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Physiological Function of Superoxide Dismutase in Glucose-Limited Chemostat Cultures of *Escherichia coli*

H. MOUSTAFA HASSAN AND IRWIN FRIDOVICH*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Conditions for continuous culture of *Escherichia coli* K-12 His⁻ Thi⁻ under glucose limitation were established. Both the capacity for respiration, at $D >$ 0.2/h, and specific activity of superoxide dismutase increased as a function of specific growth rate, whereas peroxidase and catalase were either invariant with or inversely related to this growth rate. The abrupt increase in the availability of glucose, as a means of elevating the growth rate, was followed by an increase in superoxide dismutase, which reached a plateau before there was a significant increase in the growth rate. Thus, an increase in superoxide dismutase appeared to be a prerequisite for an increase in the rate of growth. Cells that had higher levels of superoxide dismutase, because of varying specific growth rates, were more resistant to the toxicity of hyperbaric oxygen. Superoxide dismutase thus behaved like an essential defense against the toxicity of oxygen. Sensitivity towards streptonigrin increased with specific growth rate in the range of 0.09 to 0.25/h but decreased with further increases in the growth rate. Since this antibiotic has been shown to shunt electrons to oxygen, with concomitant production of O_2^-, these results indicated a progressive deficiency of reducing power at growth rates below 0.25/h and a surfeit of reducing power with progressively greater protection against O_2^- by superoxide dismutase at growth rates greater than 0.25/h.

Metabolism of and resistance to oxygen is of central importance in the physiology and the classification of microorganisms. Thus, terms such as obligate anaerobe, aerotolerant anaerobe, and facultative anaerobe are frequently used. The toxicity of oxygen has been well documented (7, 15, 22). It has been proposed that intermediates in the reduction of oxygen to water, such as O_2^- and H_2O_2, are important agents of oxygen toxicity and that enzymes which catalytically scavenge these intermediates, such as superoxide dismutases, catalase, and peroxidase, are essential defenses against this toxicity (5, 19).

Bacteria respond rapidly to changes in environment and therefore change continuously during the growth cycle in batch culture because of changes in the concentrations of nutrients and waste products (13, 17). Previous studies of the correlation between superoxide dismutases (SODs), catalase, and peroxidase and resistance to oxygen toxicity were performed in batch cultures, and the results must have been affected by the complex physiological changes attending such culture (9, 10). Interpretation of the results of such studies is, therefore, not as straightforward as could be desired.

These complexities can be overcome through the use of steady-state chemostat cultures. The chemostat (16, 20, 21) allows balanced growth over a wide range of specific growth rates in a constant environment. This technique has been used to good advantage in studying enzyme synthesis and regulatory mechanisms (13). In a like manner, we now explore the synthesis and control of those enzymes which provide defenses against oxygen toxicity in *Escherichia coli*.

MATERIALS AND METHODS

E. coli K-12 His⁻ Thi⁻ (ATCC 23794) was grown at pH 7.0 and at 37°C in a basal medium composed of (per liter): $MgSO_4 \cdot 7H_2O$, 0.2 g; citric acid · water, 2.0 g; K_2HPO_4, 10.0 g; $NaNH_4HPO_4 \cdot 4H_2O$, 3.5 g; DL-histidine, thiamine, and glucose were used at 300 mg/liter, 30 mg/liter, 5.0 g/liter, respectively, except where otherwise indicated. Batch cultures were grown on a shaker at 200 rpm. The chemostat was a water-jacketed tube having an inner diameter of 50 mm and a length of 350 mm. Magnetic stirring and forced aeration through a Kimflow gas dispersion tube (size 12-C) were maintained throughout. Inflow was controlled with a Technicon proportioning pump (Technicon Corp., Tarrytown, N.Y.), and the volume of the culture was held at 265 ml by an overflow siphon tube. Dilution rate (D/h) is expressed as f/v, where f is the rate of inflow (milliliters per hour) and v is the hold-up volume of the

chemostat. Overflow culture was collected in an ice bath, and cell density was measured in terms of absorbance at 600 nm. The chemostat culture was assumed to have reached a steady state when cell density varied by less than 2% over two generation times. Air inflow was sterilized and humidified before being bubbled into the culture.

