Biosynthesis of Oxygen-Detoxifying Enzymes in *Bdellovibrio stolpii*†

ROBERT S. VON STEIN,¹ LYNN E. BARBER,¹ AND H. MOUSTAFA HASSAN^{1,2*}

Departments of Microbiology¹ and Food Science,² North Carolina State University, Raleigh, North Carolina 27650

Received 6 May 1982/Accepted 4 August 1982

Axentially grown *Bdellovibrio stolpii* (i.e., grown independently of the host) was examined for superoxide dismutase, catalase, and peroxidase activities. Kinetics of enzyme synthesis were determined for aerobically grown cultures and for cultures exposed to 100% oxygen. Enzymatic activities varied with the age of the culture. Normally grown cultures exhibited maximum activity during the first 10 h of growth and again as the stationary phase was approached, beginning at about 48 h. Polyacrylamide gel electropherograms of cell-free extracts revealed that *B. stolpii* contained one major band (I) and two minor bands (II, III) of superoxide dismutase activity. Each of these enzymes was inactivated by H₂O₂, indicating that they were iron-containing enzymes. Manganese-containing superoxide dismutase was not detected in *B. stolpii*. Increased oxygenation did not appreciably stimulate enzyme synthesis, for only superoxide dismutase was induced, reaching maximum activity at 10 h and then rapidly falling to normal levels. Superoxide dismutase appears to be the main enzymatic defense against oxygen toxicity in *B. stolpii*. Induction of superoxide dismutase with 100% oxygen was manifested as an increase in the intensities of the two minor bands of activity, suggesting that isozyme I is constitutive, whereas isozymes II and III are inducible. The induction of isozymes II and III by 100% oxygen was prevented by an inhibitor of protein biosynthesis, chloramphenicol.

The toxicity of oxygen is due to the reactive intermediates of oxygen reduction, superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH·) (4, 12), which are formed whenever oxygen is consumed by respiring cells and provided with the opportunity to react with reduced cellular constituents (17). Living cells have developed protective enzymatic mechanisms to defend against these lethal intermediates. Elimination of the superoxide radical is achieved by the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of O₂⁻ to H₂O₂ and ground-state oxygen while catalases (CATs) and peroxidases (PERs) remove H₂O₂. Since removal of O₂⁻ will prevent generation of OH· via an iron-catalyzed Haber-Weiss reaction (9), SOD is perhaps the first line of defense against oxygen toxicity (9). Elevated levels of SOD in several procaryotes and eucaryotes (6-8) have been seen to correlate with enhanced resistance to oxygen toxicity. In *Escherichia coli*, which contains an iron superoxide

dismutase (FeSOD) (22) and a manganese superoxide dismutase (MnSOD) (15), induction of SOD with increased oxygenation results in increases in MnSOD, whereas FeSOD remains constant (6, 10). Similarly, oxygen also causes induction of CAT and PER in *E. coli* (6, 10).

The discovery of *Bdellovibrio* (19) provided an opportunity to examine a unique and previously unknown aspect of microbial life. *Bdellovibrio* is an obligate aerobic organism, requiring oxygen to successfully attack, penetrate, and lyse host cells. It is thus susceptible to the potentially toxic effects of oxygen. It is important, therefore, to examine the responses of *Bdellovibrio* to oxygen. This study was undertaken to determine the enzymes of defense, if any, available to *Bdellovibrio*, and the levels at which they are present. Additional goals were to study the induction of these enzymes during 100% O₂ exposure and to establish which type of SOD is present.

This report will present evidence that (i) *Bdellovibrio stolpii* contains CAT, PER, and SOD, and that the levels of these enzymes vary with the age of the culture; (ii) oxygen induces only

† Paper no. 8396 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, N.C.

SOD, and (iii) *B. stolpii* contains one constitutive and two inducible isozymes of iron-containing SOD.

