1977) and GSTB (Tanzer *et al.*, 1983). Suspensions of *S. mutans* and *S. sobrinus* were prepared in PBS and the media inoculated and incubated as described above.

Recovery of S. mutans from saliva on TYC agar containing sucrose 20% and bacitracin 0.2 units/ml and MSB

Twenty saliva samples were processed as before and 0.1 ml of 10^1 , 10^2 and 10^3 dilutions plated on TYC agar containing sucrose 20% and bacitracin 0.2 units/ml and on MSB in triplicate. Plates were incubated anaerobically for 48 h at 37 °C when colonies whose morphology was typical of *S. mutans* were counted on appropriate plates. The results obtained with the two media were compared statistically by a paired *t* test.

Results

Table I shows the recoveries of *S. mutans* NCTC 10449 and *S. sobrinus* strain 6715 on TYC and blood agar. The recovery of each strain on the selective medium exceeded 90% of that on blood agar.

Table II shows the effect of the sucrose and bacitracin supplements in the TYC agar on the percentage recoveries of the two strains. For *S. mutans* the recovery varied from 65% (bacitracin 0.1 units/ml,

Table I. Recovery of *S. mutans* NCTC 10449 and *S. sobrinus* strain 6715 on TYC agar and blood agar

Species	Number of colonies on		Percentage recovery on TYC
	Blood agar (n = 3)	TYC (n = 3)	
<i>S. mutans</i>	100	98	98
<i>S. sobrinus</i>	250	233	93

Table II. Recovery of *S. mutans* NCTC 10449 and *S. sobrinus* strain 6715 on TYC agar supplemented with different concentrations of sucrose and bacitracin and on blood agar

Species	Bacitracin concentration (units/ml)	Percentage of count on blood agar at sucrose concentration (%w/v)		
		10	20	30
<i>S. mutans</i> *	0.1	65	51	40
	0.2	63	56	42
	0.3	55	41	38
<i>S. sobrinus</i> †	0.1	89	73	71
	0.2	78	73	67
	0.3	50	37	49

* F values ($df_1 = 2$, $df_2 = 4$): bacitracin 6.38 (NS); sucrose 30.81 ($p < 0.01$).

† F values ($df_1 = 2$, $df_2 = 4$): bacitracin 28.01 ($p < 0.05$); sucrose 3.55 (NS).

sucrose 10%) to 38% (bacitracin 0.3 units/ml, sucrose 30%), and for *S. sobrinus* from 89% (bacitracin 0.1 units/ml, sucrose 10%) to 37% (bacitracin 0.3 units/ml, sucrose 20%). Sucrose had a significant inhibitory effect on *S. mutans* but not on *S. sobrinus*. The converse was true for bacitracin.

The five saliva samples varied widely in the degree of growth of contaminants (table III). For example, sample C showed no growth on any of the plates, whereas sample E gave a score of at least 2 on all plates. Inhibition of contaminants was observed with increasing concentrations of sucrose, while bacitracin appeared to have only a negligible effect.

The recovery of *S. mutans* on the four selective media is shown in table IV. The best recoveries were with GSTB (64%) and TYCSB (60%); MSB gave 46% recovery but MSFA did not allow growth of this organism at all. TYCSB gave the best recovery of *S. sobrinus* (73%), followed by MSFA (70%) and GSTB (63%). MSB gave only 11% recovery. The recovery of *S. mutans* from 20 saliva samples with TYCSB and MSB is shown in table V.

Table III. Growth of organisms other than *S. mutans* on supplemented TYC plates

Saliva sample	Bacitracin concentration (units/ml)	Growth* of other species at sucrose concentration (% w/v)		
		10	20	30
A	0.1	3	1	0
	0.2	3	1	0
	0.3	2	1	0
B	0.1	1	0	0
	0.2	0	0	0
	0.3	0	0	0
C	0.1	0	0	0
	0.2	0	0	0
	0.3	0	0	0
D	0.1	2	1	0
	0.2	2	1	0
	0.3	2	1	0
E	0.1	3	3	2
	0.2	2	2	2
	0.3	3	2	2

* 3 = confluent growth; 2 = intermediate; 1 = slight; 0 = no growth.

Table IV. Recovery of *S. mutans* and *S. sobrinus* on MSB, MSFA, GSTB, TYCSB and blood agar

Medium	Mean number of colonies (on three plates) of		Percentage of count on blood agar	
	<i>S. mutans</i>	<i>S. sobrinus</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
Blood agar	320	149	100	100
MSB	147	16	46	11
MSFA	no growth	105	—	70
GSTB	205	94	64	63
TYCSB	193	108	60	73

Because of the wide variation in the counts in different individuals, it was first necessary to normalise the distribution of the data to perform statistical analyses. A logarithmic transformation was used and was followed by a paired *t* test. Sample 13 yielded no *S. mutans* from a 10^1 dilution and was discarded, thus reducing *n* to 19.

