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Growth of *Peptococcus* and *Peptostreptococcus*: Effect of Variations of Culture Media on Efficiency of Recovery

R. MARSHALL,* V. K. YASUI,† R. PRABHALA, A. K. KAUFMAN, AND I. WALLACE‡

Department of Microbiology and Public Health, California State University, Los Angeles, Los Angeles, California 90032

Received 9 February 1981/Accepted 29 May 1981

Reference strains and clinical isolates of *Peptococcus* and *Peptostreptococococcus* spp. were evaluated for their growth response in supplemented thioglycolate-yeast extract media. Supplements used included various combinations of hemin, menadione, sodium bicarbonate, and Tween 80. Parallel studies were done to compare the efficiency of recovery of viable cells grown in thioglycolate-based media and Wilkins-Chalgren broth and agar. In addition, the effects of age of the medium and medium storage on viable cell yields for reference strains were determined. Reference strains grown in freshly prepared thioglycolate-yeast extract medium supplemented with sodium bicarbonate produced a 10-fold greater increase in the number of viable cells recovered after 24 h of incubation than did the same organisms cultivated in Wilkins-Chalgren medium. The efficiency of recovery of organisms when either mid-logarithmic- or mid-stationary-phase cells were used to prepare standardized inocula was similar. The results suggest that thioglycolate-yeast extract medium supplemented with sodium bicarbonate is more productive than Wilkins-Chalgren medium for the cultivation of anaerobic gram-positive cocci and may represent a suitable alternative for antimicrobial susceptibility testing of these organisms.

Many reports on the effects of oxygen on the growth of anaerobes (1, 6, 12), hemin and carbon dioxide requirements (3, 4, 8, 10, 13), various reducing agents, and different medium formulations (5, 7, 9, 10) are available. However, current literature on the efficiency of recovery of individual strains of anaerobic gram-positive cocci with standardized inocula and cultivation conditions, as well as various medium formulations, is lacking.

This research was undertaken initially to provide information on the antimicrobial susceptibility patterns of anaerobic gram-positive cocci cultivated under standardized conditions. However, only minimal cell yields were obtained in preliminary growth experiments. Therefore, efforts were directed at investigating the effects of Wilkins-Chalgren (WC) agar and broth and supplemented thioglycolate-based media on the efficiency of recovery of cells. In addition, holding and storage conditions of media and age of the inoculum were studied. The results of these efforts are reported here.

† Present address: Legionnaire's Disease Laboratory, Wadsworth Veterans Administration Hospital, Los Angeles, CA 90073.

‡ Present address: County Sanitation Department, Carson, CA 90745.

MATERIALS AND METHODS

Organisms. Reference strains used in these studies were obtained from the American Type Culture Collection, Rockville, Md. (ATCC), and the Anaerobe Laboratory, Wadsworth Veterans Administration Hospital, Los Angeles, Calif. (WAL). The following strains were used: *Peptococcus asaccharolyticus* ATCC 29743, *Peptococcus constellatus* ATCC 27823, *Peptococcus magnus* WAL 2508, *Peptococcus prevotii* ATCC 9321, *Peptococcus variabilis* ATCC 14956, *Peptostreptococcus anaerobius* ATCC 27337, *Peptostreptococcus micros* ATCC 33270, and *Peptostreptococcus productus* ATCC 27340. In addition, 54 isolates from specimens of human origin were kindly supplied by local clinical facilities; these isolates included *P. asaccharolyticus* (10 isolates), *P. magnus* (20 isolates), *P. prevotii* (16 isolates), and *P. anaerobius* (8 isolates).

After the identity of each organism was confirmed according to the criteria given by Holdeman et al. (5), cells were inoculated onto fresh 5% sheep blood agar plates and incubated anaerobically at 35°C for 48 h. Cells were harvested with sterile 20% skim milk, suspended uniformly by pipette, and then lyophilized in 0.25-ml portions. Lyophilized cells were stored at −25°C until needed and were then rehydrated and recharacterized to confirm identity and purity before use.