MATERIALS AND METHODS

Organisms and culture. The organism used in this study was facultatively host-independent *B. stolpii* (ATCC 27052), obtained from the American Type Culture Collection, Rockville, Md. Cultures were grown at pH 7.0 in peptone-yeast extract (PYE) medium containing 1% peptone (Difco Laboratories, Detroit, Mich.) and 0.3% yeast extract (Difco) at 20°C on a rotary shaker at 150 rpm. To examine the activities of SOD, CAT, and PER throughout the growth cycle, *B. stolpii* was cultured and sampled in the following manner. An inoculum was prepared by placing approximately 1 ml of a stock culture into 100 ml of PYE broth. The culture was incubated until early stationary phase was reached, at which time it was used to inoculate four 2,800-ml Fernbach flasks, each containing 1,000 ml of PYE broth. The size of the inoculum was varied to give an initial absorbancy at 595 nm of approximately 0.01. A Beckman spectrophotometer (model DUR, Beckman Instruments, Inc., Fullerton, Calif.) with a Gilford digital absorbance recorder (model 252, Gilford Instruments Laboratories Inc., Oberlin, Ohio) was used for optical density measurements. The inoculum culture was subsequently harvested and used as the zero time sample. Growth was followed turbidimetrically. Samples were removed approximately every two cell doublings for assaying.

Exposure to 100% oxygen. Three 2-liter Erlenmeyer flasks, each containing 1 liter of PYE broth, were inoculated as described above. Filtered oxygen was forced through each culture with gas diffusing stones (Fisher Scientific Co., Pittsburgh, Pa.) The flow of oxygen was adjusted so that as much oxygen as possible could be passed through each culture without causing excessive foaming of the media. Antifoam B (Sigma Chemical Co., St. Louis, Mo.) was added to control foaming of the media. A control culture without 100% O₂ was grown on a rotary shaker, using the procedures described earlier. Samples were taken at 0, 11, 25, and 48 h and assayed for growth and enzymatic activities in cell-free extracts.

Cell-free extract preparation. Culture samples were collected at 4°C by centrifugation for 15 min at 7,000 × g. Cultures of low cell density (those in early-log phase or exposed to oxygen) necessitated harvesting up to 1 liter of culture for analysis. Pelleted cells were washed once with 0.1 M potassium phosphate (pH 7.0) and suspended in 0.05 M potassium phosphate (pH 7.8) containing 10⁻⁴ M EDTA. Cell-free extracts were prepared by sonic disruption with a Quigley-Rochester sonic dismembrator (Quigley-Rochester, Inc., Rochester, N.Y.) at a power output of 90 W, applied in 30-s bursts for a total of 3 min. The temperature was maintained at 4 to 8°C by chilling in an ice-acetone bath. Cellular debris was removed by centrifuging the suspension at 27,000 × g for 1 h. Extracts which were to be used for assaying enzyme activity were dialyzed for 16 h at 4°C in 0.05 M potassium phosphate (pH 7.8) containing 10⁻⁴ M EDTA. The protein content of the cell-free extracts was measured by the biuret method (5), using bovine serum albumin as a standard.

Enzyme assays. SOD was assayed by the procedure described by McCord and Fridovich (16). Cell-free extracts were electrophoresed on 7.5% polyacrylamide disc gels (3), and SOD activity was localized on the gels by the photochemical method described by Beauchamp and Fridovich (1). Iron-containing SOD was distinguished from the manganese-containing enzyme by the method of Britton et al. (2). CAT and PER were assayed as previously described (21).

Oxygen uptake. Oxygen consumption of *B. stolpii* on various substrates was measured polarographically, using a Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). Respiration rates were expressed as nanomoles of O₂ consumed per minute per milligram of cell protein. Total cellular protein was made available for the biuret reaction by the inclusion of 2% deoxycholate in the assay mixture.

RESULTS

Host-independent growth. *B. stolpii* was grown independently of the host in PYE medium at 28°C at 150 rpm. Under these conditions, *B. stolpii* grew with a generation time of 5 h and reached stationary phase at about 48 h. The maximum absorbancy at 595 nm was 1.2 to 1.4. These cells exhibited a characteristic orange coloration.

