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A SELECTIVE MEDIUM FOR *STREPTOCOCCUS MUTANS*

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Summary—A selective medium was developed for the isolation of *Streptococcus mutans* from human dental plaque. Mitis-salivarius agar was modified by adding 0·2 units/ml bacitracin and by increasing the sucrose concentration to 20 per cent. At these concentrations, the selective agents allowed the undiminished recovery of *Strep. mutans* with maximum inhibition of the balance of the streptococcal flora normally encountered on this medium. Enterococci or yeasts were sometimes observed upon direct culturing of plaque samples from children with advanced dentinal lesions.

INTRODUCTION

THE ASSOCIATION of *Streptococcus mutans* with dental caries was first reported by CLARKE (1924) who isolated the organism, frequently in pure culture, from the depth of carious lesions. In recent years, this organism has become the subject of considerable investigation since its caries-inducing potential was demonstrated in animal models (FITZGERALD, JORDAN and STANLEY, 1960; FITZGERALD and KEYES, 1960). Studies which are concerned with elucidating the aetiologic role of *Strep. mutans* in dental caries have been hampered, however, by the lack of convenient and reliable techniques for the recognition and enumeration of the organisms in human dental plaque. A method generally used involves the culturing of plaque samples on mitis-salivarius agar which is selective for streptococci (KRASSE, 1966; JORDAN, KRASSE and MÖLLER, 1968). It is possible to differentiate *Strep. mutans* from the other streptococci on this medium on the basis of its unique colonial appearance. This procedure can be tedious when large numbers of samples are involved. Furthermore, the presence of *Strep. mutans* cannot be established on this medium with any degree of reliability when it constitutes only a small percentage of the total streptococcal flora.

Special media for the selective cultivation of *Strep. mutans* have been described previously. CARLSSON (1967) has described a medium containing sulphadimetine, which is based on the relative resistance of this species to the sulpha drugs. IKEDA and SANDHAM (1972) have reported the selective growth of *Strep. mutans* on mitis-salivarius agar containing 40 per cent sucrose. However, the concentration of these agents, that had to be used for the selection of *Strep. mutans*, either did not completely inhibit other organisms, or partially inhibited *Strep. mutans*.

In the present report, a selective medium for *Strep. mutans* is described which utilizes two selective agents, sucrose and bacitracin. The relative resistance of *Strep. mutans* to high concentrations of sucrose has been reported (GEHRING, 1968; IKEDA and SANDHAM, 1972). Bacitracin sensitivity has been used as a differential characteristic

in classification schemes for the streptococci (COLMAN, 1968). Lancefield group D streptococci (LANCEFIELD, 1933) are relatively resistant to the action of bacitracin (TOALA *et al.*, 1969). Although *Strep. mutans* does not react with group D antiserum, this species shares many characteristics of *Streptococcus bovis* (DE STOPPELAAR, VAN HOUTE and DE MOOR, 1967) which raised the possibility that *Strep. mutans* might also share the property of bacitracin-resistance of the group D streptococci. In fact, CARLSSON (1968) had reported that 7 out of 9 *Strep. mutans* strains were resistant to 5 units/ml of bacitracin. Seventy-nine per cent of the other streptococcal strains, similarly tested, were sensitive to bacitracin.

MATERIALS AND METHODS

The development of a selective medium for *Strep. mutans* was conducted in three phases. 1. Mitis-salivarius agar (Difco) was investigated for its adequacy in allowing full recovery of *Strep. mutans*. 2. Selective agents were tested separately, utilizing pure cultures to determine optimal concentrations for suppression of other streptococci without inhibition of *Strep. mutans*. 3. The medium, containing a combination of selective agents at optimal concentrations, was evaluated for its ability to support selective recovery of *Strep. mutans* from human dental plaque.

Culture media

Mitis-salvarius agar was tested, with one or more of the following additions, in an attempt to increase the recovery of *Strep. mutans* grown as pure cultures: Phytone (BBL), Polypeptone (BBL), Bacto Peptone (Difco), Myosate (BBL), Trypticase (BBL), all at 1 per cent concentration; beef extract (Difco) at 0·3 per cent and yeast extract (Difco) at 0·2 per cent. The inhibitors, crystal violet, trypan blue and potassium tellurite, in mitis-salivarius agar, were also tested for their effect on the growth of pure cultures of *Strep. mutans* by their successive deletion from the medium.

