

# Induction of Superoxide Dismutase by Molecular Oxygen

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Oxygen induces superoxide dismutase in *Streptococcus faecalis* and in *Escherichia coli* B. *S. faecalis* grown under 20 atm of O<sub>2</sub> had 16 times more of this enzyme than did anaerobically grown cells. In the case of *E. coli*, changing the conditions of growth from anaerobic to 5 atm of O<sub>2</sub> caused a 25-fold increase in the level of superoxide dismutase. Induction of this enzyme was a response to O<sub>2</sub> rather than to pressure, since 20 atm of N<sub>2</sub> was without effect. Induction of superoxide dismutase was a rapid process, and half of the maximal level was reached within 90 min after N<sub>2</sub>-grown cells of *S. faecalis* were exposed to 20 atm of O<sub>2</sub> at 37 C. *S. faecalis* did not contain perceptible levels of catalase under any of the growth conditions investigated by Stanier, Doudoroff, and Adelberg (23), and the concentration of catalase in *E. coli* was not affected by the presence of O<sub>2</sub> during growth. *S. faecalis*, which had been grown under 100% O<sub>2</sub> and which therefore contained an elevated level of superoxide dismutase, was more resistant of 46 atm of O<sub>2</sub> than were cells which had been grown under N<sub>2</sub>. *E. coli* grown under N<sub>2</sub> contained as much superoxide dismutase as did *S. faecalis* grown under 1 atm of O<sub>2</sub>. The *E. coli* which had been grown under N<sub>2</sub> was as resistant to the deleterious effects of 50 atm of O<sub>2</sub> as was *S. faecalis* which had been grown under 1 atm of O<sub>2</sub>. These results are consistent with the proposal that the peroxide radical is an important agent of the toxicity of oxygen and that superoxide dismutase may be a component of the systems which have been evolved to deal with this potential toxicity.

Oxygen is toxic (11, 12, 22). The damaging effects of O<sub>2</sub> have been demonstrated in bacteria (3, 9, 12, 22), protozoa (26), fungi (25), fish (5), human lymphocytes (21), and whole animals (8). There is, as yet, no adequate explanation for the toxicity of oxygen. Superoxide dismutase, which catalyzes the reaction: O<sub>2</sub><sup>-</sup> + O<sub>2</sub><sup>-</sup> + 2H<sup>+</sup> → H<sub>2</sub>O<sub>2</sub> + O<sub>2</sub> (17) is ubiquitous among oxygen-metabolizing organisms but is absent from obligate anaerobes (18). This enzyme may constitute an important component of the defenses against oxygen toxicity. Thus, univalent reduction of oxygen, which has been demonstrated in several enzymatic systems (7, 16, 20), generates the superoxide radical whose great chemical reactivity may be presumed to constitute a threat to the integrity of cellular components. If one or more of the reactions which generate O<sub>2</sub><sup>-</sup> were not saturated with respect to oxygen under atmospheric conditions, raising the partial pressure of oxygen would increase the intracellular flux of O<sub>2</sub><sup>-</sup>. If this flux of O<sub>2</sub><sup>-</sup> exceeded the scavenging ability

of the ambient level of superoxide dismutase, cellular damage would result. If this is indeed the situation, it appeared likely that superoxide dismutase would be induced in response to oxygen and that cells which had a high level of this enzyme would be less sensitive towards oxygen than those which had a lower level of this enzyme. This report describes experiments with *Streptococcus faecalis* and with *Escherichia coli* which appear to affirm these expectations.

