Cloned manganese superoxide dismutase reduces oxidative stress in *Escherichia coli* and *Anacystis nidulans*

(shuttle vectors/paraquat/activated oxygen/photobleaching)

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ABSTRACT The Mn superoxide dismutase gene of *Escherichia coli* was subcloned into the *E. coli*–*Anacystis nidulans* shuttle vector pSG111 to make the plasmid pMYG1. Transformation of *E. coli* HB101 with pMYG1 resulted in a 6-fold increase in superoxide dismutase activity. There was also induction of Mn superoxide dismutase in the transformants upon exposure to paraquat, as evidenced by dramatically increased levels of the Mn superoxide dismutase polypeptide in cytoplasmic extracts and a 16-fold further increase in superoxide dismutase activity. As well, the *E. coli* transformants showed resistance to paraquat-mediated inhibition of growth. *Anacystis nidulans*, a cyanobacterium that has no detectable Mn superoxide dismutase and is, consequently, very sensitive to oxidative stress, was also transformed with pMYG1. The transformants had detectable levels of Mn superoxide dismutase protein and showed resistance to paraquat-mediated inhibition of growth and photobleaching of pigments. Paraquat is known to promote formation of the superoxide radical anion, $O_2^{\cdot-}$, and thus the data have been interpreted as indicating that the cloned Mn superoxide dismutase provides protection in both *E. coli* and *A. nidulans* against damage attributable to $O_2^{\cdot-}$.

Escherichia coli exhibits symptoms of oxidative stress when exposed to redox cycling agents such as paraquat (1) and certain photoactivated dyes (2). Protection against oxidative stress has been correlated with the presence of Mn superoxide dismutase. For example, expression of the Mn superoxide dismutase gene in *E. coli* is induced during exposure to redox cycling agents, whereas the gene for Fe superoxide dismutase is expressed constitutively (3). Indeed, levels of the Fe superoxide dismutase protein decline during oxidative stress (4), and the enzyme is inactivated by H_2O_2 (5). The protective role attributed to Mn superoxide dismutase in the event of oxidative stress is further substantiated by the finding that the mutation rate under normal aerobic conditions for a Mn superoxide dismutase-minus mutant of *E. coli* was 9-fold higher than that for the corresponding wild type or for an Fe superoxide dismutase-minus mutant (6).

Cyanobacteria experience photooxidative stress when exposed to high light intensities (7, 8) or paraquat (9). Manifestations of these stresses include a reduction in growth rate and pigment photobleaching. Photooxidative injury in cyanobacteria is intensified when synthesis of superoxide dismutase and catalase is inhibited, suggesting that these enzymes provide protection against the effects of oxidative stress (8, 10). Some cyanobacteria, such as *Anacystis nidulans*, are particularly sensitive to photooxidative stress because they possess only Fe superoxide dismutase. This sensitivity is reduced in other cyanobacteria when Fe superoxide dismutase

as well as the more H_2O_2 -resistant Mn superoxide dismutase are present (11).

In the present study, the prospect that increased tolerance to oxidative stress can be achieved by genetic manipulation of Mn superoxide dismutase was examined by subcloning the *E. coli* Mn superoxide dismutase gene into an *E. coli*–*A. nidulans* shuttle vector and introducing this new vector into both *E. coli* and *A. nidulans*. The resulting transformants were tested for resistance to oxidative stress induced by exposure to paraquat.

MATERIALS AND METHODS

Culture Conditions and Plasmid Construction. *Anacystis nidulans* SPC (the R2 strain cured of pANS; ref. 12) was obtained from N. Straus (Department of Botany, University of Toronto) and was grown at 30°C in BG11 medium with 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ [1 einstein (E) = 1 mol of photons] continuous cool white illumination (13). *E. coli* HB101 was grown at 30°C in YT medium (14). The plasmid pDT1-5, which contains the *E. coli* Mn superoxide dismutase gene (15), was obtained from D. Touati (Institut Jacques Monod, Centre National de la Recherche Scientifique, Université de Paris), and the *E. coli*–*A. nidulans* shuttle vector pSG111 (16) was provided by L. Sherman (Division of Biological Sciences, University of Missouri).

