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Function and Stationary-Phase Induction of Periplasmic Copper-Zinc Superoxide Dismutase and Catalase/Peroxidase in *Caulobacter crescentus*

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Although cytosolic superoxide dismutases (SODs) are widely distributed among bacteria, only a small number of species contain a periplasmic SOD. One of these is *Caulobacter crescentus*, which has a copper-zinc SOD (CuZnSOD) in the periplasm and an iron SOD (FeSOD) in the cytosol. The function of periplasmic CuZnSOD was studied by characterizing a mutant of *C. crescentus* with an insertionally inactivated CuZnSOD gene. Wild-type and mutant strains showed identical tolerance to intracellular superoxide. However, in response to extracellular superoxide, the presence of periplasmic CuZnSOD increased survival by as much as 20-fold. This is the first demonstration that periplasmic SOD defends against external superoxide of environmental origin. This result has implications for those bacterial pathogens that contain a CuZnSOD. *C. crescentus* was shown to contain a single catalase/peroxidase which, like *Escherichia coli* KatG catalase/peroxidase, is present in both the periplasmic and cytoplasmic fractions. The growth stage dependence of *C. crescentus* catalase/peroxidase and SOD activity was studied. Although FeSOD activity was identical in exponential- and stationary-phase cultures, CuZnSOD was induced nearly 4-fold in stationary phase and the catalase/peroxidase was induced nearly 100-fold. Induction of antioxidant enzymes in the periplasm of *C. crescentus* appears to be an important attribute of the stationary-phase response and may be a useful tool for studying its regulation.

Metabolism of oxygen produces reactive and potentially toxic species. In defense against reactive oxygen, organisms contain antioxidants and enzymes that repair oxidative damage. Prominent among antioxidants are the superoxide dismutases (SODs), which catalyze the decomposition of superoxide, the first reactive species formed in the reduction of molecular oxygen (2, 11). The SODs are a family of metalloenzymes containing either iron, manganese, or both copper and zinc at the active site. Bacteria generally contain cytoplasmic Mn and FeSODs (16). A small number of gram-negative species contain a copper-zinc SOD. This is highly unusual because CuZnSOD is ordinarily found only in eukaryotes (12). In contrast to bacterial Mn and FeSODs, bacterial CuZnSOD resides in the periplasm and is absent from the cytosol (40, 45). Species containing periplasmic CuZnSOD also contain a cytosolic SOD, usually FeSOD.

CuZnSOD was originally identified in the symbiotic luminescent bacterium *Photobacterium leiognathi* (36) and subsequently found in nonsymbiotic pathogenic and nonpathogenic species. CuZnSOD genes have been cloned from *Brucella abortus*, *Caulobacter crescentus*, *Haemophilus influenzae*, *H. parainfluenzae*, *Legionella pneumophila*, and *P. leiognathi* (3, 26, 42, 45, 46). The presence of CuZnSOD in the periplasm of pathogens led to the hypothesis that it was important in defense against extracellular superoxide from the infected host. For the cattle pathogen, *B. abortus*, in vivo studies in mice with CuZnSOD mutants have led to conflicting conclusions about the role of CuZnSOD in bacterial virulence (28, 39, 48).

The importance of bacterial cytosolic Mn and FeSOD in protecting cytosolic targets from reactive oxygen damage is well established (4, 9, 10, 20, 23, 43, 47). However, little is known about the function of periplasmic CuZnSOD. To investigate CuZnSOD (SodC) function, we constructed a null mutant in *C. crescentus* (44), a nonpathogenic dimorphic pond bacterium which also contains a cytosolic FeSOD. *C. crescentus* lacking periplasmic CuZnSOD showed no decrease in aerotolerance or increased sensitivity to superoxide generated in the cytosol. This contrasted with mutants of cytosolic SODs in other species, which are nonviable aerobically or are highly sensitive to redox agents that increase superoxide in the cytosol (4, 32, 37, 43). Our results with *C. crescentus* indicated that CuZnSOD in the periplasm does not play a major role in decomposing cytosolic superoxide and suggested that CuZnSOD and FeSOD decompose superoxide originating from external and intracellular sources, respectively (44). This scenario is consistent with evidence that superoxide generated in the cytosol does not pass through the inner membrane and therefore would not be scavenged by a periplasmic CuZnSOD (17).