## MATERIALS AND METHODS

Trypticase soy broth was a product of the Bioquest Division of the Becton and Dickinson Co. Yeast extract and agar were obtained from the Difco Laboratories. *S. faecalis* was obtained from A. Proctor of the Department of Microbiology and *E. coli* B was provided by D. Hall of the Division of Genetics of Duke University Medical Center, Durham, N.C. Cells were exposed to hyperbaric O<sub>2</sub> or N<sub>2</sub> at 25 C on agar plates or in shallow liquid cultures which were subjected to continuous magnetic stirring. Pressure

was achieved and maintained in stainless steel vessels. Superoxide dismutase in the cell-free extracts was assayed as previously described (17) but with the modification (1) that  $2 \times 10^{-5}$  M cyanide was added to inhibit cytochrome *c* peroxidases, which otherwise may interfere with this assay. Cell-free extracts were prepared by sonic disruption of cell suspensions in 0.05 M potassium phosphate,  $10^{-4}$  M EDTA (pH 7.8) at 0 C for 3 min with a Branson Sonifier, model W185, operated at a power output of 90 W, followed by centrifugation for 15 min at  $27,000 \times g$ . These cell-free extracts were concentrated 10-fold by ultrafiltration over an Amicon PM-10 membrane and were then restored to their original volume by addition of 0.05 M potassium phosphate,  $10^{-4}$  M EDTA at pH 7.8. This was done to reduce the concentrations of endogenous low-molecular-weight substances.

*S. faecalis* was grown in a medium containing 30 g of Trypticase soy broth per liter and 5 g of yeast extract per liter. *E. coli* was grown in a low phosphate medium whose composition in grams per liter was: glucose, 40.5;  $\text{NH}_4\text{Cl}$ , 5.0;  $\text{Na}_2\text{HPO}_4$ , 6.0;  $\text{KH}_2\text{PO}_4$ , 3.0; tryptone (Difco), 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; NaCl, 0.02;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02; ascorbic acid, 0.02. Bacterial growth was at 37 C and was monitored turbidimetrically at 600 nm (14). The protein concentrations of cell-free extracts were estimated spectrophotometrically (24). Electrophoretic separations were performed on polyacrylamide gels, (6) and superoxide dismutase activity was localized on these gels as previously described (1). Gels which had been stained for enzymatic activity were scanned for absorbance at 400 nm with a Gilford Gel Scanner, and the amount of activity in a particular band was estimated from the area under the corresponding absorbance peak as produced by the Gel Scanner. Catalase was quantitated spectrophotometrically (2).

## RESULTS

**Induction of superoxide dismutase by oxygen.** When *S. faecalis*, which had been grown anaerobically, was the inoculum, subsequent growth was equally rapid under  $\text{N}_2$ , 0.2 atm of  $\text{O}_2$ , or 1.0 atm of  $\text{O}_2$ , but was distinctly slower under 20 atm of  $\text{O}_2$ . Thus, early stationary phase was reached in 4 to 4.5 h under the former conditions, but in 9 h under the latter condition. Inoculation involved equal dilution of the cells into fresh media in all of these experiments, and the stainless steel pressure vessel was brought to the temperature of growth, 37 C, before the freshly inoculated samples were placed in it. It is clear that 20 atm of  $\text{O}_2$  slowed the rate of growth of these cultures. Cells were grown to late exponential phase under varying partial pressures of  $\text{O}_2$  and were then collected by centrifugation. Cell-free extracts prepared from these samples were then assayed for protein content and for superoxide dismutase activity. The results of these

measurements (Fig. 1) demonstrate that growth under increasing concentrations of  $\text{O}_2$  elicited increased production of superoxide dismutase. Control experiments demonstrated that pressure, per se, was not a factor in the induction of superoxide dismutase. Cells grown under 1 atm of  $\text{N}_2$  gave extracts with the same specific activity of this enzyme as did cells grown under 20 atm of  $\text{N}_2$ .

Extracts from cells grown under  $\text{N}_2$  or under 20 atm of  $\text{O}_2$  were analyzed by electrophoresis on polyacrylamide gels (6), and the gels were then stained for superoxide dismutase. Only a single zone of this activity was seen in both cases and it always exhibited the same mobility. This suggests that the induction by  $\text{O}_2$  of superoxide dismutase in *S. faecalis* involves an increase in the enzyme which is present at low levels even in the anaerobically grown cells rather than the appearance of a new isoenzyme.