Effects of various compounds on the respiration rate of *B. stolpii*. The respiration rate of *B. stolpii* was measured in the presence of a variety of compounds (Table 1). As might be expected for an organism that relies on amino acids as a major energy source, glutamate and α-ketoglutarate increased respiration 17 and 27% over the endogenous rate, respectively, whereas glucose and ribose caused no increase. Complex sub-

TABLE 1. Effects of various compounds on the respiration rate of free *B. stolpii* suspensions

Compound added ^a	Endogenous respiration (nmol of O ₂ per min/mg of protein)	Increase over endogenous rate (nmol of O ₂ per min/mg of protein)	% Increase in respiration
Peptone	12.89	6.67	51.7
Yeast extract	10.99	8.91	81.0
PYE	6.71	9.63	143.5
Glutamate	9.26	1.55	16.8
α-Ketoglutarate	8.55	2.29	26.8
Sodium acetate	7.38	3.10	42.0
Sodium succinate	7.68	1.32	17.2
Ribose	10.28	0.01	0
Glucose	10.80	0	0

^a Peptone (0.032%) and yeast extract (0.01%) were added to the chamber containing 2.8 ml of 0.05 M potassium phosphate (pH 7.0) and 200 μl of a late-log phase cell suspension. PYE (100 μl) was added to the chamber, and the remaining compounds were added to final concentrations of 3.2 mM. Total volume was 3.1 ml.

strates, such as peptone and yeast extract, had a somewhat greater effect, increasing respiration 51 and 81%, respectively. These results agree with previously published findings (13) reporting a 20 and 50% increase in respiration with glutamate and peptone, respectively, in *B. bacteriovorus* 109J. PYE caused the largest percent increase in respiration, and this was equivalent to the sum of the individual percent increases caused by peptone and yeast extract. Although peptone and yeast extract each provided a good respirable substrate, neither served as an adequate growth medium. Thus, cultures grown in peptone or yeast extract grew more slowly and attained lower maximum cell densities than cultures grown in PYE medium. This suggested that separate growth factors were present in peptone and yeast extract, and that the combination of these factors was required for optimum growth. The presence of a growth factor in yeast extract was previously indicated by Ishiguro (14).

Kinetics of enzyme synthesis during growth. Axenically grown *B. stolpii* contained SOD, CAT, and PER activity. Figure 1 shows the levels of enzyme activity during growth in PYE medium. SOD and PER levels decreased during the first 18 h of growth and then gradually increased through stationary phase. CAT activity, however, peaked at 8 h, rapidly declined, and remained at a low level. The steady increase in pH was consistent with the use of amino acids and peptides as an energy source, thereby eliminating fermentation as the cause for the initial drop in SOD activity (11).

Identification of *B. stolpii* SOD. The locations of *B. stolpii* SOD on 7.5% polyacrylamide disc gels are shown in the densitometric scans of Fig. 2. *B. stolpii* exhibited one major band (I) and two minor bands (II and III) of SOD activity. Gels treated with 5 mM H_2O_2 demonstrated an inhibi-

FIG. 1. Changes in cell density (●), pH (■), SOD activity (×), PER activity (□), and CAT activity (△) during aerobic growth of *B. stolpii* in PYE medium.

FIG. 2. Densitometric scan of a polyacrylamide gel electropherogram stained for SOD activity. A cell-free extract from aerobically grown *B. stolpii* in PYE medium was used. The sample applied to the gel contained 0.2 mg of protein. The numbers in the figure represent R_f values.

tion of all SOD activity, indicating that these are iron-containing enzymes (2).

Effects of increased oxygenation on SOD, CAT, and PER. Cultures grown under conditions of continuous oxygenation (100% O_2) showed more than a twofold increase in generation time and a corresponding reduction in cell density. It was also noted that oxygenated cells did not show the characteristic orange coloration of normally grown cells. The significance of this observation is not known.

Exposure to 100% oxygen greatly affected the activity of SOD. At 10 h, the oxygenated culture showed an almost fourfold increase in activity. However, activity fell rapidly, and by 25 h it was similar to that of the untreated control (Fig. 3). Addition of chloramphenicol (250 μ g/ml), an inhibitor of protein biosynthesis, inhibited the growth of the organism and prevented the increase in SOD activity elicited by 100% oxygen (data not shown). Furthermore, CAT and PER levels in the oxygenated cells were not significantly higher than those of the non-oxygenated control. The effects of 100% O_2 on growth, pH, and enzyme synthesis are shown in Fig. 4.