Plasmid pMYG1 was constructed by ligating pSG111, which had been completely digested with *Eco*RI and partially digested with *Bam*HI, with an *Eco*RI–*Bam*HI fragment from pDT1-5 that contained the *E. coli* Mn superoxide dismutase gene (15) and transforming *E. coli* HB101 with the ligation mixture. pMYG1 was selected as an ampicillin-resistant, chloramphenicol-sensitive colony that showed increased superoxide dismutase activity. By contrast, both *E. coli* and *A. nidulans* transformants containing pSG111 (16) were ampicillin-resistant and chloramphenicol-resistant. *E. coli* HB101 was transformed with pMYG1 or pSG111 as described by Maniatis *et al.* (17), and *A. nidulans* was transformed with the same plasmids according to Gendel *et al.* (12). Plasmid DNA was isolated from *E. coli* according to Maniatis *et al.* (17) and from *A. nidulans* as described by Daniell *et al.* (18). Restriction fragment analyses were carried out as described (17).

Hybridization. Restriction enzyme-digested DNA was fractionated by agarose gel electrophoresis, and the fragments were denatured and electroblotted onto Biotrans nylon membranes (ICN) for 4.5 hr at 4°C in 25 mM sodium phosphate (pH 6.5) (19). The membranes were air-dried and baked at 80°C. The blots were hybridized (17) with a 1.1-kilobase *Ava* I DNA fragment from plasmid pDT1-5 containing the *E. coli* Mn superoxide dismutase gene (20). The DNA

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probe was radiolabeled with [α - ^{32} P]dCTP by using a random oligonucleotide-primed labeling kit from Pharmacia (21).

Cell-Free Synthesis of Plasmid-Encoded Proteins. Protein synthesis in an *E. coli* C600 cell-free system (22) was programmed with plasmid DNA. The reaction mixtures (50 μ l) contained, in addition to buffer, salts, tRNAs, an ATP-generating system, and *E. coli* C600 cell-free extract (12.5 μ g/ μ l), 7 μ Ci of [^{35} S]methionine (800 Ci/mmol; 1 Ci = 37 GBq) and 0.5–3.0 μ g of plasmid DNA. After 40 min of reaction at 37°C, protein was precipitated with cold 5% trichloroacetic acid and analyzed by SDS/PAGE in a 10–20% gradient polyacrylamide gel with a discontinuous buffer system (23). Protein was measured according to Bradford (24).

Cell Fractionation. Cells (200 ml) of control *E. coli* cultures or cultures grown in the presence of paraquat (0.1–8 mM) were sedimented by centrifugation at 8000 $\times$ g, resuspended in 1 ml of 10 mM potassium phosphate (pH 7.0), and lysed by sonication on ice (five 20-s bursts with 1-min intervening cooling periods with a Branson Sonifier cell disrupter). The lysate was centrifuged for 30 min at 4°C in an Eppendorf centrifuge. The supernatant was assayed for superoxide dismutase activity (25), and the membrane and supernatant fractions were analyzed by SDS/PAGE in 10–20% gradient gels (23).

A. nidulans cells were plated onto nylon membranes (diameter, 100 mm) and grown on solid BG11 medium [containing ampicillin (1 μ g/ml) if the cells had been transformed with pMYG1 or pSG111] for 4 days at 30°C in 100 μ E·m $^{-2}$ ·s $^{-1}$ light. The filters were then transferred either to fresh solid BG11 medium or to solid BG11 medium containing 10, 100, or 1000 nM paraquat and the cells were grown at 30°C for 2 days in 100 μ E·m $^{-2}$ ·s $^{-1}$ light. Cells scraped from six to eight filters were resuspended in 2 ml of lysozyme (1 mg/ml) in 10 mM potassium phosphate (pH 7.0), lysed by sonication on ice as for *E. coli*, and centrifuged for 30 min at 4°C in an Eppendorf centrifuge. The supernatant was analyzed by SDS/PAGE in a 10–20% gradient gel (23) or assayed for superoxide dismutase activity (25).