TYCSB gave a significantly higher recovery of *S. mutans* than MSB ($p < 0.05$).

Discussion

Gold *et al.* (1973) devised a medium selective for *S. mutans* consisting of mitis-salivarius agar with

Table V. Isolation of *S. mutans* from saliva on TYCSB and MSB

Saliva sample no.	Viable count (cfu/ml) of <i>S. mutans</i> on			
	TYCSB (10^4)	MSB (10^4)	$\log_{10}$ TYCSB count (10^4)	$\log_{10}$ MSB count (10^4)
1	19.0	27.0	1.28	1.43
2	8.7	6.8	0.94	0.83
3	220.0	150.5	2.34	2.18
4	59.0	66.0	1.77	1.82
5	9.2	7.8	0.96	0.89
6	32.1	20.8	1.51	1.32
7	8.6	5.7	0.93	0.76
8	19.3	12.0	1.29	1.08
9	8.5	5.6	0.93	0.75
10	6.8	6.5	0.83	0.81
11	11.4	10.5	1.06	1.02
12	1.1	1.1	0.04	0.04
14	26.7	23.9	1.43	1.38
15	24.8	15.5	1.39	1.19
16	13.5	3.6	1.13	0.56
17	65.3	61.7	1.81	1.79
18	24.0	22.3	1.38	1.35
19	95.0	72.0	1.98	1.86
20	41.0	52.0	1.61	1.72

$\log_{10}$ TYCSB count vs. $\log_{10}$ MSB count, $t = 2.738$, $p < 0.05$.

bacitracin 0.2 units/ml and a sucrose concentration of 20%. These workers reported undiminished recovery of *S. mutans* and good suppression of other organisms. However, it was subsequently reported that serotype-a strains were inhibited completely and that the various manufacturers' preparations of the base medium yielded markedly different recoveries (Emilson and Bratthall, 1976; Liljemark *et al.*, 1976; Staat, 1976). In the present investigation, it was therefore decided to add various concentrations of sucrose and bacitracin to a different base medium—TYC—as a new selective medium for the isolation of *S. mutans*.

Before evaluating the effect of these additives to TYC medium, the recovery of *S. mutans* on TYC agar alone was compared to that on blood agar. Representatives of serotypes c of *S. mutans* (NCTC 10449) and d/g of *S. sobrinus* (6715) were chosen for this comparison because these serotypes are the ones most commonly found in Europe and in the USA (Bratthall, 1972; Shklair and Keene, 1974). The percentage recovery of *S. mutans* and of *S. sobrinus* on TYC was respectively 98% and 93% of that on blood agar (table I).

The results obtained with different combinations of concentrations of sucrose and bacitracin indicated that recovery of *S. mutans* was significantly inhibited by a 30% sucrose concentration whereas

bacitracin had little effect. *S. sobrinus* exhibited the reverse effect, being inhibited by bacitracin 0.3 units/ml but not significantly by sucrose. There was no difference in the recovery of either organism with a concentration of bacitracin between 0.1 and 0.2 units/ml and the latter concentration was adopted in the further experiments.

At the concentrations tested, sucrose had a significant inhibitory effect on the numbers of organisms other than *S. mutans*, but bacitracin did not. Therefore, the higher the sucrose concentration employed, the fewer contaminants would be expected. However, as already mentioned, high sucrose concentrations also inhibited the recovery of a serotype-c strain.

Thus the optimal concentrations of sucrose and bacitracin proved to be 20% and 0.2 units/ml respectively for the purpose of achieving maximum recovery of the *S. mutans* while suppressing the growth of other organisms.

Other selective media have been created for the isolation of *S. mutans* in addition to differential media, notably MM10 (Syed and Loesche, 1972) and BCY (Ikeda and Sandham, 1972b). These did not inhibit other organisms, but allowed the detection and enumeration of *S. mutans*.

None of the media previously developed for the selective isolation of *S. mutans* has proved ideal. Carlsson (1967) utilised the sulphonamide resistance of *S. mutans* and susceptibility of the other oral streptococcal species and incorporated sulphadimidine 1 g/L into a base medium similar to mitis-salivarius agar. This proved selective for the isolation of *S. mutans* from unspecified samples from the oral cavity, although contaminants amounted to approximately 5% of the total count on ordinary mitis-salivarius agar. The total streptococcal count in saliva is usually about 1000 times greater than the *S. mutans* count and such a degree of contamination is unacceptable.

