JOURNAL OF BACTERIOLOGY, Mar. 1977, p. 1574-1583

Vol. 129, No. 3
Printed in U.S.A.

Enzymatic Defenses Against the Toxicity of Oxygen and of Streptonigrin in *Escherichia coli*

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Received for publication 21 September 1976

Anaerobically grown *Escherichia coli* K-12 contain only one superoxide dismutase and that is the iron-containing isozyme found in the periplasmic space. Exposure to oxygen caused the induction of a manganese-containing superoxide dismutase and of another, previously undescribed, superoxide dismutase, as well as of catalase and peroxidase. These inductions differed in their responsiveness towards oxygen. Thus the very low levels of oxygen present in deep, static, aerobic cultures were enough for nearly maximal induction of the manganese-superoxide dismutase. In contrast, induction of the new superoxide dismutase, catalase, and peroxidase required the much higher levels of oxygen achieved in vigorously agitated aerobic cultures. Anaerobically grown cells showed a much greater oxygen enhancement of the lethality of streptonigrin than did aerobically grown cells, in accord with the proposal that streptonigrin can serve as an intracellular source of superoxide. Anaerobically grown cells in which enzyme inductions were prevented by puromycin were damaged by exposure to air. This damage was evidenced both as a decline in viable cell count and as structural abnormalities evident under an electron microscope.

The importance of superoxide dismutase (SOD) as a defense against the toxicity of oxygen has been supported by studies of the induction of this activity in *Escherichia coli* B. *E. coli* has been reported to contain two superoxide dismutases, one of which contains manganese (12) and is found in the cell matrix (10), whereas the other contains iron (17) and is found in the periplasmic space (10). Exposure of *E. coli* B to oxygen caused an increase in manganese-containing SOD (MnSOD) which correlated with a gain in resistance towards hyperbaric oxygen (7, 10). Streptonigrin is more toxic in the presence of oxygen and seems to function as an intracellular source of O_2^- (16, J. R. White, T. O. Vaughan and W.-S. Yeh, Proc. Fed. Am. Soc. Exp. Biol. 30:1145, 1971). In accord with this view, increased levels of MnSOD in *E. coli* B also correlated with resistance towards streptonigrin (8). *E. coli* K-12 appeared to differ from *E. coli* B in its responses to oxygen. Thus, transfer from static aerobic cultures to forced aeration occasioned an induction of MnSOD in the case of *E. coli* B but not in *E. coli* K-12 (9). However, static liquid culture is not the same as completely anaerobic culture, and it appeared possible that the apparent difference between *E. coli* B and *E. coli* K-12 might be quantitative rather than qualitative. Thus *E. coli* K-12 might respond to such low concentrations of oxygen that its MnSOD was almost fully induced by the low levels of O_2 present in static liquid cultures. It thus appeared important to explore the responses of *E. coli* K-12 to oxygen. The induction of superoxide dismutases, catalase, and peroxidase by oxygen in *E. coli* K-12 and the protection afforded by these enzymes against the toxicity of oxygen and of streptonigrin form the substance of this report.

MATERIALS AND METHODS

E. coli K-12 *his*$^-$ *thi*$^-$ (ATCC 23794) was grown at 37°C in a medium containing 3% Trypticase soy broth (Baltimore Biological Laboratories) and 0.5% yeast extract (Difco). Aerobic conditions were achieved by oscillation of 100-ml cultures in 500-ml Bellco culture flasks at 200 rpm on a rotary platform shaker. Static culture entailed growth in full flasks or test tubes without agitation. Anaerobiosis involved transfer of the medium directly from the autoclave into an anaerobic jar (Bioquest) followed, after a delay of 48 h, by inoculation and growth in an anaerobic glove box. Hydrogen gas and a palladium catalyst were used to scavenge oxygen from these anaerobic chambers.

Cells were harvested at 4°C by centrifugation for 15 min at 10,000 × *g* and were washed once with 0.1 M potassium phosphate (pH 7.0). Washed cells were suspended in 0.05 M potassium phosphate, 10^{-4} M ethylenediaminetetraacetic acid (EDTA) at pH 7.8 and were disrupted for 3 min with a Branson W185 sonifier, operated at an output of 70 W. The cell suspension was kept at 4 to 6°C by working in an ice-

TABLE 1. *Effects of aeration on superoxide dismutases and catalase in E. coli K-12*

Condition of growth	Enzyme level[a]				
	Catalase	Superoxide dismutases			
		Total	MnSOD	New SOD	FeSOD
Anaerobic	4.8	17.8	0	0	17.8
Static, aerobic	5.1	22.1	10.8	0.5	10.9
Agitated, aerobic	11.2	23.5	10.0	2.6	10.9

[a] Enzyme levels are given as units of activity per milligram of protein in cell-free extracts.

