Superoxide Dismutase (*sod-1*) Null Mutants of *Neurospora crassa*: Oxidative Stress Sensitivity, Spontaneous Mutation Rate and Response to Mutagens

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ABSTRACT

Enzymatic superoxide-dismutase activity is believed to be important in defense against the toxic effects of superoxide. Although superoxide dismutases are among the best studied proteins, numerous questions remain concerning the specific biological roles of the various superoxide-dismutase types. In part, this is because the proposed damaging effects of superoxide are manifold, ranging from inactivation of certain metabolic enzymes to DNA damage. Studies with superoxide-deficient mutants have proven valuable, but surprisingly few such studies have been reported. We have constructed and characterized *Neurospora crassa* mutants that are null for *sod-1*, the gene that encodes copper-zinc superoxide dismutase. Mutant strains are sensitive to paraquat and elevated oxygen concentrations, and they exhibit an increased spontaneous mutation rate. They appear to have near wild-type sensitivities to near- and far-UV, heat shock and γ-irradiation. Unlike the equivalent *Saccharomyces cerevisiae* mutant and the *sodA sodB* double mutant of *Escherichia coli*, they do not exhibit aerobic auxotrophy. These results are discussed in the context of an attempt to identify consensus phenotypes among superoxide dismutase-deficient mutants. *N. crassa sod-1* null mutant strains were also employed in genetic and subcellular fractionation studies. Results support the hypothesis that a single gene (*sod-1*), located between *Fsr-12* and *leu-3* on linkage group I, is responsible for most or all CuZn superoxide dismutase activity in this organism.

STUDIES combining biochemical and genetic approaches have provided important confirming evidence that superoxide dismutases play protective roles in aerobic cells. These efforts have been aided by superoxide-dismutase mutants reported for *Escherichia coli*, *Saccharomyces cerevisiae* and *Drosophila melanogaster* (CARLIOZ and TOUATI 1986; BILINSKI *et al.* 1985; GRALLA and VALENTINE 1991; PHILLIPS *et al.* 1989). However, there is not as yet a consensus phenotype for superoxide dismutase deficient mutants. This is because the loss of superoxide dismutase activity has pleiotropic deleterious consequences ranging from slow growth to conditional auxotrophies to DNA damage. The recent report (ROSEN *et al.* 1993) linking familial amyotrophic lateral sclerosis with mutations in the gene for human CuZn superoxide dismutase provides important support for the conclusion that further genetic study will prove valuable in elucidating the roles of superoxide dismutases. Even among organisms with well-developed histories in genetic research, however, such progress has been hindered by the absence of efficient screening procedures for organisms with inactivated superoxide dismutase genes.

As is typical of eukaryotes, the filamentous fungus *Neurospora crassa* possesses two different, evolutionarily unrelated forms of the enzyme superoxide dismutase, which, as a scavenger of superoxide, appears to provide a frontline defense against superoxide-mediated toxicity. A copper and zinc (CuZn)-containing superoxide dismutase is assumed to be cytosolic, whereas mitochondria possess a manganese (Mn)-containing form of the enzyme. We have employed a natural gene disruption process to create mutations in the gene for *N. crassa* cytosolic CuZn superoxide dismutase (*sod-1*), which we previously cloned and characterized (CHARY *et al.* 1990). In *N. crassa*, the duplication (by transformation) of genes normally present in single copy activates a disruption process that occurs during sexual reproduction–after fertilization and prior to ascospore formation (SELKER *et al.* 1987; SELKER and GARRETT 1988). The disruption process results in the accumulation of GC to AT transition mutations in both copies of a duplicated sequence–the resident copy and the copy introduced by transformation. The process has been given the acronym RIP (for *r*epeat *i*nduced *p*oint mutations) (SELKER and GARRETT 1988). The recent discovery of this process has provided a powerful tool for performing gene manipulations in *N. crassa.*

Our report here of the construction and characterization of *N. crassa sod-1* null mutants represents one of the first applications of RIP-mediated disruption to study mutations that cause poorly characterized phenotypes. We have evaluated these mutants for sensitivity to various forms of environmental stress and for spontaneous mutagenesis. Most notable among our findings are the observations that *sod-1* mutant strains are

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hypersensitive to superoxide-generating compounds, and that they exhibit an increased rate of spontaneous mutagenesis without possessing an increased sensitivity to ionizing radiation or other mutagens. Unlike the corresponding *S. cerevisiae* mutant, *N. crassa sod-1* strains do not exhibit conditional aerobic auxotrophy, manifested as a requirement for specific amino acids when growth is on defined media in the presence of oxygen. We have also employed *sod-1* mutants to better define the intracellular localization of copper-zinc and manganese superoxide dismutases in *N. crassa*.

MATERIALS AND METHODS

***N. crassa* strains:** With the exception of mutants reported here, *N. crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC), Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas. The wild type strain employed was 74-OR23-1A. Transformations leading to the identification of *sod-1* null mutants employed strain 89601 (*qa-2, aro-9, inl*), which permits selection on minimal medium of transformants carrying a functional *qa-2* gene (VOLLMER and YANOFSKY 1986; HUGHES *et al.* 1983). Transformants carrying duplicated copies of the *sod-1* gene, in addition to functional *qa-2*, were mated with strain B369, which carries marker alleles (*leu-3, arg-1*) on linkage group IL. Null mutants were also backcrossed to strain B369 to examine segregation of *sod-1* alleles.