In the present study we directly demonstrated the involvement of periplasmic *C. crescentus* CuZnSOD in defense against environmental superoxide. In addition, we identified a single catalase/peroxidase in *C. crescentus* and demonstrated increases in the activity of it and the periplasmic CuZnSOD in the transition from exponential- to stationary-phase growth.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. $SodC^+$ and $SodC^-$ strains were, respectively, wild-type *C. crescentus* CB15 and the isogenic *sodC*::*cat* mutant, strain SGC100 (44). Media were PYE complex medium (peptone, yeast extract, 0.8 mM $MgSO_4$, 0.5 mM $CaCl_2$), M2 minimal medium (0.3% glucose, 2

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mM phosphate with 0.5 mM each Mg^{2+} and Ca^{2+}), and PIPES minimal medium (0.2% glucose, 0.2% glutamate, 0.2 mM phosphate with 0.5 mM each Mg^{2+} and Ca^{2+}) (45). Swarm plates for measurement of mobility contained M2 salts, 0.05% glucose, no Ca^{2+} and the indicated concentrations of Mg^{2+} and citrate in 0.2% agar (22). All culturing was at 30°C.

Cell extracts and cell fractionation. Cell pellets were disrupted by sonication and supernatants stored at −20°C (20, 41). Periplasm and cytoplasm fractions were prepared by spheroplasting. Cells from PYE medium were resuspended to 50 optical density units per ml in Tris and treated with lysozyme-EDTA for 30 to 40 min (45, 49). Spheroplasting was stopped by the addition of $MgCl_2$ and $CaCl_2$ to 20 mM each. Isocitrate dehydrogenase and CuZnSOD were used as markers for cytoplasm and periplasm, respectively. By limiting spheroplast formation to 65 to 70%, lysis of spheroplasts was kept to less than 15%. For cultures grown in PIPES-low-phosphate medium, glucose 6-phosphate dehydrogenase and alkaline phosphatase were used as markers.

Enzyme assays. Isocitrate dehydrogenase was assayed by the reduction of $NADP^+$ in Tris HCl (pH 8.0)–10 mM sodium isocitrate–5 mM $MnCl_2$ (7). Glucose 6-phosphate dehydrogenase activity was measured by the glucose 6-phosphate-dependent reduction of $NADP^+$ (20, 45). One unit equals 1 μmol of $NADP^+$ reduced per min.

Peroxidase was assayed by the oxidation of 0.34 mM dianisidine in 10 mM potassium phosphate (pH 6.4)–0.17% Triton X-100–1 mM hydrogen peroxide. One unit equals 1 μmol of H_2O_2 reduced per min, with $\varepsilon_M = 11.3 \times 10^4\ M^{-1}\ cm^{-1}$ and 1:1 dianisidine:H_2O_2 (5). Catalase activity was assayed in 10 mM H_2O_2–20 mM potassium phosphate–1 mM $MgCl_2$ (pH 7.2). The decrease in H_2O_2 concentration was monitored by the aminoantipyrine-phenol method (13). One unit equals 1 μmol of H_2O_2 decomposed per min.

Total SOD activity was assayed by the inhibition of pyrogallol autooxidation at pH 8.2. One unit of activity reduces ΔA_{320} per min from 0.02 to 0.01. Contributions of CuZn and FeSOD to total activity were determined by inhibition of CuZnSOD with 1 mM KCN (20, 31, 35, 41).

Specific activity was expressed as enzyme activity units per milligram of protein, determined by the bicinchoninic acid method (38).

In situ enzyme activity stains following nondenaturing electrophoresis. SOD activity was visualized as an achromatic zone from inhibition of photochemical reduction of nitroblue tetrazolium to formazan blue (41, 45). Catalase activity was visualized via inhibition of diaminobenzidine oxidation by horseradish peroxidase-H_2O_2 (6) and peroxidase activity was visualized by the catalase protocol with horseradish peroxidase omitted (14).

Uptake of ^{3}H-leucine. Uptake of L-[4,5-^{3}H]leucine (150 Ci/mmol) was measured in cultures grown to mid-log phase (optical density at 600 nm [OD_{600}] = 0.3 to 0.6) in PYE without or with sodium citrate (44). Cells were washed and resuspended in 1× M2 salts. Since magnesium and calcium reverse the sensitivity of *C. crescentus* to citrate, those salts were omitted from the M2 wash solution, so the effect of citrate could be determined. Rates of uptake were determined from the initial 3 min.