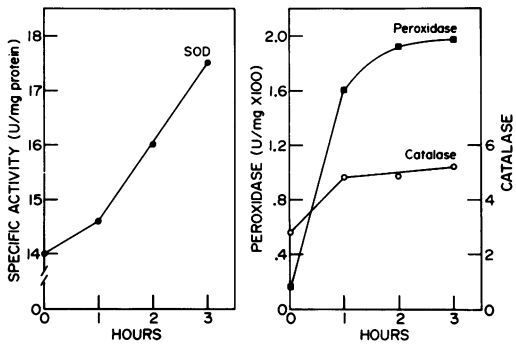

FIG. 1. *Induction of SOD (●), catalase (○), and peroxidase (■) in E. coli K-12. The cells were grown anaerobically in TSY medium. At zero time the culture was shifted to aerobic conditions and at intervals samples were removed for enzyme assays. In all cases enzyme activity is expressed as units per milligram of protein.*

salt bath and by applying the sonifier power intermittently. Cell debris was removed by centrifugation at 30,000 × *g* for 60 min, and cell-free extracts were then used directly for enzyme assays or were first dialyzed for 16 h against the phosphate-EDTA buffer. Protein concentration was estimated using the biuret method (6). SOD was assayed as previously described (13). SOD isoenzymes were estimated from the areas under densitometric scans of polyacrylamide gel electropherograms, which had been stained for SOD activity as previously described (1). Catalase was assayed by the method of Beers and Sizer (2), whereas peroxidase was assayed by the dianisidine procedure (11).

Surviving cells were determined by spreading appropriate dilutions in duplicate on TSY medium (3% Trypticase soy broth, 0.5% yeast extract) solidified with 1.5% agar and counting the colonies formed after 24 to 36 h at 37°C. In three separate experiments the standard error of the mean of such duplicate plate counts was ± 3 to 8%.

Preparation of cells for electron microscopy. Cultures were chilled and centrifuged at 4°C for 5 min at 2,000 × *g*. The cells were suspended in cold 0.05 M veronal-acetate buffer at pH 7.6 and again centrifuged. The washed cells were suspended in this buffer, containing 4% glutaraldehyde. After 2 h at room temperature, the fixed cells were centrifuged and were suspended in warm 4% agar-0.5% tryptone-0.5% NaCl and were spread on glass slides. After cooling and solidification, the agar was cut into small cubes, which were washed five times in the veronal-acetate buffer and then soaked in 0.5% uranyl acetate for 2 h at room temperature. The uranyl acetate was removed by five washes with the buffer, and the agar cubes were immersed in 2% osmium tetroxide for 2 h. The OsO_4 was removed by five washes with buffer, and the agar cubes were dehydrated by successive 10-min passages through 20, 30, 40, 50, 70, 80, 90, and 95% ethanol and by three 5-min washes in 100% ethanol. The dehydrated agar cubes were soaked for 15 min in ethanol-propylene oxide (1:1 [vol/vol]) followed by two 5-min soaks in propylene oxide. The cubes were then passed successively through propylene oxide:Epon (3:1, 1:1, and 1:3 [vol/vol]) and were then embedded in pure Epon 812 (Luft). The resin was polymerized at 60°C for 48 h, and the samples were sectioned with an LKB-Huxley ultramicrotome using a Dupont diamond knife. The sections were placed on carbon-coated copper grids and stained with uranyl acetate (15) for 5 min and with lead citrate (14) for 3 min. Stained sections were examined with a JEM-100C electron microscope (Joel Ltd., Tokyo) using 80 kV electron acceleration.

RESULTS

Effect of oxygen on SOD and catalase. *E. coli* K-12 responded to oxygen by increasing the intracellular levels of specific superoxide dismutases and of catalase. The levels of oxygen needed to elicit these responses varied from enzyme to enzyme (Table 1). The very low levels of O_2 present in static cultures was enough to cause full induction of MnSOD but only minor inductions of new SOD and of catalase. (New SOD has been isolated by H. Dougherty, Merck, Sharp and Dohme, Rahway, N.J., and appears to contain iron. Its intracellular location has not yet been studied.) Higher concentrations of dissolved oxygen, achieved by agitation of the cultures, elicited full induction of new SOD and of catalase. Iron-containing SOD (FeSOD) appeared to be constitutive, and its specific activity was modestly decreased by oxygenation of the growth medium possibly due to a deficiency of iron consequent to the induction of cytochromes and other iron-containing pro-

