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Factors Related to the Oxygen Tolerance of Anaerobic Bacteria

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The effect of atmospheric oxygen on the viability of 13 strains of anaerobic bacteria, two strains of facultative bacteria, and one aerobic organism was examined. There were great variations in oxygen tolerance among the bacteria. All facultative bacteria survived more than 72 h of exposure to atmospheric oxygen. The survival time for anaerobes ranged from less than 45 min for *Peptostreptococcus anaerobius* to more than 72 h for two *Clostridium perfringens* strains. An effort was made to relate the degree of oxygen tolerance to the activities of superoxide dismutase, catalase, and peroxidases in cell-free extracts of the bacteria. All facultative bacteria and a number of anaerobic bacteria possessed superoxide dismutase. There was a correlation between superoxide dismutase activity and oxygen tolerance, but there were notable exceptions. Polyacrylamide gel electropherograms stained for superoxide dismutase indicated that many of the anaerobic bacteria contained at least two electrophoretically distinct enzymes with superoxide dismutase activity. All facultative bacteria contained peroxidase, whereas none of the anaerobic bacteria possessed measurable amounts of this enzyme. Catalase activity was variable among the bacteria and showed no relationship to oxygen tolerance. The ability of the bacteria to reduce oxygen was also examined and related to enzyme content and oxygen tolerance. In general, organisms that survived for relatively long periods of time in the presence of oxygen but demonstrated little superoxide dismutase activity reduced little oxygen. The effects of medium composition and conditions of growth were examined for their influence on the level of the three enzymes. Bacteria grown on the surface of an enriched blood agar medium generally had more enzyme activity than bacteria grown in a liquid medium. The data indicate that superoxide dismutase activity and oxygen reduction rates are important determinants related to the tolerance of anaerobic bacteria to oxygen.

Anaerobic bacteria differ in their ability to survive in the presence of oxygen (7, 21, 29, 32, 33). It has been proposed that differences in oxygen tolerance among anaerobes may be related to the effectiveness of defense mechanisms possessed by bacteria against toxic products of oxygen reduction (24). Many toxic by-products, including the superoxide anion and hydrogen peroxide, are generated when molecular oxygen interacts with various cellular constituents (8, 9, 26). Superoxide anions can react with hydrogen peroxide in the cell to form hydroxyl radicals, which are the most potent oxidants known (14). The subsequent reaction between superoxide anions and the hydroxyl radical results in the production of singlet oxygen which is also damaging to the cell (26). The major enzymes protecting the cell against these toxic oxygen reduction products are superoxide dismutase (SOD), catalase, and peroxidases (26).

In 1971, McCord et al. (24) determined the distribution of SOD and catalase in aerobes, strict anaerobes, and aerotolerant anaerobes. All aerobic organisms exhibited both SOD and catalase activities. Aerotolerant organisms were devoid of catalase but demonstrated some SOD activity. Strict anaerobes, on the other hand, contained no SOD and generally no catalase. It was concluded that the prime physiological function of SOD is protection of oxygen-metabolizing organisms against the potentially detrimental effects of the superoxide anion.

Other investigators have detected SOD in obligate anaerobes (4, 11, 17, 23, 31). Hewitt and Morris (17) surveyed various species of clostridia and found that the SOD activity present in the organisms seemed to correlate with their oxygen

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tolerance. Tally et al. (31) examined 22 anaerobic bacteria for oxygen tolerance and SOD activity. They detected SOD in many of the anaerobic bacteria and observed a correlation between oxygen tolerance and SOD activity. However, some of the bacteria did not fit this pattern.

In this paper we report data relating oxygen tolerance of anaerobic bacteria to the activities of SOD, catalase, and peroxidases in the cells and to the capacity of the bacteria to reduce oxygen.