The medium of Ikeda and Sandham (1972a), containing 40% sucrose, is also selective for *S.*

mutans, but gave only 37% recovery of the organism compared with the basic mitis-salivarius agar. This suggests that a sucrose concentration as high as 40% is partially inhibitory to *S. mutans*, a finding confirmed by Gold *et al.* (1973). Gold *et al.* (1973) found that the degree of inhibition varied markedly with the strain studied, and this has also been confirmed in the present study.

Three other media were compared with TYCSB for their ability to recover *S. mutans* and *S. sobrinus*. The media chosen were MSB (Gold *et al.*, 1973), almost universally adopted for the selective isolation of *S. mutans*, Linke's (1977) MSFA, and GSTB as described by Tanzer *et al.* (1983).

Optimal recovery of *S. mutans* was obtained on GSTB and TYCSB (64% and 60% respectively). The recovery was only 46% on MSB and there was no growth on MSFA. This finding was confirmed on repeated testing. This is in contrast to Linke's original report (1977) where this strain was found to grow well, producing blue-purple silky colonies. However, Linke (1977) incubated the plates aerobically for 10 days whereas in this study they were incubated anaerobically for 48 h.

In contrast, MSFA proved effective in recovering *S. sobrinus* (70%) and TYCSB proved equally good (73%). GSTB gave a 63% recovery but MSB only 11%.

TYCSB therefore appears to be superior to the other media tested, although GSTB deserves further study. Linke's medium (1977) proved unreliable and also allowed growth of other mannitol-utilising organisms such as enterococci and staphylococci. TYCSB allows a more complete recovery of *S. mutans* than MSB and thus might be expected to detect these organisms in samples when present in numbers undetectable by MSB.

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REFERENCES

- Adams D, Rawle L, White S 1982 Quantitative methods used to assess bacteria in plaque. In: Frank R H, Leach S A (eds) Surface and colloid phenomena in the oral cavity: methodological aspects. IRL Press, London, pp 213-225.
- Bratthall D 1972 Demonstration of *Streptococcus mutans* strains in some selected areas of the world. *Odontologisk Revy* **23**:401-410.
- Carlsson J 1967 A medium for isolation of *Streptococcus mutans*. *Archives of Oral Biology* **12**:1657-1658.
- de Stoppelaar J D 1971 *Streptococcus mutans*, *Streptococcus sanguis* and dental caries. Thesis, Rijksuniversiteit, Utrecht.
- Emilson C G, Bratthall D 1976 Growth of *Streptococcus mutans* on various selective media. *Journal of Clinical Microbiology* **4**:95-98.
- Gold O G, Jordon H V, Van Houte J 1973 A selective medium for *Streptococcus mutans*. *Archives of Oral Biology* **18**: 1357-1364.
- Ikeda T, Sandham H J 1972a A high-sucrose medium for the identification of *Streptococcus mutans*. *Archives of Oral Biology* **17**:781-783.

- Ikeda T, Sandham H J 1972b A medium for the recognition and enumeration of *Streptococcus mutans*. *Archives of Oral Biology* **17**:601–604.
- Liljemark W F, Okrent D H, Bloomquist C G 1976 Differential recovery of *Streptococcus mutans* from various mitis-salivarius agar preparations. *Journal of Clinical Microbiology* **4**:108–109.
- Linke H A B 1977 New medium for the isolation of *Streptococcus mutans* and its differentiation from other oral streptococci. *Journal of Clinical Microbiology* **5**:604–609.
- McGhee J R, Michalek S M 1981 Immunobiology of dental caries: microbial aspects and local immunity. *Annual Review of Microbiology* **35**:595–638.
- Shklair I L, Keene H J 1974 A biochemical scheme for the separation of the five varieties of *Streptococcus mutans*. *Archives of Oral Biology* **19**:1079–1081.
- Staat R H 1976 Inhibition of *Streptococcus mutans* strains by different mitis-salivarius agar preparations. *Journal of Clinical Microbiology* **3**:378–380.
- Syed S A, Loesche W J 1972 Survival of human dental plaque flora in various transport media. *Applied Microbiology* **24**:638–644.
- Tanzer J M, Borjesson A C, Kurasz A, Laskoski L, Testa M, Krasse B 1983 GSTB, an alternative to MSB agar. *Journal of Dental Research* **62**:241. (Abstract).
- Van Palenstein Helderman W H, Ijsseldijk M, Huis in't Veld J H J 1983 A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. *Archives of Oral Biology* **28**:599–603.
- Wade W G, Llewellyn D R, Walker D M, Aldred M J 1983 A new medium for the isolation of *Streptococcus mutans*. *Journal of Dental Research* **62**:420 (Abstract).