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Characterization of the *Bacillus stearothermophilus* Manganese Superoxide Dismutase Gene and Its Ability to Complement Copper/Zinc Superoxide Dismutase Deficiency in *Saccharomyces cerevisiae*

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Recombinant clones containing the manganese superoxide dismutase (MnSOD) gene of *Bacillus stearothermophilus* were isolated with an oligonucleotide probe designed to match a part of the previously determined amino acid sequence. Complementation analyses, performed by introducing each plasmid into a superoxide dismutase-deficient mutant of *Escherichia coli*, allowed us to define the region of DNA which encodes the MnSOD structural gene and to identify a promoter region immediately upstream from the gene. These data were subsequently confirmed by DNA sequencing. Since MnSOD is normally restricted to the mitochondria in eucaryotes, we were interested (i) in determining whether *B. stearothermophilus* MnSOD could function in eucaryotic cytosol and (ii) in determining whether MnSOD could replace the structurally unrelated copper/zinc superoxide dismutase (Cu/ZnSOD) which is normally found there. To test this, the sequence encoding bacterial MnSOD was cloned into a yeast expression vector and subsequently introduced into a Cu/ZnSOD-deficient mutant of the yeast *Saccharomyces cerevisiae*. Functional expression of the protein was demonstrated, and complementation tests revealed that the protein was able to provide tolerance at wild-type levels to conditions which are normally restrictive for this mutant. Thus, in spite of the evolutionary unrelatedness of these two enzymes, Cu/ZnSOD can be functionally replaced by MnSOD in yeast cytosol.

Superoxide dismutases (SODs; EC 1.15.1.1) are a class of metalloproteins that catalyze the dismutation of superoxide radicals ($O_2^{\cdot -}$) to oxygen and hydrogen peroxide. Their presence in all aerobic organisms examined has led to suggestions that they play a critical role in protecting cells against oxidative stress. The prime culprits which mediate oxygen toxicity are thought to be superoxide radicals and hydrogen peroxide, which, in the presence of trace amounts of iron salts, can react to form hydroxyl radical ($OH^{\cdot}$), the most powerful oxidant known (18). Together with the catalases and peroxidases (which remove H_2O_2), SOD thus provides a vital defense mechanism against the formation of deleterious oxygen species. The importance of SOD has been verified by the isolation of *Escherichia coli* (13) and yeast (6, 42) mutants which are deficient in SOD enzymes. These mutants are all characterized by a hypersensitivity to conditions of oxidative stress.

Three classes of SOD have been defined on the basis of their metal cofactors: copper/zinc, manganese, and iron forms (for a review, see reference 2). Procaryotes generally contain manganese SOD (MnSOD) or iron SOD (FeSOD) or both. While FeSOD is generally found only in procaryotic species, MnSOD is also present in the mitochondria of eucaryotic cells. FeSODs and MnSODs are very similar in their primary, secondary, and tertiary structures (34). The copper/zinc enzyme (Cu/ZnSOD), however, represents a distinct class, being structurally unrelated and found only in eucaryotic species, where it is often present as several isoforms. One of these isoforms is always present in the cytosol, and additional extracellular and chloroplastic forms have been identified (22, 35, 37).

It is generally assumed that three different classes of SOD have arisen from evolution as an optimization of enzymic function to different environments in different compartments of the cell. However, human Cu/ZnSOD has been found to function in *E. coli* almost as efficiently as the endogenous MnSOD and FeSOD normally present (31). The present study was initiated to determine whether MnSOD could be active in eucaryotic cytosol, a location where it is normally absent. Also, we wanted to address the question of whether MnSOD was able to replace Cu/ZnSOD.

For these experiments, we have first isolated and characterized the gene encoding MnSOD from the thermophilic bacterium *Bacillus stearothermophilus*. Subsequently, the DNA sequence encoding MnSOD was cloned into a yeast expression vector and introduced into a Cu/ZnSOD-deficient yeast strain (6). The results of complementation experiments demonstrate the ability of procaryotic MnSOD to function in yeast cytosol and to be able to replace the cytosolic Cu/ZnSOD of yeast cells. Thus, although structurally unrelated, MnSOD has the capacity to substitute for Cu/ZnSOD.

MATERIALS AND METHODS

Bacterial and yeast strains. *B. stearothermophilus* ATCC 12980 was obtained from the American Type Culture Collection (Rockville, Md.).

For standard recombinant DNA work, *E. coli* MC1061 (14) was used as a transformation host. Complementation analyses of *E. coli* were performed with strain QC774, which contains insertions in both MnSOD (*sodA*) and FeSOD (*sodB*) genes and lacks all SOD activity (13).

The *Saccharomyces cerevisiae* strains used were DL1 (α *leu2 his3 ura3*) (42), which contains normal SOD activity, and DSCD2-4a (α *leu1 his3 ura3 sod1-1*) (kindly provided by

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T. Biliński [Zamosc College of Agriculture, Zamosc, Poland]), which lacks any detectable Cu/ZnSOD activity (6).

