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Bactericidal Effect of Anaerobic Broth Exposed to Atmospheric Oxygen Tested on *Peptostreptococcus anaerobius*

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Peptostreptococcus anaerobius strain VPI 4330-1 was used as the test organism in an evaluation of the bactericidal effect of anaerobic broth exposed to air. The test organism, grown under anaerobic conditions in Trypticase soy broth, was diluted in buffered salt solution, and about 2×10^4 cells were suspended in 10 ml of an aerated broth. Ninety percent of the cells were killed within 15 min in actinomyces broth and within 50 min in Trypticase soy broth. All cells survived for 2 h in fluid thioglycolate medium. Addition of DABCO [1,4-diazabicyclo(2.2.2)octane] or mannitol to Trypticase soy broth did not influence the death rate of the organism, whereas superoxide dismutase decreased the death rate. Addition of catalase or manganese dioxide to the broth kept all the cells viable for 2 h. Of the three broth media tested, actinomyces broth reduced oxygen at the highest rate and Trypticase soy broth reduced it at the slowest rate. Hydrogen peroxide could be demonstrated in actinomyces broth and in Trypticase soy broth but not in fluid thioglycolate medium. In addition to catalase, manganese dioxide also removed all hydrogen peroxide from Trypticase soy broth, and superoxide dismutase significantly decreased the concentration of hydrogen peroxide in the broth. The results suggest that hydrogen peroxide mediated the toxic effect of atmospheric oxygen in these broth media.

The recently increased awareness of the importance of anaerobic bacteria in human infections (2) has rekindled the interest in refining the technique for isolation of anaerobic bacteria from clinical sources. However, when the efficiencies of various methods have been compared, conflicting results have been reported (20, 25, 30, 31), and, in addition, it has been found that most anaerobes isolated from clinical specimens could survive an exposure to atmospheric oxygen for several hours (4, 6a, 8, 21, 32). This indicates that the time of exposure of a specimen to atmospheric oxygen is not the ultimate factor in determining the efficiency of a method, and that other, yet uncontrolled factors may be more important for an optimal recovery of anaerobes from clinical sources.

Addition of blood or catalase to culture media significantly increases the ability of many anaerobes to survive an exposure to atmospheric (6a, 15) or hyperbaric oxygen (16). This protecting effect of catalase indicates that hydrogen peroxide may be the main toxic factor formed in culture media exposed to oxygen (3, 7, 27), and the aim of the present study was to evaluate the relative significance of various toxic products formed from oxygen in anaerobic broth media.

MATERIALS AND METHODS

Microorganism. *Peptostreptococcus anaerobius* strain VPI 4330-1 was used as the test organism, and it was kept on blood agar plates (17) at 4°C under strictly anaerobic conditions in an anaerobic box with an atmosphere of 10% H_2 and 5% CO_2 in nitrogen (33).

Chemicals. Trypticase soy broth, actinomyces broth, and fluid thioglycolate medium were obtained from Baltimore Biological Laboratory (BBL), Cockeysville, Md. Agar was from Difco Laboratories, Detroit, Mich. Catalase (purified powder from beef liver, C10, Sigma Chemical Co., St. Louis, Mo.) was purified from superoxide dismutase by Sephadex G200 (Pharmacia, Uppsala, Sweden) gel filtration in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. Superoxide dismutase was from Truett Laboratories, Biochemical Division, Dallas, Tex. Horse blood (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) in blood agar plates (17) was hemolyzed by freeze-thawing. Polypropylene glycol 2025 was from British Drug Houses Ltd., U.K., and 1,4-diazabicyclo(2.2.2)octane (DABCO) was from Aldrich-Europe, Beerse, Belgium.

Preparation of broth media. The anaerobic broth was prepared in an anaerobic box by dissolving the dehydrated culture medium in oxygen-free water to the recommended concentration. The water was

made oxygen-free by bringing hot, just autoclaved water into the box, where it was kept for 1 day before use. The broth was then brought out from the box in tightly stoppered test tubes, autoclaved at 121°C, and cooled to the temperature wanted in the experiment.