The recovery of *Strep. mutans* from dental plaque was compared on heart-infusion agar (Difco) with 5 per cent defibrinated sheep blood, MM10 medium (SYED and LOESCHE, 1972), mitis-salivarius agar and the selective medium developed.

Selective agents

Representative *Strep. mutans* strains, and strains of other oral streptococci, were tested to determine the optimal concentration of selective agents that would allow undiminished recovery of the former, and maximum suppression of the latter.

Sucrose (Fisher Scientific Co.) was incorporated into mitis-salivarius agar before sterilization over a range of 10–40 per cent w/v. Bacitracin was incorporated in mitis-salivarius agar in increasing concentrations from 0·06 to 2·0 units/ml of the medium after sterilization. The bacitracin stock solution was made up in sterile distilled water under aseptic conditions with a minimum of agitation.

Microorganisms

One representative from each of the five serological groups of *Strep. mutans* (BRATTHALL, 1970) was used for the development of the medium. These were strains AHT (ZINNER *et al.*, 1965), FA-1 (FITZGERALD *et al.*, 1960), NCTC 10449 (EDWARDSSON, 1968), SL-1 (FITZGERALD and JORDAN, 1968) and B2 (EDWARDSSON, 1968). In addition to the above *Strep. mutans* strains, the following laboratory strains were used: *Streptococcus sanguis* H4 and *Streptococcus salivarius* H5, both isolated from human dental plaque, *Streptococcus mitis* 26 (LILJEMARK and GIBBONS, 1972) and *Streptococcus faecalis* N83.

Pure culture studies

Pure cultures were grown and maintained in trypticase soy broth (BBL). Cultures were grown under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide for 18 hr. Ten-fold dilutions were made in 9 ml phosphate buffer 0·067 M, pH 7·2. Duplicate samples of 0·1 ml were plated from 10^{-6} and 10^{-7} dilutions and spread on the surface of the medium with a sterile, bent, glass rod.

Plaque sampling

Pooled plaque samples were collected from approximal tooth surfaces of 12–14-yr-old school

children, using sterile dental floss. The floss was immediately placed in a small screw-capped vial containing 3 ml of VMG II transport medium (MÖLLER, 1966). The vials were agitated on a Vortex test-tube mixer (Scientific Industries Inc., Springfield, Mass.) for 30 sec in order to dislodge the plaque from the floss and to obtain a homogeneous suspension. Ten-fold dilutions of the suspensions were made in 0·05 per cent yeast-extract water. Duplicate 0·1 ml samples of the dilutions were plated and spread on the medium with a sterile, bent, glass rod.

All plates, from pure culture and plaque sampling studies, were incubated under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide for 24 hr at 37°C. After incubation, the plates were allowed to stand at room temperature for 24 hr. The plates were then examined, at a magnification of ×15, using a wide-field dissecting microscope.

Enumeration and identification

Total counts were determined using the Quebec colony counter. *Streptococcus mutans* colonies were identified and counted under the dissecting microscope on plates with 30–300 colonies, whenever possible. Colonial characteristics of *Strep. mutans* on blood agar with sucrose and MM10 agar were similar to the appearance of this species on mitis-salivarius agar as described by KRASSE (1966). Representative colonies typical of *Strep. mutans*, as well as those whose identity as *Strep. mutans* was doubtful, were subcultured to test the ability of the strains to ferment mannitol and sorbitol and to determine their colonial morphology on mitis-salivarius agar.

RESULTS

Initial experiments with representative strains of *Strep. mutans* suggested that mitis-salivarius agar was nutritionally adequate and non-inhibitory for this species. Comparable colony counts were obtained on mitis-salivarius agar, with and without the inhibitory agents, crystal violet, trypan blue and tellurite, which are usually incorporated in this medium. No increase in colony counts was observed when phytone, polypeptone, bacto peptone, myosate, trypticase, beef extract or yeast were added to mitis-salivarius agar. Pure cultures of *Strep. mutans*, and dental plaque samples cultured in parallel on mitis-salivarius agar and on other rich nonselective media, showed no advantage of the latter over mitis-salivarius agar. Some examples of the results from experiments, in which mitis-salivarius agar was compared to heart-infusion agar with 5 per cent defibrinated sheep blood and 5 per cent sucrose, are shown in Table 1. The data indicate that the recovery of *Strep. mutans* from dental plaque samples was similar on both media. In further studies of the development of a selective medium for *Strep. mutans*, mitis-salivarius agar was therefore used as a basal medium.