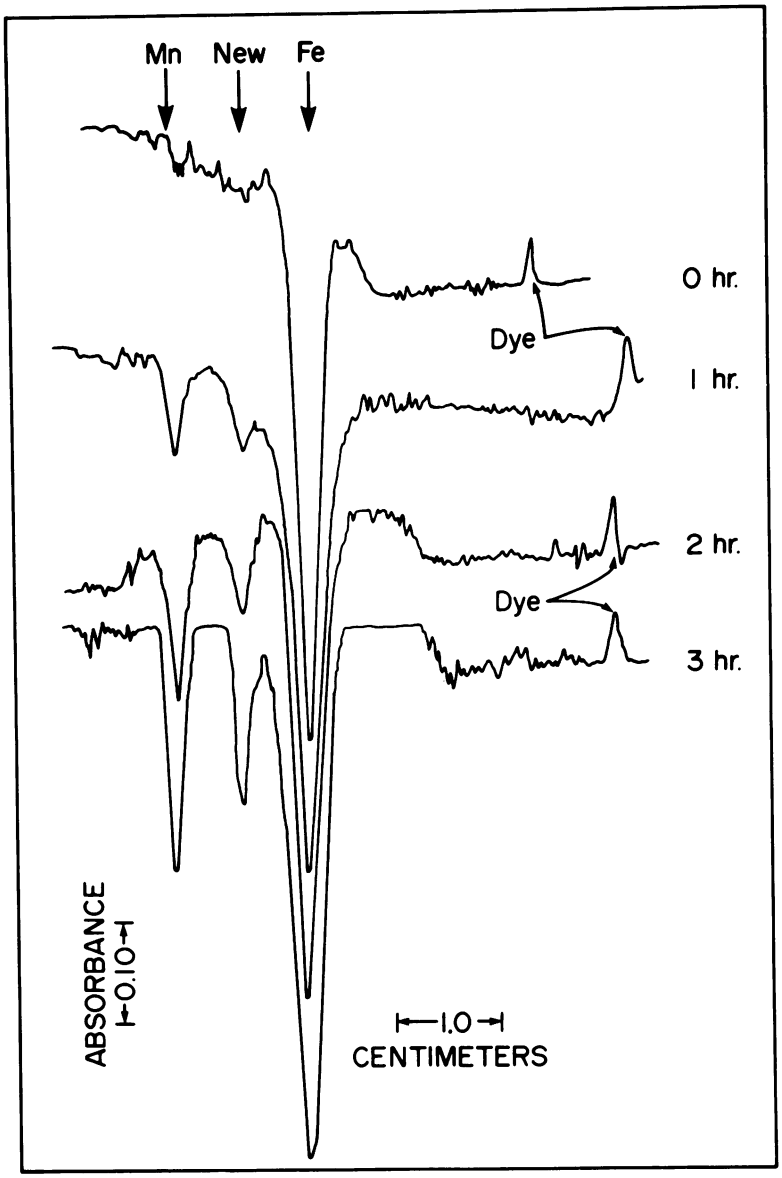

FIG. 2. *Densitometric scans of polyacrylamide gel electropherograms stained for SOD activity. The culture was grown anaerobically and shifted to aerobic conditions as in Fig. 1. At intervals after the shift, portions of the cell-free extracts were applied to 10% gels. The gels were stained for activity and scanned at 560 nm. The samples applied to the gels contained 0.247, 0.222, 0.284, and 0.346 mg of protein at 0, 1, 2, and 3 h, respectively.*

teins by oxygen or to increases in net soluble protein in aerobically grown cells. An attempt was made to measure the concentration of dissolved oxygen in deep, static liquid cultures of *E. coli* K-12. When a 1.5% inoculum was started in fresh medium at 37°C, the oxygen concentration fell linearly from the initial value of 0.20 mM to zero within 60 min, during which time the absorbance, at 600 nm had risen to 0.14, at a generation time of 33 min. This rate of growth was continued for another hour to an absorbance of 0.52, before the growth rate declined slightly to a generation time of 42 min. After 4 h the absorbance at 600 nm had reached a plateau value of 2.4. An oxygen concentration of 1.0 μM would easily have been detected by the Gilson Medical Electronics Oxygraph used in these studies. We must conclude that the

cells harvested from deep static liquid culture had grown for at least four doublings at a concentration of dissolved oxygen lower than 1.0 μM.

Rates of induction of SOD, catalase, and peroxidase. *E. coli* K-12 was grown to the late exponential growth phase under anaerobic conditions and abruptly transferred to an aerobic rotary platform shaker. At intervals thereafter samples were removed and cell-free extracts were prepared for enzyme assays. The results (Fig. 1) showed a rapid induction of SOD, catalase, and peroxidase. During the course of 3 h at 37°C, the total catalase doubled, whereas the peroxidase increased approximately 10-fold. SOD changed from 14 U/mg to 17.5 U/mg in 3 h. However, the level of SOD continued to rise almost linearly over this time and showed no indication of levelling off, whereas catalase and peroxidase activities had reached a plateau after 3 h of aerobic incubation.

