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Differential Medium for Detecting Dental Plaque Bacteria Resembling *Actinomyces viscosus* and *Actinomyces naeslundii*

R. P. ELLEN* AND I. B. BALCERZAK-RACZKOWSKI

Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada M5G 1G6

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A medium for detecting colonies of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque samples was developed. The medium (CNAC-20) contains 20.0 μg of $3CdSO_4 \cdot 8H_2O$ per ml of Columbia CNA agar base. Laboratory strains of *A. viscosus* grew on CNAC-20 in characteristic round, white, smooth, opaque colonies. Increasing the cadmium concentration impaired the growth of some *A. viscosus* strains. Stock strains of *A. naeslundii* and *A. israelii* grew in colonies of similar white, opaque morphology. The few strains of other gram-positive plaque bacteria that grew on CNAC-20 had colonies easily distinguished from those of *A. viscosus*. Most of the bacterial strains freshly isolated from *Actinomyces*-like colonies on CNAC-20 that had been inoculated with human dental plaque samples were found to have cultural characteristics consistent with previous descriptions of *A. viscosus* or *A. naeslundii*. CNAC-20 may facilitate investigations into the relationship of microaerophilic *Actinomyces* with the etiology of dental diseases.

Actinomyces viscosus and *Actinomyces naeslundii* may be involved in the etiology of periodontal disease and root surface caries. Naturally occurring periodontal disease among susceptible laboratory hamsters has been shown to result from transmissible *Odontomyces viscosus* (now classified as *A. viscosus*) infections (15, 17). Human dentogingival plaque isolates of both species have been shown to induce periodontal lesions and root surface caries in pathogen-free hamsters and monoinfected germfree rats (12, 13, 16, 20). Aside from their consistent colonization of human root carious lesions (14, 21, 23), little is known about the ecology of *A. viscosus* and *A. naeslundii* and their relationship to the incidence of naturally occurring human dental diseases. Laborious procedures currently required for the identification of oral *Actinomyces* species preclude processing the numerous plaque samples necessary for clinical investigations. This report describes a partially selective, differential plating medium on which colonies of *A. viscosus* and *A. naeslundii* may be readily identified.

MATERIALS AND METHODS

Strains and cultural conditions. The following strains were obtained from the culture collection at the University of Toronto, Faculty of Dentistry: *A. naeslundii* JS-10; *A. viscosus* RB-2; *Streptococcus sanguis* S and JC-8; *Streptococcus mitis* 15909, RE-7, and UT-18; *Streptococcus salivarius* TON-3; *Streptococcus mutans* LM-7, BHT, JC-2, Sl-1, OMZ-61, UT-21, and UT-22; and *Lactobacillus* sp. 1-7. Cultures of the following strains were obtained from Harold V. Jordan, Forsyth Dental Center: *A. naeslundii* WVU 398A; *A. viscosus* M100; *Actinomyces israelii* 10048; and *Actinomyces odontolyticus* 17982. Laboratory strains *A. viscosus* T14 and *A. naeslundii* I were obtained from Sigmund S. Socransky, Forsyth Dental Center, who also supplied *A. viscosus* T14B and *A. naeslundii* IB, which were freshly isolated from gnotobiotic rats monoinfected with T14 or I. *A. viscosus* strains A-2 and A-3 were provided by Walter J. Loesche, University of Michigan. *Bacterionema matruchotii* strains 18, 208, and 214 were obtained from Marion N. Gilmour, Eastman Dental Center. *Lactobacillus caseii* 4961, *Lactobacillus fermenti* 11581, and *Rothia dentocariosa* 19131 and 14190 were obtained from Benjamin F. Hammond, University of Pennsylvania.

Stock cultures of all strains were maintained by either monthly subculture on brain heart infusion agar (BHI; Difco) slants or by biweekly transfer in fluid thioglycollate medium (Difco). Stock cultures of *B. matruchotii* strains were maintained on half-strength BHI (6).