The aerobic broth was prepared by dissolving the dehydrated culture medium in aerated water to the recommended concentration. The broth was filtered through a 0.22-μm membrane filter (type GS, Millipore Corp., Bedford, Mass.) in room atmosphere.

Survival of *P. anaerobius* in broth media and salt solution. The organism was grown overnight at 37°C in the anaerobic box in Trypticase soy broth containing 0.5 mg of L-cysteine·HCl per ml. In the morning, 0.5 ml of the culture was inoculated into 5 ml of the same broth. When the cells were in logarithmic growth, they were diluted to a density of about 2×10^5 cells per ml in an autoclaved salt solution containing, per liter: 0.1 g of $CaCl_2$, 0.1 g of $MgSO_4$, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, and 1.0 g of NaCl (pH 7.0). The cell suspension was brought out from the box, and 0.1 ml was inoculated into 10 ml of broth medium or salt solution in a 50-ml round-bottomed flask submerged under continuous shaking into a thermostated water bath. The broth medium and the salt solution had been aerated by vigorous shaking for 30 min before the inoculation. To some of the flasks 20 μl of a superoxide dismutase (3.3 mg/ml) solution or 50 μl of a catalase (1.6 mg/ml) solution was added just before the start of aeration. Other flasks contained 100 mM mannitol, 10 mM DABCO, or 10 mg of manganese dioxide.

After the inoculation of the flasks, samples of 0.1 ml were taken at regular time intervals from the flasks and were spread over the surface of duplicate blood agar plates. Just before use, the plates were taken from the anaerobic box; plates were returned to the box immediately after inoculation. The plates were incubated for 3 days at 37°C in the box.

Oxygen consumption and hydrogen peroxide formation in the broth media. Oxygen was measured in a thermostated reaction vessel holding 10 ml (11). The Clarke electrode was connected to an oxygen monitor and pH meter (type PHA 927/PHM 27; Radiometer, Copenhagen, Denmark). Hydrogen peroxide was determined according to Dempsey et al. (10).

The oxygen consumption and the concentration of hydrogen peroxide during aeration of anaerobic Trypticase soy broth, actinomyces broth, and fluid thioglycolate medium were followed in the following way. To a 1-liter flask, 200 ml of anaerobic broth was added and was aerated by vigorous shaking in a thermostated water bath. At regular time intervals, 10-ml samples of the broth were added to the reaction vessel, which was immediately closed, and the decrease in oxygen concentration was followed for 5 min. Then 20 μl of a catalase solution (1.6 mg of protein per ml) was added to the reaction vessel. An increase in oxygen concentration after addition of catalase indicated that hydrogen peroxide was present in the broth. By comparing this increase with that caused by a subsequent addition of a standard amount of hydrogen peroxide, the concentration of hydrogen peroxide in the reaction vessel at the time for catalase addition could be calculated. With this method a concentration as low as 2 μM hydrogen peroxide can be detected.

Preparation of cell-free extract. The organism was grown in 250 ml of Trypticase soy broth (BBL) for 18 h at 37°C in the anaerobic box. The cells were harvested by centrifugation at 13,000 × *g* for 30 min at 4°C, washed twice with 0.04 M potassium phosphate buffer, pH 7.0, and suspended in 5 ml of the same buffer with 1 drop of polypropylene glycol 2025 and 10 mg of dithiothreitol. Then 4 ml of glass beads (0.10 to 0.11 mm) was added, and the cells were disintegrated for 1 min in a homogenizer (type MSK, B. Braun, Melsungen, Germany) under CO_2 cooling. Cell debris was removed by centrifugation at 40,000 × *g* for 60 min at 4°C.

Assay of enzyme activity. Superoxide dismutase activity was determined according to McCord and Fridovich (22) in a cell-free extract, which had been dialyzed against 0.04 M potassium phosphate buffer, pH 7.0, for 18 h. Activity of catalase in the cell-free extract was determined as described by Beers and Sizer (5).