Rates of induction of SOD isozymes. Anaerobically grown *E. coli* K-12 contained only FeSOD, whereas aerobically grown cells also contained MnSOD and new SOD. The rates of induction of these isozymes were also examined. Figure 2 presents densitometric scans of polyacrylamide gel electropherograms stained for SOD activity. This activity stain (1) gives achromatic zones against a blue background, so the tracings in Fig. 2 show a decrease in absorbancy at positions containing SOD activity. The samples studied were prepared as described for Fig. 1. It is evident that anaerobically grown cells contained only FeSOD and that transfer to aerobic conditions resulted in an essentially parallel induction of MnSOD and of new SOD, whereas the total amount of FeSOD remained constant. The amount of each isozyme in these samples was estimated from the areas under the activity troughs in Fig. 2. Figure 3 shows that transfer of *E. coli* K-12 from anaerobic to aerobic conditions resulted in an essentially linear increase in MnSOD and new SOD without apparent lag. The specific rates of synthesis were 1 and 0.75 U/mg of protein per h for MnSOD and new SOD, respectively. FeSOD did not increase during 3 h of aeration. Indeed, although its total activity per cell remained constant, its specific activity declined from 14 to 12.1 U/mg.

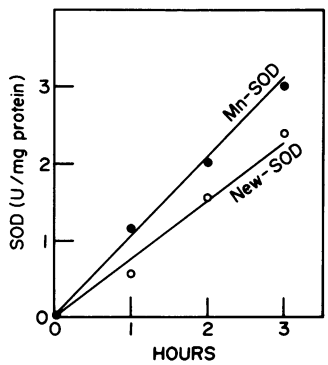

FIG. 3. *Kinetics of induction of MnSOD and new SOD. Growth and experimental conditions were the same as in Fig. 1 and 2. The specific activities of MnSOD and New SOD were estimated by measuring the areas under the peaks, of each species, from Fig. 2, and from the total SOD activity as measured in the cell-free extracts.*

Effects of puromycin and of chloramphenicol on the induction of SOD, catalase, and peroxidase. Attempts to demonstrate the importance of superoxide dismutases, catalase, and peroxidase in defense against oxygen toxicity could be foiled by the induction of these enzymes during exposure of anaerobically grown cells to the test levels of oxygen. Inhibitors of protein synthesis were therefore used to prevent induction of these enzymes. *E. coli* K-12 was grown anaerobically to the late exponential growth phase and the culture was divided into four equal portions. One portion was cooled with crushed ice and the cells were collected by centrifugation and disrupted for enzyme assays. Puromycin was added to 0.5 mg/ml to the second portion, chloramphenicol was added to 0.5 mg/ml to the third portion, and the

TABLE 2. *Effect of puromycin and chloramphenicol on the induced synthesis of SOD, catalase, and peroxidase in E. coli K-12*

Culture conditions	Exposure to air (h)	Catalase (U/mg)	Peroxidase (U/mg) × 100	Superoxide dismutase (U/mg)			
				Total	MnSOD	New SOD	FeSOD
Anaerobically grown cells	0	4.6	0.22	10.7	0	0	10.7
Anaerobically grown cells +	2	4.7	0.25	10.1	0	0	10.1
puromycin (0.5 mg/ml)	4	4.0	0.19	9.9	0	0	9.9
Anaerobically grown cells +	2	3.9	0.23	9.8	0	0	9.8
chloramphenicol (0.5 mg/ml)	4	3.6	0.18	9.1	0	0	9.1
Anaerobically grown cells	4	11.1	2.8	14.5	4.0	1.6	8.9

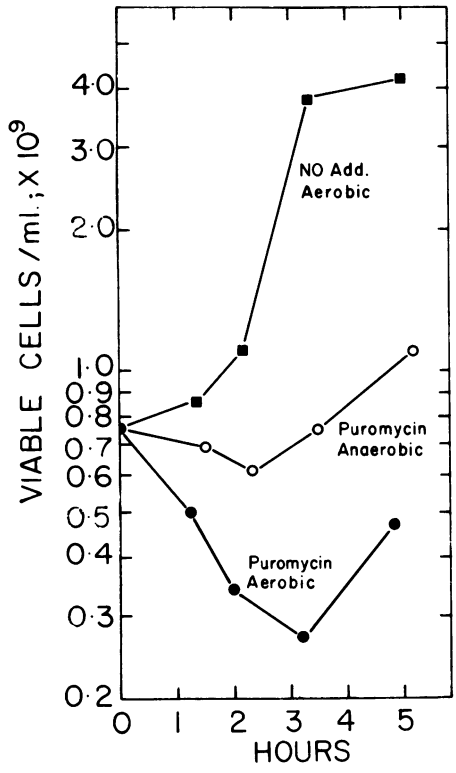

FIG. 4. *Effect of puromycin on the ability of anaerobically grown cells to tolerate oxygen. E. coli K-12 was grown anaerobically to the late exponential growth phase. Puromycin (0.5 mg/ml) was added to the culture and allowed to incubate anaerobically for another 15 min before exposure to air on a shaker. Symbols: ■, aerobic without puromycin; ○, puromycin added and the culture was kept anaerobically; ●, puromycin added and exposed to air.*