Composition of experimental medium. To prepare the partially selective medium for *A. viscosus* and *A. naeslundii*, $3CdSO_4 \cdot 8H_2O$ is added at a concentration of 20.0 μg/ml to Columbia CNA agar base (Difco). The medium can be autoclaved. Columbia CNA agar, first described for the selective cultivation of gram-positive cocci (5), contains 10 mg of colistin sulfate and 15 mg of nalidixic acid per liter to inhibit the growth of gram-negative bacteria. Moreover, streptococci other than enterococci grow

poorly on it (4). Cadmium in the range of 1.0 to 10.0 μg/ml has been found to impair the growth of many strains of dental plaque streptococci (H. J. Sandham, unpublished data). Plates containing the experimental medium, herein termed CNAC-20, are incubated at 35 C in 90% air and 10% CO_2 to encourage the growth of the microaerophilic species *A. viscosus* and *A. naeslundii* while impairing the growth of anaerobic *Actinomyces* species and other gram-positive bacteria, which prefer anaerobic conditions for primary isolation.

Investigations with laboratory strains. All the stock strains of the microorganisms listed above were compared for their ability to grow on CNAC-20. Twenty-hour cultures were grown in tryptic soy broth (Difco) containing 1.0% (wt/vol) glucose and in an atmosphere of 95% N_2 and 5% CO_2. Strains of *R. dentocariosa* were grown aerobically. Tenfold dilutions of the cultures were prepared in 0.05% yeast extract broth. One-tenth milliliter of appropriate dilutions was spread on the surface of quadruplicate plates of BHI and on duplicate plates of CNA and CNAC-20 media. Two BHI plates (control) were incubated in 95% N_2 and 5% CO_2 except for plates streaked with *R. dentocariosa*, which were incubated in air. The remaining duplicate BHI, CNA, and CNAC-20 plates were incubated in 90% air and 10% CO_2. After 48 h, the average number of colony-forming units (CFU) was calculated, and the colony morphology was noted.

The optimal concentration of cadmium sulfate for the medium was determined. Preliminary studies established 20.0 μg/ml as the minimal concentration for inhibiting the growth of the non-*Actinomyces* stock strains (R. P. Ellen, unpublished data). Twenty-hour cultures of *A. viscosus* strains T14, T14B, A-2, A-3, RB-2, and M100 were grown in tryptic soy broth. Samples of 10-fold serial dilutions were streaked in duplicate on CNA agar (control) or on CNA plates containing concentrations of $3CdSO_4 \cdot 8H_2O$ ranging from 20 to 20 μg/ml. After 48 h of incubation, the CFU were counted.

Dental plaque samples. CNAC-20 was tested for its usefulness in detecting *A. viscosus* and *A. naeslundii* colonies grown from samples of dental plaque. Twenty-five plaque samples of unknown age were collected from laboratory personnel and dental student volunteers. All samples were collected with sterile stainless-steel curettes from the gingival third of buccal or lingual enamel surfaces of first molar teeth. The samples were placed into 3.0 ml of reduced transport fluid (22). Each collection tube was immersed in an ice bath while the plaque samples were dispersed by sonication (Biosonic IV, Bronwill Scientific, Rochester, N.Y.) under a continuous flow of oxygen-free gas. Tenfold serial dilutions of each sample were streaked on duplicate plates of CNAC-20. The plates were incubated in 90% air and 10% CO_2 for 4 days.

Of the colonies that grew on CNAC-20, 217 with a colony morphology resembling that of laboratory strains of *A. viscosus* and *A. naeslundii* were isolated. Twenty-hour tryptic soy broth cultures were Gram stained. All strains of gram-positive rods, pleomorphic branching rods, and filaments were grown in pure culture for testing their biochemical reactions.