Anaerobic system. The anaerobic system used throughout this research was the GasPak jar system (BBL Microbiology Systems, Cockeysville, Md.). Be-

fore each use, the catalyst was reactivated by dry heat at 160 to 170°C for 2 h.

Preparation of standardized inocula. The reference strains used for the evaluation of age of the medium and medium supplements were grown in thioglycolate broth without indicator (BBL 135C) with 0.5% yeast extract-hemin (5 μg/ml)-menadione (0.1 μg/ml)-sodium bicarbonate (1 mg/ml), hereafter referred to as TYHMS, at 35°C for 24 h. Hemin was added to the thioglycolate-yeast extract base before autoclave sterilization. Menadione and sodium bicarbonate were filter sterilized and then added to the sterile medium base. Inocula for other studies of both reference strains and clinical isolates were cultivated with thioglycolate-yeast extract broth (TY) with sodium bicarbonate (TYS).

Before use in growth studies, each culture was adjusted to a standardized turbidity equivalent to a 0.5 McFarland no. 1 standard at 525 nm. One volume of the standardized suspension was used to inoculate nine volumes of test medium, resulting in an initial cell density of 10^4 colony-forming units (CFU) per ml. This procedure was used for all growth studies.

Media. (i) Effect of aged TYHMS agar recovery of reference strains. Aged media were prepared by adding 1.5% agar to TYHMS which, after sterilization, was cooled, supplemented, and then placed in a water bath at 53 ± 2°C for 24 h. Standardized cell suspensions were used to inoculate TYHMS. After 24 h of incubation, serial 10-fold dilutions were made from samples removed from the cultures. Portions from suitable dilutions were dispensed into sterile plastic petri dishes and mixed with the aged TYHMS agar, which had been cooled to about 42°C. To compare the efficiency of recovery when freshly prepared media were used, we repeated the procedure using TYHMS agar within 90 min after sterilization. Duplicate plates were made at each dilution used. Plates containing 30 to 300 colonies were counted. The results were recorded as the average CFU per milliliter. This experiment was repeated three times.

(ii) Evaluation of hemin, menadione, sodium bicarbonate, and Tween 80. Standardized suspensions of cells of each reference strain were inoculated into TYHMS and incubated as described previously. Test media were prepared by adding 1.5% agar to TY, and various combinations of supplements were added. Those evaluated were TY with hemin, TY with hemin and menadione, TYS, TY with Tween 80 (0.1%), TY with 1 N sodium hydroxide (used to adjust pH to 7.2 or 7.6), and TYHMS. Medium combinations were inoculated with standardized suspensions of cells of reference strains which had been washed three times in sterile saline. Cultures were incubated, and plate counts were made as before.

(iii) Evaluation of age of the inoculum on the efficiency of recovery. TYS was inoculated with standardized suspensions of washed cells of each reference strain. Samples taken at 4-h intervals over a 72-h period were diluted. Portions were dispensed into sterile plastic petri dishes and mixed with freshly prepared, cooled TYS agar. Inoculated plates were incubated for 48 h at 35°C, and plate counts were made as before. Growth curve determinations were repeated three times. The incubation time required to reach the mid-logarithmic phase of growth was determined by calculating the X intercept which corresponded to the midpoint of the regression line. The incubation time required to reach the mid-stationary phase of growth was determined by estimating the X intercept which corresponded to the midpoint of the flat portion of the graphed growth response (11, 14).

TYS was inoculated with standardized suspensions of washed cells of each of the reference strains, which had been incubated for the amount of time required to reach both phases of growth. Portions were then removed, and triplicate pour plates were made with freshly prepared TYS agar. Incubation of cultures and plate counting were as for the other studies. Three independent repetitions of this experiment were performed.

(iv) Efficiency of recovery of reference strains from WC broth. TYS was first inoculated with each of the reference strains and incubated until the mid-logarithmic phase of growth. Cells were harvested and washed three times in sterile saline. Standardized suspensions were made as before and inoculated into freshly prepared WC broth (7). After 24 h of incubation, samples were removed, and portions from suitable dilutions were dispensed into sterile plastic petri dishes. Freshly prepared WC agar (Difco Laboratories, Detroit, Mich.) was added. After solidification, plates were incubated for 48 h before colony counts were made. For comparative purposes, the same protocol was followed when freshly prepared TYS and TYS agar were used. This experiment was repeated three times.