TABLE 1. COMPARATIVE RECOVERIES OF *Streptococcus mutans* FROM HUMAN DENTAL PLAQUE ON BLOOD AGAR AND MITIS-SALIVARIUS AGAR

Plaque number	Blood agar	Mitis-salivarius agar
1	63*	83
2	380	430
3	53	50
4	45	41
5	58	67

* Mean number of *Strep. mutans* colonies on duplicate plates from 0·1 ml of a 10^{-3} dilution of plaque collected in VMG II transport medium. Individual counts never exceeded ± 13 per cent of the mean.

Studies with five strains of *Strep. mutans* representing the different serotypes demonstrated that all tolerated concentrations of sucrose up to 20 per cent in mitis-salivarius agar without any adverse effects on their growth or colonial morphology (Table 2). Strains AHT, FA-1 and NCTC 10449 grew in the presence of sucrose

concentrations up to 40 per cent with only a moderate decrease in numbers at the higher sucrose levels. However, strains SL-1 and B2 were more sensitive to the higher sucrose concentrations. The laboratory strain of *Strep. sanguis* was completely inhibited at a level of 15 per cent sucrose in mitis-salivarius agar. The *Strep. salivarius* and *Strep. mitis* laboratory strains were more resistant, being inhibited by 35 and 20 per cent sucrose respectively.

TABLE 2. EFFECT OF SUCROSE ON PURE CULTURES OF ORAL STREPTOCOCCI

Sucrose concentration in mitis-salivarius agar (per cent)	*Strep. mutans*					*Strep. sanguis*	*Strep. salivarius*	*Strep. mitis*
	AHT	FA-1	10449	SL-1	B2	H4	H5	26
5	169*	279	88	30	100	43	127	132
10	184	298	92	25	105	37	123	69
15	164	235	95	41	108	0	128	46
20	161	269	95	45	108	0	106	0
25	181	120	69	28	110	0	39	0
30	126	156	80	32	91	0	1	0
35	96	107	71	12	0	0	0	0
40	91	102	73	0	0	0	0	0

* Mean number of colonies on duplicate plates from 0·1 ml of a 10^{-6} dilution.

Pure cultures of *Strep. mutans* grew on mitis-salivarius agar containing up to 2 units/ml bacitracin, which was the maximum level tested (Table 3). With some of the strains a reduction in numbers was observed above 0·25 units/ml. *Streptococcus salivarius*, strain H5 and *Strep. mitis*, strain 26 were completely inhibited at 0·25 and 0·125 units/ml respectively. *Streptococcus sanguis*, strain H4 was more resistant to bacitracin. Although a gradual reduction in numbers occurred as the bacitracin concentration was increased, complete inhibition was not achieved below 2 units/ml. The data in Table 3 indicate that the optimum concentration of bacitracin, which would allow the selective growth of *Strep. mutans*, was between 0·125 and 0·25 units/ml.

TABLE 3. EFFECT OF BACITRACIN ON PURE CULTURE OF ORAL STREPTOCOCCI

Bacitracin concentration in mitis-salivarius agar (units/ml)	*Strep. mutans*					*Strep. sanguis*	*Strep. salivarius*	*Strep. mitis*
	AHT	FA-1	10449	SL-1	B2	H4	H5	26
0	235*	200	236	62	125	184	36	170
0·0625	232	191	231	58	119	110	28	103
0·125	262	196	220	60	107	109	18	0
0·25	237	191	197	51	112	97	0	0
0·5	245	182	212	35	72	82	0	0
1	214	181	194	42	82	8	0	0
2	205	58	183	35	75	0	0	0

* Mean number of colonies on duplicate plates from 0·1 ml of a 10^{-6} dilution.

On the basis of the data obtained with pure cultures, the recovery of *Strep. mutans* from human dental plaque samples was studied on mitis-salivarius agar, to which 0·05–0·2 units/ml of bacitracin were added, either singly, or in combination with, 20 per cent sucrose. Each sample was simultaneously cultured on mitis-salivarius agar without additions. As shown with some examples in Table 4, virtually complete inhibition of bacteria, other than *Strep. mutans*, was obtained with mitis-salivarius

agar containing 20 per cent sucrose and 0·2 units/ml bacitracin. Lower concentrations of bacitracin, alone or in combination with 20 per cent sucrose, were not completely effective.

