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Effect of Culture Medium and Carbon Dioxide Concentration on Growth of Anaerobic Bacteria Commonly Encountered in Clinical Specimens

DON R. STALONS, CLYDE THORNSBERRY, AND V. R. DOWELL, JR.

Department of Parasitology and Laboratory Practice, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514, and the Center for Disease Control, Atlanta, Georgia 30333

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Representative strains of anaerobic bacteria from human infections were used to evaluate broth media, gas mixtures, and inocula for use in developing a procedure for performing minimal inhibitory concentration antimicrobic susceptibility tests. Nine commercially available media, including two that were chemically defined, were tested. Tests were performed in atmospheres with carbon dioxide concentrations between 2.5 and 10% and also in the GasPak system (BBL) that had a disposable hydrogen-carbon dioxide generator. Growth curves on each organism grown in Schaedler broth and a 5% carbon dioxide atmosphere were used to determine growth characteristics, equate time of the particular growth phases to turbidity readings, and determine the numbers of viable organisms present in the culture. Schaedler broth proved to be most advantageous in combination with an atmosphere of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. The growth curve studies yielded valuable data on the rapidity and quantity of growth under these conditions. We believe these data have provided information which can be used as the basis for developing a standardized procedure for antimicrobic susceptibility testing for anaerobic bacteria.

The increased awareness of the association of anaerobic bacteria with human disease (5, 6) has fostered an interest in improving techniques for isolation and identification of anaerobes and methods for determining their antibiotic susceptibility. The GasPak (BBL) systems (3), prereduced anaerobically sterilized media (9), the anaerobic glove box (1), and gas-liquid chromatography for identification of metabolic products and cellular constituents (10, 11) are some of the recent notable achievements. However, little effort has been made to determine specific growth requirements of the anaerobic bacteria, to define optimal cultural conditions needed for physiological studies, or to develop a standard method for determining the antibiotic susceptibility of anaerobes.

We felt that before a standardized procedure was developed, it was necessary to obtain some basic information on medium requirements and cultural conditions which would allow optimal growth of anaerobic bacteria. The objectives of this investigation were to study growth characteristics of anaerobes commonly isolated from clinical specimens and select a broth medium and a carbon dioxide concentration suitable for use in subsequent study to develop a standardized procedure for determining the antibiotic susceptibility of these microorganisms.

(This paper is part of a dissertation presented by D. R. S. to the School of Public Health of the University of North Carolina, Chapel Hill, in partial fulfillment of the requirements for the degree of Doctor of Public Health.)

MATERIALS AND METHODS

Organisms. The bacterial strains used in this study were obtained from the stock culture collection of the Anaerobe Unit, Center for Disease Control, and from the American Type Culture Collection. Each strain was characterized by a battery of microscopic, cultural, and biochemical tests as described by Dowell and Hawkins (4) and Holdeman and Moore (8).

The majority of the studies were performed with four bacterial strains that were considered representative of the commonly encountered anaerobes. These organisms were *Bacteroides fragilis* subspecies *fragilis* (9053), *Peptostreptococcus*, CDC group 2 (11512), *Eubacterium alactolyticum* (8174), and *Clostridium perfringens* (BP6K). Strains of other anaerobes commonly encountered in human infections (4) were used to substantiate the findings obtained with the four microorganisms mentioned above, and growth curves of some were also studied.

Media. Several readily available commercial media were evaluated. These included brain heart infusion broth (BBL), thioglycolate broth (BBL, 0135C), Mueller-Hinton broth (BBL), peptone-yeast broth (Difco), Schaedler broth (BBL), Trypticase soy broth (BBL), heart-infusion broth (Difco), medium 199 tissue culture medium with Earle salts (Gibco), and Wright-Mundy broth (BBL). The bacterial cultures were isolated and maintained on Schaedler agar containing 5% defibrinated rabbit blood and 0.5% yeast extract; plate counts were also performed on this medium. Hemin, which stimulates the growth of various *Bacteroides* species, and menadione, a growth requirement of some *Bacteroides melaninogenicus* strains (7), were added to all media in final concentrations of 5 and 0.1 μg/ml, respectively.

Anaerobic systems. The anaerobic systems used were the GasPak jar (BBL), an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.), and 200-mm desiccator jars (Scientific Products no. D1435-1) that were used in an evacuation and gas replacement method. A palladium-coated alumina catalyst (Englehard Industries) was used in conjunction with all anaerobic systems.

