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Quantitative Method for Measurement of Aerotolerance of Bacteria and Its Application to Oral Indigenous Anaerobes

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An index which expressed the aerobic to anaerobic potential was made for bacteria with intermediate tolerance for oxygen. One method used for this analysis was measurement of the relative bacterial growth ratio. The other method was based on the pattern of the absorbancy versus depth plot. The index was applied to oral indigenous anaerobes.

Brock (1) classified bacteria into five groups as follows: (i) obligate aerobes, (ii) facultative anaerobes, (iii) aerotolerant anaerobes, (iv) obligate anaerobes, and (v) microaerophilic organisms. He described facultative anaerobes as bacteria that can grow with and without oxygen and aerotolerant anaerobes as bacteria that can grow in the absence of oxygen. However, the group definitions of Brock are descriptive, and classifying every bacterium is practically difficult. It is also difficult to compare the resistance for the oxygen effect from one bacterium to others.

The purpose of the present study was to provide quantitative measurements of the aerotolerance of microorganisms. Two methods were used; one involved the shaking of a liquid culture and the other involved an agar-mixed culture inoculated before the agar was solidified. Stab culture in agar was also used for comparison. Bacterial growth was quantitatively evaluated by measuring absorbancy. Oral indigenous bacteria were used; enterobacteria and other strains were used for comparison.

GAM broth (Nissui Co., Tokyo, Japan), a medium for the growth of anaerobes, and GAM semisolid medium, GAM broth containing 1.5 g of agar per liter of distilled water, were used for the cultivation of both aerobic and anaerobic strains. The composition of GAM broth was the same as that described in our previous paper (2). For stab cultures, seed cultures were stabbed into 3 ml of GAM semisolid medium in screw-cap tubes (12 mm in diameter) by using platinum needles and incubated for 1 to 2 days at 37°C. The extent of the growth was determined by eye. For the agar-mixed cultures, seed cultures grown in GAM broth were diluted with the same medium 10- to 100-fold. A 0.5-ml portion of each dilution was mixed in culture tubes with 4.5 ml of GAM semisolid medium which was kept molten at 50°C in a water bath. The mixtures were quickly cooled and incubated in air or in a jar (Tomy JK-1, Tominaga Co., Inc., Tokyo, Japan) filled with pure oxygen gas for 1 to 2 days at 37°C. The distance from the medium surface to the growth region was measured by determining the absorbancy. The absorbancy was plotted against the distance. For the shaken culture, seed cultures (0.05 ml) obtained after 24 h of incubation were inoculated into 5 ml of GAM broth in L-form culture tubes (Monod-type, 18 mm in diameter; Muto Co., Inc., Sapporo, Japan). The tubes were aerobically or anaerobically shaken at 400 rpm at 37°C for 24 h by using a Gyrotory incubator shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). The anaerobically shaken tubes were sealed after dissolved oxygen in the broth was evacuated. To determine the effects of shaking, cultures in GAM broth were also incubated in parallel without shaking. The absorbancy of the liquid medium was measured by using a Leitz model M photometer with an A filter. Observations were repeated three to nine times. The relative bacterial growth ratio (RBGR) is the absorbancy of the aerobically shaken culture divided by the absorbancy of the anaerobically shaken culture.

RBGR values obtained for the strains studied are given in Table 1. The results of the stab cultures are also shown. The RBGR value is the mean value of three to nine determinations, and the values varied from ∞ with obligate aerobes to 0 with obligate anaerobes. The RBGR of facultative anaerobes was in the range of 6.0 to 1.8, and for aerotolerant anaerobes, the range was from 1.6 to 0.2. The effect of aeration on bacterial growth can be seen by comparing aerobically shaken cultures with anaerobically shaken cultures. No growth was observed for obligate anaerobes in the presence of air. In aerotolerant anaerobes (especially with *Streptococcus mutans*), the growth rate was markedly reduced by aeration in strains that showed RBGR values below 0.3, whereas the rate was not affected in strains with RBGR values around 1.0; furthermore, growth was rather stimulated in most strains that showed RBGR values above 1.3. In contrast to the aerobically shaken cultures, little difference in growth rate was observed between unshaken and anaerobically shaken cultures for most aerobes and anaerobes. RBGR values thus obtained present a clearer classification than the usual qualitative growth indications obtained by using stab cultures. This method also yields a quantitative estimation for the classification of Brock (1).

