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Oxygen Sensitivity of Various Anaerobic Bacteria

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Anaerobes differ in their sensitivity to oxygen, as two patterns were recognizable in the organisms included in this study. Strict anaerobes were species incapable of agar surface growth at pO_2 levels greater than 0.5%. Species that were found to be strict anaerobes were *Treponema macrodentium*, *Treponema denticola*, *Treponema oralis* n. sp., *Clostridium haemolyticum*, *Selenomonas ruminatium*, *Butyrivibrio fibrisolvens*, *Succinivibrio dextrinosolvens*, and *Lachnospira multiparus*. Moderate anaerobes would include those species capable of growth in the presence of oxygen levels as high as 2 to 8%. The moderate anaerobes could be exposed to room atmosphere for 60 to 90 min without appreciable loss of viability. Species considered as moderate anaerobes were *Bacteroides fragilis*, *B. melaninogenicus*, *B. oralis*, *Fusobacteria nucleatum*, *Clostridium novyi* type A, and *Peptostreptococcus elsdenii*. The recognition of at least two general types of anaerobes would seem to have practical import in regard to the primary isolation of anaerobes from source material.

Anaerobic bacteria are usually considered to be bacteria which can grow only in the absence of oxygen (4, 8). Yet, among the anaerobes it is apparent that degrees of sensitivity to molecular oxygen exist. When atmospheric oxygen is rigidly excluded during the primary isolation of bacteria from gingival debris, i.e., in the Hungate roll tube technique (3), from 50 to 60% of the total microscopic count can be recovered as single colonies (M. Stutman and D. F. Gordon, Abstr. 181, Intern. Ass. Dental Res., 1969). When the same specimen is manipulated in room atmosphere and then incubated anaerobically in jars, only 15 to 30% of the microscopic count will grow out. When gingival debris was plated and incubated in anaerobic chambers, three to four times as many bacteria were isolated as when the same material was plated on the bench top and then incubated anaerobically in jars (1). This difference in recoveries between the methods can best be explained by assuming that certain anaerobic bacteria cannot survive even short exposures to atmospheric oxygen (1, 14). In the present study, various anaerobic bacteria were assayed for their ability to grow on agar surfaces exposed to gas atmospheres containing from 0 to 12% oxygen. The species employed included both Brewer jar and roll tube isolates and are listed in Table 1.

MATERIALS AND METHODS

All manipulations of the cultures were performed in an anaerobic chamber (9) under a 90% nitrogen and 10% hydrogen (forming gas) atmosphere. Any traces of oxygen in the commercial grades of gases employed were removed by an inline Deoxo Filter (Engelhard Industries). The chamber oxygen levels as measured by a coulometric cell (Lockwood MacLorie, Inc.) varied from 2 to 20 ppm. Agar plates containing either Brain Heart Infusion supplemented with 10% horse blood and 0.5 μg of menadione per ml or supplemented PPLO medium (13) were streaked with the various cultures and then placed into jars while in the chamber. A brass lid, containing a neoprene O-ring in a recessed groove on its under side (InterTech Inc., Natick, Mass.), was then placed on the jar and connected via tubing to a vacuum line. The jar was evacuated to 27 mm of Hg, which secured the O-ring seal, and then filled with forming gas, 10% carbon dioxide, and a variable amount of air to give oxygen levels ranging from 0 to 12%. In those jars that were to be kept completely anaerobic, stainless-steel mesh packets containing Deoxo catalyst were placed in the jars. Also included in each of these jars was a tube containing a mixture of 1 part of 40% pyrogallol and approximately 10 parts of 0.1 N NaOH presaturated with Na_2CO_3. This indicator was observed to remain colorless throughout the incubation period. When the rumen strains were under investigation, the CO_2 level was increased to 20 to 25% at the expense of the forming gas. The jars were then removed from the chamber and incubated for 7 days at 37 C; they were then opened and growth and purity were noted. The results of the agar surface streakings were evaluated as growth, light growth, or no growth. All strains were tested a minimum of three times.