Instrumentation and equipment. Volatile acid products of the bacterial strains in peptone-yeast extract-glucose medium were identified with a Beckman GC-2A chromatograph (4). Turbidity measurements were made in disposable tubes (16 by 125 mm; Pyrex) with a Coleman Jr. spectrophotometer at 540 nm. A Beckman Expandomatic pH meter equipped with a Corning combination electrode (no. 476050) was used for pH determinations.

Inoculum. All tests were performed with an inoculum of 0.05 ml of each culture that had been adjusted to the turbidity of a MacFarland no. 1 nephelometer standard which yields a concentration of approximately 3.0×10^8 organisms per ml. At each inoculation step, Gram stains of the microorganisms were inspected to minimize the possibility of contamination.

Media and atmosphere evaluation. In addition to evaluating the different media to determine which one best supports the growth of the anaerobes, we also determined the optimal gaseous environment by varying the carbon dioxide concentration. Three to five tubes containing 6 ml of each medium were inoculated with each test culture and incubated in each of the anaerobic atmospheres. Immediately after inoculation and after a 24-h incubation period at 37 C, the cultures and control tubes of uninoculated medium were measured for turbidity and pH. The gaseous environments used were: (i) that of the GasPak system with a disposable hydrogen-carbon dioxide generator; (ii) 10% carbon dioxide, 80% nitrogen, and 10% hydrogen; (iii) 7.5% carbon dioxide, 82.5% nitrogen, and 10% hydrogen; (iv) 5% carbon dioxide, 85% nitrogen, and 10% hydrogen; and (v) 2.5% carbon dioxide, 87.5% nitrogen, and 10% hydrogen. (Gas mixtures were obtained from the Mathieson Co.)

Growth curve studies. Using the optimal broth and atmosphere determined by these studies, we plotted growth curves for each organism to determine the rapidity and degree of growth in terms of turbidity and numbers of organisms produced within specified periods of time. Twenty-four culture tubes (16 by 125 mm) containing 6.0 ml of Schaedler broth were inoculated as previously described with 0.05 ml of a culture adjusted to a MacFarland no. 1 nephelometer standard. Sequential determinations of turbidity were made at 1-, 2-, and/or 4-h intervals to establish the lag, exponential, and stationary phases of growth for each organism.

Plate counts. During the growth curve studies, numbers of viable organisms were determined during the exponential phase of the four representative anaerobic bacteria (*B. fragilis* subsp. *fragilis*, *Peptostreptococcus* CDC group 2, *E. alactolyticum*, and *C. perfringens*). The culture was diluted 10^{-1} through 10^{-8} in Schaedler broth with 0.5 ml of culture and 4.5 ml of medium used at each dilution step. A 0.1-ml amount of each dilution was spread over the surface of Schaedler blood agar with a glass spreader, and the plates were incubated anaerobically at 37 C for 24 to 48 h. After incubation, colony counts were performed on plates containing 30 to 300 colonies, and the number of viable bacteria per milliliter was calculated.

RESULTS

In the initial study of media and varying atmospheres, the four representative anaerobes (*B. fragilis* subsp. *fragilis*, *Peptostreptococcus* CDC group 2, *E. alactolyticum*, and *C. perfringens*) were used. Nine different media, including two chemically defined ones, and five concentrations of carbon dioxide, including that of the GasPak jar, were evaluated to determine the optimal medium and gaseous environment for each culture. The chemically defined media (medium 199 and Wright-Mundy) proved unsatisfactory at the start and were dropped from the study. Turbidity and pH changes in the cultures were measured to determine the effects of varying the medium and cultural conditions (Tables 1-4).

In each of the 45 media-carbon dioxide combinations, triplicate cultures of each representative strain were studied. Relative turbidity and pH measurements were made on each culture, and the percentage of change in turbidity was calculated after a 24-h incubation period to give an indication of growth. No change in pH was detected immediately after inoculation; only after the log-linear growth phase of the organism was there a pH change that could have conceivably affected antibiotic activity if antibiotic had been present.