MATERIALS AND METHODS

Microorganisms. The anaerobic and facultative bacteria used in these studies were isolated from human feces and from clinical specimens. Both the fecal and clinical isolates were handled in such a way to minimize exposure to oxygen. All isolation techniques and subsequent incubation were performed in an anaerobic glove box isolator maintained at 37°C (1). Fecal specimens, collected in a specimen cup, were placed immediately into a GasPak jar (Bioquest, Div. of Becton, Dickinson & Co., Cockeysville, Md.) which had been activated for at least 2 h. The anaerobic jar containing the specimen was then placed into an anaerobic glove box isolator within 5 min after collection. Inside the anaerobic isolator the specimen was removed from the jar and homogenized in a prereduced Virginia Polytechnic Institute (VPI) salts diluent (18), and approximately 1 g of the homogenate was added to 9 ml of the salts diluent. A 10-fold dilution series was then performed. For nonselective isolation of anaerobic bacteria, 1 ml of each dilution was flooded on enriched brucella blood agar (BBA) containing (per liter): brucella agar (Baltimore Biological Laboratory, Cockeysville, Md.), 43 g; hemin, 0.005 g; cysteine hydrochloride, 0.5 g; sodium carbonate, 0.42 g; menadione, 0.005 g; and sheep blood, 50 ml. For selective isolation of *Bacteroides* species and *Fusobacterium* species, various dilutions of the fecal homogenate were inoculated on enriched BBA containing 100 μg of kanamycin and 7.5 μg of vancomycin per ml and also on enriched BBA containing 100 μg of neomycin per ml. For the isolation of *Clostridium* species, dilutions contained in screw-cap tubes were removed from the anaerobic isolator and placed in an 80°C water bath for 10 min. They were then brought back into the isolator, and each dilution was plated on egg yolk agar (18). Facultative anaerobes and aerobic bacteria were isolated from enriched BBA plates which were inoculated with each fecal dilution inside the anaerobic isolator, after which they were incubated aerobically at 37°C. Anaerobic bacteria isolated from feces or obtained from clinical specimens were identified on the basis of morphology, antibiotic sensitivity patterns, and volatile fatty acid production and by cultural and biochemical tests described by Holdeman and Moore (18) and Sutter et al. (30). The microorganisms were transferred only two times after primary isolation before storage to minimize the possibility of selecting for mutants. The bacteria were stored in peptone-yeast extract-glucose (PYG) broth containing 10% glycerol at −70°C.

Oxygen tolerance determination. Determination of the oxygen tolerance of each organism was performed as previously described (29). A 48-h PYG broth (18) culture of each organism was swabbed on the surface of two prereduced BBA plates with a cotton-tipped applicator. Plates were incubated inside the anaerobic chamber for 48 h at 37°C. Approximately 5.0 ml of prereduced VPI salts diluent (18) was flooded on the surface of each plate, and the growth was loosened with a sterile bent glass rod. Some of the cell suspension was then taken up in a tuberculin syringe, and two vessels containing VPI salts diluent were inoculated so that each contained approximately 10^7 viable bacteria per ml. Both vessels were kept inside the anaerobic isolator so that all manipulations were performed under oxygen-free conditions. The VPI salts diluent was maintained under anaerobic conditions in the control vessel but was aerated in the test vessel by utilizing air lines connected to the outside of the anaerobic isolator. The pH of the diluent in the control vessel was 7.8, and the oxidation-reduction potential was −301 mV. In the test vessel, which was aerated, the pH of the diluent was 7.2, and the oxidation-reduction potential was +101 mV. Samples were removed at zero time and at 20 and 45 min and 1, 1.5, 2, 3, 4, 8, 24, 48, and 72 h after inoculation to determine viable populations. Tenfold serial dilutions were prepared from each sample in prereduced VPI salts diluent. A 1-ml quantity of each dilution was flooded on the surface of a prereduced enriched BBA plate. Plates were allowed to incubate anaerobically until colonies were clearly distinguishable, after which colony counts were made. Plates inoculated with *Pseudomonas aeruginosa* were incubated aerobically, however. The procedure was repeated two times for each organism, and the mean values of the results were recorded. Variability was minimal between the two trials for each organism. Oxygen tolerance was expressed as the first time interval after inoculation when no viable cells were recovered from a 1.0-ml sample removed from the bacterial suspension exposed to air in the test vessel. Oxygen tolerance values of the anaerobic bacteria ranged from 45 min to over 72 h. *Escherichia coli* and *P. aeruginosa* were also examined for oxygen tolerance. These organisms were grown aerobically on enriched BBA before they were brought into the anaerobic isolator and inoculated into the vessels.