Nitrate and nitrite reduction, gelatin liquefaction (tube), catalase activity, and Voges-Proskauer tests were performed according to methods outlined by Cowan and Steel (3). Each isolate was tested for its ability to grow on Rogosa SL agar (Difco). The abilities of each strain to grow under aerobic and relatively anaerobic conditions were compared. Duplicate streaked plates of tryptic soy agar containing 5.0% sheep blood were incubated either in air or in 95% N_2 and 5% CO_2. Acid production from glucose, lactose, galactose, raffinose, arabinose, and xylose was detected by using a basal medium described by Holmberg and Hallander (8) to which filter-sterilized carbohydrates were added aseptically. Duplicate tubes of the glucose broth were used for the oxidation-fermentation (O-F) test of Hugh and Leifson (10).

RESULTS

Growth of stock strains on CNAC-20. In an atmosphere of 90% air and 10% CO_2, all stock strains of *A. viscosus* grew on CNAC-20 to the same degree as on BHI and CNA plates (Table 1). Some strains grew better on BHI incubated under more anaerobic conditions. The characteristic colonies formed by *A. viscosus* strains on CNAC-20 were 1 to 3 mm in diameter, white, opaque, round, slightly convex, and smooth (Fig. 1). They were easily detected with the unaided eye. Although strains I and IB did not grow on CNAC-20, the colony morphologies of the *A. naeslundii* strains that grew resembled those of *A. viscosus*. Colonies of *A. israelii* 10048 were also similar.

In contrast, none of the other gram-positive rods that grew on CNAC-20 had colony morphologies resembling the white, smooth colones of *A. viscosus* (Table 1). All three *B. matruchotii* strains grew on CNAC-20. However, it was necessary to use a microscope to detect their small, rough, elevated colonies. *R. dentocariosa* 14190 grew on CNAC-20 in colonies that were similar in size to those of *A. viscosus*. However, the *Rothia* colonies were more convex, with darker yellow centers and less smooth surface textures.

A. odontolyticus 17982, *R. dentocariosa* 19131, and the *Lactobacillus* species tested did not grow on CNAC-20, even at low dilutions yielding BHI and CNA plates too crowded to count.

All but one of the 13 stock strains of oral streptococci failed to grow on CNAC-20 (Table 2). *S. mitis* strain UT-18 grew in tiny, rough, white colonies easily distinguished from typical *Actinomyces* colonies.

TABLE 1. *Relative growth[a] and colony morphology of stock gram-positive rods and filamentous bacteria on BHI, CNA, and CNAC-20 media*

Microorganism	BHI (control)[b] (N_2 + CO_2)	BHI (air + CO_2)	CNA (air + CO_2)	CNAC-20 (air + CO_2)	Colony morphology
Actinomyces viscosus					
T14	100	95	92	120	1.0–3.0 mm; round; white opaque; slightly convex; smooth
T14B	100	98	81	84	
A-2	100	70	69	76	
A-3	100	54	58	78	
M100	100	21	21	14	
A. naeslundii					
398A	100	94	128	101	Same as *A. viscosus*
JS10	100	81	75	79	
I	100	93	95	0[c]	
IB	100	101	110	0	
A. israelii 10048	100	90	66	16	Same as *A. viscosus*
A. odontolyticus 17982	100	16	15	0	
Bacterionema matruchotii					
18	100	84	94	110	Microscopic, rough, raised
208	100	100	101	97	
214	100	51	57	40	
Rothia dentocariosa					
19131	100	108	110	0	
14190	100	91	76	68	Convex, dark yellow center, white edge, "orange peel" surface
Lactobacillus caesii 4961	100		95	0	
L. fermenti 11581	100	105	109	0	
Lactobacillus 1-7	100	106		0	

[a] Relative growth = {[average number of CFU on experimental medium]/[average number of CFU on BHI (control)]} × 100.

[b] *Rothia* strains grown aerobically.

[c] No colonies detected at lowest dilution tested.

Increasing the concentration of cadmium sulfate in CNAC impaired the growth of some *A. viscosus* strains (Table 3). Although strains T14, A-2, and M100 grew equally well at all concentrations tested, the growth of strains T14B, A-3, and RB-2 was partially inhibited at concentrations greater than 20 μg/ml.