A mean percentage increase in turbidity was calculated for each of the four representative strains from triplicate cultures of each in the 35 media-carbon dioxide combinations (excluding the synthetic media 199 and Wright-Mundy

TABLE 1. *Turbidity and pH changes produced by Bacteroides fragilis subsp. fragilis in commonly used commercial broth media; incubation at 37 C for 24 h*

Medium	Turbidity[a] in CO_2 atmosphere[b] of:					Change in pH in CO_2 atmosphere[b] of:				
	2.5%	5.0%	7.5%	10.0%	GasPak	2.5%	5.0%	7.5%	10.0%	GasPak
Brain heart infusion	2	6	1	7	2	0.04[c]	0.09	0.08	0.10	0.13
Thioglycolate	2	1	0	0	4	0.03	0.08	0.07	0.00	0.07
Mueller-Hinton	5	55	5	10	5	0.03	0.73	0.02	0.04	0.12
Peptone-yeast	5	2	9	9	3	0.04	0.04	0.02	0.02	0.12
Schaedler	51	77	49	52	52	1.99	1.83	1.84	1.67	1.95
Tryticase soy broth	1	3	3	1	7	0.07	0.09	0.11	0.11	0.17
Heart infusion	3	15	5	9	5	0.00	0.05	0.08	0.09	0.18
Wright-Mundy	2	3	2	2	1	0.00	0.03	0.03	0.03	0.01
Medium 199 tissue culture without indicator	11	6	4	4	7	0.44	0.10	0.00	0.04	0.13

[a] Expressed as 100 − % turbidity at 540 nm.
[b] CO_2 concentration in combination with 10% hydrogen and a balance of nitrogen.
[c] pH units.

TABLE 2. *Turbidity and pH changes produced by Peptostreptococcus CDC group 2 in commonly used commercial broth media; incubation at 37 C for 24 h*[a]

Medium	Turbidity in CO_2 atmosphere of:					Change in pH in CO_2 atmosphere of:				
	2.5%	5.0%	7.5%	10.0%	GasPak	2.5%	5.0%	7.5%	10.0%	GasPak
Brain heart infusion	36	35	26	26	37	0.12	0.15	0.13	0.11	0.23
Thioglycolate	13	19	21	18	20	0.24	0.34	0.40	0.39	0.43
Mueller-Hinton	23	22	23	24	26	0.13	0.20	0.21	0.22	0.36
Peptone-yeast	12	6	13	5	5	0.03	0.03	0.10	0.10	0.15
Schaedler	19	55	60	62	65	0.19	0.53	0.72	0.75	1.05
Trypticase soy broth	22	26	25	29	28	0.17	0.23	0.21	0.28	0.35
Heart infusion	15	10	3	4	5	0.09	0.10	0.09	0.14	0.15
Wright-Mundy	1	1	1	1	1	0.00	0.01	0.00	0.00	0.00
Medium 199 tissue culture without indicator	1	1	1	1	1	0.04	0.01	0.00	0.00	0.02

[a] All determinations were as in Table 1.

TABLE 3. *Turbidity and pH changes produced by Eubacterium alactolyticum in commonly used commercial broth media; incubation at 37 C for 24 h*[a]

Medium	Turbidity in CO_2 atmosphere of:					Change in pH in CO_2 atmosphere of:				
	2.5%	5.0%	7.5%	10.0%	GasPak	2.5%	5.0%	7.5%	10.0%	GasPak
Brain heart infusion	1	7	3	2	0	0.00	0.03	0.02	0.00	0.02
Thioglycolate	4	8	2	3	2	0.12	0.21	0.20	0.20	0.12
Mueller-Hinton	4	5	2	4	1	0.04	0.02	0.02	0.02	0.00
Peptone-yeast	4	4	1	0	2	0.02	0.00	0.02	0.03	0.01
Schaedler	7	22	4	11	6	0.08	0.12	0.08	0.07	0.08
Trypticase soy broth	4	6	0	3	1	0.04	0.05	0.02	0.01	0.03
Heart infusion	4	3	0	0	0	0.02	0.03	0.01	0.01	0.02
Wright-Mundy	1	1	1	1	1	0.00	0.00	0.01	0.00	0.01
Medium 199 tissue culture without indicator	1	2	1	1	1	0.02	0.02	0.01	0.02	0.01

[a] All determinations were as in Table 1.

broth). For each organism, these 35 combinations were ranked according to the mean change in turbidity—the lower the number in this ranking process, the greater the growth response.

In the case of *B. fragilis* subsp. *fragilis*, the

greatest increase in turbidity was noted in a Schaedler broth-5% carbon dioxide combination; the second largest increase was observed with a Mueller-Hinton-5% carbon dioxide combination. Five of the six largest growth responses of *B. fragilis* subsp. *fragilis* were associated with Schaedler medium and various carbon dioxide concentrations. The probability of observing such a result due to chance alone is less than 0.0002.