Cell-free extract preparation. Cell-free extracts, prepared from the bacteria, were used to examine SOD, catalase, and peroxidase activities. Organisms were grown on prereduced enriched BBA plates which were incubated inside the anaerobic isolator for 48 h. The plates were flooded with 5.0 ml of cold 50 mM potassium phosphate buffer at pH 7.8 to remove the growth. The cell suspension was centrifuged at 15,000 × *g* for 10 min in a refrigerated centrifuge. The supernatant was discarded, and the cells were washed several times in phosphate buffer to remove traces of extracellular components that might interfere with the enzyme assays. The cellular suspension was kept at 4°C to prevent any induction of enzymatic activity that might occur during handling and preparation of the cell-free extracts. The packed cells were suspended in phosphate buffer at pH 7.8 containing 0.1 mM

ethylenediaminetetraacetic acid, placed in a 25-ml rosette cell (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), and sonically treated in an ice bath by a Branson Sonifier (Branson Ultrasonics Corp., Danbury, Conn.) with a microtip adaptor at a 100-W power setting. The sonic treatment was performed in 2-min bursts, each followed by a 3-min cooling period. Cell breakage was continued until 90% or more of the cells were ruptured. The temperature of the suspension did not exceed 11°C during this process. Cellular debris was then removed by centrifuging the suspension twice at 20,000 × *g* for 20 min. The protein content of the cell-free extracts was measured by the method of Lowry et al. (22).

Enzyme assays. The procedure of Marklund and Marklund (25) was used to determine the SOD levels of the cell-free extracts. This procedure is based on the ability of SOD to inhibit the autoxidation of pyrogallol. The rate of autoxidation was measured as the increase in absorbance at 420 nm with a Beckman DU spectrophotometer (model 2400; Beckman Instruments, Inc., Fullerton, Calif.), using a cuvette with a 1-cm light path. The spectrophotometer was attached to a Gilford digital absorbance recorder (model 222; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). A constant temperature of 25°C was maintained during measurements with a Haake temperature control unit (Haake Instruments, Inc., Saddle Brook, N.J.) which was attached directly to the cuvette holder. Because peroxidase can oxidize pyrogallol in the presence of hydrogen peroxide, 0.2 μmol of bovine liver catalase (catalog no. C-40; Sigma Chemical Co., St. Louis, Mo.) was added to the cell-free extract during the SOD assay to eliminate hydrogen peroxide. The catalase preparation was found to possess SOD activity (0.23 U of SOD per μmol of catalase) but at a level which did not interfere with the assay (15). A 1-U amount of SOD was defined as the amount of enzyme required to inhibit the rate of autoxidation of 0.2 mM pyrogallol by 50%. Activity was expressed as units of SOD per milligram of protein of the cell-free extract. The sensitivity of the SOD assay was approximately 0.20 U of SOD (20 ng of bovine blood SOD). The catalase content of the cell-free extracts was determined by the method of Beers and Sizer (3). A 1-U amount of catalase was defined as the amount of enzyme required to decompose 1 μmol of hydrogen peroxide per min at 25°C. Units were expressed in reference to the protein content of the cell-free extracts. The sensitivity of the catalase assay was approximately 1.5 U of catalase (40 ng of bovine liver catalase). The procedure described in the *Worthington Enzyme Manual* (34) was used to determine the content of peroxidases in the cell-free extracts. A 1-U amount of peroxidase was defined as the amount of enzyme required to decompose 1 μmol of hydrogen peroxide per min at 25°C. The sensitivity of the peroxidase assay was approximately 0.001 U of peroxidase (17 ng of horseradish peroxidase).

Oxygen reduction studies. Cell suspensions used for the oxygen reduction analyses were prepared from bacteria grown on prereduced BBA plates as described previously. These plates were incubated anaerobically for 48 h, after which the growth was removed by flooding the plates with VPI diluent lacking cysteine. Aerobically grown *E. coli* and *P. aeruginosa* were also examined for oxygen reduction. The cell suspensions were placed in screw-cap tubes and removed from the anaerobic isolator for oxygen reduction measurements. The rate at which oxygen was reduced at 37°C by the bacteria was determined with a YSI Biological oxygen monitor (model 53; Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio) connected to polarographic Clark oxygen probe electrodes. This entire procedure was repeated three times for each bacterial strain, and the average value was determined. Variability between trials was minimal for each organism. The rate of oxygen reduction by the cell suspension was expressed in terms of the cell-free extract protein content. The rate of oxygen reduction was also determined in relation to the total cell and viable cell counts. All three methods of expressing the rate of oxygen reduction yielded a similar relationship between the organisms. As a control, the rate of oxygen reduction by VPI salts diluent (without cysteine), which was used to flood the surface of sterile prereduced enriched BBA plates, was measured.