Design of oligonucleotide probe. The oligonucleotide mixture 3′-TACTTITAIGTIGTITGITT(C/T)GTIGT-5′ was designed against amino acids 23 to 31 of the previously determined amino acid sequence of MnSOD from *B. stearothermophilus* (12). It was made in the anticoding orientation. Deoxyinosines were used at the third codon position for amino acids with two or more coding alternatives, except for lysine, for which both T and C were used. The applications for hybridization probes of deoxyinosines at ambiguous codon positions have been reported previously (32).

The oligonucleotide mixture was chemically synthesized with an Applied Biosystems 380A DNA synthesizer, on the basis of the phosphoramide triester method (3). To screen the library, the oligonucleotides were end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (27) to a specific activity of approximately 2×10^8 dpm/μg of DNA.

Construction and screening of library. Chromosomal DNA was isolated from *B. stearothermophilus* ATCC 12980 by the method of Mielenz (29). Following partial *Sau*3A digestion, fragments ranging from 2 to 5 kilobases (kb) were isolated from 1% agarose gels. These fragments were cloned into the unique *Bam*HI site of pUC9 (43) and introduced into *E. coli* MC1061. Approximately 90% of the transformed cells contained a recombinant plasmid with an estimated average insert size of 2.5 kb.

To screen the library, 10,000 colonies were placed individually into the wells of microdilution plates and grown overnight at 37°C. Subsequently, replicas were made on nylon filters (Hybond-N; Amersham Corp., Arlington Heights, Ill.) which were further treated as described previously (20). The prehybridization and hybridization mixtures contained 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, and 0.05% sodium PP_i. Prehybridization was performed for 2 h at 42°C, and hybridization was carried out overnight at the same temperature. The filters were then washed three times in 6× SSC–0.1% sodium dodecyl sulfate at 50°C for 30 min each. The filters were autoradiographed on XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens.

Analysis of positive clones. For complementation analyses, plasmids derived from each of the hybridizing clones were introduced into SOD-deficient *E. coli* QC774 by $CaCl_2$ transformation (26), and recombinant clones were selected on Luria broth plates containing 100 μg of ampicillin per ml. Five colonies from each plate were streaked onto minimal medium (30) on which survival was dependent upon SOD activity (13), and the presence or absence of growth was scored after 48 h at 37°C.

Crude protein extracts were obtained from *E. coli* and *B. stearothermophilus* by a combination of osmotic shock (25) and freezing and thawing. Protein concentrations were measured by the protein-dye binding assay (9) with a kit supplied by Bio-Rad Laboratories (Richmond, Calif.). Samples were separated on nondenaturing 10% polyacrylamide gels, and SOD activity on these gels was visualized by the in situ staining technique of Beauchamp and Fridovich (4). Inhibitor studies performed as described previously (7) confirmed the identity of MnSOD (data not shown).

Analysis of recombinant DNA was performed by standard procedures (27). The DNA sequences on both strands were determined by the procedure of Maxam and Gilbert (28). Nucleotide and amino acid comparison analyses were performed with the help of the Intelligenetics software package for molecular biologists.

Expression of *B. stearothermophilus* MnSOD in yeast. The MnSOD-coding region was isolated as a 730-base-pair *Sma*I-*Bal*I fragment from clone 3A. The *Sma*I site was unique to the pUC9 polylinker, 7 nucleotides upstream from the MnSOD initiation codon. *Bal*I recognizes a single restriction site, located approximately 100 nucleotides downstream from the stop codon (Fig. 1). This fragment was cloned into the dephosphorylated *Sma*I site of the yeast expression vector pEMBLyex4 (15) to generate pSALSOD1. Such a transcriptional fusion permits MnSOD expression to be controlled by the galactose-inducible hybrid *GAL-CYC1* promoter (21) situated upstream from the MnSOD initiation codon. The MnSOD sequence was flanked at the 3′ end by the transcription termination signals of the 2μm *FLP* gene. This plasmid was introduced into yeast strains DL1 and DSCD2-4a by lithium acetate transformation (24), and transformants were selected with the plasmid-encoded *URA3* gene.

Yeast cells were grown at 28°C in minimal medium (SD; 0.67% yeast nitrogen base, 0.15% Casamino Acids) with 2% glucose (SDgluc) or 2% galactose (SDgal) as the carbon source. Nonrecombinant cells were grown in the same medium supplemented with 0.002% uracil. Crude protein extracts were obtained by disruption of cells with glass beads essentially as described previously (42), and the extracts were run on nondenaturing protein gels for SOD activity staining (see above).

Complementation analyses in yeast. The ability of pSALSOD1 to restore wild-type growth to the Cu/ZnSOD-deficient mutant DSCD2-4a was tested by analyzing its sensitivities to oxygen, paraquat, and hydrogen peroxide.

(i) Sensitivity to oxygen. For growth in 100% oxygen, cells pregrown on SDgluc or SDgal plates were restreaked onto the same medium and placed in an anaerobic jar which was flushed with 100% O_2. Growth was scored after 3 days of incubation at 28°C.