Specific growth rates in batch cultures (k/h) were calculated by linear regression from the rate of change of cell density (absorbancy at 600 nm) from the expression

$$\ln x/x_0 = k\ (t - t_0)$$

where x_0 and x are the cell densities at times t_0 and t, respectively. In steady-state chemostat cultures, cell density is invariant with time and the specific growth rate is equal to the specific dilution rate (k/h = D/h) (15, 20, 21).

The respiration rates of the culture were determined using portions that were measured at 37°C with a Clark type of polarographic oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) and are expressed as n-atoms of O_2 consumed per minute per milligram of cell protein. Protein was estimated by a biuret procedure (6). Cell suspensions were treated with an equal volume of 20% deoxycholate prior to application of the biuret assay.

Enzyme assays were performed on cell-free extracts. Culture samples (40 to 50 ml) were chilled to 4°C, collected by centrifugation, and washed once with 0.1 M potassium phosphate at pH 7.0. Washed cells were suspended in 50 mM potassium phosphate and 0.1 mM ethylenediaminetetraacetic acid at pH 7.8 and disrupted for 3 min with a Branson sonifier (model W185) at a power output of 70 W. An ice-salt bath and intermittent application of power combined to prevent warming during sonic treatment. Sonically treated materials were clarified at 30,000 × g for 60 min. SOD was assayed in solution and on polyacrylamide gels as previously described (1, 18). Catalase was assayed according to Beers and Sizer (2). Peroxidase was measured with the dianisidine method (11). Cell survival was estimated by dilution and plating as previously described (14).

RESULTS

Effects of nutrients on growth and enzyme synthesis in batch culture. *E. coli* K-12 His^- Thi^- was used. Therefore, histidine, thiamine, and the carbon source (glucose) could all serve to limit its growth. The effects of all three of these nutrients on plateau cell density in batch cultures were explored. Figure 1 shows that DL-histidine was limiting in the range 0 to 40 mg/liter, thiamine at 0 to 0.1 mg/liter, and glucose at 0 to 5 mM. Table 1 presents specific growth rates, generation times, and levels of SOD at specified levels of these nutrients.

Enzyme synthesis in a glucose-limited chemostat. A continuous culture was established in a medium containing excess DL-histidine (150 mg/liter) and thiamine (30 mg/liter)

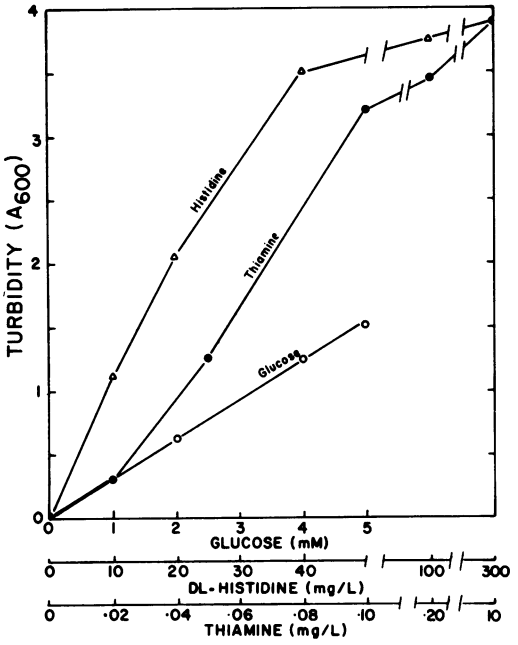

FIG. 1. *Effect of different concentrations of* DL-*histidine, thiamine, and glucose on the plateau cell density of E. coli K-12 grown in batch cultures at 37°C. When one nutrient was varied, the other two were present in excess (*DL-*histidine, 300 mg/liter; thiamine, 30 mg/liter; and glucose, 5 g/liter).*

but limiting glucose (4 mM). Specific dilution rates were systematically varied, and the steady-state cell densities, rates of respiration, and enzyme activities were measured. The results are given as a function of specific growth rates in Fig. 2. One experimental point in Fig. 2B, that for SOD at k = 0.535/h, was made on cells growing in log-phase batch culture in the glucose-limited medium. The remaining points were all from a steady-state chemostat culture. The specific activity for peroxidase declined with increasing specific growth rates, whereas that for catalase was invariant until the rate exceeded 0.4/h, and it then declined. Both the capacity for respiration and specific activity of SOD increased in parallel with more rapid growth (Fig. 2B). The relationship between the specific activity of SOD and the specific growth rate was linear and extrapolated to 1.5 U/mg of protein at zero growth. This may be taken as an indication that SOD, unlike glutamic dehydrogenase and glutamic-oxalacetic transaminase (13), is essential even for nongrowing cells.