Polyacrylamide gel electrophoresis analysis for SOD. Polyacrylamide gel electrophoresis in a 7.5% lower gel was performed on the cell-free extract of each organism to separate enzymes with SOD activity (5). Cell-free extracts were prepared as described earlier. Extracts containing approximately 150 μg of protein, 15 mg of sucrose, and bromophenol blue marker dye were added to the top of the gel. Various concentrations of cell-free extract were examined to achieve optimum visualization and band separation. Electrophoresis was then performed at 2 mA/tube until the bromophenol blue marker dye had passed through most of the gel. The presence of enzymes with SOD activity was visualized on the gels by the procedure of Beauchamp and Fridovich (2).

Effect of medium composition and condition of growth on enzyme content. The SOD, catalase, and peroxidase activities of the bacterial extracts were determined both when the bacteria were grown in PYG broth supplemented with hemin and menadione (18) and when they were grown on prereduced enriched BBA plates. A 500-ml PYG culture of the organism was incubated for 48 h at 37°C in the anaerobic glove box isolator. Growth was stopped by cooling to 4°C. The cells were centrifuged at 15,000 × *g* for 15 min and washed several times in 50 mM phosphate buffer at pH 7.8. Cell-free extracts, prepared as described above, of bacteria grown in PYG and of bacteria grown on prereduced enriched BBA plates were assayed for SOD, catalase, and peroxidases.

RESULTS

Oxygen tolerance, oxygen reduction, and activities of SOD, catalase, and peroxidase in the bacteria. Table 1 shows the oxygen tolerance of the bacteria, their SOD, catalase, and peroxidase activities, and the rate at which they reduced oxygen. The organisms listed in Table 1 are divided into four groups, depending on the length of time they survived in the presence of oxygen. Although there was some cell death in the anaerobic control vessel, it was not

TABLE 1. *Oxygen tolerance, oxygen reduction, and the activities of SOD, catalase, and peroxidase in anaerobic, facultative, and aerobic bacteria*

ORGANISMS[a]	OXYGEN TOLERANCE[b]	ENZYME LEVELS (units/mg protein cell-free extract)			OXYGEN REDUCTION (μl O_2/min/mg of protein)
		SOD	CATALASE	PEROXIDASE	
INTOLERANT ANAEROBES					
Peptostreptococcus anaerobius	45 min	0[c]	0	0	.46
Clostridium aminovalericum	45 min	0	0	0	.19
Eubacterium lentum	1 h	3.6	0	0	.15
Fusobacterium nucleatum	1 h	0	0	0	.13
Bacteroides melaninogenicus	2 h	0	0	0	.44
MODERATELY TOLERANT ANAEROBES					
Bacteroides fragilis	4 h	7.0	15.2	0	.36
Propionibacterium acnes (clinical)	8 h	.9	103.2	0	.37
Propionibacterium acnes	8 h	.3	136.2	0	.37
Bacteroides vulgatus	8 h	12.5	0	0	.56
TOLERANT ANAEROBES					
Bifidobacterium adolescentis	48 h	.3	0	0	.06
Bacteroides fragilis (clinical)	48 h	6.8	7.1	0	.40
Clostridium perfringens (clinical)	>72 h	1.4	0	0	.02
Clostridium perfringens	>72 h	.4	0	0	.03
AEROBIC AND FACULTATIVE BACTERIA					
Lactobacillus plantarum	>72 h	49.7	32.2	.227	.84
Pseudomonas aeruginosa	>72 h	10.9	112.9	.003	1.48
Escherichia coli (anaerobically grown)	>72 h	15.3	11.0	.047	.31
Escherichia coli (aerobically grown)	>72 h	33.4	46.0	.026	.25

[a] Unless stated otherwise, organisms were isolated from the fecal flora.

[b] First time interval after inoculation when no viable cells could be recovered from a culture suspension that had been exposed to air.