Plasmid construction: Plasmid pCN102 (see Figure 1) was constructed for *sod-1* disruption experiments. We first subcloned a 2.0-kbp *Xba*I-*Hin*dIII fragment carrying the entire *sod-1* coding region from pCN24 (CHARY *et al.* 1990) into the multiple cloning site of pUC18. This new plasmid was designated pCN101 and has been deposited at the Fungal Genetics Stock Center. To obtain pCN102, we subcloned into pCN101 an approximately 2.5-kbp *Bam*HI fragment carrying the *qa-2* gene, which had previously been inserted into the *Bam*HI site of pBR325. This latter *qa-2* plasmid construct was obtained from MARC ORBACH, University of Arizona.

***N. crassa* transformation and growth conditions:** The preparation of competent *N. crassa* cells and transformation procedures were performed as described by VOLLMER and YANOFSKY (1986). Sexual crosses were performed on synthetic cross medium following the general procedures of DAVIS and DE SERRES (1970). Medium was supplemented, when necessary, with appropriate amino acids at 1.0 mM.

Other experiments employed VOGEL's (1964) minimal medium. Routinely, this medium contained 1.5% sucrose and 80 mg/liter myoinositol. Plates employed in survivorship experiments also contained 1.5% agar and 2% sorbose (sorbose medium). In certain instances plates were additionally supplemented with 1% tryptone and 0.5% yeast extract (NIYT medium).

Molecular genetic methods: Agarose gel electrophoresis, DNA blot hybridizations, DNA labeling with ^{32}P and molecular cloning methods were as described previously (CHARY *et al.* 1990).

Preparation of cellular and mitochondrial extracts, and assay of superoxide dismutase activity: The identification of *sod-1* null mutant strains required the screening of cellular extracts from many ascospore-derived progeny on polyacrylamide gels stained for superoxide-dismutase activity. To facilitate this process we developed a simple and rapid procedure, employing partial cell wall digestion, for preparing protein extracts from *N. crassa* conidia and mycelia. Strains to be examined were grown on agar plates or in slant tubes for several days. Aerial growth, usually a combination of conidia and mycelium, was transferred with a sterile loop to a 1.5-ml microcentrifuge tube. Typically, the fungal mass occupied a volume of 200–400 μl. To this was added 400–500 μl of 5 mg/ml NovoZym™ 234 (Novo BioLabs, Bagsvaerd, Denmark) in 1.0 M sorbitol, and the mixture was vortexed and incubated for 1–2 hr at 30°. The cells were pelleted by centrifugation (5–10 min) and the supernatant discarded. Care was taken to remove as much supernatant as possible. The cells were resuspended in 50 mM potassium phosphate buffer (pH 7.5) with vigorous vortexing, and were then subjected to one or more freeze-thaw cycles, accompanied by vortexing. The cell debris was pelleted and the supernatant saved for analysis.

Mitochondria were prepared following the Bead Beater/sucrose method (CRAMER *et al.* 1983; TAYLOR and NATVIG 1987). For superoxide dismutase assays, mitochondria were disrupted in 50 mM potassium phosphate buffer (pH 7.5). Two different mitochondrial preparations were assayed–the 15,000 × *g* pellet (crude fraction) and the purified mitochondria from a 1.2 M/1.6 M sucrose step gradient.

Superoxide dismutase bands on native polyacrylamide slab gels were visualized using the in-gel activity assay of BEAUCHAMP and FRIDOVICH (1971). Bands representing putative Mn superoxide dismutases were not reduced in intensity when gels were pre-soaked in 2.0 mM KCN, an inhibitor of CuZn superoxide dismutases (BEAUCHAMP and FRIDOVICH 1971).

Total protein in cellular and mitochondrial extracts was assayed by the method of BRADFORD (1976).

Assays of sensitivity to elevated oxygen and paraquat: To test the sensitivities of wild type and mutant strains to elevated oxygen tensions and to the superoxide-generating compound paraquat, conidia were germinated for 2 h in minimal medium before plating (10^2–10^3 conidia/plate) onto sorbose medium. To test sensitivity to elevated oxygen, plates were incubated for 2–4 days in an anaerojar adjusted to 98% oxygen (NATVIG 1982). To test sensitivity to paraquat, sorbose plates contained paraquat at final concentrations from 5 to 100 μM.

Mutation rate assay and sensitivity to mutagens: Spontaneous mutation estimates were made by measuring forward mutations at the *N. crassa mtr* locus encoding neutral amino acid permease, essentially as described by STADLER *et al.* (1991). Mutations at the *mtr* locus were detected on the basis of the resistance of strains carrying mutations to the toxic amino acid analog *p*-fluorophenylalanine (FPA). For each strain examined, 9 or 11 individual colonies were used to inoculate small minimal-medium slant tubes. Conidial suspensions from 5-day-old slant cultures were filtered through glass wool and counted by hemacytometer. For detection of *mtr* mutants, 5×10^6 conidia were plated in sorbose medium supplemented with 30 mg/liter FPA. Colonies were counted after incubation at 33° for 4–5 days. For viability estimates, 200 conidia were spread onto minimal sorbose plates and incubated at 33°. Colonies were counted after 24 hr for wild type and 2–3 days for the *sod-1* strains. Mutation rates were calculated by a formula utilizing median mutant frequencies (DRAKE 1991). The median *mtr* mutant frequencies used were corrected for viability and the proportion of uninucleate conidia (since *mtr* mutants are recessive).