The response of *E. coli* to changes in the partial pressure of  $\text{O}_2$  was examined under comparable conditions. *E. coli*, when grown anaerobically, was found to contain more superoxide dismutase than *S. faecalis* under the same conditions. Anaerobically grown *S. faecalis* gave extracts whose specific activity was 0.8, whereas *E. coli* gave specific activities of 3.8. It was nevertheless true that oxygen induced the superoxide dismutase of *E. coli*. This is demonstrated by the results in Table 1. *E. coli* contains two electrophoretically distinct superoxide dismutases (1), and these were affected very differently by the partial pressure of  $\text{O}_2$  under which the cells were grown. Thus isoenzyme I, which was the slower moving species, was strikingly induced by  $\text{O}_2$  whereas isoenzyme II, which showed more rapid cathodic migration, was induced to a much smaller extent. Polyacrylamide gel electropherograms of extracts of *E. coli*, which had been grown under different partial pressure of  $\text{O}_2$ , were stained for superoxide dismutase activity, and the amount of each isoenzyme was then estimated from the area under the corresponding peaks obtained with a gel scanning densitometer. The ratios of the amounts of isoenzyme I to isoenzyme II, so estimated, are presented in Table 2.

**Rate of induction of superoxide dismutase.** *S. faecalis*, which had been grown anaerobically, was transferred to fresh broth and incubated under 20 atm of  $\text{O}_2$ . The doubling time under these conditions was 1.2 h. At intervals the turbidity of the culture and the superoxide dismutase activity of cell-free extracts, prepared from samples of these cells,

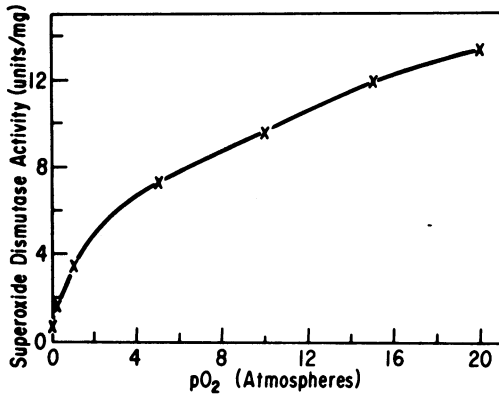


FIG. 1. Effect of oxygen upon the level of superoxide dismutase in *S. faecalis*. *S. faecalis*, grown under N<sub>2</sub>, was used to inoculate broth cultures which were then grown to late exponential phase at 37 C under the O<sub>2</sub> tensions indicated on the abscissa. These cells were harvested, and cell-free extracts prepared from them were assayed for superoxide dismutase and for protein. The specific activity of these extracts is here graphed as a function of the O<sub>2</sub> tension during growth.

TABLE 1. *E. coli* superoxide dismutase and catalase levels<sup>a</sup>

Growth conditions	Superoxide dismutase units (mg <sup>-1</sup> )	Catalase units (mg <sup>-1</sup> )
Nitrogen	3.8	11.3
Air	13.5	12.5
1 atm O <sub>2</sub>	21.2	— <sup>b</sup>
5 atm O <sub>2</sub> (45 min)	42.5	— <sup>b</sup>
5 atm O <sub>2</sub> (19 h)	92.8	12.6

<sup>a</sup> Cell-free extracts from *E. coli* grown under the indicated condition were assayed for superoxide dismutase and catalase activity as described in Materials and Methods. Catalase was measured on fresh cell-free extracts which had never been frozen.

<sup>b</sup> Catalase was not measured under these conditions.

were measured. The results of these manipulations (Fig. 2) demonstrate that exposure of *S. faecalis* to O<sub>2</sub> caused a marked increase in the content of superoxide dismutase. It is also apparent that this enzyme induction was more rapid than was the proliferation of cells under the same conditions. Thus, the amount of superoxide dismutase activity increased nearly 10-fold within 2.5 h whereas the cells could have increased in number by only fourfold in the same interval had they been growing exponentially. There was, in fact, some lag in the cell growth, so that the 10-fold increase in enzyme activity, seen in the first 2.5 h under 20 atm of