Electrophoresis of cell-free extracts showed that isozymes II and III were induced by oxygen, whereas isozyme I remained relatively unchanged (Fig. 5). This induction was prevented by adding chloramphenicol (250 μ g/ml) during exposure of the culture to 100% oxygen (data not shown). Gels treated with H_2O_2 displayed complete inhibition of all SOD activity, indicating that each of the bands consisted of FeSOD.

DISCUSSION

B. stolpii was found to contain catalase, peroxidase, and superoxide dismutase. These enzymes seemed to play an important role in protecting the cells against the toxic intermediates of oxygen reduction. The levels of these

FIG. 3. Effects of 100% oxygen on the biosynthesis of SOD, PER, and CAT in *B. stolpii* grown in PYE medium. Control lines represent enzyme activities of culture grown under normal aeration.

enzymes varied with the age of the culture. Maximum enzymatic activity occurred during the first 10 h of growth and increased again as stationary phase was approached. However, during mid-log phase and into late-log phase, enzymatic activity was at its lowest level. At this time, the cells must rely on an alternate means of defense. A likely explanation is that *B. stolpii* is capable of repairing damaged cellular components during its rapid, mid-log phase growth. When the rate of growth is slowed, as occurs during early-log and stationary phases, the efficiency of the repair process is reduced, and hence, the cell boosts its SOD levels to provide greater protection.

Polyacrylamide gel electrophoresis of cell-free extracts revealed that *B. stolpii* contains three isozymes of superoxide dismutase. Each of the three forms was inactivated by 5 mM H₂O₂, but not by 1 mM NaCN, which is indicative of iron-containing SODs. These findings agree with previously published work by Britton et al. (2), in which all of the gram-negative bacteria examined contained FeSOD. An iron-containing SOD was also reported in *Rhizobium japonicum* (20).

However, unlike most of these bacteria, *B. stolpii* was not found to possess a manganese-containing SOD.

Induction of SOD with 100% O₂ was manifested as an increase in the intensities of the two minor bands of activity, whereas isozyme I was seen as the major band of activity under normal conditions. This inductive effect of oxygen was prevented by the inclusion of chloramphenicol in the growth medium. Thus, the increase in SOD activity appeared to be due to de novo synthesis rather than to an activation of pre-existing protein. Therefore, isozyme I appeared to be constitutive, whereas isozymes II and III were inducible. Induction of an FeSOD has been previously reported in *Bacteroides fragilis* (18). This anaerobic bacterium was found to only contain two electrophoretically distinct iron superoxide dismutases. Exposure of *B. fragilis* to low levels of oxygen caused a three- to fivefold increase in the specific activity of FeSOD. Thus, *B. stolpii* is similar to *B. fragilis* in terms of its ability to induce an FeSOD. This is in sharp contrast to *E. coli*, in which the FeSOD is constitutive and not induced by oxygen.

FIG. 4. Effects of 100% oxygen on growth (●), pH (■), SOD (×), PER (□), and CAT (Δ) in *B. stolpii* grown in PYE medium.

FIG. 5. Effects of 100% oxygen on the relative amounts of SOD isozymes in *B. stolpii* grown in PYE medium. Equal amounts of protein (0.1 mg) were applied to the gels.

The appearance of three separate bands of FeSOD activity in *B. stolpii* is paralleled in *Neisseria subflava*, which was found to contain four separate and tightly migrating bands of FeSOD (2). Although the occurrence of multiple bands of FeSOD is indeed an unusual phenomenon, it is not unique to *Bdellovibrio*.

Exposure of *B. stolpii* to an atmosphere of 100% O₂ resulted in a reduction of growth. Additionally, CAT and PER levels were not appreciably increased in comparison with those of the control, and SOD activity was elevated only during the initial phase of growth. This large increase in SOD activity occurred at 10 h and was a response to the high concentration of O₂. Early in growth under continuous oxygenation, the cell density and the ratio of available O₂ per cell are relatively high, resulting in an elevated level of SOD. As growth continues and the cell density increases, the concentration of available O₂ per cell decreases and SOD activity is subsequently reduced. The threat created by increased oxygenation is perhaps diminished by a combination of the initially high SOD activity and a simultaneous decrease in respiration and growth. In this way, the cell is able to preclude significant damage by preventing the accumulation of O₂⁻ and other reduced-oxygen intermediates.