Sensitivity to mutagens was assayed in spot tests (SCHROEDER 1988). A dilute suspension of conidia was made in sterile water and drops were placed on medium containing 0.015% methyl methanesulfonate (MMS), or drops were placed on medium and the plate exposed to 570 J/m^2 far UV from two Westinghouse G25T8 sterilamps. For gamma irradiation, 1 ml suspensions on ice were given a dose of 14.5 krad from a ^{60}Co source, and drops were spotted on sorbose medium. Conidial germination and growth were examined after 48 and 72 hr at 30°.

Ionizing radiation sensitivity was also examined in survival curves by diluting conidia to 2×10^6 per ml in sterile water and exposing 1-ml samples in 1.5-ml microcentrifuge tubes on ice

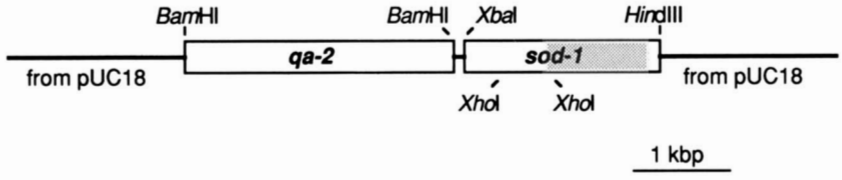

FIGURE 1.—Plasmid pCN102 employed in *sod-1* disruption experiments. The protein-coding region of *sod-1* is represented by shading.

to irradiation from a ^{60}Co source. After irradiation, the samples were kept on ice from 30 to 90 min before dilution to 600–6000 conidia/ml. Duplicate samples were taken and the samples were spread over three plates to give a total of 100–900 survivors per sample. Colonies were counted after 3–6 days at 30°. Dose rates were 1 krad/min (two experiments) or 5 krad/min (one experiment). The results from the two different dosage regimes were very similar and have been combined.

Sensitivity to heat shock and near UV: Sensitivity to heat shock was examined using two different methods. First, we employed a slightly modified form of the replica-plating method of KAPOOR *et al.* (1990). Conidia were plated onto sorbose medium and grown for 60 h at 30°, at which time they were incubated at 58° for periods from 10 to 60 min. Plates were then overlayed with moistened paper filters and incubated overnight at 30°. The filters were removed to new plates and these plates, along with the original plates, were monitored over several days for the appearance of colonies. A second procedure was modified from that of PLESOFSKY-VIG and BRAMBL (1985). Conidia were suspended in 5 ml minimal medium in glass culture tubes and incubated with shaking for 1 h at 30°. The tubes were then transferred to a shaking 50° water bath. Samples were removed immediately prior to the heat shock and after 15, 30 and 60 min at 50°. Samples were serially diluted in minimal medium and 100 μl sub-samples were spread onto N sorbose plates at a final density of approximately 100, 500 and 1000 conidia per plate. Colonies were counted after 4–7 days.

Sensitivity to near UV-irradiation was examined by exposing plated conidia on open petri plates to two F15T8 BLB black-light bulbs (HARTMAN and EISENSTARK 1978) at a distance of 20 cm. Plates were held in a shallow water bath to prevent overheating. Near-UV intensity was measured with a model 730 Radiometer (Optronics Laboratories, Inc., Orlando, Florida).

RESULTS

Identification of *sod-1* null mutants created by RIP-mediated disruption: Plasmid pCN102 (Figure 1), carrying the *sod-1*-coding region and the *N. crassa qa-2* gene, was used to transform the *N. crassa qa-2 aro-9* mutant strain 89601. Twenty *qa-2*$^+$ transformants were selected and passed through five single-conidium transfers in an attempt to obtain homokaryotic strains for gene disruption experiments. All 20 transformants were crossed with strain B369. Simultaneously, transformants were analyzed by DNA blot hybridization using appropriate restriction enzymes to determine the number of *sod-1* copies. Five strains were targeted for additional analysis on the basis of possessing one or two additional *sod-1* copies. Ascospores derived from crosses involving these five strains were germinated on plates containing NIYT medium, and approximately 20 progeny derived from each of the five transformants were analyzed for superoxide dismutase activity on polyacrylamide gels. Among the strains in this analysis, we identified four mutant strains (designated 3B, 3B2, 33 and 3C), all of which were derived from a single transformant, T3 (Figure 2A). Blot hybridization analysis of *Xho*I-digested DNAs revealed a loss of *Xho*I sites in the duplicated region, which provides strong circumstantial evidence for RIP-mediated disruption (Figure 2B).

A sexual cross employing mutant strain 3C and its non-transformant parent (B369) resulted in apparent Mendelian segregation of the *sod-1* null allele (Table 1). We included several *sod-1* progeny from this cross in subsequent experiments. Among these were strains 3C1, 3C2 and 3C5.