RESULTS

Survival of *P. anaerobius* in broth media and salt solution exposed to air. *P. anaerobius* VPI 4330-1 was inoculated into three different anaerobic broth media, which had been autoclaved for 10 min, cooled to 25°C, and aerated for 30 min before the inoculation. Ninety percent of the cells were killed within 15 min in actinomyces broth and within 50 min in Trypticase soy broth (Fig. 1). No significant decrease in the number of viable cells could be detected after storage of the organism for 2 h in fluid thioglycolate medium (Fig. 1).

The death rate of the organism was much

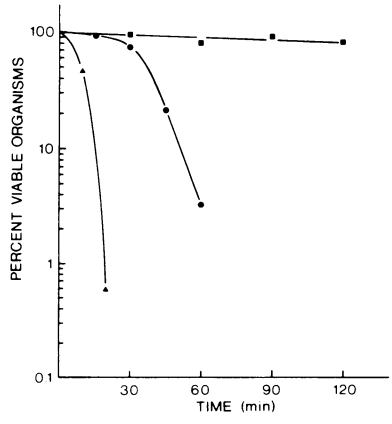

FIG. 1. *Survival of P. anaerobius VPI 4330-1 at 25°C in anaerobic broth media, autoclaved for 10 min and aerated for 30 min before addition of cells. Symbols:* (▲) *actinomyces broth;* (●) *Trypticase soy broth;* (■) *fluid thioglycolate medium.*

slower in aerobic Trypticase soy broth than in aerated anaerobic broth (Fig. 2). After 1 h, more than 95% of the cells were killed in the aerated anaerobic broth, whereas less than 25% were killed in the aerobic broth (Fig. 2). Addition of superoxide dismutase to these media significantly decreased the death rate (Fig. 2). With catalase in the media no decrease in the number of viable cells could be detected during the 2-h experimental period (Fig. 2). Addition of manganese dioxide to the media had the same effect as catalase. Addition of DABCO or mannitol to the media did not influence the survival of the organism.

The death rate was significantly increased when survival was tested in media at 37°C instead of at 25°C (Fig. 3). Also, at 37°C catalase fully protected the organism (Fig. 3).

When the organism was inoculated into anaerobic Trypticase soy broth media that had been autoclaved for 10, 20, 40, and 60 min, cooled to 25°C, and aerated for 30 min before the inoculation, the death rate of the organism was increased with increasing sterilization time. The death rate of the organism was also increased by increasing the concentration of ingredients in the broth. By increasing the aeration time of the anaerobic broth before the inoculation, the death rate was decreased.

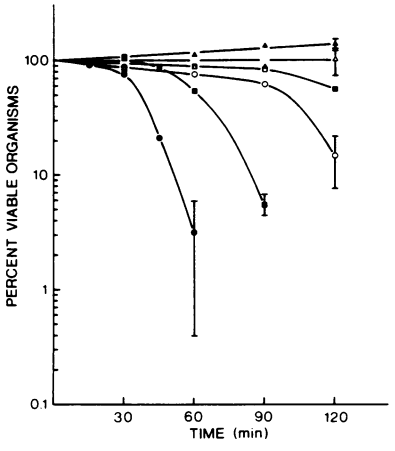

FIG. 2. *Effect of catalase and superoxide dismutase (SOD) on the survival of P. anaerobius VPI 4330-1 in aerated anaerobic or aerobic Trypticase soy broth (TSB) at 25°C. Symbols:* (●) *anaerobic TSB;* (■) *anaerobic TSB with SOD (6.6 μg/ml);* (▲) *anaerobic TSB with catalase (8.0 μg/ml);* (○) *aerobic TSB;* (□) *aerobic TSB with SOD;* (△) *aerobic TSB with catalase. Aerobic TSB was prepared and filter sterilized in room atmosphere. Anaerobic TSB was prepared in an anaerobic box, autoclaved for 10 min, and then aerated for 30 min before addition of the cells. Catalase and SOD were added at the start of the aeration. Means and standard deviations are given for three independent experiments.*