With the strain of *Peptostreptococcus* CDC group 2, the Schaedler broth-GasPak and 10% carbon dioxide combination yielded the greatest amount of growth. For the *E. alactolyticum* and the *C. perfringens* strains, the Schaedler broth-5% carbon dioxide combination again produced the greatest percentage of increase in relative turbidity.

The results of this ranking of growth response are summarized in Tables 5 and 6. These summaries were obtained by ordering the rank totals for the seven media and the five carbon dioxide concentrations. For example, the Schaedler medium consistently yielded the largest increase in relative turbidity when the results were averaged over all levels of carbon dioxide concentration (Table 5). The probability of observing such a result due to chance alone is less than 0.003. This is very strong evidence that the Schaedler broth does in fact yield a higher growth response than the other six media. When the results were averaged over the seven media, the largest increase in relative turbidity was associated with the 5% carbon dioxide concentration (Table 6).

Growth curves of the four representative organisms (*B. fragilis* subsp. *fragilis*, *Peptostreptococcus* CDC group 2, *E. alactolyticum*, and *C. perfringens*) were plotted to study the growth phases of these organisms. The slope and duration of the exponential growth phase, the maximal turbidity, and the numbers of organisms at a relative turbidimetric value were determined (Fig. 1-4). It should be noted that, for different strains of each species, lag-phase times are likely to vary. For example, nearly 10 h separated the lag times of seven strains of *B. fragilis* subsp. *fragilis*, including strain 9053; the longest lag time was 16 h. However, the slopes of the log-linear phases of growth and the maximal turbidities produced by each strain were nearly identical.

Additional growth curves were also plotted to include some of the commonly isolated anaerobic pathogens. These data (Fig. 5) clearly show that the Schaedler medium in combination with the 5% carbon dioxide concentration universally supported the growth of these organisms. Moreover, the rates of growth were such that a mid-log-linear phase could be obtained, in the majority of cases, before 12 to 24 h had elapsed. It is possible that a specific turbidity reading from any of the curves in Fig. 5 may be equated to approximate numbers of organisms by referring to the representative organism of Fig. 1 through 4; e.g., at a given concentration, two organisms of a similar size and conformation probably would yield comparable turbidities. The practicality of this assumption will be determined in future studies.

DISCUSSION

The need for a medium and an atmosphere that will support optimal growth of the anaerobic bacteria commonly associated with human disease cannot be overemphasized if standardization of procedures is to be attained. From the data obtained in this investigation, Schaedler broth in combination with an atmosphere of 5%

TABLE 4. *Turbidity and pH changes produced by Clostridium perfringens in commonly used commercial broth media; incubation at 37 C for 24 h*[a]

Medium	Turbidity in CO_2 atmosphere of:					Change in pH in CO_2 atmosphere of:				
	2.5%	5.0%	7.5%	10.0%	GasPak	2.5%	5.0%	7.5%	10.0%	GasPak
Brain heart infusion	78	77	75	76	75	0.80	0.73	0.70	0.64	0.62
Thioglycolate	78	79	81	77	77	1.94	1.88	1.91	1.88	1.86
Mueller-Hinton	68	74	81	77	77	1.04	0.90	0.80	0.64	0.77
Peptone-yeast	68	73	71	75	68	0.63	0.47	0.41	0.41	0.39
Schaedler	86	89	81	84	81	1.61	1.43	1.28	1.26	1.32
Trypticase soy broth	66	64	69	72	69	1.01	0.90	0.82	0.81	0.89
Heart infusion	52	55	53	52	54	0.29	0.24	0.21	0.14	0.18
Wright-Mundy	1	2	2	1	1	0.08	0.04	0.03	0.03	0.05
Medium 199 tissue culture without indicator	25	23	26	25	25	1.21	0.90	0.85	0.68	0.91

[a] All determinations were as in Table 1.

TABLE 5. *Ranking of the growth response of four representative anaerobes in seven media and five CO_2 concentrations*

Medium	Organism[a]			
	Bacteroides fragilis subsp. *fragilis*	*Peptostreptococcus* (CDC group 2)	*Eubacterium alactolyticum*	*Clostridium perfringens*
Brain heart infusion	5	5	2	3
Thioglycolate	7	2	5	2
Mueller-Hinton	2	3	4	6
Peptone-yeast	4	6	6	4
Schaedler	1	1	1	1
Trypticase soy broth	6	4	3	5
Heart infusion	3	7	7	7

[a] Lowest number indicates the most favorable ranking with regard to growth response.