Exposure to extracellular superoxide. Extracellular superoxide was generated by the action of xanthine oxidase on hypoxanthine (15, 29). Overnight cultures in complex or minimal medium (PYE or PIPES) were washed and suspended in 1× M2 salts without Ca^{2+} or Mg^{2+}. The reaction mixture contained ≈10^4 cells per ml in 20 mM sodium phosphate (pH 7.8)–0.1 mM hypoxanthine, with 1 U of catalase (Boehringer) per ml to decompose H_2O_2 formed by xanthine oxidase and ensure that observed effects are attributable to superoxide. Superoxide production was initiated by the addition of xanthine oxidase (Sigma, Grade III) to a final concentration of 0.02 to 0.07 U (15 to 45 μg) per ml. Aliquots were diluted into 3 ml of top agar and plated on PYE plates for determination of survival.

RESULTS AND DISCUSSION

Effects of varying divalent metal ions. A $SodC^-$ mutant of *C. crescentus*, lacking periplasmic CuZnSOD and containing cytoplasmic FeSOD, was previously constructed in this laboratory (44). Reasoning that periplasmic SOD may protect periplasmic or membrane targets, we compared functions of the cell envelope in $SodC^+$ and $SodC^-$ strains. No differences were found in phage plaquing, uptake of leucine, or tolerance to hyperosmotic stress under standard culture conditions. However, the $SodC^-$ strain showed an increased calcium and/or magnesium requirement for optimal growth when the concentration of divalent cations was reduced in the medium or when citrate was added as a divalent cation chelator (44). In the present study, the effect of divalent cations was studied in more detail. PYE medium was constituted with decreasing amounts of either magnesium or calcium chloride. Comparisons were made to PYE with 0.5 mM Mg^{2+} and Ca^{2+}. Since nutrients in PYE contain some Mg^{2+} and Ca^{2+}, cited concentrations are nominal, not absolute.

TABLE 1. Dependence of doubling time on citrate concentration[a]

Citrate (mM)	Doubling time (min)	
	$SodC^+$	$SodC^-$
0	105	103
1.1	103	108
1.4	105	103
1.6	112	174
2.2	240	460

[a] PYE medium with the indicated concentration of citrate.

With calcium as the sole added divalent metal, no change in doubling time was seen for the $SodC^-$ strain from 0.5 to 0.025 mM Ca^{2+}. However, with Mg^{2+} as the sole added divalent metal, growth rate of the $SodC^-$ strain was not sustained and doubling time was about 30% higher at 0.025 mM Mg^{2+}. For $SodC^+$ *C. crescentus*, Mg^{2+} or Ca^{2+} added individually was as effective as both and doubling time was unchanged over the above concentration range. These data suggested that magnesium-dependent processes might be important cellular targets protected by periplasmic CuZnSOD, e.g., Mg^{2+} transport or reactions of MgATP.

CuZn and FeSOD activities were determined over a wide range of cation concentrations for exponential cultures of the $SodC^+$ strain. In PYE medium, CuZn and FeSOD activities are approximately equal (CuZn:FeSOD = 0.8 to 1.0). Reducing Mg^{2+} and Ca^{2+} concentrations 20-fold to 0.025 mM had no effect on total SOD activity or the CuZnSOD:FeSOD ratio. Increasing 20-fold to 10 mM cation was also without effect on the ratio of CuZn:FeSOD although total SOD activity was reduced 10 to 40%. In sum, there was no evidence of preferential induction of CuZnSOD in response to changes in Mg^{2+} or Ca^{2+} concentration in complex medium.

Effect of citrate on cell growth and function. We previously observed that the plating efficiency of *C. crescentus* on complex medium was reduced 5 to 6 orders of magnitude by 2 to 4 mM sodium citrate (44). Addition of Mg^{2+} or Ca^{2+} but not Co^{2+}, Cu^{2+}, Fe^{2+}, or Zn^{2+} reversed the effect of citrate. (In the present study, we found that 1 mM strontium chloride also rescues *C. crescentus* from 4 mM sodium citrate, increasing plating efficiency on PYE medium from 0.001 to 110%.) Sensitivity to citrate was greater for the $SodC^-$ strain, consistent with its increased Mg^{2+}/Ca^{2+} requirement for optimal growth in minimal medium. Evidently *C. crescentus* is sensitive to citrate because available divalent cations are reduced by chelation (8).