SOD synthesis during transition to unrestricted growth. Since the specific activity of SOD was linearly correlated with specific growth rate in steady-state cultures (Fig. 2B), it

TABLE 1. *Maximum specific growth rates (k_{max}) during balanced, unrestricted growth in batch cultures*[a]

Medium	Concn of growth-limiting factor	k/h	Generation time (min)[b]	SOD (U/mg)[c]
Glucose-minimal		0.558	75	5.58
Glucose limiting	4 mM	0.533	78	6.36
Thiamine limiting	0.05 mg/liter	0.442	94	6.64
Histidine limiting	10 mg/liter	0.531	78.4	8.55

[a] Unrestricted growth = logarithmic growth phase where all nutrients are still in excess.
[b] Generation time = ln 2/k.
[c] SOD measurements were at 6 to 7 h (log phase).

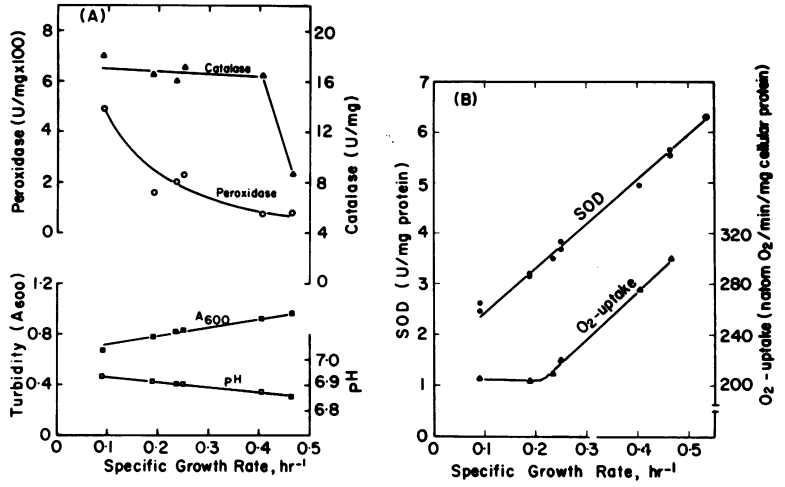

FIG. 2. *Effects of dilution rates on growth. Symbols: absorbancy at 600 nm (■), pH (□), respiration rates (▲), and the specific activities of catalase (△), peroxidase (○), and SOD (●). The point for SOD at k = 0.535/h was measured on log-phase cells grown in batch culture in a glucose-limited (4 mM) minimal medium.*

seemed important to observe its changes during abrupt transition from a glucose-limited chemostat culture (k = 0.25/h) to unrestricted batch culture (k = 0.533/h). Figure 3 demonstrates that the specific growth rate lagged for 55 min after the transition, whereas the specific activity of SOD increased progressively for 55 min after the transition to the level that is characteristic of the higher rate of growth. The specific activity of SOD was thus increased to that which is appropriate for the higher growth rate, before the growth rate itself increased.

Correlations between SOD and oxygen tolerance. The results in Fig. 2 demonstrated that SOD increased with the specific growth rate and in parallel with increased respiration, whereas peroxidase declined and catalase was invariant over most of the range studied. This, in accord with previous data (8, 9), suggests that SOD is an essential and limiting defense against an intermediate of oxygen reduction, presumably O_2^-. The correlation between specific growth rate and SOD in a glucose-limited chemostat culture also provided an opportunity to correlate SOD content with oxygen tolerance. Cells in steady-state growth at different dilution rates were taken from the chemostat and exposed for 2 h to 20 atm of O_2 or N_2 at 37°C in the presence of 0.5 mg of chloramphenicol per ml, which inhibits the induction of SOD, catalase, and peroxidase (14). Survival after this hyperbaric exposure was measured in terms of the ability to give rise to visible colonies after dilution and plating on nutrient agar. The data in Fig. 4 demonstrate that tolerance towards hyperbaric oxygen did increase with increasing specific growth rate (Fig. 4A) and SOD content (Fig. 4B). Exposure to 20 atm of N_2 had no effect, thus controlling for possible lethality due to pressure changes or to the chloramphenicol that was present to prevent protein synthesis during the test exposures.