(ii) Sensitivity to paraquat. To test sensitivity to paraquat, cells were pregrown in SDgal and inoculated at an A_{600} of 0.05 into fresh SDgal liquid media. Paraquat (175 μM) was added to the cultures when the A_{600} had reached approximately 0.15, and growth was then monitored for 24 h.

(iii) Sensitivity to hydrogen peroxide. Cells grown in SDgal to stationary phase were inoculated into fresh media and grown to an A_{600} of 0.5. These cells were then diluted to a concentration of 10^6/ml in SDgal, and hydrogen peroxide was added to a final concentration of 25 mM. The cells were incubated at 28°C, and samples were taken after 20, 40, and 60 min. Cells were immediately spun down and washed with water before being suspended in water and plated at several dilutions on SDgal solid media. Viability was measured after 4 days by comparing the number of colonies formed with the number formed from an untreated culture.

RESULTS

Isolation and characterization of the MnSOD gene from *B. stearothermophilus*. On the basis of the previously determined amino acid sequence of *B. stearothermophilus* MnSOD (12), an oligonucleotide mixture was synthesized to serve as a probe for isolation of the corresponding gene.

A library containing partial *Sau*3A fragments derived from the chromosomal DNA of *B. stearothermophilus* was constructed in *E. coli*, in the plasmid pUC9 (43). A total of 10,000 colonies were screened with the end-labeled oligonucleotide mixture; 7 colonies (denoted 1A to 7A) gave a positive signal.

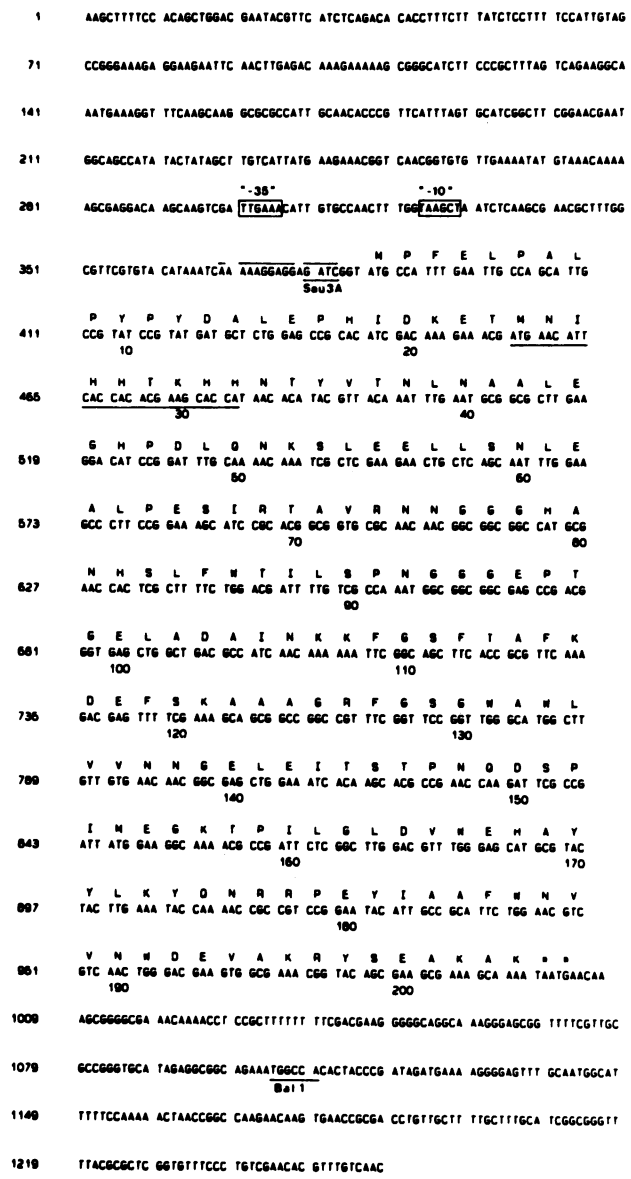

FIG. 1. Sequence of MnSOD gene from *B. stearothermophilus*. The sequence shown is from the *Hin*dIII site 386 nucleotides upstream from the MnSOD-coding sequence to the *Hin*cII site 260 nucleotides downstream from the termination codons (Fig. 2a). Nucleotides are numbered on the left, and the predicted coding sequence, extending from position 387 (start codon ATG) to position 998 (stop codons TAA and TGA, marked with asterisks), has been translated into the corresponding amino acid sequence, shown above the nucleotide sequence in the one-letter notation. The sequence homologous to the oligonucleotide probe is underlined. The positions of the *Sau*3A (nucleotides 380 to 383) and *Bal*I (nucleotides 1104 to 1109) sites are indicated. Putative "−35" and "−10" promoter elements have been boxed. The nucleotides complementary to the 3′ end of 16S rRNA from *B. stearothermophilus* (41), located 4 to 18 nucleotides upstream from the initiation codon, are indicated by lines drawn above the homologous nucleotides.