In the present study, citrate was used to implicate Mg^{2+}/Ca^{2+}-dependent processes that involve periplasmic CuZn SOD. $SodC^+$ and $SodC^-$ strains were compared in mobility, uptake of leucine, and response to hyperosmotic stress. The doubling time data in Table 1 demonstrate that the $SodC^-$ strain is more sensitive to citrate in PYE liquid medium over the same range (1.5 to 2 mM citrate) as on plates (44).

No differential effect on motility of $SodC^+$ and $SodC^-$ strains was seen on decreasing Mg^{2+} concentration from 0.5 to 0.05 mM or adding 1.1 or 1.6 mM citrate to swarm plates. Uptake of leucine, as a representative organic nutrient, behaved similarly. Rates of uptake were comparable for $SodC^+$ and $SodC^-$ in the absence of citrate and there was no differential effect at 1.1 and 1.6 mM citrate. Thus, the increased requirement for Mg^{2+}/Ca^{2+} in the $SodC^-$ strain did not have major effects on cell motility or leucine transport.

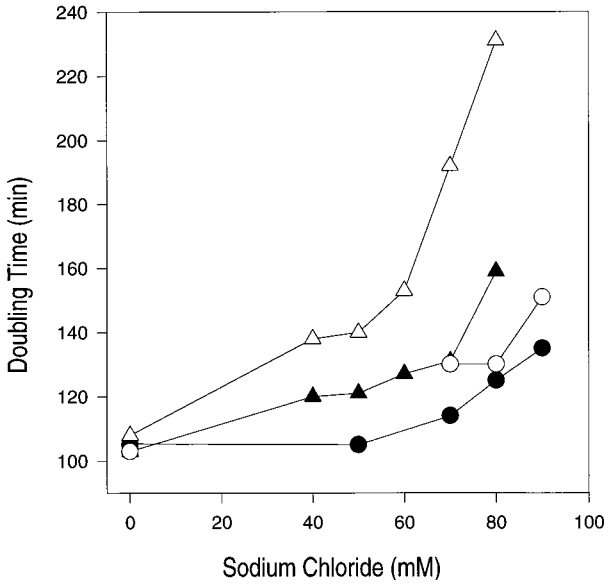

FIG. 1. Effect of hyperosmolarity on growth rate. Cultures were outgrown for 2 h in PYE and then NaCl was added to the indicated concentration for determination of doubling time. When present, citrate was added 2 h after the 2-h outgrowth and then NaCl was added 2 h later. Circles, no citrate; triangles, 1.1 mM sodium citrate; filled symbols, SodC$^+$; open symbols, SodC$^-$.

Differences were seen in tolerance to hyperosmotic stress. *C. crescentus* is sensitive to sodium chloride and shows increases in doubling time when 70 to 90 mM NaCl is added to PYE (Fig. 1). By comparison, complex media for *E. coli* contain 85 to 170 mM NaCl. With 1.1 mM citrate substantial differences were seen in the effect of NaCl on doubling times of SodC$^+$ and SodC$^-$ strains, even though this citrate concentration had no effect in the absence of NaCl (Table 1).

The general robustness of the SodC$^-$ mutant and its maintenance of plaquing, leucine uptake, and motility suggest that periplasmic CuZnSOD is not involved in global protection of inner or outer membranes against lipid peroxidation. In addition, major alteration to periplasmic proteins was not evident on comparing periplasmic extracts by sodium dodecyl sulfate acrylamide gel electrophoresis. Rather, our data on the effect of Mg^{2+} on growth and on tolerance to NaCl suggest that periplasmic CuZnSOD may be critical for specific processes. As more is learned about magnesium homeostasis and maintenance of osmotolerance in *C. crescentus*, the membrane and/or periplasmic targets protected by CuZnSOD may be identified.