Correlations between SOD and resistance to the oxygen enhancement of streptonigrin lethality. The lethality of streptonigrin is enhanced by oxygen, and this has been attributed to the intracellular production of O_2^- during the redox cycling of this *p*-quinone antibiotic (23).

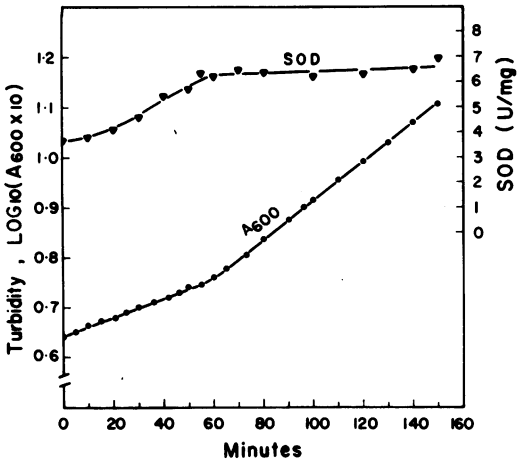

FIG. 3. *Changes in cell density (absorbancy at 600 nm [A_{600}]) and in the specific activity of SOD during transition from restricted to unrestricted growth conditions. Before termination of the chemostat, a steady-state condition at D = 0.25/h was established. At time zero, the flow of fresh medium was stopped, and the culture in the growth vessel was diluted with 265 ml of prewarmed minimal medium containing 8 mM glucose to bring the final concentration of glucose to about 4 mM. Immediately after the transition, 30-ml samples were removed at intervals for measurements of absorbancy at 600 nm and enzyme assays. For the SOD assays, the cells were chilled by pouring on crushed ice containing chloramphenicol (final concentration, 0.5 mg/ml). SOD assays were carried out on the cell-free extracts as described in Material and Methods.*

In that case, SOD should provide resistance against the oxygen enhancement of streptonigrin lethality. Correlations supporting this deduction have been reported from studies with batch cultures of *E. coli* B (9) and *E. coli* K-12 (14). We looked for comparable positive correlations between SOD and resistance towards streptonigrin in chemostat cultures growing at different rates.

Cell suspensions were removed from the glucose-limited chemostat at different steady-state growth rates, diluted to approximately 10^8 cells/ml (10-fold dilution) with a glucose (4 mM) and salts medium containing 2 μg of streptonigrin per ml, and then incubated on a rotary shaker operated at 200 rpm and at 37°C. At intervals, samples were taken for assessment of survivors by plating and counting. The results are shown in Fig. 5. Sensitivity to streptonigrin increased with specific growth rate in the range of 0.09 to 0.25/h and then decreased at growth rates higher than 0.25/h. The anticipated result was a progressive decrease in sensitivity with increasing growth rate because of the increasing content of SOD with increasing growth rate.

Effects of growth rate on the SOD isozymes. *E. coli* contains three varieties of SOD that can be resolved by electrophoresis on acrylamide gels and estimated by densitometry after staining such gels for SOD activity (1). The results shown in Fig. 6 indicate that all of the forms of SOD increase with a specific growth