Dental plaque isolates resembling A. viscosus and A. naeslundii. Plates of CNAC-20 inoculated with dental plaque samples contained round, white, smooth, opaque colonies similar to those produced by stock strains of *Actinomyces* (Fig. 2). Of the 217 of these colonies randomly selected from CNAC-20, 210 (97%) were gram-positive rods. Most of these demonstrated branched "Y" and "V" forms typical of *Actinomyces* species. Two hundred and five strains which continued to grow in vitro were tested for several characteristics to determine their probable identities (Table 4).

Of the 205 isolates tested, 172 (84%) demonstrated the following combination of cultural characteristics completely consistent with previous descriptions of *A. viscosus* or *A. naeslundii* (1, 8): nitrate reduction positive, gelatin liquefaction negative, Voges-Proskauer test negative, O-F test +/+, +/±, or ±/+. All of the 172 strains grew well on blood agar plates incubated under either aerobic conditions or in 95% N_2 and 5% CO_2. None of the isolates grew on Rogosa SL agar. Of these 172 isolates, 126 were catalase positive and thus resembled *A. viscosus*. The remaining 46 catalase-negative isolates may be considered similar to *A. naeslun-*

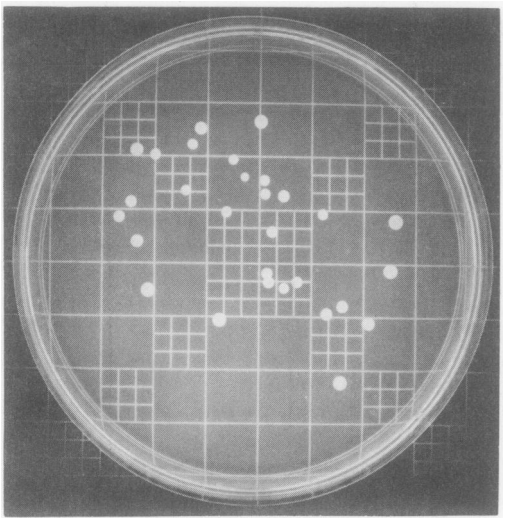

FIG. 1. *CNAC-20 medium inoculated with diluted sample of a pure culture of Actinomyces viscosus strain T14B and viewed on Quebec colony counter.*

TABLE 2. *Relative growth[a] of stock oral streptococci on BHI, CNA, and CNAC-20 media*

Microorganism	BHI (control) (N_2 + CO_2)	BHI (air + CO_2)	CNA (air + CO_2)	CNAC-20 (air + CO_2)
Streptococcus sanguis				
S	100		98	0[b]
JC-8	100	51	40	0
S. mitis				
15909	100	100	100	0
RE-7	100	112	85	0
UT-18	100	96	91	20
S. mutans				
LM-7	100	91	103	0
BHT	100	81	84	0
JC-2	100	98	97	0
SL-1	100	95	70	0
OMZ-61	100	98	91	0
UT-21	100	85	138	0
UT-22	100	157	126	0
S. salivarius TON-3	100	112	78	0

[a] Relative growth expressed as in Table 1.
[b] No colonies detected at lowest dilution tested.

dii. Of the 33 strains inconsistent with the above description, 21 were eliminated solely for being either nitrate negative or O-F +/−. Eleven strains (5%) were nitrate negative and may resemble the 9% of *A. naeslundii* and *A. viscosus* strains described as nitrate negative in *Bergey's Manual of Determinative Bacteriology* (1). The 10 isolates that were O-F +/− satisfied all other criteria tested, including acid production from all of the carbohydrates except pentoses. If these nitrate-negative and O-F +/− strains were considered to resemble *Actinomyces*, the *A. viscosus*- and *A. naeslundii*-like strains would number 193 (94%).