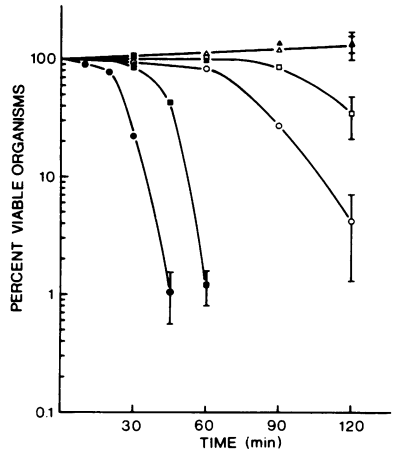

FIG. 3. *Effect of catalase and superoxide dismutase on the survival of P. anaerobius VPI 4330-1 in aerated anaerobic or aerobic Trypticase soy broth at 37°C. Symbols and experimental conditions are identical to those given in Fig. 2. Means and standard deviations are given for three independent experiments.*

When the organism was inoculated into aerobic salt solution, more than 60% of the cells were killed within 30 min. However, in the following 90 min less than 20% of the cells of the inoculum were killed (Fig. 4). The protecting effect of catalase and superoxide dismutase in the salt solution was similar to that in Trypticase soy both. In salt solutions containing 10 and 25 μM hydrogen peroxide the death rate was increased (Fig. 4). Addition of filter-sterilized glucose to a final concentration of 55 mM in the salt solution did not influence the death rate of the organism.

Oxygen consumption and concentration of hydrogen peroxide in various uninoculated broth media. There were great differences in the rates of oxygen consumption by the three anaerobic broth media, which had been autoclaved for 10 min and cooled to 25°C before the aeration (Fig. 5). Actinomyces broth had the most rapid rate of oxygen consumption and Trypticase soy broth had the slowest. After 1 h the rate of oxygen consumption by Trypticase soy broth had decreased below the detection level (less than 1 nmol of oxygen/min per ml). After 4 h of aeration there was a significant oxygen consumption by actinomyces broth as well as by fluid thioglycolate medium (Fig. 5). Hydrogen peroxide could be demonstrated in actinomyces broth and in Trypticase soy broth, but not in fluid thioglycolate medium (Fig. 6).

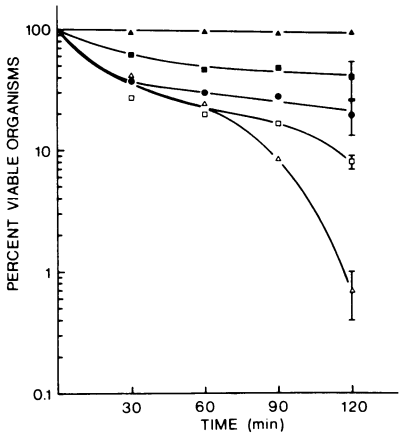

FIG. 4. *Effect of catalase and superoxide dismutase (SOD) on the survival of P. anaerobius VPI 4330-1 in aerobic salt solution (SS) at 25°C. Symbols:* (●) *SS;* (■) *SS with SOD (6.6 μg/ml);* (▲) *SS with catalase (8.0 μg/ml);* (□) *SS with 10 μM H_2O_2;* (△) *SS with 25 μM H_2O_2. Means and standard deviations are given for two independent experiments.*

Addition of superoxide dismutase to the anaerobic Trypticase soy broth significantly decreased the concentration of hydrogen peroxide in the medium (Fig. 7). The concentration of hydrogen peroxide was lower at 37°C than at 25°C (Fig. 7). A higher amount of hydrogen peroxide was formed in aerated anaerobic Trypticase soy broth when it had been autoclaved for 30 min (Fig. 7) instead of 10 min (Fig. 6). Also, in the aerobic Trypticase soy broth hydrogen peroxide could be detected. Two hours after the preparation of the medium the hydrogen peroxide concentration reached a steady-state level of 4 μM, and this had not changed 24 h later. No hydrogen peroxide could be detected in the aerated salt solution or in media treated with manganese dioxide. Addition of cells to a final concentration of 10^6 per ml in the aerobic Trypticase soy broth and the aerobic salt solution did not result in any oxygen consumption or increase in the amount of hydrogen peroxide in these solutions.