O<sub>2</sub>, was actually accompanied by a twofold increase in turbidity. During the exponential phase of cell proliferation, under the conditions shown in Fig. 2, the amount of superoxide dismutase per milliliter of culture was a linear function of the turbidity of the culture. These results indicate that the increase in superoxide dismutase activity, observed upon transferring *S. faecalis* from anaerobic conditions to 20 atm of O<sub>2</sub>, was due to enzyme induction rather than to selection of an enzyme-rich strain. It was also shown that *S. faecalis*, grown to stationary phase under N<sub>2</sub>, exhibited a fourfold increase in the specific activity of superoxide dismutase during a 30-min exposure to 20 atm of O<sub>2</sub>. *E. coli*, under the same conditions, showed a tenfold gain in their content of this enzyme during 45 min of exposure to O<sub>2</sub>.

**Catalase.** Early attempts to explain the inability of obligate anaerobes to grow in air proposed that H<sub>2</sub>O<sub>2</sub> was the toxic product of oxygen metabolism and that the presence of catalase distinguished O<sub>2</sub>-tolerant from O<sub>2</sub>-in-

TABLE 2. Relative amounts of *E. coli* isoenzymes<sup>a</sup>

Condition of growth	Isoenzyme I/ isozyme II
Nitrogen	0.4
Air	1.2
5 atm O <sub>2</sub>	7.6

<sup>a</sup> Cell-free extracts of *E. coli* grown under the indicated condition were subjected to electrophoresis on 10% acrylamide gels and stained for activity as described in Materials and Methods. The gels were scanned and the relative areas for each peak were normalized for isoenzyme II as 1 area unit.

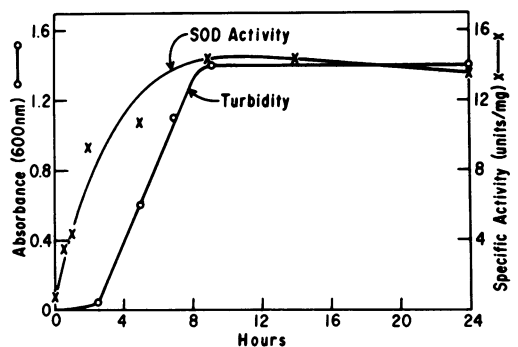


FIG. 2. Rate of induction of superoxide dismutase in *S. faecalis* under 20 atm of O<sub>2</sub>. *S. faecalis*, grown under N<sub>2</sub>, was used to inoculate (10<sup>3</sup> cells per ml) broth cultures which were then incubated at 37 C under 20 atm of O<sub>2</sub>. Growth was followed by measuring optical density (O) while superoxide dismutase (x) was assayed on cell-free extracts prepared at the indicated intervals.

tolerant cells (4, 19). Subsequent discoveries of acatalasic aerobes (10, 13) weakened the impact of this hypothesis. It was nevertheless considered important to assay cell-free extracts for catalase. *S. faecalis*, whether grown under  $N_2$  or under 20 atm of  $O_2$ , did not contain detectable catalase. They may contain peroxidases, which serve to scavenge  $H_2O_2$ , but these were not investigated. *E. coli* did contain catalase, but the specific activity of this enzyme was not significantly changed in cells grown under 5 atm of  $O_2$  as compared to cells grown under  $N_2$ . Thus, extracts of oxygen-grown *E. coli* contained 12.6 units of catalase per mg of protein, whereas the corresponding figure for  $N_2$ -grown cells was 11.3. It is thus apparent that catalase, unlike superoxide dismutase, was not induced by  $O_2$ .