(v) Evaluation of refrigerator storage of media on the efficiency of recovery of reference strains. The efficiency of recovery of reference strains from WC agar and broth was evaluated by preparing enough media for 15 days of testing and inoculating both media on the day of preparation (day 0) with standardized suspensions of washed, mid-logarithmic-phase cells of each strain. Pour plates were made after 24 h of incubation with fresh WC agar and counted for CFU per milliliter after 48 h of incubation. This protocol was repeated daily for 15 consecutive days with media which had been stored in GasPak jars at 4°C. An identical procedure was followed in a parallel experiment except that freshly prepared WC agar and broth were used for comparative purposes. Two repetitions of this experiment were completed.

RESULTS

Results of early experiments with standardized inocula indicated that the recovery of reference strains was poor after 24 h of incubation in TYHMS which had been stored at 4°C for more than 8 h after preparation. In addition, TYHMS agar which had been stored at 4 or 22°C, regardless of time, and remelted before inoculation was incapable of supporting growth of the strains used. Because we were interested in performing growth studies over prolonged times, we wanted to evaluate the effect on the

efficiency of recovery of cells of TYHMS agar which was held in a water bath at 53 ± 2°C during these studies. For comparative purposes, a parallel study in which freshly prepared TYHMS agar was used at each sampling time was done (Table 1). We did not attempt to determine the reason for the difference in growth-promoting abilities of the fresh versus aged media.

It was noted that *P. productus* was the only reference strain to be stimulated by medium supplementation. In fact, the average range of CFU per milliliter for this organism in TY, TY with Tween 80, TY with hemin, and TY with hemin and menadione was 1.1×10^5 to 1.9×10^5, whereas that in TYHMS and TYS was 4.6×10^7 to 5.3×10^7. To determine whether this result was dependent on a pH effect or on something else, we inoculated standardized suspensions of *P. productus* into TYS at pH 7.2 and 7.6 and into TY with 1 N sodium hydroxide at pH 7.2 and 7.6. After 24 h of incubation, portions were removed and diluted. Colony counts were made after suitable incubation of pour plates. The average CFU per milliliter in TYS at both pH levels was 1.5×10^7, whereas the CFU per milliliter in the TY with 1 N sodium hydroxide at both pH levels was 3.0×10^5. After 24 h of incubation in broth, pH values of the spent media were 6.0 for TYS and 6.9 for TY with 1 N sodium hydroxide. All subsequent growth studies were performed with TYS to ensure adequate recovery of all reference strains.

The efficiency of recovery of cells from TY with Tween 80 was not different from that from TYS for reference strains other than *P. productus*. It was interesting to note, however, that colonies produced on TY with Tween 80 agar were two to three times larger than usual for the *Peptococcus* species, whereas colonies produced by the *Peptostreptococcus* species were much smaller than usual.

Analysis of growth curves revealed that *Peptococcus* species required 12 ± 0.16 h to reach the mid-logarithmic stage of growth, whereas *Peptostreptococcus* species required 10 ± 0.52 h. To reach the mid-stationary phase of growth, *Peptococcus* species required 24 ± 0.82 h, whereas *Peptostreptococcus* species required 18 ± 0.61 h. We found no significant difference in the number of CFU per milliliter recovered for the reference strains after 24 h of incubation when either mid-logarithmic- or mid-stationary-phase inocula were used.

To compare the relative productivity of TYS and WC broth for the cultivation of reference strains, we carried out a parallel study using freshly prepared media of both formulations at each sampling time. Clearly, the recovery of cells was better with TYS (Table 2). However, as previously mentioned, refrigerator storage of this medium destroys its growth-promoting abilities. A study was designed to compare the efficiency of recovery of cells from freshly prepared and stored WC agar and broth. The storage conditions were the same as those used for TYS. A decrease of 1 log unit of cell recovery for each test strain resulted when WC agar which had been stored for 6 days and WC broth which had been stored for 4 days were used, as compared with cell recovery when fresh media were used.