[c] No demonstrable enzyme activity.

significant when compared with the bacteria exposed to oxygen. Organisms that survived less than 2 h of exposure to oxygen are designated intolerant anaerobic bacteria. *Eubacterium lentum* was the only organism in this group possessing any activity of the enzymes examined, and this was SOD activity. All four of the moderately tolerant anaerobic bacteria (i.e., those surviving 4 to 8 h of oxygen exposure) possessed SOD, three possessed catalase, and no peroxidase activity was detected in any of the four species. Interestingly, the SOD activity of the most tolerant of the anaerobic bacteria (those which survived from 48 to more than 72 h in the presence of oxygen) was no greater than that observed in the moderately tolerant group. Only one of four of the most tolerant anaerobes possessed catalase, and none possessed peroxidase.

Overall, the enzyme activity was greatest in the group of bacteria capable of aerobic growth. Generally, higher levels of SOD and catalase were detected in these organisms than in the anaerobes. All of the aerobic and facultative bacteria contained peroxidase. When *E. coli*, grown anaerobically, was subsequently exposed to oxygen, there was a 3-log decrease in viable cell count by 72 h (29). On the other hand, aerobically incubated *E. coli* showed no decrease in viable cell count when exposed to oxygen in the test reaction vessel. In both instances, the anaerobic control vessel showed no decrease in viable cell count during the 72-h experimental period. *E. coli* grown anaerobically contained less SOD and catalase but slightly more peroxidase than when grown aerobically (Table 1).

The rate at which each of the organisms reduced oxygen was measured next. The results represent the average of three determinations (Table 1). They are expressed as microliters of oxygen reduced per minute per milligram of protein of cell-free extract. The rate in which the anaerobic bacteria reduced oxygen ranged from 0.02 μl of oxygen per min per mg of protein to 0.56 μl of oxygen per min per mg of protein. It is apparent that oxygen-tolerant anaerobes which demonstrated little SOD activity, such as the *Clostridium perfringens* strains and *Bifidobacterium adolescentis*, reduced oxygen at a much slower rate than did the other tolerant anaerobic organisms. The clinical isolate of *Bacteriodes fragilis* was a tolerant organism that reduced oxygen at a relatively rapid rate. Its SOD activity was greater than that detected with other tolerant anaerobic organisms. In gen-

eral, bacteria with little SOD activity that reduced oxygen at a slow rate survived for longer periods of time in the presence of oxygen than comparable organisms that reduced oxygen at a more rapid rate. The rate of oxygen reduction by facultative and aerobic bacteria was relatively rapid, ranging from 0.25 μl of oxygen per min per mg of protein for aerobically grown *E. coli* to 1.48 μl of oxygen per min per mg of protein for *P. aeruginosa*. The rate of oxygen reduction was also determined in relation to the total cell and viable cell counts. All three methods of expressing the rate of oxygen reduction yielded a similar relationship between the organisms. The VPI salts diluent in which the bacteria were suspended did not reduce oxygen even after exposure to the surface of a sterile prereduced BBA plate.

Polyacrylamide gel electrophoresis analysis for SOD. Figure 1 depicts SOD electropherograms of the bacteria that gave positive reactions. It can be seen that many anaerobic bacteria contain at least two electrophoretically distinct enzymes with SOD activity. Bands of SOD activity in the acrylamide gels were present in the cell-free extracts of only those bacteria which also demonstrated SOD activity by inhibiting pyrogallol oxidation. However, cell-free extracts prepared from the two strains of *Propionibacterium acnes* and from the *B. adolescentis* strain produced no bands of SOD activity, although the extracts inhibited pyrogallol oxidation. This was the case even after 3.0 mg of extract protein was applied to the gels.