fourth portion was left untreated. These cell suspensions were then exposed to oxygen by agitation at 200 rpm on a platform shaker. Aliquots were taken after 2 and 4 h of aeration, and the cells were collected by centrifugation and were disrupted by sonic oscillation. The resultant cell-free extracts were assayed for SOD, catalase, and peroxidase. Table 2 shows that aeration of anaerobically grown *E. coli* K-12 caused a 2.4-fold increase in catalase, a 12.7-fold increase in peroxidase, and the appearance of MnSOD and of new SOD, and that either puromycin or chloramphenicol completely suppressed these enzyme inductions.

SOD, catalase, peroxidase, and oxygen toxicity. It has already been shown that increased intracellular levels of SOD, achieved by growth under oxygen, correlated with protection against hyperbaric oxygen in the cases of *Streptococcus faecalis*, *E. coli* B, and *Saccharomyces cerevisiae* (7–9). *E. coli* K-12 was thought to be different in that transfer from static culture to forced aeration did not cause induction of SOD and did not provide resistance to hyperbaric oxygen (9). Since it is now evident that *E. coli* K-12 does induce MnSOD and new SOD, in response to oxygen, but differs from *E. coli* B only in that it responds maximally to very low levels of oxygen, it was mandatory that the correlation between enzyme levels and oxygen tolerance in *E. coli* K-12 be reinvestigated. *E. coli* K-12 was grown to the late exponential growth phase in the absence of oxygen. Puromycin (0.5 mg/ml), as an inhibitor of protein synthesis, was added to two cultures under anaerobic conditions. After 15 min, one of these cultures was transferred to an aerobic rotatory platform shaker, whereas the other culture was maintained in the anaerobic incubator. A third control culture was transferred from anaerobic to aerobic conditions in the absence of puromycin. Figure 4 demonstrates that anaerobically grown cells experienced 66% mortality in 3 h when exposed to air in the presence of puromycin (lower line); whereas puromycin, in the absence of oxygen (middle line), caused very little cell death. The top line shows that anaerobically grown cells, transferred to air in the absence of puromycin, were able to multiply rapidly to stationary phase. Anaerobically grown cells, which initially contained no MnSOD or new SOD, escaped serious damage when ex-

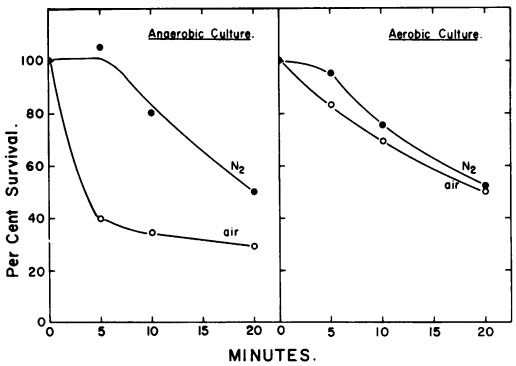

FIG. 5. *Effect of streptonigrin on the viability of aerobically and anaerobically grown cultures of E. coli K-12. The late exponential phase cells from aerobically and anaerobically grown cells were diluted to approximately 10^8 cells/ml in sterile TSY medium containing 2 μg of streptonigrin per ml, and incubated at 37°C in the presence or absence of air. At intervals, samples were removed for plating on TSY agar, and viable counts were made after 24 h of incubation at 37°C.*