To confirm that these clones contained the MnSOD gene, we tested their activities by complementation analyses with a SOD-deficient strain of *E. coli* which is unable to grow aerobically on minimal medium (13). Plasmids from each of the positively hybridizing clones were introduced into this strain, and transformants were plated on minimal medium.

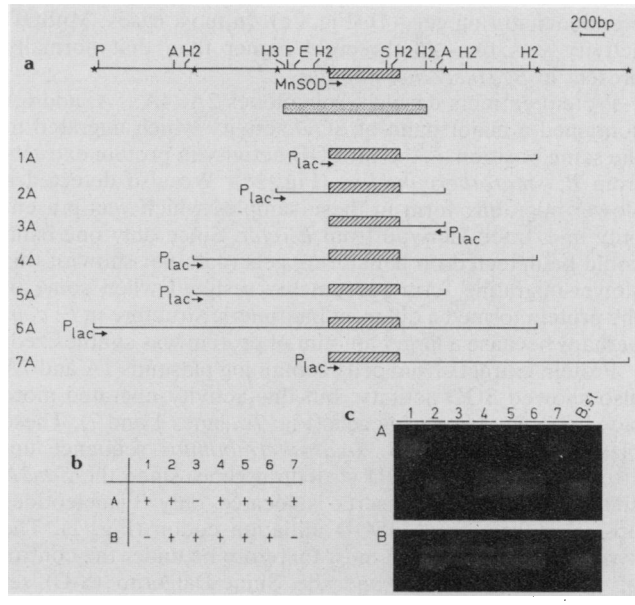

FIG. 2. Analysis of recombinant clones containing *B. stearothermophilus* MnSOD. (a) Physical map of the region of the *B. stearothermophilus* chromosome containing the MnSOD gene and alignment of each of the recombinant clones. Symbols: ▨, MnSOD open reading frame; →, direction of transcription; ▩, region of sequence shown in Fig. 1; ★, *Sau*3A sites which delimit the various clones. For each of the plasmids 1A to 7A, the position of the pUC9-encoded *lacZ* promoter is shown (P_{lac}). Abbreviations: A, *Alw*N1; E, *Eco*RI; H2, *Hin*cII; H3, *Hin*dIII; P, *Pvu*II; S, *Sph*I; bp, base pairs. (b) Complementation analysis of each of the seven recombinant clones in the SOD-deficient *E. coli* mutant. Series A represents the original plasmids on the basis of pUC9, and series B represents the inserts recloned into pUC18 in which the *lacZ* promoter is on the opposite side of the insert (see text for details). The presence and absence of growth on minimal media are denoted as + and −, respectively. (c) SOD activity in extracts of *E. coli* transformed with each of the recombinant plasmids. Samples containing 40 μg of total protein were separated on nondenaturing 10% acrylamide gels and stained for SOD activity (see Materials and Methods). Series and clones are denoted as in panel b. Lane B.st shows the SOD activity in a protein extract derived from *B. stearothermophilus*.

The introduction of six of the seven plasmids into this strain led to the restoration of growth on minimal medium (Fig. 2b). Restriction enzyme analysis revealed that of these, clone 2A contained the smallest insert (1.45 kb). The nucleotide sequence of this insert was determined (Fig. 1). The sequence encoding MnSOD was found within a 1.05-kb *Sau*3A fragment, which was common to all seven hybridizing clones (Fig. 2). A sequence showing a very high degree of complementarity to the 3′ end of *B. stearothermophilus* 16S rRNA (40, 41) is located 3 nucleotides upstream from the ATG initiation codon. This sequence presumably functions as an efficient ribosome-binding site for the RNA transcript. The reading frame ends with two consecutive stop codons (TAA and TGA). Translation of the DNA sequence into the corresponding amino acid sequence revealed complete identity with the amino acid sequence for *B. stearothermophilus* MnSOD previously determined by Brock and Walker (12).

The alignment of each of the hybridizing clones is shown in Fig. 2, together with the results of the complementation analysis of SOD-deficient *E. coli*. The presence or absence of SOD activity within these different bacterial clones was confirmed by assaying total protein extracts for SOD activity

on nondenaturing gels (4) (Fig. 2c). In most cases, MnSOD activity was two- to threefold higher than that normally present in *B. stearothermophilus*.

Protein extracts derived from clones 2A, 4A, 5A, and 6A contained a major band of SOD activity which migrated to the same position as the MnSOD activity in protein extracts from *B. stearothermophilus* (Fig. 2c). We also detected a slower-migrating form in these samples which was present only in extracts derived from *E. coli*. Since only one band could be detected on denaturing gels (data not shown), this slower-migrating form presumably resulted when some of the protein formed a different multimeric structure in *E. coli*, perhaps because a larger amount of protein was synthesized.