TABLE 6. *Summary of the order of CO_2 concentrations in terms of increase in turbidity averaged for the seven media*[a]

CO_2 concn	Organism			
	Bacteroides fragilis subsp. *fragilis*	*Peptostreptococcus* (CDC group 2)	*Eubacterium alactolyticum*	*Clostridium perfringens*
2.5%	5	5	2	2
5.0%	1	2	1	1
7.5%	4	4	4	3
10.0%	2	3	3	4
GasPak	3	1	5	5

[a] See Brewer and Allgeier (3).

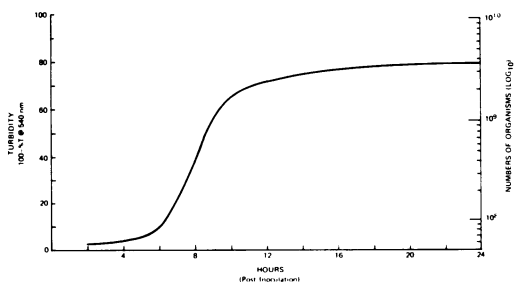

FIG. 1. *Growth curve of Bacteroides fragilis subsp. fragilis in Schaedler broth at 37 C.*

carbon dioxide, 10% hydrogen, and 85% nitrogen was clearly best for obtaining good growth of the organisms tested within 24 h of incubation.

The literature reveals that a number of different media have been used by various investigators in their attempts to develop a susceptibility test method for anaerobes (C. Thornsberry, 1972, Proc. Int. Conf., in press). Undoubtedly the use of different media has contributed to the variations in results reported by different workers. Since the anaerobes have received so much attention in recent years and since these bacteria are now more frequently isolated from clinical specimens, there is an increased demand for antimicrobial susceptibility tests. Consequently, there has been an increasing need to standardize testing methods in much the same way that the Kirby-Bauer (2) method has been standardized for testing the commonly encountered facultative anaerobic organisms. For susceptibility tests to be performed, adequate growth is necessary, preferably within 18 to 24 h. The data from this study show that, for most of the anaerobes commonly isolated from clinical specimens, Schaedler broth and an atmosphere of 5% carbon dioxide meet this need. However, some of the more fastidious organisms such as the anaerobic cocci do not grow luxuriantly under these conditions without further supplementation (Fig. 5). With organisms that grow slowly or produce less turbidity, it could be difficult to differentiate between growth and no growth, regardless of the method used. We believe that in the development of standard methods for anaerobes, the use of this medium and this carbon dioxide atmosphere will be of great value.

When the minimal inhibitory concentrations of selected antibiotics are determined, the inoculum concentration must be carefully considered because the number of viable organisms in the inoculum directly affects the amount of antibiotic required to inhibit or kill the organism. It is possible that the data from this study (Fig. 1–4) could be used to estimate the number of viable anaerobes in a particular culture at a

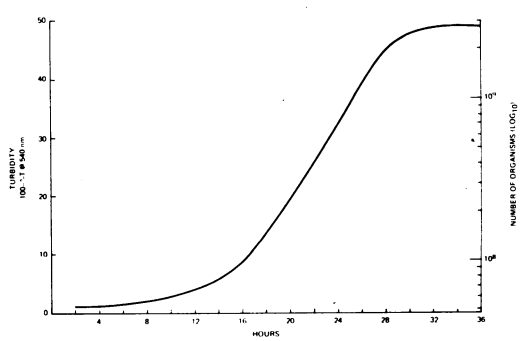

FIG. 2. *Growth curve of Peptostreptococcus (CDC group 2) in Schaedler broth at 37 C.*