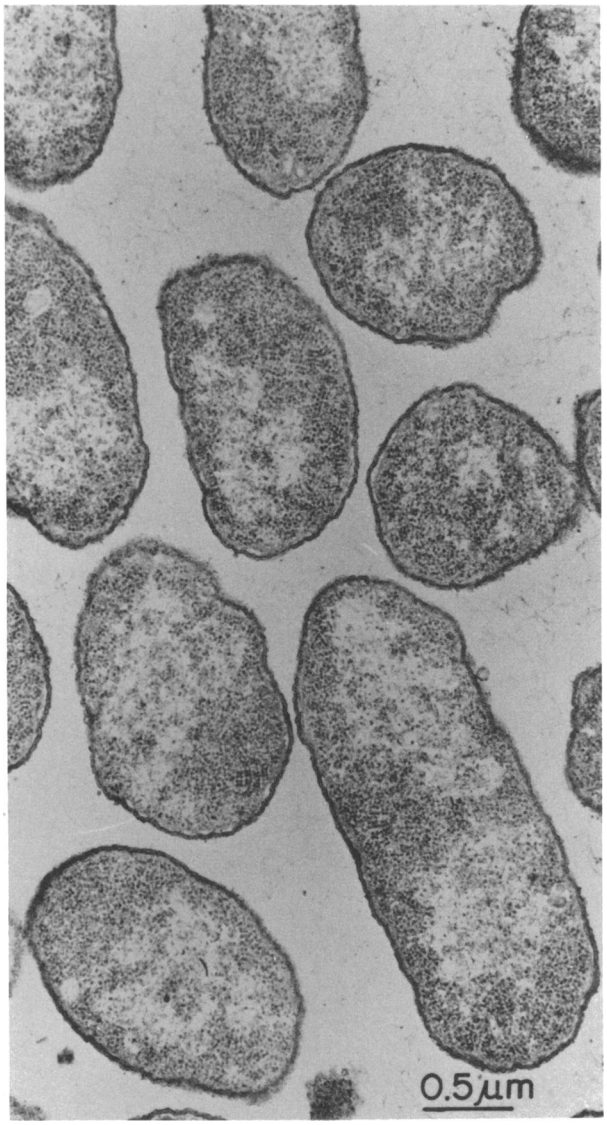

FIG. 6. *Electron micrograph of a thin section of E. coli K-12. The cells were grown anaerobically, treated with puromycin (0.5 mg/ml), and fixed and handled without exposure to oxygen.*

posed to air in the absence of puromycin, presumably because they were free to induce the protective superoxide dismutases, catalase, and peroxidase, and puromycin made these cells oxygen sensitive by preventing these enzyme inductions. In that case aerobically grown cells, exposed to air in the presence of puromycin, should suffer no damage since they already contain high levels of the protective enzymes. This was tested and found to be true. Thus *E. coli* K-12 grown aerobically were treated with 0.5 mg of puromycin per ml, and aerobic incubation was continued. No cell lethality was observed. Indeed, after a lag of 1 h the cells grew exponentially probably due to the presence of some puromycin-resistant cells.

SOD and susceptibility to streptonigrin. If the oxygen enhancement of the lethality of streptonigrin is due to O_2^- generated inside the cells by cyclical reduction and autoxidation of this paraquinone antibiotic, then *E. coli* K-12 containing MnSOD, by virtue of oxygen induction, should be more resistant than comparable cells lacking this enzyme. Cultures grown to