RESULTS

The *E. coli* gene for Mn superoxide dismutase was introduced into both *E. coli* and *A. nidulans* in the *E. coli*–*A. nidulans* shuttle vector pMYG1. Plasmid pMYG1 was constructed by ligating the *E. coli* Mn superoxide dismutase gene from pDT1-5 into the shuttle vector pSG111. Published maps of pDT1-5 (15) and pSG111 (16) together with *Eco*RI, *Bam*HI, and *Xho*I restriction fragment analyses of pMYG1, pDT1-5, and pSG111 and the results of cell-free translations of pMYG1 fragments were used to deduce a restriction endonuclease map for pMYG1 (Fig. 1). *Eco*RI restriction fragment patterns for pMYG1 isolated from *E. coli* HB101/pMYG1 and *A. nidulans* SPC/pMYG1 were identical, indicating that the plasmid is not subject to rearrangement in *A. nidulans*. The isolated plasmids also hybridized strongly to the *E. coli* Mn superoxide dismutase gene probe. Furthermore, pMYG1 from either *E. coli* HB101/pMYG1 or *A. nidulans* SPC/pMYG1 and the plasmid pDT1-5 directed the synthesis of the 20.5-kDa Mn superoxide dismutase polypeptide when used to program protein synthesis in an *E. coli* cell-free system (Fig. 2). pMYG1 also programmed the synthesis of β -lactamase (29.5 kDa) but not the 21.3-kDa chloramphenicol acetyltransferase polypeptide encoded by the parent shuttle vector pSG111 (Fig. 2). The 27.4-kDa β -lactamase cleavage product was also detectable in some gels (Fig. 2B).

Superoxide dismutase specific activity was 6- to 7-fold higher in *E. coli* HB101/pMYG1, which contained the cloned Mn superoxide dismutase gene, than in HB101/pSG111. When cells of either transformant were exposed to paraquat

FIG. 1. Restriction endonuclease map of pMYG1. Thick line, *A. nidulans* plasmid DNA; thin line, *E. coli* plasmid DNA; gray bar, *E. coli* chromosomal DNA containing the Mn superoxide dismutase (MnSOD) gene; Amp ^{r} , ampicillin-resistance gene; Ori E, *E. coli* replication origin; Ori A, *A. nidulans* replication origin; kb, kilobases.

(0.5–5.0 mM), the activity of the enzyme rose 10- to 100-fold, although HB101/pMYG1 cells always displayed a higher level of superoxide dismutase activity than HB101/pSG111 cells (Table 1). SDS/PAGE analysis of cytoplasmic proteins confirmed that the increased superoxide dismutase activity upon exposure to paraquat reflected induction of Mn superoxide dismutase. The 20.5-kDa Mn superoxide dismutase polypeptide was induced in both *E. coli* HB101/pSG111 and HB101/pMYG1 during a 9-hr exposure to 0.5 mM paraquat (Fig. 3, lanes 2, 4, 6, and 8), and for HB101/pMYG1 became the major cytoplasmic protein as a result of the paraquat treatment (Fig. 3, lane 8). By contrast, Mn superoxide dismutase was not induced in *A. nidulans* SPC/pMYG1 during treatment with paraquat. The 20.5-kDa polypeptide corresponding to Mn superoxide dismutase was not detect-