Periplasmic CuZnSOD and defense against extracellular superoxide. We previously showed that SodC$^+$ and SodC$^-$ strains of *C. crescentus* are equally sensitive to paraquat, a redox cycling drug that produces superoxide in the cytosol. In the present study, it was shown that cytosolic superoxide produced from paraquat does not alter CuZnSOD activity although activity of cytosolic FeSOD was decreased by 30 to 40% (Table 2). Clearly periplasmic CuZnSOD is not a defense against cytoplasmic superoxide. To evaluate its role in defense against extracellular superoxide, superoxide radical was generated by xanthine oxidase acting on purines. Killing curves for cultures grown in minimal medium show similar initial rates and then a 10-fold greater survival for SodC$^+$ after about 15 min (Fig. 2). A nearly fourfold greater survival was found for SodC$^+$ *C. crescentus* after growth in complex medium in the presence of 1.1 mM citrate (Fig. 3). A survival increase of

TABLE 2. Effect of paraquat on SOD activity[a]

SOD	SOD activity[b] at paraquat concn		
	0	0.2	0.5
CuZnSOD	110	104	130
FeSOD	100	56	70
CuZn/FeSOD	1.1	2.0	1.9
Total SOD	210	160	200

[a] SodC$^+$ *C. crescentus* was grown to early log phase in PYE, and then paraquat was added and cells were harvested 5 h later. Compared to a control culture without paraquat, doubling times in 0.2 and 0.5 mM paraquat were increased by 60 and 70%, respectively. Values are the averages of two or three independent cultures.

[b] SOD activity units per milligram of protein.

≈20-fold was found for SodC$^+$ after growth in complex medium with 1.4 mM citrate (data not shown). Doubling times in complex medium are identical for SodC$^+$ and SodC$^-$ strains in 1.1 and 1.4 mM citrate (Table 1). To our knowledge, this is the first direct demonstration that periplasmic CuZnSOD defends against extracellular superoxide.

The xanthine oxidase system required resuspension of cells in buffer to observe significant rates of killing. To challenge with external superoxide in complex medium, we chose pyrogallol, 1,2,3-trihydroxybenzene, whose spontaneous autooxidation to generate superoxide radical is the basis for an SOD assay (31). It was unknown whether pyrogallol would remain external to *C. crescentus* and exclusively generate superoxide extracellularly or whether it could be internalized and generate cytosolic superoxide as well. We reasoned that even if pyrogallol is internalized and autooxidizes in the cytosol, differences in killing would be indicative of periplasmic CuZnSOD, because SodC$^+$ and SodC$^-$ strains have comparable activities of FeSOD in the cytosol (data not shown).

The results of using pyrogallol as a source of superoxide in complex medium are shown in Fig. 4. SodC$^-$ *C. crescentus* was

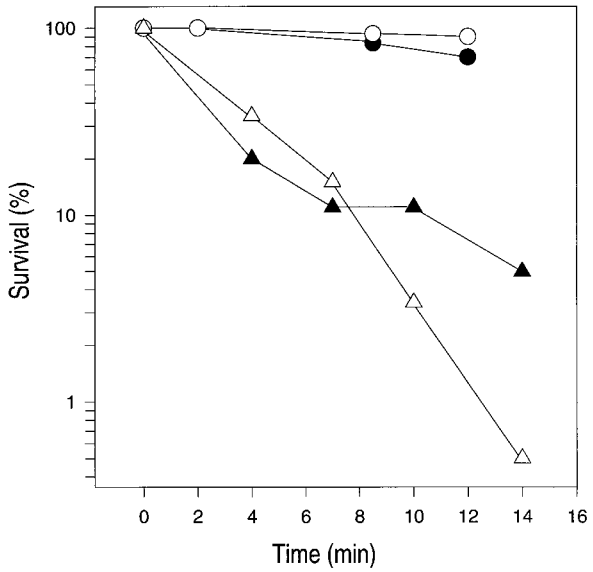

FIG. 2. External superoxide exposure of cultures grown in minimal medium. Cultures were grown overnight in PIPES medium and then resuspended in buffer and exposed to superoxide from xanthine oxidase acting on hypoxanthine. After the indicated times, aliquots were plated for survival on PYE plates. Circles, no superoxide; triangles, superoxide added; filled symbols, SodC$^+$; open symbols, SodC$^-$.