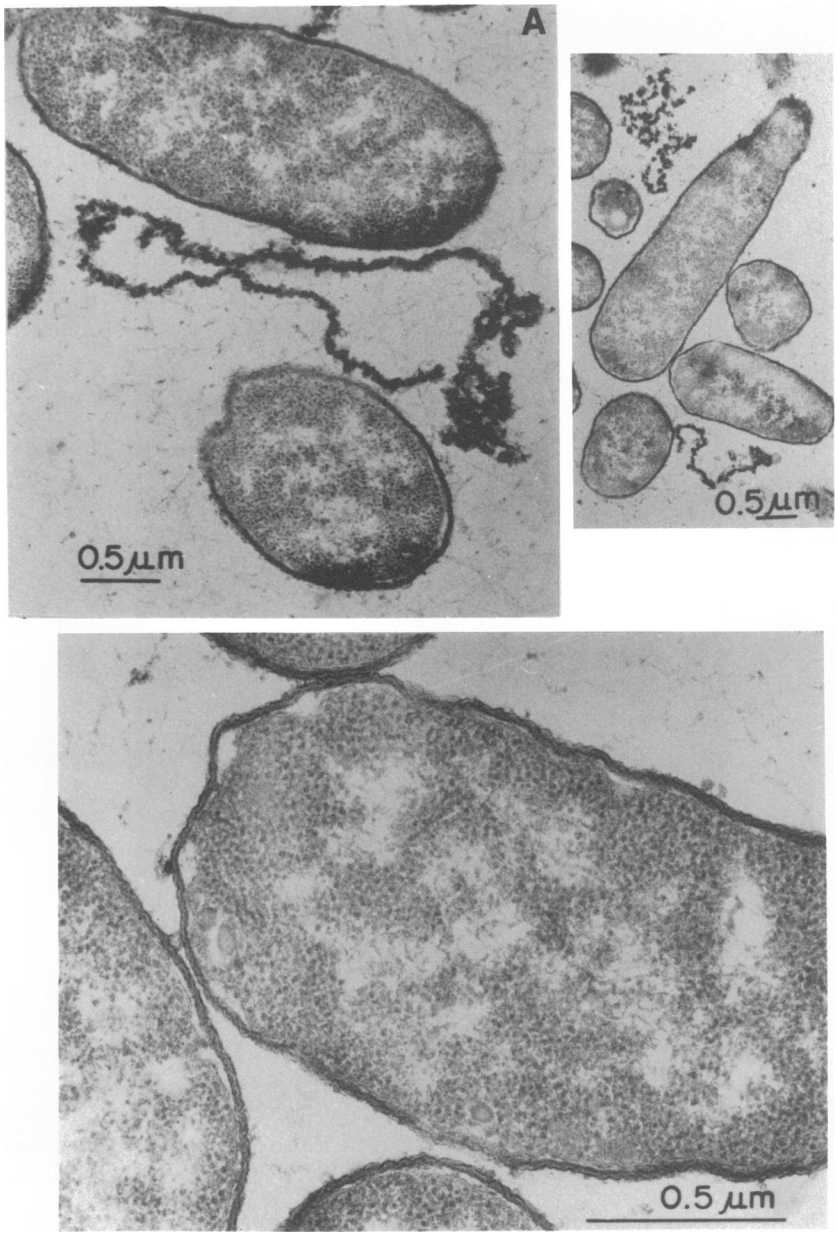

FIG. 7. *(A and B) Electron micrographs of thin sections of E. coli K-12 grown anaerobically and transferred to air in the presence of puromycin to prevent de novo protein synthesis. Cell fragments and membrane damage are apparent.*

the late exponential growth phase anaerobically or aerobically were diluted to 10^8 cells/ml in sterile TSY medium containing 2 μg of streptonigrin per ml and were incubated at 37°C, either in the absence or presence of air. At intervals, samples were removed for dilution and plating. Figure 5 demonstrates that anaerobically grown cells were very susceptible to the oxygen enhancement of the toxicity of streptonigrin, whereas aerobically grown cells were much less sensitive to this oxygen enhancement.

Visualizations of oxygen damage by electron microscopy. Exposure of anerobically

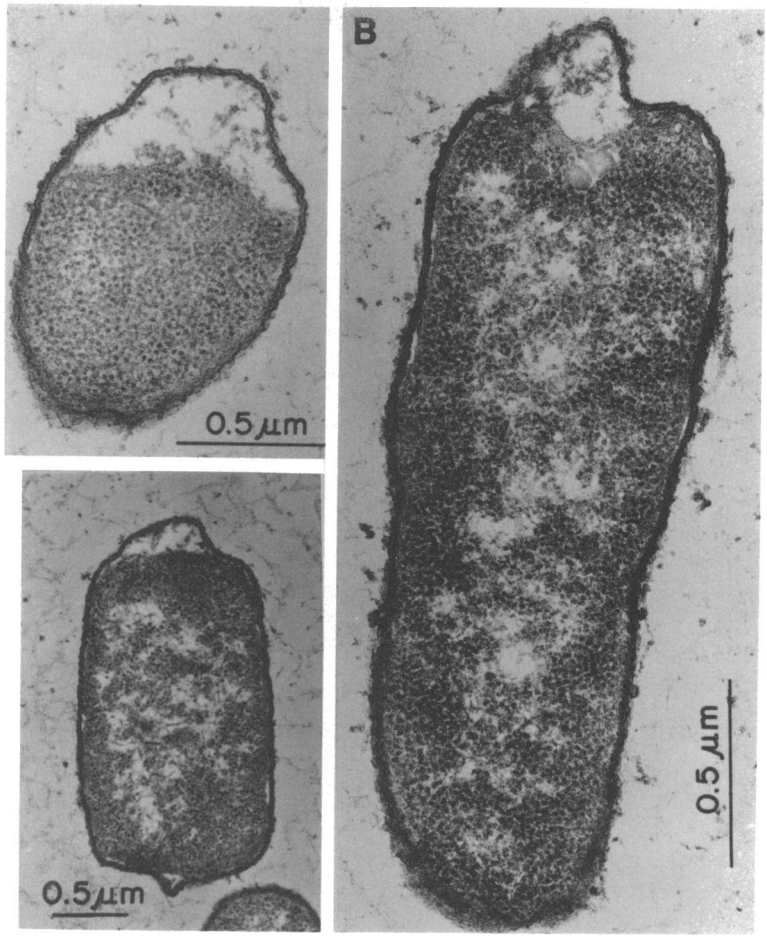

FIG. 7B

grown cells to air, in the presence of puromycin, resulted in some loss of viability (Fig. 4). Presumably this was due to the toxic actions of O_2^- and H_2O_2. It appeared possible to see the results of the oxygen damage at high magnification. Anaerobically grown *E. coli* K-12 were exposed to air, for 3 h, in the presence and absence of puromycin, and a control lot was treated with puromycin anaerobically as described in the legend of Fig. 4. The cells were then prepared for electron microscopy, as described above. Figure 6 illustrates that anaerobic treatment with puromycin did not cause visible abnormalities. In contrast, exposure to air in the presence of puromycin caused occasional cell breakage and a general damage to the cytoplasmic membrane which led to the appearance of gaps between the cytoplasm and the cell wall, most frequently at the poles of the cell (Fig. 7A and B). Exposure to air, in the absence of puromycin, did not cause structural aberrations (Fig. 8).