Assay of enzyme activity. No catalase or superoxide dismutase activity could be demonstrated in cell-free extracts of *P. anaerobicus* VPI 4330-1.

DISCUSSION

When molecular oxygen is reduced to water by chemical or enzymatic reactions, a variety of potentially damaging products such as superoxide anions, hydrogen peroxide, hydroxyl free radicals, and singlet oxygen may be formed (6, 13). Two of the three anaerobic broth media exposed to air in the present study had a potent bactericidal effect on *P. anaerobius* VPI 4330-1. The protecting effect provided by the high-molecular-weight enzyme catalase and the insoluble substance manganese dioxide suggests that the toxic effect of oxygen was mediated by hydrogen peroxide produced in the broth media as well as in the salt solution. The fact that hydrogen peroxide could be demonstrated in the broth media but not in the salt solution does not exclude an involvement of hydrogen peroxide in the salt solution. Hydrogen peroxide may be formed by interactions between the cells and the oxygen in the salt solution and may execute

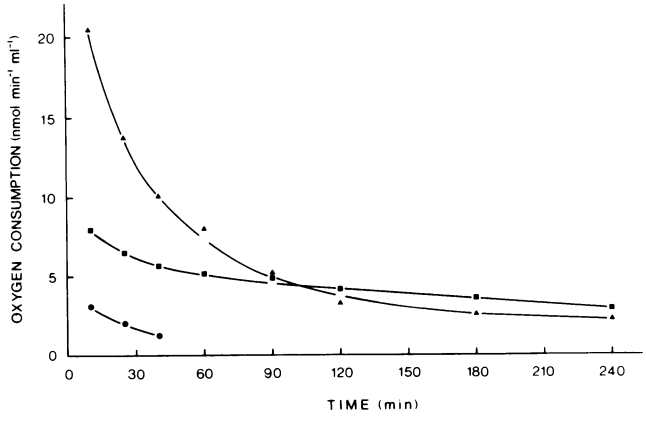

FIG. 5. *Change in the rate of oxygen consumption during continuous aeration at 25°C of anaerobic broth autoclaved for 10 min. Symbols:* (▲) *actinomyces broth;* (●) *Trypticase soy broth;* (■) *fluid thioglycolate medium. At times indicated, samples of the aerated broth were transferred to a closed oxygen electrode reaction vessel. The rate of consumption was read 5 min after the transfer.*

its killing effect on the cells before reaching a detectable level in the solution. The partial protection afforded by superoxide dismutase may not necessarily mean that superoxide anions were involved. The effect of superoxide dismutase may be ascribed to the lowered concentration of hydrogen peroxide in the media (Fig. 7). This lowered hydrogen peroxide concentration was not caused by the presence of any catalase in the superoxide dismutase preparation and cannot yet be explained. The lack of protecting effect of a singlet oxygen quencher, DABCO (29), or a scavenger of hydroxyl free radicals, mannitol (14), does not exclude an involvement of singlet oxygen or hydroxyl free radicals in the toxic effect of oxygen, although the present study and recent works by DeChatelet et al. (9) and Harmon and Kautter (15) focus the interest on the effect of hydrogen peroxide.

The present study gives no evidence for an intracellular production of toxic products from oxygen in *P. anaerobius* VPI 4330-1. No oxygen consumption by the organism could be demonstrated. The death rate was similar in aerobic salt solution and aerobic Trypticase soy broth, and the death rate was not increased by supplementing the salt solution with glucose. Again, it has to be observed that extracellular catalase protected the organism under all experimental conditions.