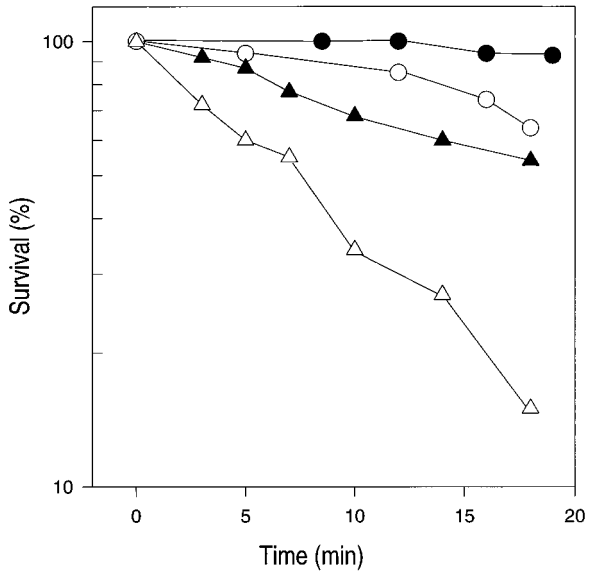

FIG. 3. External superoxide exposure of cultures grown in complex medium with citrate. Cultures were outgrown in PYE containing 1.1 mM sodium citrate to OD_{600} = 0.8 and then exposed to external superoxide and plated as described in the legend to Fig. 2. Symbols are as in Fig. 2.

more sensitive to pyrogallol by 2- to 20-fold in exponential and early stationary phase, at two pyrogallol concentrations. These data corroborate results with xanthine oxidase as a generator of external superoxide. Data from the two methods argue strongly that periplasmic CuZnSOD plays an important role in defense against extracellular superoxide.

External superoxide is likely of environmental origin. Although *C. crescentus* is not a pathogen and does not encounter superoxide from respiratory burst activity of host cells, it may be exposed to reactive oxygen in its natural environment, freshwater ponds. Plant-derived phenols and polyphenols, such as tannic acid, are found in ponds. Polyphenols are capable of generating superoxide radical by autooxidation, an example being the triphenol, pyrogallol. In addition, *C. crescentus* adheres to blue-green algae (cyanobacteria) (1, 27) and during daylight encounters high local concentrations of oxygen from algal photosynthesis. The natural environment of *C. crescentus*, containing high $[O_2]$ and compounds that can react with O_2 to generate superoxide, provides a rationale for its periplasmic CuZnSOD. Pyrogallol may thus mimic superoxide-generating toxins encountered by *C. crescentus* in nature.

Growth stage-dependent expression of CuZnSOD. Caulobacters, adapted to survive in nutrient poor environments, are expected to spend prolonged periods in a nongrowing state. However, little is known about changes in gene expression on entrance and exit from stationary phase. SOD activities were determined in $SodC^+$ *C. crescentus* for exponential and 20-h cultures in complex medium. CuZnSOD activity increased 3.7-fold in stationary phase while FeSOD activity was unchanged (Table 3). Thus in the transition from exponential to stationary phase the ratio of periplasm:cytoplasm SOD activity increases nearly fourfold.

Growth stage-dependent expression and intracellular localizations of catalase. Although catalase activity has been demonstrated in *C. crescentus* colonies (34), little is known about the enzyme(s). Activity stains for catalase and peroxidase identified the same closely spaced doublet in nondenaturing gels (Fig. 5). *C. crescentus* appears to contain a single enzyme with both catalatic and peroxidatic activity. The explanation for catalase/peroxidase isozymes is unknown. The KatG catalase/peroxidase of *E. coli* shows a similar doublet (21).

Peroxidase assays showed a 9-fold increase in specific activity after 24 h, compared to exponential *C. crescentus* cultures, and a ≈100-fold increase after 50 to 70 h (Table 4). (Assaying the catalase activity by disappearance of H_2O_2 revealed comparable increases in the transition from exponential growth to stationary phase.) On activity gels, exponential-phase catalase had the same mobility as that in Fig. 5. All cultures were homogeneous in colony morphology, eliminating the possibility that the activity increase in stationary phase was from a slowly growing contaminant. This stationary-phase induction of *C. crescentus* catalase/peroxidase differs from the well known induction of *E. coli* KatE catalase in two ways. First, the mag-