In other experiments, 6 ml of either thioglycollate broth (Bioquest) or supplemented PPLO broth, both containing reduced resazurin, was added to sterile petri plates so as to give a large surface to volume ratio. These plates were inoculated with pure cultures and treated as were the agar plates, except that growth

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TABLE 1. *Oxygen sensitivity of various anaerobic bacteria*

Strict anaerobes (maximum growth at $pO_2 < 0.5\%$)	Moderate anaerobes (maximum growth at $pO_2 < 3\%$)	Microaerophiles (maximum growth at $pO_2 > 0.3$ and $< 20\%$)
Treponema macrodentium strain TM1	*Bacteroides fragilis* NTCC strain 9343, C, F, B, H, 11, 16 (7)	*Vibrio sputorum* (6) var. *sputorum* strains JVS, ER33; var. *bubulum* strains 8010, 9977
T. denticola strains (MRB, TRRD)	*B. melaninogenicus* strains BE1, 49, 9GBK, CR2A, 1GBK (10)	*Vibrio fetus* strain 270H (6)
T. oralis n. sp. strains Richards 1	*B. oralis* strains J1, 9B, R53, 7CM (7)	
Clostridium haemolyticum ATCC strains 9650, 9652[a]	*Fusobacteria nucleatum* strains 7CF, 13BF, 82F (5)	
Selenomonas ruminatium strain GA192[b] (3)[c]	*Clostridium novyi* type A ATCC strain 19402[a]	
Butyrivibrio fibrisolvens strain D1[b] (3)	*Peptostreptococcus elsdenii* strain B159[b] (3)	
Succinivibrio dextrinosolvens strain 24[b] (3)		
Lachnospira multiparus strain 40[b] (3)		

[a] Described in Bergey's Manual of Determinative Bacteriology, 7th ed., The Williams & Wilkins Co., Baltimore.

[b] Roll tube isolates.

[c] Numbers in parentheses are references describing species.

at the end of 7 days was quantitated by measuring absorbancy at 520 nm with a Bausch & Lomb Spectronic 20 colorimeter.

RESULTS

The results listed in Tables 1 and 2 are a compilation of the agar surface streakings and the quantitative absorbancy readings obtained from growth in the shallow broth plates. Three general oxygen sensitivity patterns for organisms capable of growth under strictly anaerobic conditions were recognized.

Strict anaerobes. The group designated as strict anaerobes did not exhibit agar surface growth at oxygen tensions greater than 0.5% when streaked on the PPLO medium. This medium contains enough cysteine to lower its E_h to about -180 mv (13). In the shallow broth plates of the same medium, maximal growth for most strains occurred in the range of 0 to 0.4% oxygen, with reduced or no growth over the range of 0.4 to 0.7% oxygen. On occasion some growth did occur for *Clostridium haemolyticum* and *Lachnospira multiparus* at oxygen tensions as high as 1%. These two species were the only strict anaerobes capable of surface growth on the 10% blood-Brain Heart Infusion medium. The *Treponema* species appeared to be the most sensitive of the strains tested, as they did not grow at pO_2 greater than 0.1%. In fact, of 19 spirochete strains tested, only 4 would grow on agar surfaces.

Moderate anaerobes. The organisms listed as moderate anaerobes in Tables 1 and 2 grew routinely at oxygen tensions greater than 0.5%. The limiting pO_2 for agar surface growth on 10% blood-Brain Heart Infusion medium of these species varied from 2 to 8%. Growth in broth showed a similar end point variation, but most strains tested appeared to grow maximally in oxygen tensions up to and including 3% oxygen. This level was arbitrarily chosen, therefore, as an upper limit for gaseous oxygen beyond which most moderate anaerobes would show reduced growth. This group of anaerobes included several *Bacteroides* species, *Fusobacterium nucleatum*, *Clostridium novyi* type A, and *Peptostreptococcus elsdenii*. *P. elsdenii* was the most oxygen-sensitive species in this group, whereas *Bacteroides fragilis* as noted by Drasar (2) appeared to be the most oxygen tolerant. In some cases, strain variation was observed within a species. Thus, *Bacteroides melaninogenicus* strain BE1 grew optimally in broth at pO_2 levels as high as 6%, whereas *B. melaninogenicus* strains 9GBK and 1GBK did not grow well at pO_2 levels greater than 2%.