Results with the agar-mixed culture method are shown in Fig. 1. The growth characteristics of obligate aerobes, facultative anaerobes, aerotolerant anaerobes, and obligate anaerobes could be clearly visualized by this method. Growth-inhibited zones with the agar-mixed culture were clearly observed below the medium surface for aerotolerant anaerobes and obligate anaerobes. The depth was less in air than in pure oxygen, and it was also less in cultures with an inoculation of 10^7 CFU than in cultures inoculated with 10^4 CFU. The oxidation-reduction potential in the medium de-

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TABLE 1. Bacterial growth in stab and shaken cultures

Organism type and strain[a]	Growth in broth (A_{630})			RBGR[b]	Growth on semisolid agar	
	Not shaken	Shaken in air	Anaerobically shaken		Surface[c]	Depth[d]
Obligate aerobes						
Micrococcus luteus NCTC 2665	0.02	0.15	0.00	∞	++	−
Neisseria mucosa S-11[a]	0.05	2.00	0.04	50.0 ± 1.4	++	−
Pseudomonas aeruginosa S-2	0.09	0.07	0.01	7.0 ± 0.1	++	−
Facultative anaerobes						
Escherichia coli IID 861	0.58	3.05	0.51	6.0 ± 1.2	++	+
Escherichia coli K-67	0.52	2.75	0.49	5.6 ± 2.0	++	+
Escherichia coli B/r	0.46	1.53	0.52	2.9 ± 0.1	++	+
Proteus vulgaris IID 874	0.36	0.93	0.42	2.2 ± 0.0	++	+
Staphylococcus aureus IID 671	0.44	0.95	0.48	2.0 ± 0.1	++	+
Salmonella enteritidis IID 604	0.61	1.08	0.61	1.8 ± 0.2	++	+
Aerotolerant anaerobes						
Streptococcus faecalis ATCC 9756	0.83	1.32	0.82	1.6 ± 0.8	+	+
Streptococcus mutans MT 703	0.23	0.18	0.13	1.4 ± 0.2	+	+
Streptococcus mutans Pk 1	0.59	1.42	0.98	1.4 ± 0.3	+	+
Streptococcus faecalis ATCC 19433	0.96	1.42	1.08	1.3 ± 0.6	+	+
Streptococcus pyogenes IID 689	0.54	0.35	0.28	1.3 ± 0.4	+	+
Streptococcus mutans OMZ 175	0.41	0.54	0.47	1.1 ± 0.5	+	+
Streptococcus mutans B 13	0.42	0.52	0.46	1.1 ± 0.5	+	+
Streptococcus sanguis S-9	0.25	0.24	0.24	1.0 ± 0.4	+	+
Streptococcus mutans Ingbritt	0.14	0.15	0.16	1.0 ± 0.2	+	+
Streptococcus mutans 1089	0.15	0.14	0.18	0.8 ± 0.5	+	+
Streptococcus mutans BHT	0.35	0.14	0.33	0.4 ± 0.0	+	+
Streptococcus salivarius S-6	0.54	0.13	0.52	0.3 ± 0.0	+	+
Lactobacillus acidophilus ATCC 4356	0.20	0.06	0.18	0.3 ± 0.1	+	+
Streptococcus sanguis ATCC 10557	0.39	0.13	0.38	0.3 ± 0.1	+	+
Streptococcus mitior (*mitis*) S-8	0.21	0.05	0.25	0.2 ± 0.1	+	+
Obligate anaerobes						
Veillonella alcalescens ATCC 17748	0.10	0.02	0.12	0.2 ± 0.1	−	+
Propionibacterium acnes ATCC 11827	0.97	0.01	0.99	0.0 ± 0.1	−	+
Fusobacterium nucleatum IID 891	0.43	0.01	0.37	0.0 ± 0.1	−	+

[a] Strains of S series were isolated by our laboratory.
[b] Mean of three to nine determinations ± the standard deviation.
[c] The extent of growth was examined by eye. ++, Growth at the whole surface area; +, growth in a small region around the stabbed point; −, no growth.
[d] +, Growth at >5 mm below the surface region; −, no growth.

termined by resazurin changed with E_h +0.051 V in the zone where the growth was inhibited. The result did not completely agree with the order of aerotolerance obtained by RBGR values. As the depth varied with the inoculated concentration of organisms, the growth in agar-mixed cultures may involve complicated effects of oxygen consumption and accumulation of metabolites.