In view of these results, the selective medium (MSB) was formulated to contain 0·2 units of bacitracin/ml of medium and 20 per cent sucrose and was prepared as follows: To 1000 ml reconstituted mitis-salivarius agar, 150 g of sucrose was added. The medium was heated to dissolve the components and then autoclaved at 121°C for 15 min. The medium was then cooled to 45°C after which 1 ml of each of the sterile stock solutions (1 per cent tellurite and 200 units/ml bacitracin) was added. The

TABLE 4. SELECTIVE RECOVERY OF *Strep. mutans* FROM HUMAN DENTAL PLAQUE ON MITIS-SALIVARIUS AGAR CONTAINING VARIOUS CONCENTRATIONS OF BACITRACIN AND SUCROSE

Additions to mitis-salivarius agar	Plaque number 1		2		3		4		5	
	Strep. mutans	Other bacteria	*Strep. mutans*	Other bacteria	*Strep. mutans*	Other bacteria	*Strep. mutans*	Other bacteria	*Strep. mutans*	Other bacteria
None	N.D.*	> 300	N.D.	> 300	N.D.	> 300	N.D.	> 300	N.D.	> 300
0·05 unit/ml Bacitracin and 20% sucrose	> 300	> 100	> 300	35†	> 300	7	> 300	13	N.D.	> 1000
0·1 unit/ml Bacitracin	N.D.	> 300	> 300	93	> 300	80	> 300	100	N.D.	> 300
0·1 unit/mlBacitracin and 20% sucrose	> 300	80	> 300	43	> 300	7	> 300	27	N.D.	> 300
0·2 unit/ml Bacitracin	N.D.	> 300	> 300	60	> 300	57	> 300	65	N.D.	> 300
0·2 unit/ml Bacitracin and 20% sucrose	> 300	0	> 300	0	> 300	0	> 300	0	1	7

* N.D. Not detectable, plates too crowded.
† Mean number of colonies on duplicate plates from 0·1 ml of VMG II transport medium containing plaque material.

bacitracin solution is stable for 1 week, if stored in the refrigerator. The flask containing the medium was gently swirled to mix the contents thoroughly without foaming. Plates were poured with approximately 20 ml of the medium and then permitted to dry for 24 hr at room temperature. After drying, plates were stored in the refrigerator until use.

The comparative recoveries of *Strep. mutans*, from diluted dental plaque samples, cultured in parallel on MM10 agar, mitis-salivarius and MSB agar, the selective medium with 20 per cent sucrose and 0·2 units/ml bacitracin, are shown in Table 5. No differences were observed in the number of *Strep. mutans* colonies that could be cultivated on MSB agar as compared to MM10 agar. The colonial morphology of

TABLE 5. COMPARATIVE RECOVERIES OF *Strep. mutans* FROM HUMAN DENTAL PLAQUE ON MM10 AGAR, MITIS-SALIVARIUS AGAR AND MITIS-SALIVARIUS WITH SUCROSE AND BACITRACIN (MSB)

Plaque number	MM 10		Mitis-salivarius		MSB	
	Strep. mutans	Other bacteria	*Strep. mutans*	Other bacteria	*Strep, mutans*	Other bacteria
1	3*	228	7	96	6	1
2	26	> 300	N.D.†	> 300	24	0
3	117	> 300	106	> 300	100	0
4	15	121	12	24	37	0
5	47	> 300	46	> 300	40	0

* Mean number of colonies on duplicate plates from 0·1 ml of a 10^{-3} dilution of plaque in VMG II transport medium Individual counts of *Strep. mutans* varied less than ±30 per cent from the mean except in plaque number 1 (±50 per cent) due to the low numbers of *Strep. mutans*.
† N.D. Not detectable, plates too crowded.

Strep. mutans, on the three media, was similar. The superiority of MSB agar over mitis-salivarius agar, in recovering small numbers of *Strep. mutans*, is clearly demonstrated in the case of plaque sample 2. *Streptococcus mutans* could not be detected on the crowded mitis-salivarius plate. Bacteria, other than *Strep. mutans*, were virtually all inhibited when MSB agar was used. When undiluted plaque samples were cultured, MSB agar was clearly superior to the other two media. Confluent bacterial growth on MM10 agar and mitis-salivarius agar prevented detection of *Strep. mutans* colonies. Other experiments involving dental plaque, saliva and samples from the dorsum of the tongue, from many different subjects have shown that even a few colony-forming units of *Strep. mutans*, present in total populations of 10^7–10^8 bacteria, can be easily detected on the MSB medium.