DISCUSSION

Exposure of *E. coli* K-12 to oxygen caused induction of MnSOD, new SOD, catalase, and peroxidase. The extremely low levels of dissolved oxygen present in static liquid cultures were sufficient to elicit maximal induction of MnSOD. In contrast, the much higher levels of oxygenation caused by vigorous agitation were required to achieve significant inductions of new SOD, catalase, and peroxidase. FeSOD was constitutive and was not induced by oxygen. Inhibitors of protein synthesis, such as puromycin or chloramphenicol, completely prevented the induction of these enzymes in response to oxygenation.

When anaerobically grown cells, devoid of MnSOD and new SOD and containing rela-

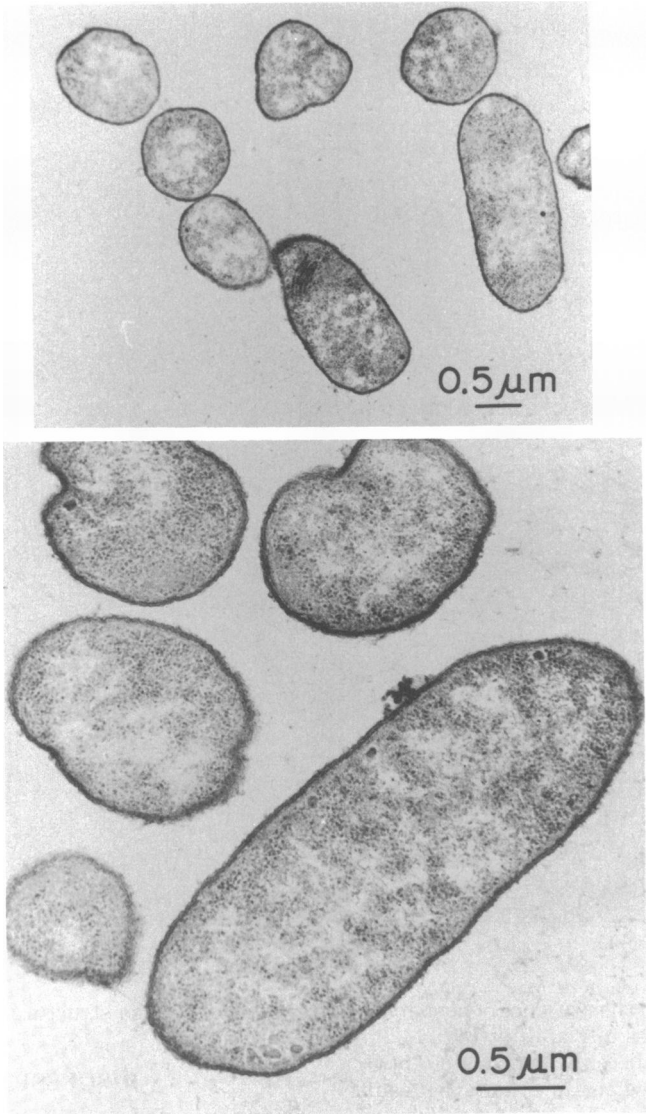

FIG. 8. *Electron micrographs of thin sections of E. coli K-12 that were grown anaerobically, shifted to air in the absence of puromycin, and allowed to induce the enzymes required for protection against oxygen toxicity.*

tively low levels of catalase and of peroxidase, were exposed to air in the presence of puromycin, they experienced cell damage which was visualized by electron microscopy and which correlated with a loss of viability. This was not due to puromycin per se, since anaerobic treatment with puromycin was without effect. Furthermore aerobically grown cells rich in MnSOD, new SOD, catalase, and peroxidase tolerated aerobic exposure to puromycin without loss of viability.

We conclude that O_2^- and H_2O_2 are agents of oxygen toxicity in *E. coli* K-12 and that the superoxide dismutases, catalase, and peroxidase are important defenses which scavenge these reactive intermediates of oxygen reduction. Since increases in MnSOD and new SOD also correlated with resistance towards the oxygen enhancement of streptonigrin lethality, it appears that this antibiotic does act like an intracellular source of O_2^-. These results obtained with *E. coli* K-12 are in accord with those previously reported for other organisms (3–5, 7–10).

ACKNOWLEDGMENTS

We thank Gerda Michalski for her expert assistance in the preparation of electron micrographs. The streptonigrin used in these studies was provided by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

This work was supported by Public Health Service research grants GM-10287 and HL-17603 from the National Institute of General Medical Science and the National Heart and Lung Institute, respectively, and research grant DAHC-0474-G-0194 from the United States Army Research Office, Research Triangle Park, N.C.

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