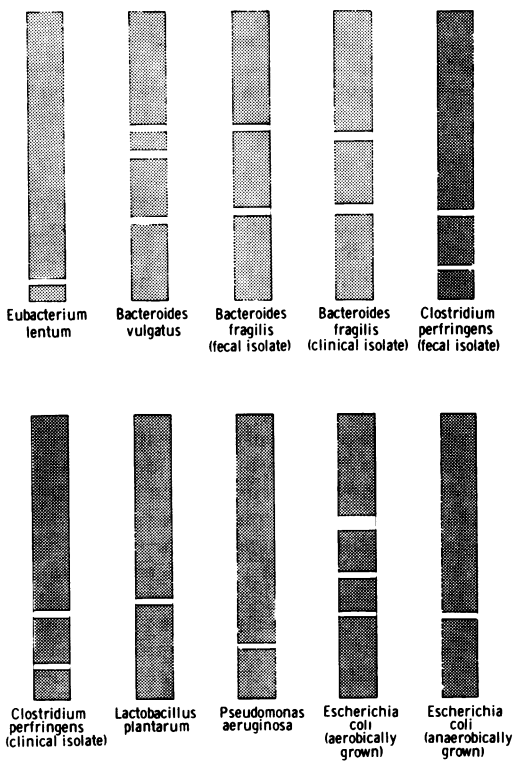

FIG. 1. *SOD electropherograms of 10 anaerobic, facultative, and aerobic bacteria. The polyacrylamide gel electrophoresis tubes were inoculated with cell-free extracts of the organisms. Only those organisms that gave positive reactions are depicted.*

The R_f values of the enzymes from the clinical and fecal strains of *B. fragilis* and from the *C. perfringens* strains were very similar. *Bacteroides vulgatus* contained three electrophoretically distinct enzymes, two of which had R_f values that were similar to those found in the *B. fragilis* strains. *E. lentum* was the only anaerobe containing a single band with SOD activity. *Lactobacillus plantarum, P. aeruginosa,* and *E. coli,* when grown anaerobically, contained only one electrophoretically distinct SOD. On the other hand, when *E. coli* was grown aerobically, it possessed at least three distinct enzymes with SOD activity.

Effect of medium composition and conditions of growth on enzyme activities. The effect of medium composition and conditions of growth on the SOD (Fig. 2), catalase (Fig. 3), and peroxidase (Fig. 4) activities of the organisms was examined next. For each organism, assays were repeated three times, and average values of the results are presented. Variability was minimal among the three trials for each organism. Peroxidase could not be detected in any of the anaerobic organisms, no matter which medium was used for growth. For 11 of 13 organisms, the activities of all three enzymes were greater when the bacteria were grown on enriched BBA plates as compared with growth in PYG broth. The two exceptions were *B. adolescentis,* which contained a greater amount of SOD when grown in PYG broth (Fig. 2), and *P. aeruginosa,* which contained more catalase when grown in PYG broth (Fig. 3). SOD activity in the clinical isolate of *P. acnes* and catalase activity in anaerobically grown *E. coli* were not detected when the organisms were grown in PYG broth but were detected when the organisms were grown on enriched BBA plates.

DISCUSSION

There were great variations in the length of time the anaerobic bacteria survived in the presence of molecular oxygen. Because resting cell suspensions were used in our studies, variables inherent as a result of bacterial multiplication were avoided. The VPI salts diluent in which the bacteria were suspended contained cysteine.

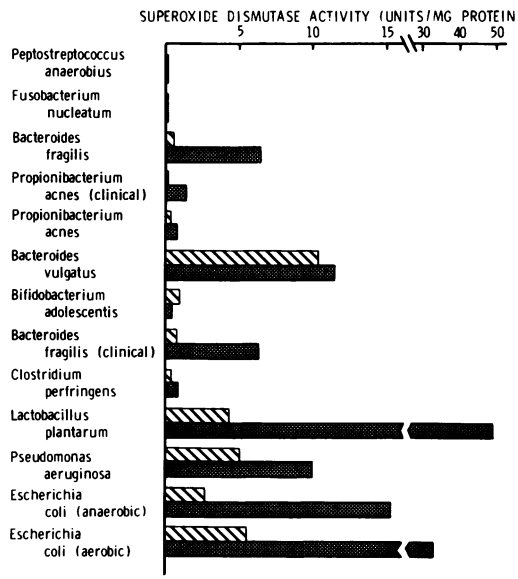

FIG. 2. *Effect of medium composition and conditions of growth on the activity of SOD in 13 anaerobic, facultative, and aerobic bacteria. The lined bars represent SOD activity of the cell-free extracts of bacteria grown in PYG broth. The dotted bars represent SOD activity of the cell-free extracts of bacteria grown on enriched BBA plates.*