Induction of the two minor bands of FeSOD during exposure to 100% O₂ suggests that separate controls exist over the synthesis of isozyme I and isozymes II and III. Additionally, the migration of each of these isozymes to different locations on polyacrylamide gels might indicate differences in their molecular structures. However, further investigation is needed to answer these questions.

LITERATURE CITED

1. Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**:276-287.
2. Britton, L., D. P. Malinowski, and I. Fridovich. 1978. Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. *J. Bacteriol.* **134**:229-236.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404-427.
4. Fridovich, I. 1978. The biology of oxygen radicals. *Science* **201**:875-880.
5. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751-766.
6. Gregory, E. M., and I. Fridovich. 1973. Induction of superoxide dismutase by molecular oxygen. *J. Bacteriol.* **114**:543-548.
7. Gregory, E. M., and I. Fridovich. 1973. Oxygen toxicity and the superoxide dismutase. *J. Bacteriol.* **114**:1193-1197.
8. Gregory, E. M., S. A. Goscin, and I. Fridovich. 1974. Superoxide dismutase and oxygen toxicity in a eukaryote. *J. Bacteriol.* **117**:456-460.
9. Halliwell, B. 1979. Oxygen free-radicals in living systems: dangerous but useful? p. 195-221. In M. Shilo (ed.), *Strategies of microbial life in extreme environments*. Dahlem Konferenzen, Berlin.
10. Hassan, H. M., and I. Fridovich. 1977. Enzymatic defenses against the toxicity of oxygen and of streptonigrin in *Escherichia coli*. *J. Bacteriol.* **129**:1574-1583.
11. Hassan, H. M., and I. Fridovich. 1977. Regulation of superoxide dismutase synthesis in *Escherichia coli*: glucose effect. *J. Bacteriol.* **132**:505-510.
12. Hassan, H. M., and I. Fridovich. 1979. Superoxide dismutase and its role for survival in the presence of oxygen, p. 179-193. In M. Shilo (ed.), *Strategies of microbial life in extreme environments*. Dahlem Konferenzen, Berlin.
13. Hespell, R. B., R. A. Rosson, M. F. Thomasow, and S. C. Rittenberg. 1973. Respiration of *Bdellovibrio bacteriovorus* strain 109J and its energy substrates for intraperiplasmic growth. *J. Bacteriol.* **113**:1280-1288.
14. Ishiguro, E. E. 1974. Minimum nutritional requirements for growth of host-independent derivatives of *Bdellovibrio bacteriovorus* strain 109J Davis. *Can. J. Microbiol.* **20**:263-265.
15. Keele, B. B., Jr., J. M. McCord, and I. Fridovich. 1970. Superoxide dismutase from *Escherichia coli* B: a new manganese containing enzyme. *J. Biol. Chem.* **245**:6176-6181.
16. McCord, J. M., and I. Fridovich. 1968. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* **243**:5753-5760.
17. Morris, J. G. 1979. Nature of oxygen toxicity in anaerobic microorganisms, p. 149-162. In M. Shilo (ed.), *Strategies of microbial life in extreme environments*. Dahlem Konferenzen, Berlin.
18. Privalle, C. T., and E. M. Gregory. 1979. Superoxide dismutase and O₂ lethality in *Bacteroides fragilis*. *J. Bacteriol.* **138**:139-145.
19. Stolp, H., and M. P. Starr. 1963. *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic and bacteriolytic microorganism. Antonie van Leeuwenhoek *J. Microbiol. Serol.* **29**:217-248.
20. Stowers, M. D., and G. H. Elkan. 1981. An inducible iron-containing superoxide dismutase in *Rhizobium japonicum*. *Can. J. Microbiol.* **27**:1202-1208.
21. Worthington Biochemicals Corp. 1977. Worthington enzyme manual, p. 63-70. Worthington Biochemicals Corp., Freehold, N.J.
22. Yost, F. J., Jr., and I. Fridovich. 1973. An iron containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.* **248**:4905-4908.