As described above, a quantitative index was obtained that is useful for the classification of microorganisms when they are cultured under well-defined conditions and their growth is measured quantitatively. The shaken culture with a liquid medium is suitable for making culture conditions as constant as possible.

In conclusion, RBGR is a simple means of giving a quantitative indication of oxygen relation. The plot of absorbancy versus depth may also be useful. Both RBGR and

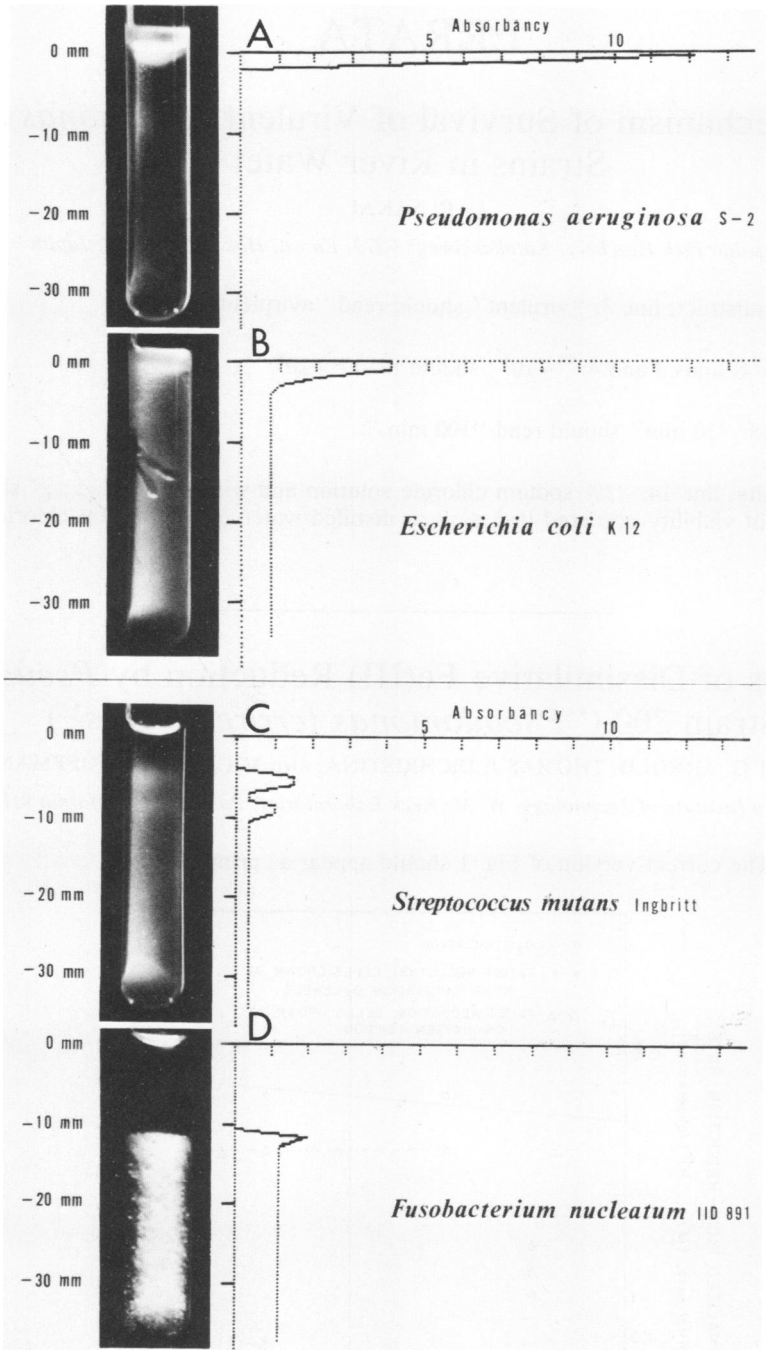

FIG. 1. Growth characteristics and absorbancy patterns of organisms in agar-mixed cultures. The distance from the medium surface to the growth region was determined by measuring the absorbancy, and the absorbancy was plotted against the distance. Organisms shown are representative of obligate aerobes (A), facultative anaerobes (B), aerotolerant anaerobes (C), and obligate anaerobes (D).

absorbancy plot may lend themselves to automated systems analysis.

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