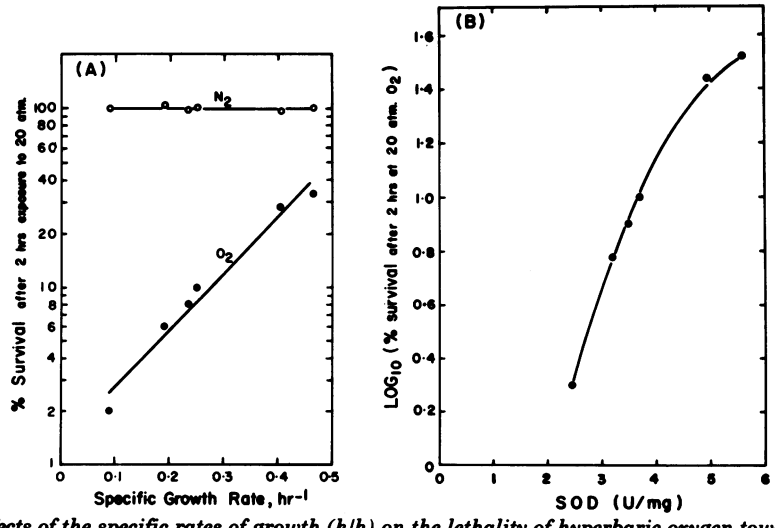

FIG. 4. *Effects of the specific rates of growth (k/h) on the lethality of hyperbaric oxygen towards E. coli K-12. Cells in the steady state were removed from the chemostat at the different dilution rates and diluted to a cell density of about 10^7 cells/ml in fresh minimal medium containing 0.5 mg of chloramphenicol per ml. The cells were then placed in a shallow petri dish and exposed to 20 atm of O_2 or N_2 at 37°C. After 2 h of exposure, the pressure was released, the cultures were diluted and plated onto Trypticase soy-yeast extract agar plates, and colony counts were made after 24 h of incubation at 37°C. (A) Presents survival under hyperbaric O_2 or N_2 as a function of specific growth rate, whereas (B) presents this survival as a function of the SOD content of the cells.*

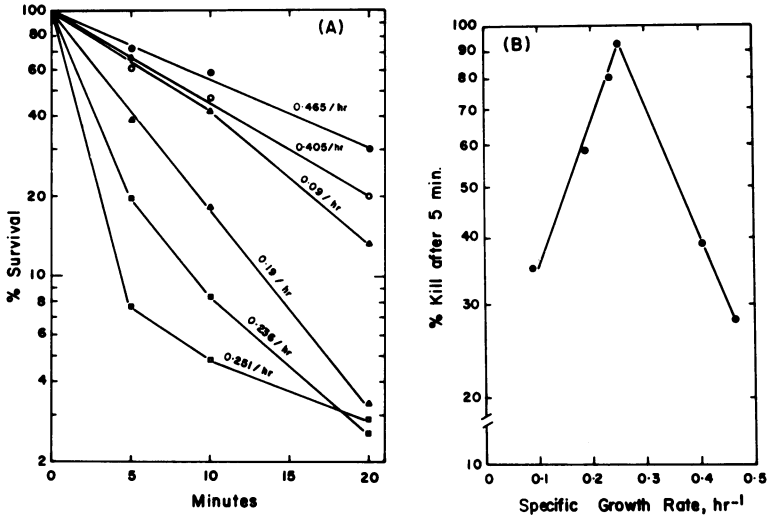

FIG. 5. *Effects of the specific growth rates (k/h) on the lethality of streptonigrin towards E. coli K-12. As in Fig. 4, cells were removed from the chemostat at the different dilution rates, then diluted 10-fold to about 10^8 cells/ml in the same growth medium containing 2 μg of streptonigrin per ml, and incubated at 37°C on a rotary shaker at 200 rpm. At various intervals, samples were removed, diluted, and plated on Trypticase soy-yeast agar. Colony counts were made after 24 h of incubation at 37°C. (A) Percentage surviving after 5, 10, and 20 min of exposure of the different types of cells to streptonigrin; the numbers on the lines represent the specific growth rates of the cultures used. (B) Percentage killed after 5 min of exposure to streptonigrin as a function of the specific growth rates (k/h).*

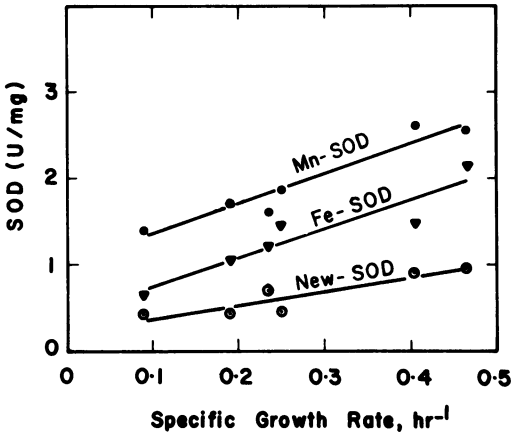

FIG. 6. *Effects of specific growth rates on the different SOD isozymes. The three isozymes were resolved from densitometric scans of polyacrylamide gel electropherograms stained for activity (1). The relative activities of MnSOD, NewSOD, and FeSOD were estimated by measuring the areas under the peaks of each species. The specific activities were calculated in relation to total SOD as measured in the cell-free extracts (18). The lines shown were calculated by linear regression analysis.*

rate but that FeSOD and MnSOD increase more rapidly than NewSOD.