Protein extracts from cells containing plasmids 1A and 7A also showed SOD activity, but this activity migrated more slowly on nondenaturing gels (Fig. 2c, lanes 1 and 7). These plasmids contain no *B. stearothermophilus* sequence upstream from the MnSOD structural gene, since the *Sau*3A site bordering these inserts is located only 3 nucleotides upstream from the MnSOD initiation codon (Fig. 1). The synthesis of this protein must therefore be under the control of the *lacZ* promoter and the Shine-Dalgarno (S-D) sequences situated immediately upstream from the MnSOD sequence (Fig. 2a). Analysis of the DNA sequence revealed that the unusual migration of this protein is most likely due to the synthesis of a fusion protein which contains the first 14 amino acids of the pUC9-encoded β-galactosidase gene. In agreement with the complementation data, we found no MnSOD activity in protein samples derived from cells containing plasmid 3A (Fig. 2c, lane 3).

Further information concerning the gene structure was obtained by determining which clones were dependent on the *lacZ* promoter for expression of MnSOD. This was determined by taking each insert as a *Sma*I-*Pst*I fragment from pUC9 and cloning it into *Sma*I-*Pst*I-digested pUC18 (44). Restriction sites for *Sma*I and *Pst*I were unique to the pUC9 polylinker in each case, so each insert could be easily manipulated in this manner. Such constructions thus contained the *lacZ* promoter on the opposite side of the insert, compared with the original clones in pUC9. Clone 1A inverted in this manner was designated 1B, clone 2A was designated 2B, etc.

These clones were again introduced into the SOD-deficient *E. coli* strain, and the results of the complementation tests and SOD activity stains are summarized in Fig. 2b and c. These results confirmed that the SOD expression from clones 1A and 7A depended on the *lacZ* promoter present upstream, since activity was lost when the inserts were inverted (Fig. 2c, lanes 1 and 7).

Clones 2, 4, 5, and 6 produced an active MnSOD protein regardless of the insert orientation with respect to the *lacZ* promoter. These clones must therefore contain promoter sequences derived from *B. stearothermophilus*. The amounts of MnSOD synthesized from the clones were approximately the same. Of these, clone 2 contained the least DNA sequence upstream from the MnSOD-coding region (450 base pairs), which appears to be sufficient to obtain expression in *E. coli*. Analysis of this sequence revealed the presence of one pair of putative −10 and −35 RNA polymerase recognition sequences which were separated by a gap of 17 nucleotides and which were homologous to the consensus for *E. coli* and vegetative *Bacillus* promoters (Fig. 1). The −10 box is situated 57 nucleotides upstream from the ATG initiation codon.

The inversion of clone 3A to clone 3B resulted in complementation and the appearance of three bands of MnSOD activity in protein extracts, each of approximately equal intensity (Fig. 2c, lane 3). Two of these bands migrated in the gel to the same position as those synthesized by clones 2, 4, 5, and 6, indicating that translation initiation begins at the MnSOD ATG. The origin of the third form of MnSOD activity present in protein extracts from this clone was not further studied experimentally but is considered further in the Discussion.

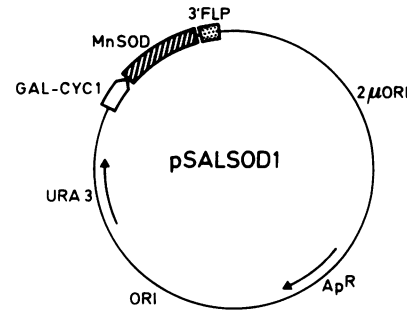

FIG. 3. Structure of yeast expression vector pSALSOD1. The *B. stearothermophilus* MnSOD-coding region (▨) is flanked at the 5′ end by the galactose-inducible *GAL-CYC1* hybrid promoter (▷) and at the 3′ end by the transcription termination signals of the 2μm *FLP* gene (▣). The plasmid is 9.6 kb and is not drawn to scale. Abbreviations: Ap^R, ampicillin resistance gene; URA 3, selection for uracil prototrophy in yeast; ORI, pBR322-derived origin of replication; 2μORI, origin of replication from the yeast 2μm plasmid.

MnSOD of *B. stearothermophilus* is functional in yeast cytosol. In eucaryotes, MnSOD is normally located only within the mitochondria (2). To determine whether there was some fundamental biochemical block prohibiting MnSOD activity in eucaryotic cytoplasm, we attempted to express bacterial MnSOD in yeast cells. The sequence encoding MnSOD was cloned into the yeast expression vector pEMBLyex4 (15), in the appropriate orientation to give expression from the galactose-inducible *GAL-CYC1* hybrid promoter (see Materials and Methods). The resulting plasmid, pSALSOD1, is illustrated in Fig. 3. This was introduced into the yeast strain DL1 (which contains a wild-type SOD profile) by lithium acetate transformation (24), and transformants were selected with the plasmid-encoded *URA3* gene.

Following growth in media containing glucose or galactose as a carbon source, protein extracts were analyzed for SOD activity. pSALSOD1-containing cells synthesized a protein with MnSOD activity, and the appearance of this protein was dependent upon growth of the cells in galactose-containing media (Fig. 4a). The protein migrated in the gel to the same position as that of MnSOD activity in protein extracts from *B. stearothermophilus*. We conclude that bacterial MnSOD is active in yeast cytosol.