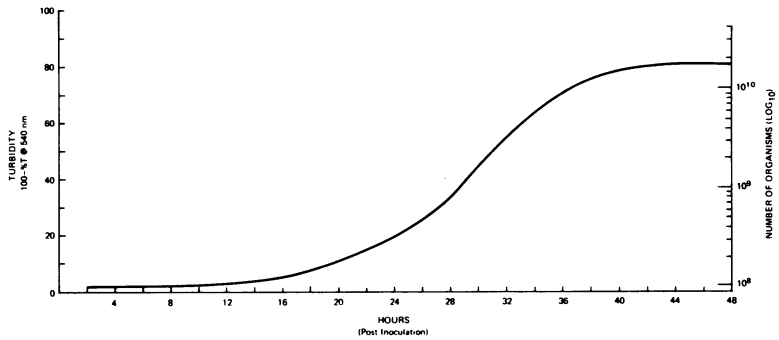

FIG. 3. *Growth curve of Eubacterium alactolyticum in Schaedler broth at 37 C.*

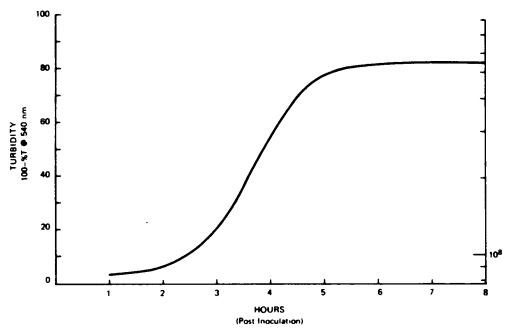

FIG. 4. *Growth curve of Clostridium perfringens in Schaedler broth at 37 C.*

given turbidity, within a range suitable for performing susceptibility tests. This possibility will be evaluated by further studies. The organisms used in this investigation are representative of most of the anaerobic bacteria with regard to morphology, biochemical nature, and size. These data provide a basis for the determination of actual numbers of organisms to be used in the inoculum for subsequent susceptibility testing.

Although good bacterial growth is the basic requirement for a culture medium that is to be used for antimicrobial susceptibility tests, other factors may also influence this choice. Some media contain components that influence the

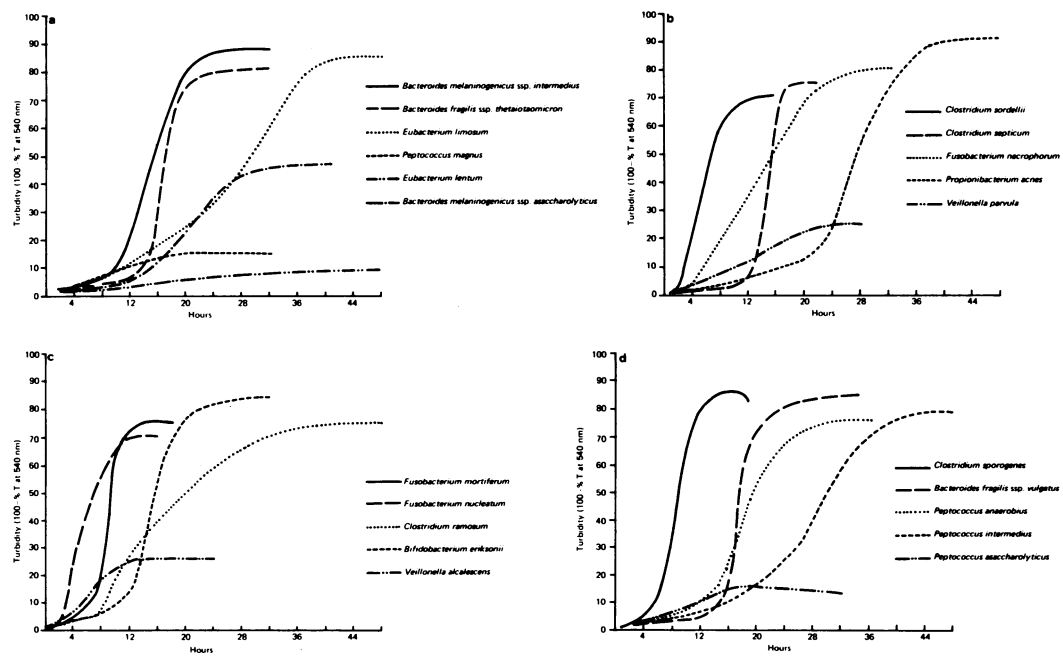

FIG. 5. *Growth curves of selected anaerobic bacteria in Schaedler broth and 5% carbon dioxide.*

interaction of the bacteria and antimicrobic(s) so that the susceptibility results are drastically altered, as, for example, the action many media exert on sulfonamide activity. Therefore, Schaedler broth and an atmosphere of 5% carbon dioxide will be further studied in antimicrobial susceptibility tests to determine whether this medium and these cultural conditions have any adverse effects on the antibiotics or on the susceptibility of the bacteria to the antimicrobics.

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