Microaerophiles. The vibrio species tested differed from the anaerobes in that growth on agar surfaces and in broth under strictly anaerobic conditions was less than that observed in the presence of small amounts of oxygen. *Vibrio sputorum* grew routinely in the presence of 5 to 10% oxygen and *Vibrio fetus* tolerated even higher levels of oxygen. This pattern of growth with peak growth at low oxygen tensions would be typical for microaerophilic bacteria.

The organisms designated as strict anaerobes, i.e., agar surface growth at $pO_2 < 0.5\%$ (Table 1) were isolated by either the roll tube technique or in the case of *Treponema* and *C. haemolyticum* strains by procedures involving minimal atmos-

TABLE 2. *Oxygen sensitivity of various anaerobic bacteria*

Species	Oxygen in gas atmosphere (%)												
	0	0.1	0.3	0.5	0.7	1.0	2.0	3.0	4.0	6.0	8.0	10	12
Treponema macrodentium	++[a]	++	0	0									
T. denticola	++	++	0	0									
T. oralis n. sp.	++	++	0	0									
Selenomonas ruminatium	++	++	+	+	0	0							
Butyrivibrio fibrisolvens	++	++	++	+	0	0							
Succinivibrio dextrinosolvens	++	++	++	+	0	0							
Lachnospira multiparus	++	++		++	+	0	0	0					
Clostridium haemolyticum	++	++	++	++	+	0	0	0					
Peptostreptococcus elsdenii	++	++	++	++	++	++	+	0	0				
C. novyi type A	++	++	++	++	++	++	+	0	0				
Bacteroides oralis	++	++	++	++	++	++	++	++	+, V	+, V	0	0	0
B. melaninogenicus	++	++	++	++	++	++	++	++, V	++, V	+, V	+, V	0	0
Fusobacteria nucleatum	++	++	++	++	++	++	++	++	++	++	+, V	0	0
Bacteroides fragilis	++	++	++	++	++	++	++	++	++	+, V	+, V	0	0
Vibrio sputorum	+	+	+	++	++	++	++	++	++	++	++	++	+, V
V. fetus	+	+	+	++	++	++	++	++	++	++	++	++	++

[a] Symbols: ++ Growth; +, slight growth; V, growth varied with strain or length of incubation (or both); 0, no growth.

TABLE 3. *Effect of length of room atmospheric exposure on subsequent anaerobic growth*

Species	Length of room exposure (min)[a]								
	0	20	40	60	80	100	300	360	480
Strict anaerobes									
T. macrodentium	100		75	39	0				
T. denticola	100		63	31	0				
T. oralis	100		45	40	0				
B. fibrisolvens	100	64		32	9	0			
S. dextrinosolvens	100	72	22	9	0.3	0			
Moderate anaerobes									
B. oralis	100				80		36	14	0
F. nucleatum	100					79	37	21	0
B. fragilis	100			100		95	100	103	85

[a] Results expressed as percentage of maximal growth. Each value is the average of 8 to 12 observations reported as a percentage of the growth which occurred under strictly anaerobic conditions.