Each of the strains carrying the *sod-1* null mutation lacked detectable CuZn superoxide dismutase activity in either whole cell or mitochondrial extracts (Figure 3), as determined by loss of the known CuZn superoxide-dismutase band and the absence of any cyanide-inhibitable activity. Strain 3C was found to lack a transcript for *sod-1* (W. DVORACHEK, K. SYLVESTER and D. NATVIG, unpublished). The *sod-1* alleles in mutant strains are stable. Despite several attempts, we have been unable to obtain either reversions in *sod-1* or second-site suppressors of paraquat sensitivity. The inability to obtain suppressors of the *N. crassa sod-1* phenotype is a different result from that obtained with superoxide-dismutase mutants of *S. cerevisiae*, wherein suppressor mutations appear to involve respiratory-chain components (LIU *et al.* 1992). This difference may reflect the obligately aerobic nature of *N. crassa*.

We consistently observed greater activities for mitochondrial Mn superoxide dismutase in cell extracts derived from *sod-1* mutant strains than in those derived from wild type, suggesting that Mn superoxide dismutase is up-regulated in *sod-1* mutants (Figure 3). Increased expression of the *N. crassa* gene for mitochondrial Mn superoxide dismutase in *sod-1* mutants is further supported by RNA blot hybridization experiments employing a cloned gene for *N. crassa* Mn superoxide dismutase (W. DVORACHEK, K. SYLVESTER and D. NATVIG, unpublished).

Genetic analysis of *sod-1* null allele: Previously, using RFLP analysis, we reported that *sod-1* maps to *N. crassa* linkage group IL (CHARY *et al.* 1990). In the present study we employed a cross of the *sod-1* mutant strain 3C to strain B369 (*leu-3 arg-1*) to examine Mendelian segregation of this allele. This cross also provided additional information regarding the map location of the *sod-1* gene. Although the progeny from this cross were skewed in favor of one parental type, possibly due to the slow growth of the *leu-3 arg-1* double mutant, the two *sod-1* alleles segregated approximately 1:1 (38 *sod-1*, 30

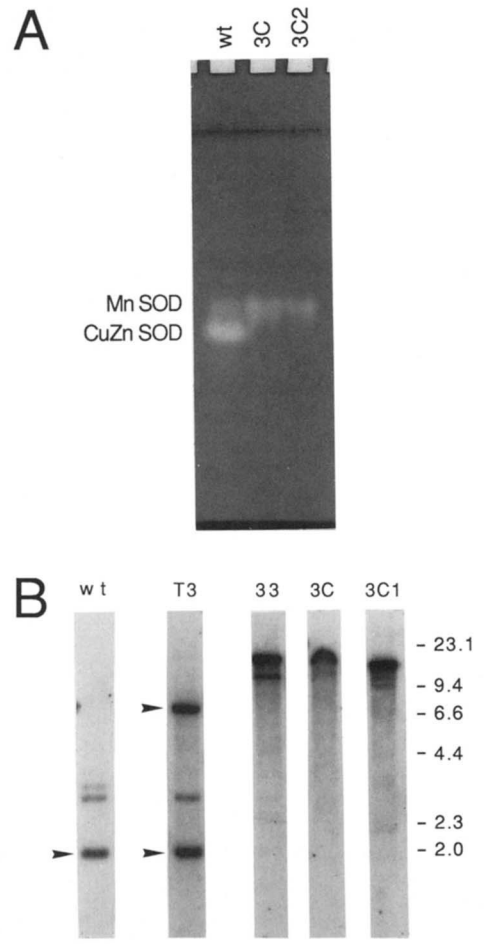

FIGURE 2.—Biochemical and molecular-genetic characterization of *sod-1* null mutant strains. (A) Cellular extracts (200–300 μg total protein) from wild type *N. crassa* and two *sod-1* mutant strains were electrophoresed and examined for superoxide dismutase activity. The lower band (CuZn superoxide dismutase) is absent in lanes containing extracts from the two mutant strains, 3C and 3C2. The remaining activity bands are presumed to represent Mn superoxide dismutase based on the the observation that they were insensitive to 2.0 mM potassium cyanide. (B) Blot hybridization analysis of genomic DNAs digested with restriction endonuclease *Xho*I (CTCGAG). DNAs were blotted and probed with labeled pCN101, which contains the same *sod-1* coding region as pCN102, but which lacks the *qa-2* gene. In the wild type the *sod-1* region is represented by a major *Xho*I band of approximately 2.0 kbp. This fragment results from an *Xho*I site that is 60 bp downstream of the translational start triplet of *sod-1* and another site that is downstream of the cloned *sod-1* region. The transformant T3 possesses a second major band representing a second copy of *sod-1* at another site (upper arrow). An approximately 500-bp fragment that was present in both wild type and T3 lanes (created by the two *Xho*I sites illustrated in Figure 1) is not shown. The fainter band seen in wild type and T3 lanes represents sequence that overlaps by perhaps a few hundred base pairs the cloned *sod-1* fragment (distal to the upstream *Xho*I site contained within the clone). The three remaining lanes are *sod-1* strains that were identified by the lack of CuZn superoxide dismutase activity. The altered migration of fragments in these lanes, resulting from loss of *Xho*I sites, is presumed to indicate RIP-mediated disruption of sequences associated with *sod-1*. Strains 33 and 3C are progeny derived from a cross between T3 and strain B369. Strains 3C1 and 3C2 were among the progeny of a cross between 3C its B369 parent.