FIG. 2. Autoradiograms showing cell-free expression of pMYG1. (A) pMYG1 isolated from *E. coli*. Lane 1, 50 μ g of cell-free extract, 2 μ g of pSG111; lane 2, 50 μ g of cell-free extract, 2 μ g of pMYG1 isolated from *E. coli*; lane 3, 50 μ g of cell-free extract, 2 μ g of pDT1-5; lane 4, 50 μ g of cell-free extract, no DNA. (B) pMYG1 isolated from *A. nidulans*. Lane 1, 67 μ g of cell-free extract, no DNA; lane 2, 33 μ g of cell-free extract, 2 μ g of pSG111; lane 3, 33 μ g of cell-free extract, 2 μ g of pDT1-5; lane 4, 67 μ g of cell-free extract, <0.5 μ g of pMYG1 isolated from *A. nidulans*. Arrows, Mn superoxide dismutase polypeptide. Molecular size markers (kDa) are indicated. Levels of [^{35} S]methionine incorporated into trichloroacetic acid-precipitable material in the absence of exogenous DNA were 9000 dpm in A and 40,000 dpm in B.

Table 1. Superoxide dismutase activity of transformed *E. coli* HB101

Paraquat, mM	Units per mg of cytoplasmic protein	
	HB101/pSG111	HB101/pMYG1
0.0	5.6	36.9
0.5	69.6	689
5.0	601	2841

Cells (50 ml) were grown to midlogarithmic phase of growth, lysed in 2 ml of lysozyme (4 mg/ml) in 10 mM potassium phosphate (pH 7.8), and centrifuged at $16,000 \times g$ for 10 min. The enzyme assay mixture contained 40 μ l (0.02 unit) of xanthine oxidase, 1 ml of 0.3 mM xanthine in 150 mM NaHCO_3 (pH 10.2), 0.5 ml of 0.6 mM Na_2EDTA , 0.5 ml of 0.15 mM nitroblue tetrazolium and either 1 ml of cell extract or 1 ml of 10 mM potassium phosphate, pH 7.8. The rate of reduction of nitroblue tetrazolium in the presence of cell extract was adjusted to 25–50% of the control containing no cell extract by diluting the lysates to ensure that activity was proportional to enzyme concentration. Enzyme activity was calculated in terms of inhibition of nitroblue tetrazolium reduction as described (25).

able in *A. nidulans* SPC/pSG111 (Fig. 4, lanes 1–4), and although it was discernible in *A. nidulans* SPC/pMYG1, it did not change during exposure to levels of paraquat (10–1000 nM) (Fig. 4, lanes 5–8) that caused photobleaching of pigments. Nor was there any increase in measured superoxide dismutase activity in SPC/pMYG1 following exposure to paraquat. Values ranged from 54.2 units/mg of cytoplasmic protein in the absence of paraquat to 51.7 units/mg of cytoplasmic protein in the presence of 1000 nM paraquat.

The amplified levels of Mn superoxide dismutase in *E. coli* HB101/pMYG1 provided protection against inhibition of growth in the presence of paraquat. When HB101/pSG111 and HB101/pMYG1 were grown for 11.5 hr in the presence of various concentrations of paraquat, there was no effect on the growth of either transformant at concentrations up to 0.4 mM (Fig. 5). However, as the concentration of paraquat was further increased, the inhibitory effect on the growth of HB101/pSG111, which did not contain the cloned Mn superoxide dismutase gene, became pronounced such that at 1 mM paraquat the culture density for HB101/pSG111 was only $\approx 35\%$ of that for HB101/pMYG1 (Fig. 5). This effect was also manifested in terms of growth rate. In the presence of 1 mM paraquat, the doubling time of HB101/pMYG1 was 2.5

FIG. 3. Levels of Mn superoxide dismutase polypeptide in cytoplasmic fractions of transformed *E. coli* HB101 grown in the presence or absence of paraquat. Lane 1, 15 μ g of HB101/pSG111 membrane protein, no paraquat; lane 2, 15 μ g of HB101/pSG111 cytoplasmic protein, no paraquat; lane 3, 15 μ g of HB101/pMYG1 membrane protein, no paraquat; lane 4, 15 μ g of HB101/pMYG1 cytoplasmic protein, no paraquat; lanes 5–8, same as lanes 1–4 except that cells were exposed to 0.5 mM paraquat for 9 hr and only 10 μ g of protein was applied per lane. Long arrows, Mn superoxide dismutase polypeptide; short arrow, chloramphenicol acetyltransferase polypeptide. The gel was stained with Coomassie blue. Molecular size markers (kDa) are indicated.