Because TYS was superior to WC agar and broth in the efficiency of recovery of reference strains used, we were interested in determining whether clinical isolates would respond in the same way with cultivation conditions identical to those used for the reference strains. Viable

TABLE 1. *Efficiency of recovery of cells from TYHMS agar after 24 h of anaerobic incubation at 35°C*

Reference strain	CFU/ml[a]	
	FM	AM
Peptococcus asaccharolyticus (ATCC 29743)	5.9×10^9	1.4×10^5
P. constellatus (ATCC 27823)	4.1×10^6	1.1×10^3
P. magnus (WAL 2508)	2.4×10^6	<100
P. prevotii (ATCC 9321)	2.2×10^8	2.3×10^7
P. variabilis (ATCC 14955)	6.7×10^7	6.4×10^6
Peptostreptococcus anaerobius (ATCC 27337)	3.0×10^8	3.0×10^4
P. micros (ATCC 33270)	2.4×10^8	4.7×10^5
P. productus (ATCC 27340)	4.6×10^7	<100

[a] Averaged over three replications. FM, Medium inoculated within 90 min after sterilization; AM, medium prepared and maintained at 53 ± 2°C in a water bath for 24 h before inoculation.

TABLE 2. *Comparison of the recovery of cells of reference strains from TYS and WC broth after 24 h of anaerobic incubation at 35°C*[a]

Organism	CFU/ml	
	TYS	WC
Peptococcus asaccharolyticus (ATCC 29743)	7.8×10^9	5.1×10^8
P. constellatus (ATCC 27823)	6.8×10^6	2.5×10^5
P. magnus (WAL 2508)	6.2×10^6	3.5×10^5
P. prevotii (ATCC 9321)	2.5×10^8	1.7×10^7
P. variabilis (ATCC 14955)	7.1×10^7	6.7×10^6
Peptostreptococcus anaerobius (ATCC 27337)	2.1×10^8	1.8×10^8
P. micros (ATCC 33270)	2.4×10^8	9.8×10^7
P. productus (ATCC 27340)	5.9×10^7	8.9×10^6

[a] Both TYS and WC broth were inoculated within 90 min after sterilization.

cell counts made on portions of standardized suspensions of cells of each of 54 isolates resulted in a recovery of 10^4 to 10^5 CFU/ml after 24 h of incubation. The results were consistent with previous data.

DISCUSSION

The studies described herein were initiated to assess the efficiency of recovery of strains of anaerobic gram-positive cocci in the genera *Peptococcus* and *Peptostreptococcus*. Although these organisms are usually considered to be in the slow-growing group of anaerobes (6), our results with both reference strains and clinical isolates clearly show that sufficient cell density can be achieved within 24 h with thioglycolate-based media.

The preparation, holding, and storage of media for use in cultivating these organisms is critical. Indeed, freshly prepared media should be used whenever possible for efficient recovery. It is clear that storage of media in GasPak jars at refrigerator temperatures is not an efficient method if these organisms are to be cultivated. Although others have found that the length of exposure to atmospheric oxygen and extended autoclaving times can adversely affect recovery of anaerobes (2), procedures for medium storage such that efficient recovery of cells can be ensured need to be carefully studied.

Supplementation of TY appears unnecessary, with the exception of stimulation of growth of *P. productus* by sodium bicarbonate. Because this organism is rarely encountered in clinical laboratories, the need for sodium bicarbonate supplementation of TY is questionable. Preparation of TY for use in the recovery of anaerobic gram-positive cocci would be easily accomplished in most laboratories with commercially available materials.

Recently, the National Committee for Clinical Laboratory Standards proposed a procedure for susceptibility testing of anaerobes with WC agar (7). Our data show that for anaerobic gram-positive cocci, TY results in more efficient recovery than that resulting from WC agar or broth. Because WC broth is not yet commercially available and because agar dilution procedures are cumbersome, it is recommended that, for ease of handling, TY is more efficient with *Peptococcus* and *Peptostreptococcus* species.

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