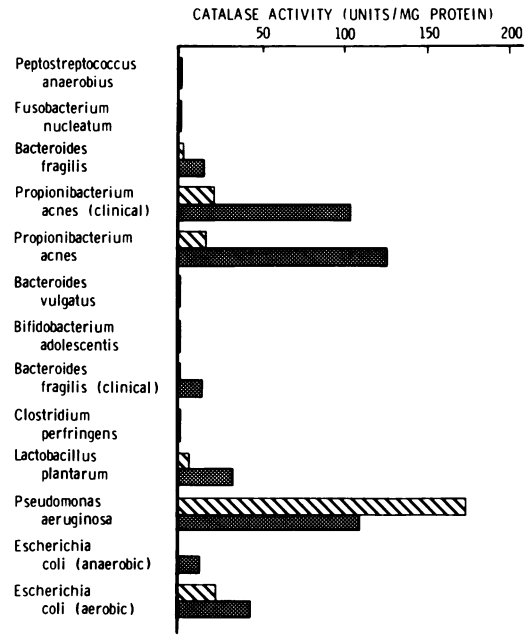

FIG. 3. *Effect of medium composition and conditions of growth on the activity of catalase in 13 anaerobic, facultative, and aerobic bacteria. The lined bars represent catalase activity of the cell-free extracts of bacteria grown in PYG broth. The dotted bars represent catalase activity of cell-free extracts of bacteria grown on enriched BBA plates.*

Although oxidized reducing agents, such as cysteine, can be toxic for some fastidious anaerobes (18), we obtained no evidence for this effect because survival patterns of the bacteria did not change during aeration when cysteine was deleted from the diluent. The oxidation-reduction potential of the diluent increased during aeration of the bacterial suspensions. However, high oxidation-reduction potential is not, in itself, a factor responsible for repression of the multiplication of anaerobes (6, 20, 27, 28, 33). Cell death, observed in our studies, appears to be mediated through molecular oxygen or oxygen reduction products rather than through oxidized cysteine or adverse oxidation-reduction potentials.

A major objective of this investigation was to relate the oxygen tolerance of the anaerobic bacteria to the activities of SOD, catalase, and peroxidase in the cells and to the rate at which the organisms reduce oxygen. Recent evidence suggests that SOD is widely distributed among anaerobic bacteria (4, 11, 17, 23, 31). In our study, facultative, aerobic, and anaerobic bacteria exhibited SOD activity, although it tended to be greater in the facultative and aerobic bacteria. Catalase activity was variable among the organisms. It was present in all the facultative bacteria but was also present in some anaerobes at high levels. Peroxidase was detected only in

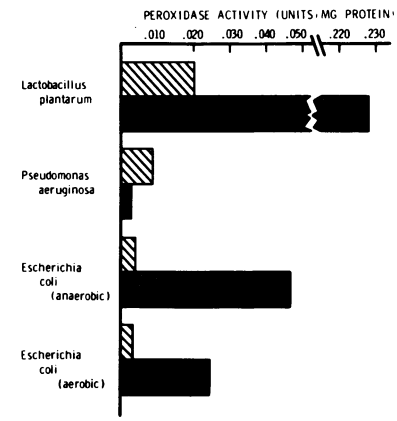

FIG. 4. *Effect of medium composition and conditions of growth on the activity of peroxidases in four facultative and aerobic bacteria. The lined bars represent peroxidase activity of the cell-free extracts of bacteria grown in PYG broth. The dotted bars represent peroxidase activity of cell-free extracts of bacteria grown on enriched BBA plates. This figure shows the peroxidase activity of aerobic organisms only, because the enzyme could not be detected in any of the anaerobic organisms, no matter which medium was used for growth.*

facultative and aerobic bacteria. Among the anaerobes, there was some correlation between enzyme activity and oxygen tolerance. With one exception, which was *E. lentum,* organisms that were extremely sensitive to the effects of oxygen possessed no measurable SOD. The most oxygen-tolerant anaerobes possessed at least some SOD. The tolerance of *C. perfringens* to oxygen was not due to sporulation because no spores could be detected through staining or heat shock (80°C for 10 min). We concluded that, although a distinction can be made between facultative bacteria and anaerobes on the basis of the activities of the protective enzymes in the cells, differences in oxygen tolerance among anaerobes cannot be accounted for solely on this basis, and other factors must be involved.