The 205 isolates tested were generally saccharolytic, producing acid from a variety of carbohydrates (Table 4). All isolates produced acid from glucose. Most produced acid from lactose, galactose, and raffinose, which oral microaerophilic gram-positive pleomorphic rods other than *Actinomyces* usually fail to ferment (1, 8). Similarly to *A. viscosus* and *A. naeslundii*, almost all of the fresh isolates failed to produce acid from arabinose and xylose.

Fifty small, grey, translucent colonies that did not resemble those of *Actinomyces* species were randomly selected from the CNAC-20 plates that had been inoculated with dental plaque samples (Fig. 2). These isolates were found to be gram-positive streptococci, subsequently identified as either *S. sanguis* or *S. mitis* by their colony morphology on mitis-salivarius agar.

DISCUSSION

To elucidate the ecology of *A. viscosus* and *A. naeslundii* and their relationship to the incidence of dental diseases, more rapid methods for their identification will be required. In this report, we have described a partially selective, differential medium that may be useful in detecting colonies of *Actinomyces* growing among colonies of other gram-positive plaque species. Using CNAC-20, gram-positive pleomorphic rods resembling *A. viscosus* and *A. naeslundii* were isolated by selecting colonies similar to those produced on the medium by stock strains of *A. viscosus*. Laboratory strains of the other oral gram-positive rods tested (*Bacterionema*, *Rothia*, and lactobacilli) either failed to grow on CNAC-20 or grew in colonies distinct from those produced by *Actinomyces* species. Moreover, very few, if any, of the *A. viscosus*-like colonies selected from CNAC-20 plates inoculated with plaque samples could be identified as *Bacterionema*, *Rothia*, or *Lactobacillus* species. Although stock strains of oral streptococci generally did not grow on CNAC-20, plates inoculated with plaque samples contained isolates resembling *S. sanguis* and *S. mitis*. However, their small, gray, translucent colonies on CNAC-20 were easily distinguished from the larger white, opaque colonies of *Actinomyces* species.

Incubating CNAC-20 plates in an atmos-

TABLE 3. *Growth of Actinomyces viscosus laboratory strains on CNA agar containing various concentrations of cadmium sulfate*

Strain of *A. viscosus*	Growth at given concn of $3CdSO_4 \cdot 8H_2O$ (μg/ml)					
	0 (control)	20.0	22.5	25.0	27.5	30.0
T14	8.1×10^6[a]	7.9×10^6	8.9×10^6	6.8×10^6	8.2×10^6	8.8×10^6
T14B	5.9×10^7	5.9×10^7	5.9×10^7	5.5×10^6	4.5×10^6	3.4×10^6
A-2	1.6×10^6	1.4×10^6	1.4×10^6	1.4×10^6	1.4×10^6	1.3×10^6
A-3	1.2×10^8	1.2×10^8	7.4×10^6	3.8×10^5	ND[b]	ND
RB-2	1.0×10^8	1.3×10^8	1.7×10^7	2.9×10^7	ND	ND
M100	7.0×10^6	7.7×10^6	—[c]	—	—	8.3×10^6

[a] Average number of CFU per milliliter of 20-h culture.
[b] ND, Not detected.
[c] —, Not tested.

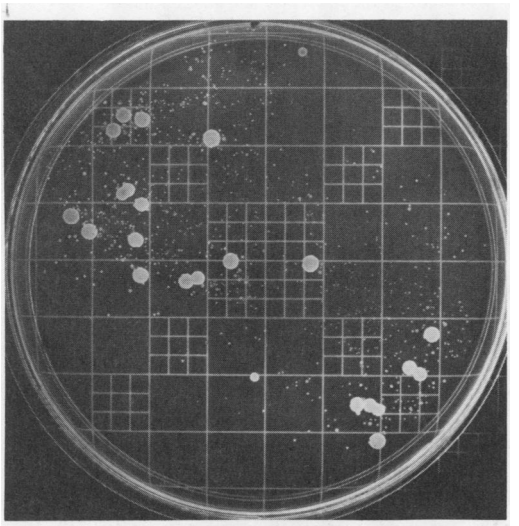

FIG. 2. *CNAC-20 medium inoculated with diluted sample of human dental plaque and viewed on Quebec colony counter. White, opaque colonies, 1 to 3 mm in diameter, contain gram-positive filamentous rods resembling A. viscosus or A. naeslundii.*