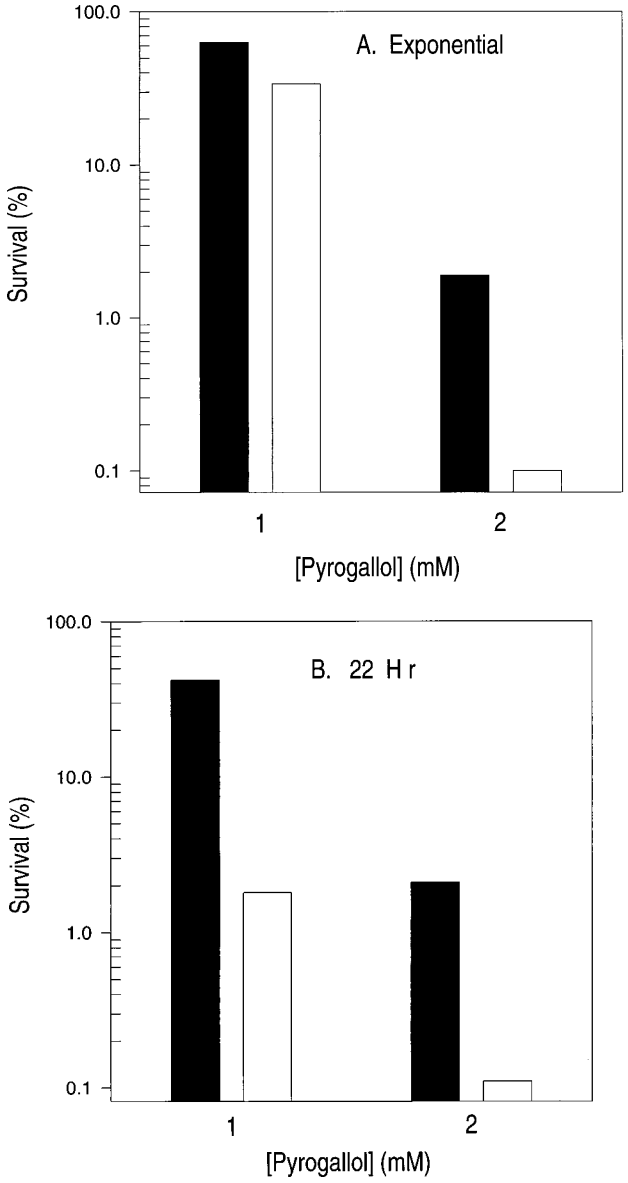

FIG. 4. Pyrogallol killing. Cultures in PYE medium were either treated directly with pyrogallol (panel B) or diluted and outgrown in PYE to OD_{600} of 0.2 to 0.3 (panel A) before treatment. After 1 h at 30°C with the indicated pyrogallol concentration, survival was determined by plating on PYE. Filled bars, $SodC^+$; open bars, $SodC^-$.

TABLE 3. Growth stage-dependent expression of SOD activity

SOD	SOD activity[a]	
	Exponential[b]	20 h
Total SOD	180	430
FeSOD	91	92
CuZnSOD	93	340
CuZn/FeSOD	1.0	3.7

[a] SOD activity in units per milligram of protein from growth in PYE medium.
[b] Mid-to-late exponential phase, $OD_{600} \approx 0.5$ to 0.7, about 7 h after dilution of an overnight culture into fresh medium.

TABLE 4. Growth stage-dependent expression of peroxidase activity[a]

Growth (h)	Peroxidase activity	
	Specific[b]	Relative
6 (log)	1.7×10^{-4}	1.0
24	1.6×10^{-3}	9 ± 2
55	1.6×10^{-2}	92 ± 13
66	1.6×10^{-2}	95 ± 8

[a] Sonic extracts were prepared from *C. crescentus* grown in rich medium (PYE, doubling time ≈ 100 min). Cultures were started at approximately the same OD and grown for the indicated time. Peroxidase activity was determined by the dianisidine assay.
[b] Micromoles of H_2O_2 reacted per minute per milligram of protein.

nitude is much greater: 100-fold compared to ≈6-fold for *E. coli* KatE in LB and smaller increases in minimal media (30). Secondly, the enzyme induced in *E. coli* stationary phase is exclusively a catalase and is a catalase/peroxidase in *C. crescentus*. Similar to *E. coli*, resistance of *C. crescentus* to killing by H_2O_2 increased dramatically in stationary phase (Fig. 6). This resistance cannot be uniquely attributed to increased *C. crescentus* catalase/peroxidase, as other enzymes are also induced in stationary phase.

The intracellular localization of the catalase/peroxidase was evaluated by spheroplasting. In a 40-h stationary-phase culture of wild-type *C. crescentus* an equal distribution was found by dianisidine assay: 49 ± 6% and 51 ± 6% in periplasm and cytosol, respectively. The isozymes seen on the activity gel did not appear to be differentially compartmentalized; both were found in each compartment. *E. coli* KatG catalase/peroxidase is similarly found in periplasmic and cytosolic fractions, being partially periplasmic and associated with the outer surface of the inner membrane (18). It will be of interest to determine if the distribution between cytosol and periplasm is growth stage dependent and whether *C. crescentus* catalase/peroxidase is induced by H_2O_2 like *E. coli* KatG.