DISCUSSION

Enzymes, such as glutamic dehydrogenase, whose activities are essential for growth are needed in progressively greater amounts as the growth rate is increased. Thus, it is perfectly reasonable that the level of glutamic dehydrogenase should be a linear function of specific growth rate and should extrapolate to zero at the limit of no growth (13). In contrast, SOD plays a defensive role, and it is not clear, à priori, that it should be needed in greater amounts as growth rate is increased. However, SOD activity did increase with specific growth rate (Fig. 2B). This suggests that the rate of production of O_2^- by *E. coli* K-12 increased with the growth rate. Moreover, extrapolation indicated a substantial retention of SOD at zero growth rate. This indicates the continuing need for defense against O_2^- under conditions of cell maintenance.

E. coli B-SG1, in a glucose-limited chemostat, has been reported (13) to show constant respiration capacity at growth rates from 0.3 to 0.5/h. In contrast, *E. coli* W, in glycerol- or ammonia-limited growth, exhibited a linear increase in respiration with growth in the range of 0.05 to 0.23/h (4). *E. coli* K-12 has now been found to exhibit both of these behaviors, depending on the growth rate. Thus, respiration was independent of growth rates below 0.24/h but was proportional to higher growth rates. A linear relationship between the rates of respiration and specific growth has been demonstrated for energy-limited chemostat cultures (12).

At growth rates in the range of 0.1 to 0.2/h,

oxygen uptake did not increase with specific growth rate, although SOD did so. Since SOD functions to scavenge O_2^- and is probably induced in response to O_2^-, we can propose that the fraction of oxygen consumed, which was univalently reduced, decreased in this range of growth rates.

The salient observation in the present report is that resistance towards the lethality of hyperbaric oxygen increased with specific growth rate in parallel with increases in content of SOD (Fig. 2B and 4). The correlation between SOD and resistance towards oxygen toxicity, which has previously been seen under the conditions of batch culture (9, 10), has now also been demonstrated under the more controlled conditions of glucose-limited chemostat culture. The work in batch culture involved changing the levels of SOD by changing the availability of oxygen and was open to the criticism that oxygen can induce changes other than the induction of SOD and that one or more of these other changes might actually have been responsible for the resistance towards oxygen, which was attributed to SOD. Oxygenation was constant and nonlimiting in the presently described chemostat culture, and the correlation between content of SOD and resistance towards oxygen can now be made with greater assurance than was previously possible (9, 10). Furthermore, the levels of catalase and peroxidase were either constant or diminished with increasing specific growth rate (Fig. 2A). This indicates that these H_2O_2-scavenging enzymes, unlike SOD, were not limiting factors in the resistance of *E. coli* K-12 towards oxygen toxicity. The critical importance of SOD was emphasized by the observation that the abrupt step-up in glucose supply did not increase the specific growth rate until the level of SOD had been increased to that which is characteristic of the greater growth rate.

The biphasic effect of growth rate upon sensitivity to streptonigrin requires special comment. There are data which indicate that the oxygen enhancement of streptonigrin toxicity is due to the redox cycling of this antibiotic, with the concomitant production of O_2^- (3). In this case, a source of reducing power is essential for the full expression of the toxicity of streptonigrin. Thus, the *p*-quinone form of the antibiotic must be reduced to the semiquinone or the hydroquinone before it can react with O_2 to generate O_2^-. In the glucose-limited chemostat culture, low dilution rates correspond to a limited supply of reducing power. Increasing the dilution rate, and hence the growth rate, would increase the supply of reducing power and thus increase the rate of reduction of streptonigrin and the rate of production of O_2^-. This would result in a lethality which increased with increasing growth rate, as shown in the rising limb of the line in Fig. 5B. Ultimately, one must reach saturation with respect to reducing power when most of the antibiotic is maintained in the reduced state. At this point, the rate of production of O_2^- would be limited by the rate of reaction of O_2 with the reduced streptonigrin. Further increases in dilution rate would then not increase the redox cycling of the antibiotic but would continue to be associated with progressive increase in the level of SOD (Fig. 2B), and the increased SOD would provide greater protection against O_2^-. Increases in dilution rate, above the point of saturation with reducing power derived from glucose, would then be associated with diminished sensitivity towards streptonigrin, and this is shown by the falling limb of the line in Fig. 5B.

ACKNOWLEDGMENTS

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