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The Overlapping Roles of Manganese and Cu/Zn SOD in Oxidative Stress Protection

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Abstract

In various organisms, high intracellular manganese provides protection against oxidative damage through unknown pathways. Herein we use a genetic approach in *S. cerevisiae* to analyze factors that promote manganese as an anti-oxidant in cells lacking Cu/Zn superoxide dismutase (*sod1*Δ). Unlike certain bacterial systems [1], oxygen resistance in yeast correlates with high intracellular manganese without a lowering of iron. This manganese for anti-oxidant protection is provided by the Nramp transporters Smf1p and Smf2p, with Smf1p playing a major role. In fact, loss of manganese transport by Smf1p together with loss of the Pmr1p manganese pump is lethal to *sod1*Δ cells in spite of normal manganese SOD2 activity. Manganese-phosphate complexes are excellent superoxide dimustase mimics *in vitro* [2], yet through genetic disruption of phosphate transport and storage, we observed no requirement for phosphate in manganese suppression of oxidative damage. If anything, elevated phosphate correlated with profound oxidative stress in *sod1*Δ mutants. The efficacy of manganese as an anti-oxidant was drastically reduced in cells that hyper-accumulate phosphate without effects on MnSOD activity. Non-SOD manganese can provide a critical backup for Cu/Zn SOD1, but only under appropriate physiologic conditions.

Keywords

Manganese; SOD1; Yeast; Phosphate; Nramp

INTRODUCTION

Superoxide dimustases (SOD) are the only eukaryotic enzymes known to remove toxic superoxide anions. Typically, the mitochondrial matrix harbors a manganese SOD2, while superoxide scavenging in all other compartments is covered by a single highly abundant Cu/Zn SOD1. In spite of the predicted importance of Cu/Zn SOD1, various genetic models have indicated that SOD1 is not essential for aerobic life, but does help guard against extensive oxidative damage. For example, mouse $SOD1^{-/-}$ mutants survive to adulthood, yet exhibit a high incidence of fatal liver carcinomas and loss in muscle mass [3–6]. *Drosophila* mutants of SOD1 survive throughout development, but have dramatically reduced lifespans as adults [7]. In *S. cerevisiae, sod1*Δ mutants are viable in atmospheric oxygen, yet exhibit numerous

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markers of oxidative stress, including damage to the lysine and methionine biosynthetic pathways, defects in vacuolar morphology and increased pools of labile iron [8–13].

A number of studies have indicated that high intracellular manganese can compensate for loss of SOD and provide protection against oxidative stress. Archibald and Fridovich observed that strains of *Lactobaccilus planarum* that lack SOD enzymes are oxygen tolerant due to intracellular accumulation of mM manganese [14–18]. More recently, elegant work by M. Daley has demonstrated that radiation and oxygen resistance in bacteria correlates with a high manganese to iron ratio [1,19,20]. In the nematode *C. elegans*, treatment with high manganese can prolong life and provide resistance to stress [21] and in the bakers yeast *S. cerevisiae*, elevated manganese is particularly effective in compensating for loss of Cu/Zn SOD1. Supplementing yeast cells with millimolar quantities of manganese salts will reverse all the aforementioned aerobic growth defects of *sod1*Δ mutants [22–24]. Moreover, *sod1*Δ deficiency can be reversed without manganese supplements when cells have mutations in either the *PMR1* or *BSD2* genes, both of which lead to increased accumulation of cellular manganese [23,25–27].

Yeast cells express two Nramp metal transporters for manganese, Smf1p and Smf2p [28–30]. Smf1p is the more abundant of the two [30], yet earlier studies indicated that the less abundant Smf2p transporter accounts for much of the intracellular manganese and for activation of manganese enzymes in the mitochondria, Golgi and cytosol [31,32]. The function of Smf1p was unclear. A possible role for Smf1p and/or Smf2p in manganese suppression of oxidative damage had not been rigorously tested, but studies with the aforementioned *bsd2*Δ suppressors of *sod1*Δ mutants suggest one or both transporters may be key. Bsd2p normally functions to down-regulate Smf1p and Smf2p by helping to target these proteins to the vacuole for degradation in a manner dependent on the Rsp5p ubiquitin ligase [27,33–36]. As such, the steady state levels of Smf1p and Smf2p are greatly elevated in *bsd2*Δ mutants, correlating with suppression of oxidative damage [27,33].

The nature of the anti-oxidant that accumulates with high manganese is not known, but it is not MnSOD. In bacteria and yeast cells that lack manganese containing SOD molecules, elevated manganese can still suppress oxidative damage [23,26,37,38]. It is possible that specific manganese binding metabolites in cells can function as SOD-mimics. For example, recent studies by Valentine and Cabelli have shown that ortho-, but not pyro-phosphate complexes of manganese can serve as excellent SOD mimics [2] and a compound of this nature may serve as the manganese anti-oxidant *in vivo*. A manganese dependent superoxide scavenging activity has been identified in lysates from bacteria and yeast cells that hyperaccumulate the metal [14,18,24,37], yet the precise compound has not been purified, perhaps due to re-distribution of manganese upon cell lysis [37].

Herein we employed a genetic strategy in the bakers yeast to analyze manganese suppression of oxidative damage. We observed that the Nramp metal transporter Smf1p serves as an important source of the manganese anti-oxidant, and that under certain instances, Smf1p is needed for aerobic viability of yeast *sod1*Δ mutants. Surprisingly, we observed no requirement for cellular phosphate in manganese suppression of oxidative damage. If anything, accumulation of high intracellular phosphate correlated with a loss in manganese anti-oxidant activity. Proper manganese bioavailability is critical for aerobic survival in cells lacking SOD1.