MnSOD can complement structurally unrelated yeast Cu/ZnSOD. *S. cerevisiae* possesses nuclear-encoded Cu/Zn and Mn classes of SOD. Cu/ZnSOD is found in the cytosol, while MnSOD is located within the mitochondrial matrix (19, 36). A mutant which lacks Cu/ZnSOD activity has been isolated (6). It is characterized by its inability to grow in 100% oxygen and is hypersensitive to hydrogen peroxide and to the superoxide-generating herbicide paraquat. The introduction of plasmid pSALSOD1 into this strain would thus allow us to determine whether MnSOD is able to remedy a deficiency in the structurally dissimilar Cu/ZnSOD enzyme.

The transformation of this strain with pSALSOD1 again

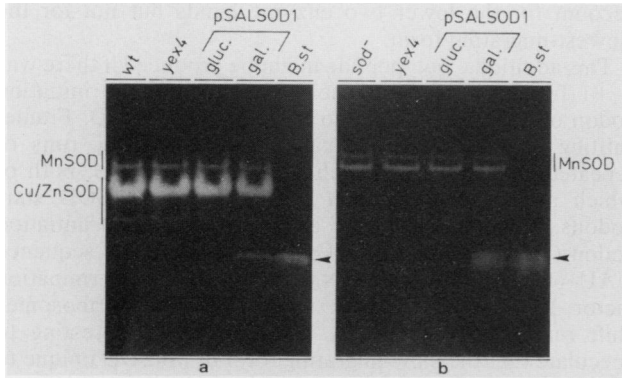

FIG. 4. Expression of *B. stearothermophilus* MnSOD in yeast. Total protein extracts were separated on nondenaturing gels and stained for SOD activity. (a) Expression of bacterial MnSOD in strain DL1. Each lane contains 40 μg of total protein. The endogenous MnSOD and Cu/ZnSOD activities of yeast are indicated. Abbreviations: wt, strain DL1; yex4, DL1 containing plasmid pEMBLyex4; gluc., DL1 containing pSALSOD1 grown in SDgluc media; gal., DL1 containing pSALSOD1 grown in SDgal media; B.st, 40 μg of total protein from *B. stearothermophilus*. Endogenous yeast MnSOD and Cu/ZnSOD are indicated. (b) Expression of bacterial MnSOD in Cu/ZnSOD-deficient strain DSCD2-4a. Each lane contains 80 μg of total protein. Abbreviations are as in panel a but refer to plasmids present in this mutant (denoted as sod^-). The position of the *B. stearothermophilus* MnSOD is indicated (◄).

resulted in the appearance of MnSOD activity which was galactose dependent (Fig. 4b), because of the *GAL-CYC1* promoter which directs synthesis of MnSOD from pSALSOD1.

Transformants were then tested for oxygen sensitivity by streaking them onto glucose- or galactose-containing media and incubating them at 28°C in an atmosphere of 100% oxygen. Expression of bacterial MnSOD in the Cu/ZnSOD-deficient mutant resulted in the restoration of a wild-type phenotype (Fig. 5a). This phenomenon was strictly correlated with growth on galactose, confirming that the synthesis of MnSOD was required for this to occur.

Similarly, bacterial MnSOD was able to provide protection against concentrations of paraquat and hydrogen peroxide which are normally toxic to cells lacking Cu/ZnSOD activity (Fig. 5b and c). Interestingly, a yeast cell containing MnSOD within the cytosol is better protected against hydrogen peroxide than wild-type cells, which contain only Cu/ZnSOD. This can be explained by the fact that Cu/ZnSOD is inactivated by hydrogen peroxide, whereas MnSOD is not (23). Wild-type cells treated with 25 mM hydrogen peroxide for 40 min indeed showed greatly reduced activity of Cu/ZnSOD but not MnSOD (data not shown), confirming that this inactivation can and does occur in vivo.