TABLE 4. *Characteristics of 205 strains of gram-positive rods and filaments isolated from dental plaque as A. viscosus-like colonies on CNAC-20 medium*

Characteristic	No. of isolates positive
Nitrate reduction	194
Nitrite reduction	144
Gelatin liquefaction	0
Growth on Rogosa SL agar	0
Voges-Proskauer test	0
Catalase test	149
Acid produced from:	
Glucose	205
Lactose	134
Galactose	161
Raffinose	163
Arabinose	0
Xylose	6

phere of 90% air and 10% CO_2 would be expected to favor the growth of *Actinomyces* species capable of aerobic growth. All of the plaque isolates tested were found to grow on blood agar equally well under both aerobic and relatively anaerobic conditions. *A. viscosus* was originally described as an aerobic, catalase-positive microorganism (9). The ability of all the stock *A. viscosus* strains to grow on CNAC-20 under aerobic conditions and the predominance of catalase-positive strains among the *Actinomyces*-like bacteria isolated on CNAC-20 from dental plaque indicate that CNAC-20 should be useful to enumerate *A. viscosus* in clinical samples. The failure of two stock *A. naeslundii* strains to grow on CNAC-20 and the low proportions of catalase-negative strains among the *Actinomyces*-like plaque isolates suggest that CNAC-20 may impair the recovery of some *A. naeslundii* CFU, especially under aerobic primary isolation conditions. A selective medium for relatively anaerobic actinomycetes has recently been developed by D. Beighton and G. Colman (Aust. Div., Int. Assoc. Dent. Res. Abstr. 41, 1974). While probably yielding a greater recovery of *A. naeslundii*, their medium also supports the growth of a wider variety of microorganisms including propionibacteria, bifidobacteria, and other true actinomycetes.

The classification of *A. naeslundii* and *A. viscosus* as two separate species on the basis of the catalase test may be artificial. Recent taxonomy studies have stressed the similarity of the two (8; E. D. Fillery et al., Int. Assoc. Dent. Res. Abstr. L-218, 1975). Moreover, the ability of the "two" species to induce periodontal and root surface lesions in animals indicates a common disease-related characteristic. It follows

that simply enumerating them together, perhaps as a total microaerophilic *Actinomyces* count on CNAC-20, may suffice to study the relevance of *A. viscosus*/*A. naeslundii*'s prevalence to the incidence of human dental diseases. If a separate *A. viscosus* and *A. naeslundii* count were necessary, their rapid differentiation on CNAC-20 might be accomplished by using either a direct catalase test or the agar plate immunofluorescence technique (G. J. Hageage and R. Harr, Int. Assoc. Dent. Res. Abstr. 241, 1972). Alternatively, typical *Actinomyces*-like colonies may be isolated from CNAC-20 plates and identified by standard cultural and serological methods.

Research into the bacteriological etiology of dental enamel caries has received significant stimuli through the development of selective plating media for lactobacilli (18) and the cariogenic streptococcus *S. mutans* (2, 7, 11). Without selective media, it is extremely difficult to enumerate microorganisms that constitute only low to moderate proportions of the cultivable flora colonizing the tooth surface or the gingival crevice. Consequently, the specific microbial etiology of periodontal disease is poorly understood (19). The current evidence that *Actinomyces* species contribute to periodontal and dental root surface pathology is probably more substantial than the evidence that implicated *S. mutans* in coronal caries before the development of mitis-salivarius agar. A selective culture medium, such as CNAC-20, may provide a similar impetus to investigations seeking to confirm or disprove the pathogenic role for *Actinomyces* in the etiology of human periodontal and root surface lesions.

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