pheric exposure (11, 13). These strict anaerobes cannot be routinely passed as surface colonies if the streaking is performed in room atmosphere. The moderate anaerobes (Table 1) were mainly isolated by techniques involving anaerobic jars and can be routinely passed by bench streaking followed by incubation in anaerobic jars. This would suggest that the strict anaerobes are more sensitive than the moderate anaerobes to atmospheric levels of oxygen. Experiments were performed to determine how long an agar medium inoculated with cells of representative species could be exposed to atmospheric levels of oxygen and still allow growth. Cultures of *Succinivibrio dextrinosolvens*, *Butyrivibrio fibrisolvens*, *Treponema macrodentium*, *Treponema denticola*, *Bacteroides oralis*, *B. fragilis* and *F. nucleatum* were serially diluted within the chamber, and 0.05-ml drops (8 to 12 drops per strain) from high dilutions were plated on the enriched PPLO medium. One series of inoculated plates was maintained completely anaerobic, whereas the other plates were removed from the chamber and exposed to room atmosphere for periods ranging from 20 min to 8 hr before being incubated under an anaerobic environment. The strict anaerobes were either very susceptible to atmospheric oxygen or their growth was inhibited because the medium was modified by exposure to air (Table 3). After 20 to 40 min of exposure, there was about a 30 to 70% reduction in colony counts, and after 60 to 100 min there was a complete inhibition of growth. The moderate anaerobes, *B. oralis* and *F. nucleatum*, showed only 20% reduction at 80 to 100 min and complete inhibition did not appear until after 8 hr. *B. fragilis* appeared to be relatively indifferent to atmospheric exposure with a slight decline in counts appearing after 8 hr.

DISCUSSION

The present results demonstrate that certain anaerobic bacteria vary in regard to their ability to grow on agar surfaces in the presence of molecular oxygen. This would mean that isolation techniques employing continuous anaerobiosis, i.e., roll tube or anaerobic chambers, would permit cultivation of all anaerobes whose nutrient requirements are met by the medium employed, whereas anaerobic jar techniques, depending on the length of atmospheric exposure, would probably discriminate against strict anaerobes and only allow growth of less oxygen-sensitive anaerobes.

In terms of nomenclature, the groupings recognized in this study are defined in the hope that they represent valid clusters of anaerobic species along an oxygen-sensitivity continuum. At one end of the continuum would be the strict anaerobes. These are bacteria which do not exhibit agar surface growth even on reduced medium at oxygen tensions greater than 0.5%. All the strains tested were stock strains which, as an inevitable consequence of laboratory passage, may be strains selected or adapted for some oxygen tolerance. The maximal oxygen tolerance of this group may have to be lowered if the oxygen sensitivity of fresh isolates of the same species proves to be much lower.

The large group of anaerobes which grow routinely on blood-agar medium after atmospheric manipulation and anaerobic incubation should be differentiated from the strict anaerobes. It is suggested that anaerobic bacteria capable of agar surface growth on unreduced medium at oxygen tensions greater than 0.5%, but not in the presence of air, be considered as moderate

anaerobes. The inhibitory level of oxygen for most of these strains will probably be less than 10%. This should distinguish moderate anaerobic bacteria from organisms such as *C. histolyticum*, *C. tertium*, etc., which can just barely grow on the surface of blood-agar exposed to air and which are referred to as aerotolerant anaerobes (8).

Another category is needed to place organisms such as *V. fetus* and *V. sputorum* which grow optimally in the presence of reduced amounts of oxygen. *V. sputorum* is biochemically and serologically related to *V. fetus* (6), but it is more oxygen sensitive, not growing above 10 to 15% oxygen. This may be related to the observation that *V. sputorum* is catalase negative, wheras *V. fetus* is catalase positive. In the absence of oxygen, *V. fetus* and *V. sputorum* grow poorly, a finding which differentiates these organisms from anaerobic bacteria. Those bacteria which exhibit minimal growth both in the presence and absence of air, but which grow maximally at intermediate oxygen levels, are best described as microaerophiles (8). Further study may show that microaerophiles are closer to strict aerobes than to anaerobes in their energy metabolism.

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