FIG. 4. Coomassie blue- and silver-stained gel for cytoplasmic extracts of transformed *A. nidulans* SPC exposed to paraquat for 2 days. Lane 1, SPC/pSG111, no paraquat; lane 2, SPC/pSG111, 10 nM paraquat; lane 3, SPC/pSG111, 100 nM paraquat; lane 4, SPC/pSG111, 1000 nM paraquat; lane 5, SPC/pMYG1, no paraquat; lane 6, SPC/pMYG1, 10 nM paraquat; lane 7, SPC/pMYG1, 100 nM paraquat; lane 8, SPC/pMYG1, 1000 nM paraquat. Thirty micrograms of protein was applied to each lane. Arrows, 20.5-kDa Mn superoxide dismutase polypeptide. Molecular size markers (kDa) are indicated.

hr. By contrast, HB101/pSG111 cells showed biphasic growth kinetics in the presence of 1 mM paraquat; for the initial phase of growth the doubling time was 2.5 hr, but it then increased to 12 hr.

Notwithstanding the fact that Mn superoxide dismutase was not induced in *A. nidulans* by exposure to paraquat, protection against the deleterious effects of this redox cycling agent was provided by the cloned Mn superoxide dismutase. In terms of growth, the doubling time for SPC/pSG111 in the presence of 2 μ M paraquat was 42 hr, whereas that for SPC/pMYG1 was 26 hr. The cloned Mn superoxide dismutase also conferred protection against paraquat-mediated photobleaching of pigments (Fig. 6). Nine-day-old cultures of *A. nidulans* SPC/pSG111 and SPC/pMYG1 both featured

FIG. 5. Effect of paraquat on the growth of transformed *E. coli* HB101 in liquid culture. The cells were grown for 11.5 hr. Δ , HB101/pSG111; $\circ$, HB101/pMYG1.

FIG. 6. Absorbance spectra of transformed *A. nidulans* SPC grown in the presence or absence of 10 nM paraquat for 9 days. Spectra were determined on 1-ml aliquots of culture: A, SPC/pMYG1, paraquat; B, SPC/pMYG1, no paraquat; C, SPC/pSG111, no paraquat; D, SPC/pSG111, paraquat.

prominent absorption peaks at 450, 630, and 660 nm (spectra B and C). When *A. nidulans* SPC/pMYG1 was grown for 9 days in the presence of 10 nM paraquat, these absorption peaks remained intact (spectrum A). However, when *A. nidulans* SPC/pSG111, which did not have a cloned Mn superoxide dismutase gene, was exposed to paraquat under the same conditions, the absorption peaks at 450 nm and 630 nm in particular were reduced (spectrum D), indicating that there had been photobleaching of pigments.

DISCUSSION

Several lines of evidence indicate that the integrity of pMYG1 is retained when it is introduced into *A. nidulans* SPC. *EcoRI* restriction patterns of the plasmid isolated from *E. coli* HB101/pMYG1 or from *A. nidulans* SPC/pMYG1 are identical, and the plasmid isolated from either transformant hybridizes strongly to a Mn superoxide dismutase gene probe. As well, plasmid isolated from either transformant directs the synthesis of the 20.5-kDa Mn superoxide dismutase polypeptide when used to program an *E. coli* cell-free system, and the same protein is induced when *E. coli* HB101/pMYG1 is exposed to paraquat and synthesized as a new polypeptide in *A. nidulans* SPC/pMYG1. The Mn superoxide dismutase gene that was used in the construction of pMYG1 was obtained from the plasmid pDT1-5 (15), which also directed synthesis of the 20.5-kDa Mn superoxide dismutase polypeptide in the cell-free system. Immunological confirmation of the identity of this polypeptide was obtained previously (15). The stability of pMYG1 in *A. nidulans* SPC/pMYG1 is of particular significance in light of a previ-

ous report (26) of plasmid instability and rearrangement in *A. nidulans*. In addition to antibiotic-resistance genes, two other genes, the *E. coli lacZ* gene and the gene for the allophycocyanin apoprotein from *Cyanophora paradoxa*, have also been stably introduced into cyanobacteria on plasmid vectors (12, 16, 27-29).