TABLE 1

Segregation of the *sod-1* mutant allele from strain 3C in a cross with strain B369 (*leu-3 arg-1*)

Phenotype[a]	No. of progeny	Presumptive explanation
Sod^-, Leu^+, Arg^+	34	Parental
Sod^+, Leu^-, Arg^-	18	Parental
Sod^-, Leu^+, Arg^-	1	Single crossover
Sod^+, Leu^-, Arg^+	4	Single crossover
Sod^-, Leu^-, Arg^-	3	Single crossover
Sod^+, Leu^+, Arg^+	5	Single crossover
Sod^+, Leu^+, Arg^-	3	Double crossover[b]
Sod^-, Leu^-, Arg^+	0	Double crossover

[a] Sod phenotype was determined by extraction and activity-gel analysis of all isolates.

[b] Gene conversion at one locus is a plausible alternative, given the high frequency of this class.

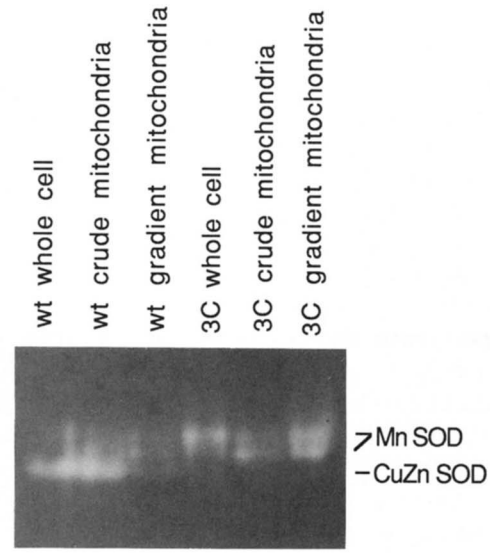

FIGURE 3.—Activity gel presenting the distribution of CuZn and Mn superoxide dismutases in cellular fractions. Each lane received approximately 250 μg total protein from the indicated cell fraction.

sod-1$^+$) (Table 1). Taken with our previous RFLP mapping data, our current results place the *sod-1* gene between *leu-3* and *Fsr-12*, outside the region between *leu-3* and *arg-1* [see recent *N. crassa* map updates of METZENBERG and GROTELUESCHEN (1992), wherein our data are reiterated, and PERKINS (1992)].

Superoxide dismutase activities associated with subcellular fractions of wild-type and *sod-1* null mutant strains: HENRY *et al.* (1980) reported that *N. crassa* possesses both mitochondrial and cytosolic CuZn superoxide dismutases. Our results suggest that, if this is the case, the mitochondrial CuZn superoxide dismutase may also be a product of the *sod-1* gene. We have not detected a second CuZn superoxide-dismutase activity in either mycelial or mitochondrial extracts from the *sod-1* strains (Figure 3).

Growth phenotype and sensitivity of *sod-1* null mutants to oxidative stress conditions: Because the *S. cerevisiae* CuZn superoxide-dismutase null mutant

TABLE 2

Mutation rates at the *N. crassa mtr* locus

Strain	Mean no. of colonies per 200 conidia (no FPA)	Mean no. of colonies per 5×10^6 viable conidia (FPA)	Mutation rate (*mtr*$^-$/nucleus/generation)
Wild type	183 ± 19 (n = 11)[a]	1.9 ± 1.4	3.0×10^{-7}
sod-1 (3C)	41 ± 16 (n = 9)	50 ± 23	6.0×10^{-6}
sod-1 (3C1)	43 ± 11 (n = 9)	60 ± 28	8.1×10^{-6}

[a] n = number of single-conidium isolates examined for a given strain.

(BILINSKI *et al.* 1985) and the *E. coli sodA sodB* double mutant (CARLIOZ and TOUATI 1986) require supplemental amino acids for aerobic growth on defined media (aerobic auxotrophy), we performed our initial screens for *sod-1* strains on complex NIYT medium. Unlike the mutants of *S. cerevisiae* and *E. coli*, however, the *N. crassa sod-1* mutants grew well on minimal medium. Although conidial survival (Table 2) and mycelial growth rate of *sod-1* strains were reduced relative to wild type on either minimal or NIYT medium, the survivorship of conidia from *sod-1* mutant strains was the same on minimal and NIYT.

All of the *N. crassa sod-1* strains examined exhibited an extreme sensitivity to the superoxide generating compound paraquat. In minimal-medium plates, paraquat concentrations as low as 25 μM, a concentration to which wild type is resistant, resulted in complete inhibition (up to 10^3 conidia/plate) (Figure 4). Similarly, filter paper disks saturated with 100–500 mM paraquat produced large zones of inhibition when placed on plates opposite *sod-1* mutant strains. We have been able to employ this filter-disk assay as a rapid screen for *sod-1* strains.

Wild-type and *sod-1* strains also differed in sensitivity to 98% oxygen, exhibiting conidial survival values of approximately 40–50% and zero, respectively, except when conidia were plated at high densities. High densities (>10^6 conidia/plate) produced a protective neighbor effect, resulting in luxuriant growth of *sod-1* strains.

***N. crassa sod-1* null mutants exhibit an increased spontaneous mutation rate:** The two *sod-1* strains, 3C and 3C1, both exhibited a 20–30 fold increase in the proportion of conidia possessing spontaneous mutations at the *mtr* locus (Table 2). This estimate is conservative, because very small colonies were not counted. Small colonies were more numerous on plates with *sod-1* strains than on plates with wild type. This likely resulted from the slower growth rate of the *sod-1* mycelium.