#### Deleterious effects of hyperbaric $O_2$ . *S.*

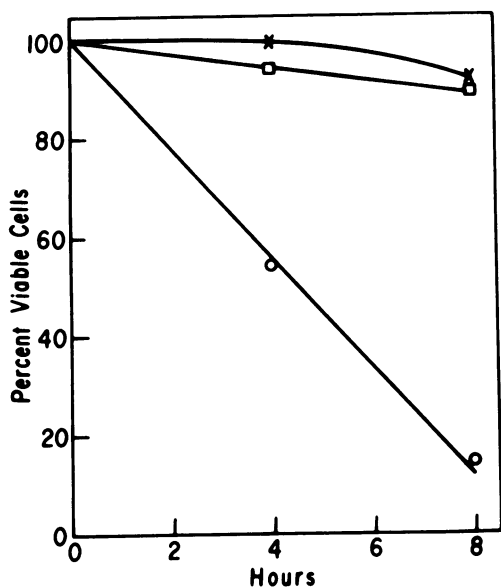


FIG. 3. Deleterious effects of hyperbaric  $O_2$  on *S. faecalis*. *S. faecalis*, grown under  $N_2$  and containing 0.8 units of superoxide dismutase per mg of soluble protein (○) or grown under  $O_2$  and containing 4.8 times more of this enzyme (×), were diluted into fresh broth ( $10^7$  cells per ml) containing 0.5 mg of puromycin per ml and were incubated at 37 C under 46 atm of  $O_2$ . At intervals portions were removed, diluted with fresh broth which was free of puromycin, and plated onto agar plates. After incubation overnight at 37 C under 20 atm of  $O_2$ , colonies were counted and the percentage of survivors of the original inoculum was calculated. In a control experiment the effects of 46 atm of  $N_2$  upon the survival of  $N_2$ -grown cells was determined (□). These measurements are of cells which produce colonies after a 12-h incubation on agar plates under 20 atm of oxygen.

*faecalis*, grown anaerobically or alternately grown under 1 atm of  $O_2$ , was suspended in fresh medium containing 0.5 mg of puromycin per ml. This inhibitor of protein synthesis was used to prevent the induction of superoxide dismutase during the subsequent exposure of these cells to hyperbaric oxygen. It was observed that, in the presence of puromycin, the level of superoxide dismutase in these cells slowly decreased during incubation under oxygen, and the half-time of this decrease was approximately 4 h. These suspensions of cells were exposed to 46 atm of  $O_2$  at 25 C, and at intervals samples were removed from the pressure vessel and, after suitable dilution, were plated onto agar. To control for the possible damaging effects of pressure, per se, a suspension of  $N_2$ -grown cells was exposed to 46 atm of  $N_2$  and then plated under otherwise identical conditions. Exposure to hyperbaric  $O_2$  was seen to cause a progressive increase in frequency of aberrant colonies of *S. faecalis* on the agar plates. These colonies were aberrant in the sense that they were smaller, after 12 h of growth at 37 C, than were the normal colonies. Aberrant colonies were produced under hyperbaric  $O_2$  but not under hyperbaric  $N_2$  and, furthermore, the rate of production of aberrant colonies was much faster in the case of  $N_2$ -grown, low-superoxide dismutase cells than in the case of  $O_2$ -grown, high-superoxide dismutase cells. To obtain quantitative data, it was necessary to count the proportion of aberrant colonies, but this was inconvenient because such counting required the decision, with respect to each colony, of whether it was normal or aberrant. The situation was simplified by the discovery that 20 atm of  $O_2$  suppressed the growth of the aberrant colonies but not the normal colonies. The procedure, then, was to expose cells to 46 atm of  $O_2$  and, at intervals, to spread diluted samples onto sterile agar plates which were incubated for 12 h at 37 C under 20 atm of  $O_2$  and then counted. Figure 3 presents the results of the experiment. It is apparent that cells which had been induced, with respect to superoxide dismutase, by previous growth under 100%  $O_2$  were more resistant towards the deleterious effects of 46 atm of  $O_2$  than were cells which had been grown under  $N_2$  and which therefore contained less superoxide dismutase. It is also clear that 46 atm of  $N_2$  was without effect of these cells. The appearance of normal colonies of *S. faecalis* may be compared with that of the aberrant colonies in Fig. 4.