Paraquat is a redox cycling agent that is known to generate O_2^- (30), and the fact that Mn superoxide dismutase was strongly induced when *E. coli* HB101/pMYG1 was exposed to paraquat indicates that the upstream regulatory region of the subcloned gene is functional in pMYG1. This notwithstanding, there was no induction of the enzyme when *A. nidulans* SPC/pMYG1 was exposed to paraquat. This suggests that the transcription and translation signals for the Mn superoxide dismutase gene in pMYG1 may not be well recognized in *A. nidulans*. Even in the absence of paraquat, Mn superoxide dismutase was less strongly expressed in *A. nidulans* SPC/pMYG1 than in *E. coli* HB101/pMYG1. This has been noted previously in relation to the expression in cyanobacteria of *E. coli* drug-resistance genes subcloned into *E. coli*-cyanobacterial shuttle vectors (16, 29).

Of particular interest is the finding that the presence of the cloned Mn superoxide dismutase confers protection against paraquat-mediated oxidative stress in both *E. coli* and *A. nidulans*. Specifically, in *E. coli* HB101/pMYG1, the amplified levels of the Mn superoxide dismutase polypeptide correlated with enhanced growth tolerance to paraquat. Hassan and Fridovich (3) and Touati (31) also observed an increased biosynthesis of Mn superoxide dismutase in *E. coli* cells exposed to paraquat. This protective effect in *E. coli* HB101/pMYG1 contrasts with a previous report (32) that *E. coli* DK1/pDT1-5, containing the same cloned Mn superoxide dismutase, showed decreased resistance to paraquat during growth on solid LB media containing 0.1-0.2 mM paraquat. The reason for this discrepancy is not clear, but it is conceivable that during growth of *E. coli* DK1/pDT1-5 on solid LB media containing paraquat the dismutation of O_2^- by excess superoxide dismutase generated more H_2O_2 than could be accommodated by available catalase. It is noteworthy in this context that a 6-fold increase in superoxide dismutase activity in mouse L cells containing a cloned Cu/Zn superoxide dismutase gene did not confer protection against the effects of paraquat, whereas protection was obtained in transformants with a smaller increase (3.6-fold) in superoxide dismutase activity (33).

Although *A. nidulans* SPC/pMYG1 was less resistant to paraquat than *E. coli* HB101/pMYG1, possibly because there was no induction of Mn superoxide dismutase in the cyanobacterial transformant, the presence of the cloned Mn superoxide dismutase gene in *A. nidulans* did, nonetheless, confer some protection against paraquat-mediated photooxidative stress. This was evident from a comparison of the growth responses of *A. nidulans* SPC/pSG111 and SPC/pMYG1 to paraquat and from the fact that SPC/pMYG1 showed greater resistance to pigment photobleaching in the presence of paraquat. A decline in phycocyanin and chlorophyll was noted previously for cyanobacteria exposed to conditions of high oxygen tension (7, 34). As well, reduction in the absorption peaks at 450, 680, and 630 nm has been observed for *A. nidulans* following heat shock (35). Exposure to paraquat clearly reduced these absorption peaks in *A. nidulans* SPC/pSG111. By contrast, in *A. nidulans* SPC/pMYG1 there was no reduction in the absorption peaks in the presence of paraquat, indicating that the cloned Mn superoxide dismutase had provided protection against this manifestation of photooxidative stress. The pigments bilirubin and biliverdin are also bleached by O_2^- (36). Although regulation of the *E. coli* Mn superoxide dismutase gene in *A. nidulans* is not fully understood, the experiments described here suggest that similar protection against photooxidative stress might be

achieved in higher plants by targeting a cloned Mn superoxide dismutase expressed from a plant promoter to chloroplasts.

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