The *sod-1* mutants do not exhibit increased sensitivity to gamma rays: Survivorship of *sod-1* null mutant strains exposed to gamma irradiation was approximately that of wild type with either the spot test or the conidial plating assay (Table 3; Figure 5). In the conidial plating experiment there was a slight indication that the *sod-1* mutant strain 3C1 in fact might be more resistant to γ-irradiation than the wild-type strain (Figure 5). We note that because the conidia used in these experiments were in a non-dividing resting stage, it is possible that different sensitivities would be observed during rapid growth.

The *sod-1* mutants do not exhibit substantial increased sensitivity to far and near UV, heat shock or chemical mutagens: Survivorship of *sod-1* strains ex-

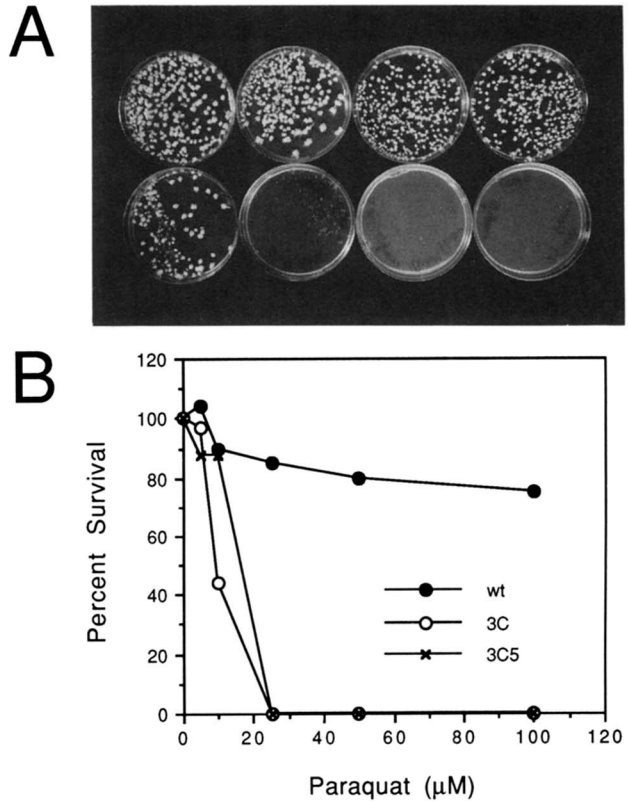

FIGURE 4.—Sensitivity of *sod-1* null mutant strains to paraquat. (A) Effects of paraquat on conidial plating efficiency. Top row from left to right: Wild type *N. crassa* conidia plated on sorbose plates containing 0, 5, 10 and 25 μM paraquat. Bottom row: same series for *sod-1* strain 3C. (B) Survival curve for conidia plated on sorbose plates containing paraquat. Conidia from wild type and two *sod-1* strains (3C and 3C5) were plated (10^2–10^3 cells/plate) onto sorbose medium containing varying concentrations of paraquat. Each value represents the average of three plates. All values are standardized to the respective no-paraquat value (defined as 100% survival).

TABLE 3

Growth of *N. crassa* strains in mutagen spot tests

Strain	No treatment	Mutagen far UV	γ-Rays	MMS
Wild type	f	f	f	f
sod-1 (3C)	m	m	m	m
sod-1 (3C1)	m	m	m	m
uvs-6	f	vs	ms	vs

Growth was scored with a dissecting scope using the following scale: fast (f), moderate (m), moderate to slow (ms) and very slow (vs).

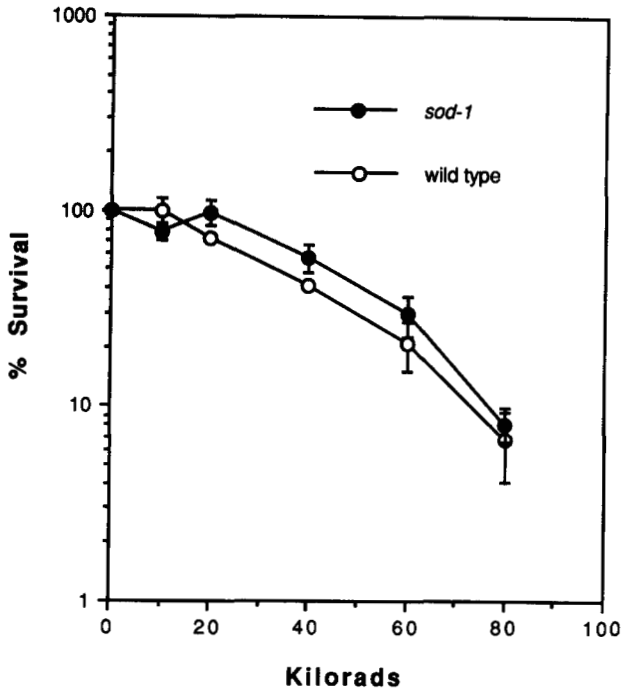

FIGURE 5.—Survival of *N. crassa* wild type and *sod-1* null mutant strains upon exposure to ionizing radiation. Results are presented for three separate experiments. Vertical bars indicate standard deviations. Standard deviations not shown are contained within the symbols.