The rate at which bacteria reduce oxygen to form toxic products probably is an important factor related to their survival in air. Gregory and Fridovich (10) reported that a strain of *L. plantarum,* which lacked catalase and SOD, was unduly resistant to oxygen simply because it utilized no oxygen and therefore produced no toxic oxygen intermediates. When we related our oxygen reduction data to SOD levels and oxygen tolerance, an interesting pattern emerged. In general, organisms which possessed little SOD activity and which reduced oxygen at a slow rate could survive for longer periods of time in the presence of air than organisms with little SOD activity that readily reduced oxygen (Table 1). However, the clinical isolate of *B. fragilis* survived for 48 h in the presence of oxygen, although it reduced oxygen at a relatively rapid rate, but it contained more SOD than the other tolerant anaerobic organisms.

It appears that the continuous spectrum of oxygen tolerance among obligately anaerobic and facultative bacteria is due partly, at least, to activities of the protective enzymes in the cell and the rate at which the cells reduce oxygen. Clearly other factors could be involved, such as: (i) the location of the enzymes in the cell (surface versus cytoplasm), (ii) the rate at which cells form toxic products (superoxide anions, hydroxyl radicals, and singlet oxygen) of oxygen reduction, and (iii) the differential sensitivities of key cellular components.

A number of the anaerobes which we examined contained more than one electrophoretically distinct enzyme with SOD activity. Carlsson et al. (4) demonstrated that *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* each contain two electrophoretically distinct enzymes. The roles of these isoenzymes need to be studied further to determine how they are involved in protecting anaerobic bacteria against the toxic effects of oxygen. It is known that facultative anaerobic *E. coli* contain at least two electrophoretically distinct SOD enzymes (13). An iron-containing SOD protects the organisms against exogenously produced superoxide anions, and a manganese-containing SOD protects them against endogenously produced superoxide anions. Protection against exogenously produced superoxide anions could be particularly important for anaerobic bacteria which reduce little or no oxygen internally and could also be advantageous for anaerobic bacteria that establish infections in well-oxygenated tissues. Finally, SOD activity could be detected in cell-free extracts prepared from two strains of *P. acnes* and one strain of *B. adolescentis* by the procedure of Marklund and Marklund (25), involving inhibition of the autoxidation of pyrogallol, but not with acrylamide gels. There is no apparent explanation for this discrepancy.

We have shown that the conditions of growth greatly affect the levels of SOD, catalase, and peroxidase in the bacteria. Gregory et al. (12) reported that glucose and other sugars repress the synthesis of catalase in *B. fragilis.* Other workers have shown SOD activity to vary under different cultural conditions (11, 16, 31, 35). Variables of this kind need to be considered when results of studies from different laboratories are compared. Different growth conditions and bacterial strain variation may explain the discrepancy in the SOD activity of *L. plantarum* reported here and elsewhere (24, 36).

An interesting phenomenon was observed when the oxygen tolerance, protective enzyme activity, and oxygen reduction rate of aerobically and anaerobically grown *E. coli* were compared. In both cases, the organisms survived aeration for more than 72 h (Table 1). In a previous study, we showed that a decrease in viable count of approximately 100-fold occurred when anaerobically grown *E. coli* were exposed to oxygen (29). This was not observed when the oragnisms were allowed to multiply under aerobic conditions before being suspended in the salt diluent and exposed to oxygen in the test vessel. In the present study, we found that *E. coli* incubated anaerobically contained less SOD and catalase than did *E. coli* incubated aerobically. When grown under anaerobic conditions and subsequently exposed to oxygen, the organisms reduced oxygen at a greater rate than when grown under aerobic conditions. The decrease in enzyme activity and concomitant increase in oxygen reduction rate probably both contribute to the greater sensitivity to oxygen of anaerobically grown organisms than aerobically grown organisms. If so, this has important implications in relation to the isolation of facultative bacteria from anaerobic environments. Oxygen-sensitive

facultative pathogenic organisms present in low numbers in patient materials may fail to grow unless appropriate anaerobic procedures are employed in processing the specimens in the diagnostic laboratory. This is supported by the work of Holdeman and Moore (19), who found strains of *E. coli* and viridans streptococci which failed to grow aerobically upon initial isolation from clinical specimens.

ACKNOWLEDGMENTS

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