In all cases, the same levels of complementation were

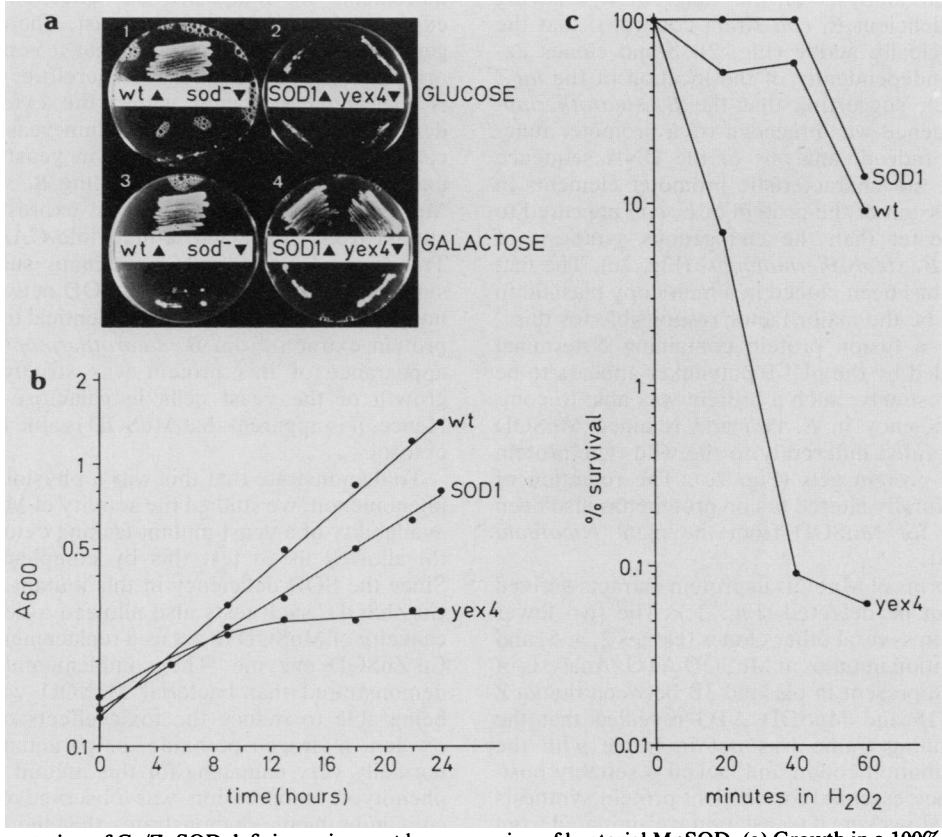

FIG. 5. Complementation of Cu/ZnSOD deficiency in yeast by expression of bacterial MnSOD. (a) Growth in a 100% oxygen atmosphere. Strains were streaked on solid medium containing SDgluc (plates 1 and 2) or SDgal (plates 3 and 4) and incubated at 28°C in 100% oxygen. Plates 1 and 3 (supplemented with 0.002% uracil), Wild-type strain DL1 (wt) (top) and Cu/ZnSOD-deficient mutant (sod^-) (bottom); plates 2 and 4, Cu/ZnSOD-deficient mutant containing pSALSOD1 (SOD1) (top) and Cu/ZnSOD-deficient mutant containing pEMBLyex4 (yex4) (bottom). (b) Growth of yeast strains in SDgal in the presence of 175 μM paraquat (added at time zero). Abbreviations: wt, strain DL1; SOD1, Cu/ZnSOD-deficient mutant bearing pSALSOD1; yex4, Cu/ZnSOD-deficient mutant carrying plasmid pEMBLyex4. (c) Percent survival of SDgal-grown yeast strains incubated in 25 mM H_2O_2 for 20, 40, and 60 min. Abbreviations are as in panel b.

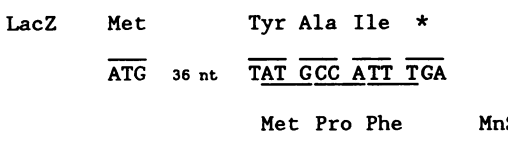

FIG. 6. Reinitiation of synthesis at MnSOD ATG by ribosomes terminating the *lacZ* peptide.

observed regardless of the presence or absence of manganese (data not shown).

DISCUSSION

SOD-deficient mutants of *E. coli* and yeast have proved very useful for studying various aspects of SOD biology (5, 8, 31). In this work, we have used such mutants to obtain information about the MnSOD gene of *B. stearothermophilus*. The gene was initially isolated with an oligonucleotide probe designed against a part of the amino acid sequence previously reported by Brock and Walker (12). DNA sequencing showed that this gene encodes a protein with complete sequence identity to this published sequence. Two other sequences for *B. stearothermophilus* MnSOD have been published (1, 11), each of which differs by one amino acid from the Brock and Walker (12) sequence. These differences may be due to the isolation of the protein from different strains of *B. stearothermophilus*.

Complementation analysis of the different hybridizing clones in a SOD-deficient *E. coli* strain confirmed that the protein was biologically active (Fig. 2). Some clones expressed MnSOD independently of the location of the *lacZ* promoter (Fig. 2c), suggesting that the *B. stearothermophilus*-coding sequence was preceded by a promoter functional in *E. coli*. Indeed, analysis of the DNA sequence showed that there are characteristic promoter elements in this region. Expression of the protein in *E. coli* appeared to be severalfold greater than the endogenous synthesis of MnSOD found in *B. stearothermophilus* (Fig. 2c). The fact that the sequence has been cloned in a multicopy plasmid in *E. coli* is likely to be the major factor responsible for this.

In some cases, a fusion protein containing N-terminal amino acids encoded by the pUC9 polylinker appears to be synthesized. Interestingly, such a protein was able to complement SOD deficiency in *E. coli* and retained MnSOD activity which migrated differently to the wild-type protein on nondenaturing protein gels (Fig. 2c). The retention of activity by a structurally altered fusion protein has also been recently reported for MnSOD from the plant *Nicotiana plumbaginifolia* (8).