Occasionally enterococci and yeasts were encountered when undiluted samples of plaque, from advanced dentinal lesions, saliva or samples from the dorsum of the tongue, were cultured. The colonies of the enterococci were dark blue to brown and flat while the yeasts appeared as large white to light blue matte colonies. All were easily differentiated from *Strep. mutans* by colonial appearance. A laboratory strain of *Strep. faecalis* (N83) was capable of uninhibited growth on MSB medium.

DISCUSSION

Previous selective media relied on single selective agents to which *Strep. mutans* was relatively resistant. In contrast, the present work was based on the assumption that different selective agents for *Strep. mutans* exhibiting different inhibitory actions could be utilized to complement each other. This approach might permit the use of the individual agents, in combination, whereby concentrations of each agent, non-inhibitory to *Strep. mutans*, would be sufficient to obtain complete suppression of other bacteria.

Optimum levels of sucrose and bacitracin were determined with pure cultures, on the basis of maximum inhibition of other streptococci without inhibition of *Strep. mutans*. At the optimal sucrose concentration (20 per cent) finally selected, *Strep. sanguis* and *Strep. mitis* were effectively inhibited but *Strep. salivarius* was not completely suppressed. In contrast, the optimal concentration of bacitracin selected (0·2 units/ml) was sufficient for the complete elimination of *Strep. salivarius* and *Strep. mitis* but studies with pure cultures indicated that *Strep. sanguis* was not completely inhibited at this concentration. Neither of these agents, at their optimum levels, interfered with the growth of *Strep. mutans*. A combination of sucrose and bacitracin incorporated in mitis-salivarius agar, at the optimal levels determined as above, was effective in inhibiting bacteria other than *Strep. mutans* when human dental plaque samples were cultured.

Mitis-salivarius agar seemed a logical starting point for the development of a selective medium for the isolation of *Strep. mutans* since the medium is already selective for the streptococci and because the colonial appearance of *Strep. mutans* is quite distinctive on this medium. In addition, mitis-salivarius agar is commerically available which would assure its ready accessibility, particularly to smaller laboratories with limited facilities for medium preparation.

To date, the only interfering organisms detected on MSB agar have been low numbers of enterococci and yeasts which developed when undiluted samples from various sites in the mouth, and plaques from children with advanced dentinal lesions, were cultured. These organisms are rarely encountered in routine plaque sampling at the dilutions commonly employed and are easily differentiated on the basis of their colonial morphology. Thus they should not constitute a problem in a routine use of the selective medium.

Development of a selective medium for *Strep. mutans* allows the isolation of this species from samples when present in low numbers relative to the total population. Studies on the transmission, epidemiology and general ecology of this species should be more feasible now than with the less sensitive cultural methods previously available, In fact, it may be necessary to re-evaluate previous studies on the presence and distribution of *Strep. mutans* in human populations (KRASSE *et al.*, 1968; JORDAN. ENGLANDER and LIM, 1969; DE STOPPELAAR, VAN HOUTE and BACKER DIRKS, 1969). Also, the scope of these clinical studies can be significantly expanded since a reliable selective medium should permit the processing of larger numbers of samples by less skilled personnel.

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Résumé—Un milieu sélectif pour l'isolement du *Streptococcus mutans* de la plaque dentaire humaine a été mis au point. Le mitis salivarius agar a été modifié en ajoutant 0,2 unités/ml de bacitracine et en augmentant la concentration de saccharose de 20 pour cent. A ces concentrations, les agents sélectifs permettent d'obtenir un maximum de *Strep. mutans*, avec une inhibition élevée du reste de la flore streptococcique normalement présente dans le milieu. Les entérocoques et les levures sont parfois présents en culture directe d'échantillons de plaques d'enfants présentant des lesions dentinaires avancées.

Zusammenfassung—Zur Isolation von *Streptococcus mutans* aus menschlicher Zahnplaque wurde ein selektives Medium entwickelt. Durch Zusatz von 0,2 E./ml Bacitracin und durch Erhöhung der Rohrzuckerkonzentration auf 20 Prozent wurde der Mitis-Salivarius Agar modifiziert. Bei diesen Konzentrationen der selektiven Wirkstoffe war es möglich, *Strep. mutans* unvermindert zurückzugewinnen bei gleichzeitiger maximaler Hemmung des in diesem Medium normalerweise vorhandenen Gleichgewichts der Streptokokkenflora. Bei direkter Kultivierung von Plaqueproben von Kindern mit fortgeschrittenen Dentinläsionen wurden gelegentlich Enterokokken oder Hefen beobachtet.

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