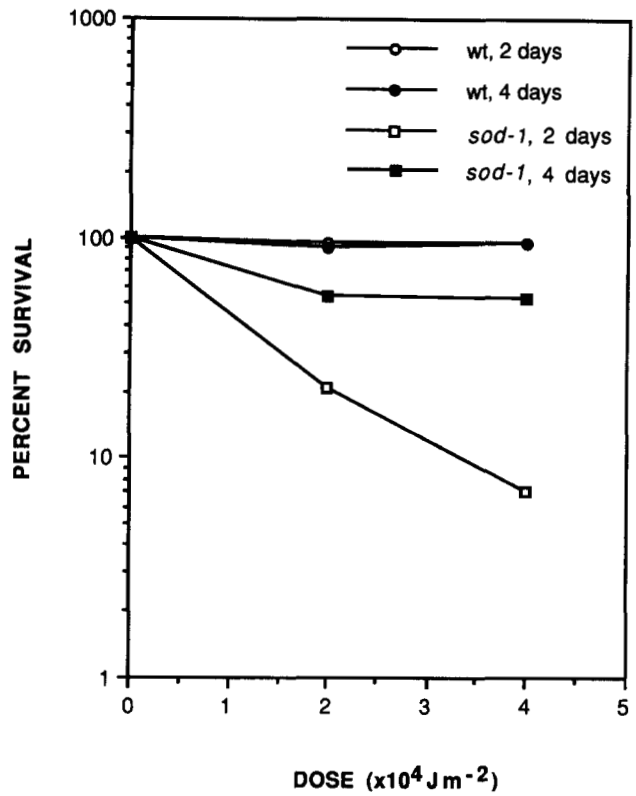

FIGURE 6.—Sensitivity of mutant and wild type strains to near-UV irradiation. Plated conidia were exposed to near UV as described in MATERIALS AND METHODS. After irradiation, plates were incubated in the dark for either 2 or 4 days. Each value represents the average of three plates.

posed to far UV and MMS in spot tests was not substantially different from that of wild type (Table 3). Attempts to examine the sensitivity of *sod-1* mutant strains to heat shock and near-UV irradiation were complicated by the fact that both agents appear to have transient static effects on *sod-1* conidia, and in the case of heat shock, replicated *sod-1* mycelia. As a result, in several experiments mutant strains appeared to be more sensitive than wild type to one or the other agent, as measured by the apparent number of surviving colonies 2–3 days post treatment. With both near-UV and heat shock, this effect was greatly diminished if colonies were allowed to grow for 4–6 days before counting. The results for one such near-UV experiment are presented (Figure 6). When we controlled for the slow growth of treated *sod-1* mutants, we consistently observed reduced survival of *sod-1* mutants upon heat shock and exposure to near UV. Survival was typically depressed no more than 25% relative to wild type, however.

DISCUSSION

In the following discussion we have focused on comparative aspects of the *N. crassa sod-1* mutant phenotype. It is, however, important to note that Mn superoxide dismutase appears to be up-regulated in *sod-1* strains. This may lessen the consequences of *sod-1* inactivation.

Mutant phenotypes and the biological role of superoxide-dismutase: Genetic studies have contributed strong evidence that enzymic superoxide degradation, first observed as an *in vitro* activity, is important *in vivo*. Supporting evidence includes the following. (1) Superoxide dismutase null mutants are universally sensitive to conditions of superoxide stress (CARLIOZ and TOUATI 1986; BILINSKI *et al.* 1985; PHILLIPS *et al.* 1989; this work). (2) Superoxide dismutases in *E. coli* are inducible under conditions of superoxide stress (HASSAN and FRIDOVICH 1977; TOUATI 1988). (3) CuZn and Mn superoxide dismutases can substitute for one another in reversing the oxygen-sensitive phenotypes of superoxide-dismutase mutants (NATVIG *et al.* 1987; BOWLER *et al.* 1990). (4) Mutations in either Mn/Fe or CuZn superoxide dismutase genes lead to increased rates of spontaneous mutagenesis in *E. coli* (FARR *et al.* 1986), *S. cerevisiae* (GRALLA and VALENTINE 1991) and *N. crassa* (this report).

In addition to providing supporting evidence for the importance of superoxide dismutases as scavengers of superoxide, studies with superoxide-deficient mutants have helped define specific areas where additional study is needed. Since superoxide and its by-products have been implicated in damage to a wide variety of cellular components, it is perhaps not surprising that mutations in superoxide-dismutase genes cause pleiotropic effects. As a consequence, however, it is now important to explore the phenotypes of diverse mutants to determine which characteristics are of general significance.

There is not yet a consensus phenotype among superoxide-dismutase mutants, although similarities exist. An increased sensitivity to paraquat and other agents of oxidative stress, an increased rate of spontaneous mutation, and a decreased growth rate are characteristics shared by mutants of *E. coli* (CARLIOZ and TOUATI 1986; FARR *et al.* 1986;), *S. cerevisiae* (BILINSKI *et al.* 1985; GRALLA and VALENTINE 1991) and *N. crassa* (this report). The *D. melanogaster* CuZn superoxide-dismutase mutant also shows hypersensitivity to paraquat (PHILLIPS *et al.* 1989).