Three distinct forms of MnSOD in protein extracts derived from clone 3B can be detected (Fig. 2c). The two lower bands are present in several other clones (clones 2, 4, 5, and 6), in which translation initiates at MnSOD ATG. Analysis of the DNA sequence present in plasmid 3B between the *lacZ* promoter of pUC18 and MnSOD ATG revealed that the MnSOD open reading frame was not in frame with the β-galactosidase initiation codon and lacked a suitably positioned S-D sequence essential for efficient protein synthesis (data not shown). However, if translation was initiated from the pUC18-encoded *lacZ* ATG (which is likely due to the presence of its upstream S-D sequence), translational coupling (33) would permit ribosomes terminating the *lacZ* peptide to reinitiate synthesis at MnSOD ATG (Fig. 6). This translation would give rise to a polypeptide identical to the MnSOD synthesized in *B. stearothermophilus* and may thus account for the lower two enzyme bands but not for the slowest-migrating form.

This additional polypeptide might be produced if there was a +1 frameshift event somewhere between the initiation codon of *lacZ* and the amino terminus of MnSOD. Frameshifting of this type (+1) can be influenced by runs of repeated bases (38, 39), such as CCC· or GGGG, both of which are present between the *lacZ* and MnSOD start codons. Also, the sequence around the MnSOD initiation codon (UAU GCC AUU UGA) is similar to the sequence UAU---CUU UGA in mRNA of the *E. coli* termination factor, RF-2, in which approximately 30% of the ribosomes shift reading frames to +1 (16, 17). It is interesting to speculate that the slow-migrating form of MnSOD unique to clone 3B arises from such an event.

The isolation of the gene encoding *B. stearothermophilus* MnSOD allowed us to study some intriguing questions concerning SOD biology and function. Three different classes of SOD enzymes have arisen during evolution; MnSOD and FeSOD are essentially procaryotic, and Cu/ZnSOD, which is structurally unrelated to these two, is found only in eucaryotic organisms. MnSOD is also found within the mitochondrial matrix of eucaryotes. We were interested in determining whether there was a physiological factor prohibiting the expression of MnSOD in eucaryotic cytosol, a location where it is normally not found. Such an obstruction would account for the evolution of a structurally unrelated SOD, such as Cu/ZnSOD, to supersede this requirement. We addressed this question by attempting to express bacterial MnSOD in yeast. The choice of bacterial gene was dictated by the fact that it is naturally devoid of organelle-targeting sequences; therefore, its protein product is expected to remain within the cytoplasm. Hence, a demonstration of its activity within yeast cells would indicate that the protein functions in yeast cytosol. For this experiment, the sequence encoding *B. stearothermophilus* MnSOD was cloned into a yeast expression vector downstream from the galactose-inducible *GAL-CYC1* promoter. Transformed yeast cells containing such a construction synthesized a protein with MnSOD activity migrating on a nondenaturing gel to a position identical to that of MnSOD in protein extracts from *B. stearothermophilus* (Fig. 4). The appearance of this protein was strictly dependent upon growth of the yeast cells in galactose-containing media. Hence, it is apparent that MnSOD is able to function in yeast cytosol.

To demonstrate that this was a physiologically significant phenomenon, we studied the activity of MnSOD in vivo. The availability of a yeast mutant lacking cytosolic SOD activity (6) allowed us to test this by complementation analysis. Since the SOD deficiency in this mutant is due to a lack of Cu/ZnSOD, such tests also allowed a determination of the capacity of MnSOD to act as a replacement for the unrelated Cu/ZnSOD enzyme. The complementation tests (Fig. 5) demonstrated that bacterial MnSOD was active in vivo, being able to reduce the toxic effects of growth in 100% oxygen, hydrogen peroxide, or paraquat, all of which are normally very damaging for this mutant. The fact that this phenotypic suppression was observed only on galactose-containing media demonstrates that the *GAL-CYC1* hybrid promoter is very tightly controlled, making it ideal for such experiments. Since expression of MnSOD restored growth to approximately wild-type levels, it is clear that MnSOD is able to efficiently compensate for Cu/ZnSOD deficiency. Indeed, it appears to better protect yeast cells against hydrogen peroxide (Fig. 5c). Cu/ZnSOD is known to be

inactivated by hydrogen peroxide, apparently as the result of the reduction of the Cu^{2+} metal at the active site followed by the destruction of an adjacent histidine residue (10, 23). The MnSOD enzyme is insensitive to this reduction and thus appears better able to protect against hydrogen peroxide toxicity.

Recently, it has been shown that human Cu/ZnSOD can restore a wild-type phenotype to SOD-deficient *E. coli* (31). All published results thus demonstrate that the in vivo capacities of all three enzymes are equivalent, meaning that the different SOD enzymes can be liberally interchanged with each other without significantly perturbing normal growth. However, it must be expected that further investigations will reveal reasons for the evolution of these three classes of functionally similar but structurally different enzymes.

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