The defect which was progressively intro-

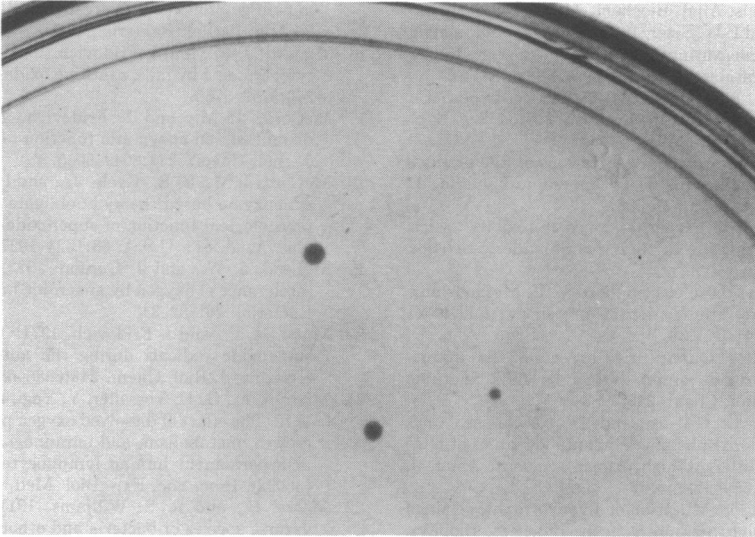


FIG. 4. Appearance of normal and aberrant colonies of *S. faecalis*. N<sub>2</sub>-grown cells were exposed to hyperbaric O<sub>2</sub> as described in the legend of Fig. 3 but after dilution and plating were grown out under air rather than under 20 atm of O<sub>2</sub>. The difference in colony size is apparent.

duced into anaerobically grown *S. faecalis*, during exposure to 46 atm of O<sub>2</sub> in the presence of puromycin, was a persistent one. Thus, cells taken from an aberrant colony and spread onto sterile agar, gave rise to aberrant colonies when grown in air. Whatever the nature of this defect, it diminished the growth rate in air and virtually halted growth under 20 atm of O<sub>2</sub>.

When *E. coli* were exposed to 46 atm of O<sub>2</sub>, under conditions which had caused the appearance of aberrant colonies of *S. faecalis*, no changes in colony morphology were observed. The resistance of *E. coli* to hyperbaric O<sub>2</sub> was also demonstrated by the fact that 80 to 85% of the cells gave rise to colonies after 8 h of exposure to 46 atm of O<sub>2</sub> at 25 C.

### DISCUSSION

Superoxide dismutase was induced by O<sub>2</sub> in both *S. faecalis* and *E. coli*, whereas catalase was not detectable in *S. faecalis* and was unaffected by O<sub>2</sub> in *E. coli*. *S. faecalis*, which had been grown under N<sub>2</sub> and which had a low level of superoxide dismutase, suffered some damage when exposed to 46 atm of O<sub>2</sub> at 25 C. This damage was progressive with time of exposure to the hyperbaric O<sub>2</sub>. Damaged cells formed colonies which were smaller than normal if incubated in air for 12 h at 37 C and did not form colonies if incubated under 20 atm of O<sub>2</sub> for 12 h at 37 C. *S. faecalis*, in which superoxide dismutase had been induced by growth

in 100% O<sub>2</sub>, was resistant towards this effect of hyperbaric oxygen. These results are consistent with the proposal that superoxide radical is an agent of oxygen toxicity and that superoxide dismutase is an important factor in the defense against the toxicity of O<sub>2</sub>. It is, of course, possible that growth in the presence of 100% O<sub>2</sub> induced enzymes other than superoxide dismutase and that these were actually responsible for the effects observed. We cannot, at present, exclude this possibility, but we can state that catalase was not induced by O<sub>2</sub> in these organisms and, therefore, was not involved in the protection afforded by prior growth under 100% O<sub>2</sub>. Growth under hyperbaric O<sub>2</sub> has been reported (15) to raise the level of catalase in *E. coli*, but this was not the case under the conditions reported above. It is pertinent that *E. coli*, which contained as much superoxide dismutase when grown under N<sub>2</sub> as did *S. faecalis* when grown under 1 atm of O<sub>2</sub>, was very resistant to the deleterious effects of 46 atm of O<sub>2</sub>.

### ACKNOWLEDGMENTS

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