Summary and significance. CuZnSODs have been found in pathogens of humans (*H. parainfluenzae* and *L. pneumophila*) and domestic cattle (*B. abortus*). A potential role in bacterial virulence has been suggested since these CuZnSODs reside in the periplasm and are positioned for defense against host-derived superoxide. Our in vitro studies with a CuZnSOD mutant of *C. crescentus* clearly show that periplasmic CuZnSOD is critical in defense against external superoxide, suggesting that CuZnSODs in pathogens may be important for virulence.

Although the stationary-phase response has been well characterized in *E. coli* and to a lesser degree in *Vibrio* spp., *Salmonella typhimurium*, and *Pseudomonas putida*, the phenomenon has not been widely characterized (19, 24, 25). Analysis of the stationary-phase response of *C. crescentus* is of interest because caulobacters are likely in a nondividing state for prolonged intervals and because alterations in the cell cycle occur in stationary phase (34). Development of cellular resistance to oxidative stress is a classic trait of the stationary phase. In this study, resistance to hydrogen peroxide was demonstrated and stationary-phase induction of two antioxidant enzymes was

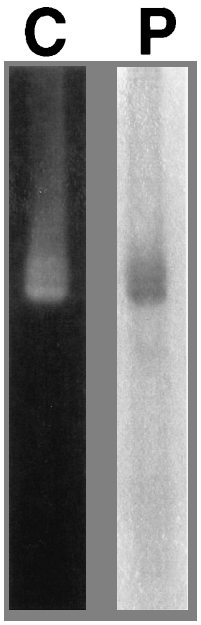

FIG. 5. Analysis for catalase and peroxidase activity in cell extracts. Extracts from wild-type *C. crescentus*, grown for 55 h in PYE medium, were electrophoresed on a polyacrylamide gel under nondenaturing conditions. Identical aliquots (270 μg of protein) were stained for catalase (C) or peroxidase (P) activity.

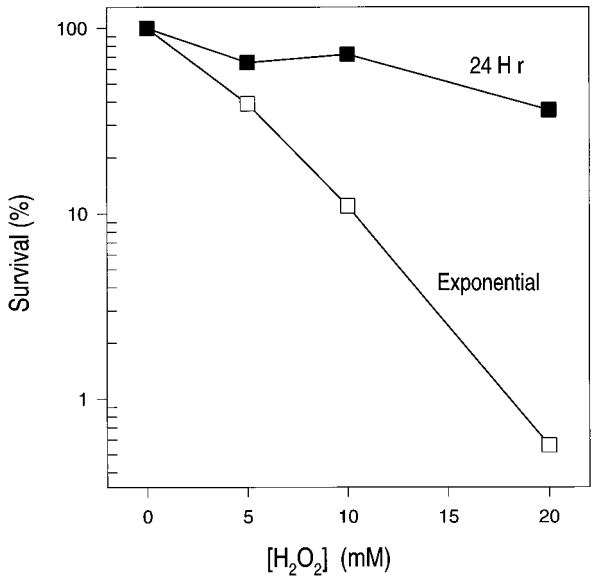

FIG. 6. Hydrogen peroxide killing. Wild-type *C. crescentus* was grown 22 h in PYE medium, treated with the indicated concentration of H_2O_2 for 15 min at 30°C, and then plated on PYE medium after addition of bovine catalase to 2 μg/ml. For log-phase killing, cultures were grown to $OD_{600} = 0.2$ to 0.4 and then resuspended in fresh PYE medium to the same concentration as the 22-h culture ($OD_{600} = 1.2$). Exposure to H_2O_2, termination of the reaction, and plating for survival were as for the 22-h culture.

identified: the periplasmic CuZnSOD and a previously uncharacterized catalase/peroxidase found in both periplasm and cytosol. The nearly 100-fold induction of catalase/peroxidase should be useful for identifying genes controlling entrance into stationary phase. Experiments to clone the *C. crescentus* catalase/peroxidase gene and identify homolog(s) to *E. coli rpoS* are underway.

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