The *E. coli* Mn/Fe superoxide dismutase double mutant (*sodA sodB*) and the *S. cerevisiae* CuZn superoxide dismutase mutant both exhibit aerobic auxotrophy on minimal medium (CARLIOZ and TOUATI 1986; BILINSKI *et al.* 1985), although the specific reasons appear to be different for the two organisms. The *E. coli* mutant exhibits a requirement for branched-chain amino acids, which has been attributed to superoxide sensitivity of the iron-sulfur center of α,β-dihydroxyisovalerate dehydratase (KUO *et al.* 1987). The *S. cerevisiae sod1* mutant requires lysine and either cysteine or methionine. The cysteine/methionine requirement is apparently not a true auxotrophy, but rather results from the metabolic consequences of sulfur assimilation, which increases in the absence of sulfur-containing amino acids (CHANG and KOSMAN 1990). It has been proposed that *sod1* strains suffer from the superoxide-mediated production of toxic sulfur radicals formed during sulfur assimilation (GRALLA and KOSMAN 1992). Lysine auxotrophy may result from the superoxide-mediated inactivation of α-amino adipate transaminase (LIU *et al.* 1992). It is not clear why the *N. crassa sod-1* mutant fails to exhibit aerobic auxotrophy.

The significance of the most striking characteristic of superoxide-dismutase mutants, sensitivity to paraquat and other agents of oxidative stress, is difficult to assess in the context of natural environments. This phenotype likely reflects an increased level of intracellular oxidative damage in mutant cells growing under aerobic conditions, but sensitivity to such agents *per se* is unlikely to have direct ecological significance for most organisms.

The fact that the fungal and *E. coli* mutants grow under standard laboratory conditions, aerobic auxotrophy notwithstanding, together with the pleiotropic nature of mutations in superoxide dismutase genes, invites an evolutionary interpretation of the role of superoxide dismutase. It is possible that in many organisms a given superoxide dismutase (CuZn, Fe or Mn) increases fitness, while not being required for viability. This hypothesis is certainly consistent with the reduced mycelial growth rate and conidial survival of *N. crassa sod-1* strains. It may also obviate the need to postulate crucial universal superoxide targets that can receive protection only from superoxide dismutases.

This hypothesis is also consistent with the observed increases in spontaneous mutation rate observed among superoxide dismutase mutants. The mutation rate observed for the *N. crassa sod-1* mutant may be outside the natural range for microorganisms (*cf.* DRAKE 1991). It is difficult to imagine that an increase in mutation rate of the magnitude observed here would not put such strains at a disadvantage in nature.

Significance of results obtained with γ-irradiation: A correlation between intracellular superoxide dismutase activity and sensitivity to ionizing radiation has not been established. In *D. melanogaster*, resistance to ionizing radiation was found to increase with increasing CuZn superoxide dismutase activity (PENG *et al.* 1986). In contrast, *E. coli* mutants lacking superoxide dismutase exhibited a resistance to gamma-irradiation greater than that of wild type, whereas strains overexpressing superoxide dismutase were more sensitive than wild type (SCOTT *et al.* 1989).

An important cause of superoxide-mediated damage in aerobic cells likely results from the metal-catalyzed reaction of superoxide (formed as a result of oxidative metabolism) with hydrogen peroxide to form toxic hydroxyl radicals (reviewed by FARR and KOGOMA 1991). The hydroxyl radical has also long been implicated in damage caused by ionizing radiation, suggesting overlap in the damage pathways of ionizing radiation and aerobic metabolism. Moreover, the production of hydroxyl radicals resulting directly from the radiolysis of water can be exacerbated in the presence of oxygen as a result of radiation-mediated superoxide production, followed by the metal-catalyzed interaction of superoxide with hydrogen peroxide (TOTTER 1980).

Damage from ionizing radiation may therefore be either superoxide-dependent or -independent (with the hydroxyl radical being an agent in either case). Evidence for both damage pathways exists for *N. crassa* in the demonstration that conidial respiratory activity resulted in reduced (by up to 50%) sensitivity to ionizing radiation, due to depletion of intracellular oxygen (KOLMARK 1965). Although we maintained conidia on ice at low density to promote aerobic conditions, a superoxide-independent damage pathway nevertheless might predominate in *N. crassa* cells under our experimental conditions.

Even if correct, the dual-pathway explanation provides only a partial explanation for our results. Superoxide dismutase-deficient mutants would still be expected to display some increase, albeit perhaps moderate, in superoxide-mediated damage due to ionizing radiation. Instead, in both our study and the *E. coli* study (SCOTT *et al.* 1989) no such response was observed.

One possible explanation for the results obtained with ionizing radiation is that superoxide dismutase-deficient mutants are induced for the repair of radiation-caused damage and/or for the synthesis of active-oxygen scavengers other than CuZn superoxide dismutase (see FARR and KOGOMA 1991). This hypothesis, however, forces additional interpretation of the increased rate of spontaneous mutagenesis observed for *sod-1* mutants. It is possible that disparate contributions are made by

"error-free" and "error-prone" DNA repair mechanisms under different conditions of oxidative stress, with superoxide-dismutase mutants being induced for error-prone repair.

In summary, after more than two decades of research the biological role of superoxide dismutase remains incompletely resolved, if not controversial. Important questions remain as to which targets of superoxide damage are of greatest significance and as to the importance of any given superoxide dismutase relative to other defense mechanisms. Studies that combine genetic and biochemical approaches are helping to resolve these issues.

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