It has been recognized for many years that

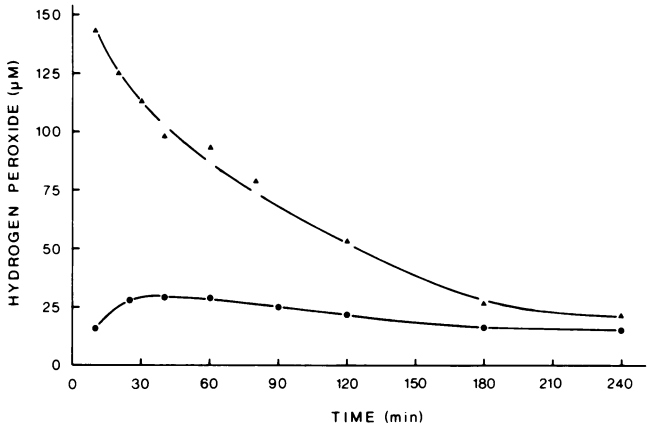

FIG. 6. *Change in the concentraton of hydrogen peroxide during continuous aeration at 25°C of anaerobic broth media autoclaved for 10 min. Symbols: (▲) actinomyces broth; (●) Trypticase soy broth. Hydrogen peroxide was not detected in fluid thioglycolate medium.*

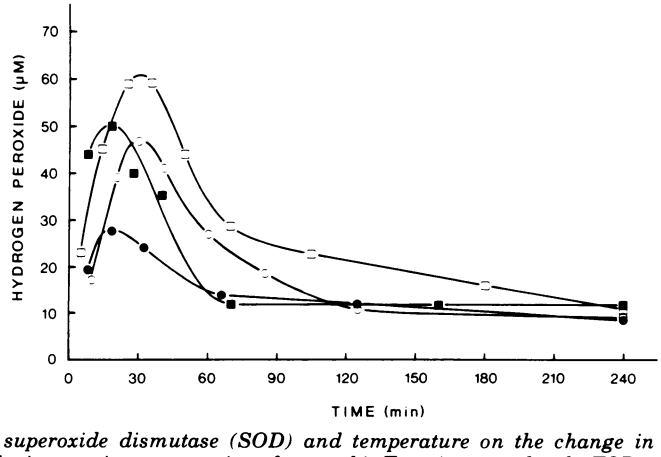

FIG. 7. *Effect of superoxide dismutase (SOD) and temperature on the change in the concentration of hydrogen peroxide during continuous aeration of anaerobic Trypticase soy broth (TSB) autoclaved for 30 min. Symbols: (□) TSB at 25°C; (○) TSB with SOD (6.6 μg/ml) at 25°C; (■) TSB at 37°C; (●) TSB with SOD at 37°C.*

hydrogen peroxide is toxic for bacteria (24), and a number of biological effects have been ascribed to it (23), but it is not clear why it is so (13, 26). The present study suggests that hydrogen peroxide caused damage to some structure of *P. anaerobius* VPI 4330-1 that can be affected by the extracellular catalase and manganase dioxide, i.e., the cell envelope. It is known that transitional metal complexes may generate free radicals from hydrogen peroxide and that the hydroxyl free radicals (12, 19, 28), as well as high oxygen tension (1), could initiate lipid peroxidation in membranes. That oxygen exposure of bacteria may damage the protoplasm membrane is supported by the finding that oxygen may interfere with the transport function of the membrane (34) and affect the membrane site of deoxyribonucleic acid initiation (18).

It is interesting to note that no hydrogen peroxide accumulated in fluid thioglycolate medium (BBL), whereas hydrogen peroxide was formed in Trypticase soy broth (BBL) and actinomyces broth (BBL), and also in Brewer thioglycolate medium (Difco), brain heart infusion (BBL), and Schaedler broth (BBL) when these media were aerated (unpublished data). One important difference between fluid thioglycolate medium (BBL) and the other media is that fluid thioglycolate medium has a low content of phosphate. Work in progress shows that products formed when glucose is heated together with phosphate at a pH higher than 7 account for most of the hydrogen peroxide-forming capacity of the media (unpublished data).

Although the toxic effect of hydrogen peroxide formed in bacteriological media exposed to air has been known for some time (3, 7, 15, 27), this has, to a limited extent been considered in studies designated for elucidating the mechanism of oxygen toxicity and in the work to improve clinical methods for handling anaerobic bacteria. The present study underlines the importance of designing anaerobic bacteriological media in such a way that no toxic products are accumulated in the media if they are exposed to oxygen.

ACKNOWLEDGMENT

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