MATERIALS AND METHODS

Yeast strains, growth conditions and plasmids

Many yeast strains in this study were derived from EG103 (MATa, *leu2-3, 112, his3*Δ*1, GAL +, trp1-289a, ura3-52*) and include the previously reported *sod1*Δ*::TRP1* KS107 [10],

sod1Δ::TRP1 pmr1Δ::LEU2 KS111 [10], *sod1Δ::TRP1 bsd2Δ::HIS3* XL110 [27], *sod1Δ::TRP1 bsd2Δ::HIS3 smf1Δ::URA3* XL110 strains [27] and the *sod1::URA3 sod2::TRP1 pmr1-1* VCSUP1 strain that contains a fully inactive allele of *PMR1* [23]. Construction of the SL109 *sod1Δ::TRP1 smf1Δ::URA3* [27], SL113 *sod1Δ::TRP1 pmr1Δ::LEU2 smf1Δ::URA3*, AR001 *sod1Δ::TRP1 pmr1Δ::LEU2 smf2Δ::HIS3*, MC123 *sod1Δ::LEU2 smf2Δ::HIS3* and MC127 *sod1Δ::TRP1 bsd2Δ::LEU2 smf2Δ::HIS3* strains were carried out using the previously published plasmids for deleting *SOD1* [39], *BSD2* [26, 27], *PMR1* [23], *SMF1* and *SMF2* [27]. All other strains for this study were derived from BY4741 (MATa, *leu2Δ0, met15Δ0, ura3Δ0, his3Δ1*) and include commercially available *kanMX4* deletion derivatives (Open Biosystems, www.openbiosystems.com) of *bsd2, pmr1, pho80* and *vph1*, all verified by colony purification and gene sequencing. LJ283 (*sod1Δ::LEU2 pmr1Δ::kanMX4*) and LJ284 (*sod1Δ::LEU2*) were obtained by deleting *SOD1* from the *pmr1Δ::kanMX4* strain and BY4741 parent as described [39]. LJ346 (*vtc4Δ::URA3*), LJ285 (*sod1Δ::LEU2 pmr1Δ::kanMX4 vtc4Δ::URA3*) and LJ286 (*sod1Δ::LEU2 vtc4Δ::URA3*) represent *vtc4Δ::URA3* derivatives of BY4741, LJ283 and LJ284, all created by using the *vtc4* gene deletion plasmid pΔVTC4 described below. A *pho84Δ::HIS3* version of BY4741 (strain LJ297) was created using the pLJ089 plasmid (see below). RS001 (*sod1Δ::LEU2 pho84Δ::HIS3*), RS002 and LR156 (*sod1Δ::LEU2 pho80Δ::kanMX4*) and MC130 (*sod1Δ::LEU2 vph1Δ::kanMX4*) represent *sod1Δ::LEU2* [39] derivatives of LJ297, the *pho80Δ::kanMX4* and *vph1Δ::kanMX4* strains respectively.

The *vtc4Δ::URA3* plasmid pΔVTC4 was constructed by PCR amplifying *VTC4* sequences −449 to +157 and +1217 to +2574 using primers that introduced *Hin*DIII sites at −449 and +2574, a *Bam*HI site at +157 and a *Sal*I site at +1217. The PCR products were digested at these sites and ligated in a trimolecular reaction to the *Sal*I and *Bam*HI sites of the *URA3* integrating vector pRS306 [40]. The resulting plasmid, pΔVTC4, was linearized with *Hin*DIII and used to delete chromosomal *VTC4* sequences +158 to +1216. To create the *pho84::HIS3* plasmid pLJ089, a *Xho*I-*Not*I fragment from plasmid pLJ246 [41] containing *PHO84* upstream and downstream sequences was ligated into pRS403 cut with the same enzymes.

Yeast cells were grown at 30°C either in enriched yeast extract, peptone, dextrose medium (YPD) or synthetic complete (SC) medium lacking lysine as needed [42]. Experiments with *sod1Δ pho84Δ* mutants used a modified synthetic medium prepared with phosphate-free yeast nitrogen base (QBiogene) and supplemented with 1 mM monobasic potassium phosphate. This medium promoted the phosphate deficiency phenotype of a *pho84* strain which is less apparent in standard SC medium containing ≈7 mM phosphate (Jensen and Culotta, unpublished). For anaerobic growth, medium was supplemented with 15 mg/L ergosterol and 0.5% Tween-80. All experiments employed cells freshly obtained from frozen stocks and cultured on YPD + ergosterol in oxygen depleted, CO_2 enriched culture jars (GasPak, Becton Dickinson). For growth tests under "microaerobic" conditions, including assays for lysine and methionine dependence, cells were seeded in 1 ml cultures at $O.D._{600}$ = 0.05 and incubated at 30°C in air without shaking for 16 hours. For spot tests of oxygen sensitivity, 10^4 and 10^3 cells were spotted onto YPD plates and allowed to grow in air for 3 days. In liquid culture tests for oxygen sensitivity, cells were pre-grown in liquid YPD + ergosterol anaerobically for 16 hours for ≈ 4 doubling times. Cells were then diluted back to an $O.D._{600}$ = 0.1 or 0.5 in 5 ml YPD or SC medium and allowed to grow in air with shaking at 220 RPM for defined time points.

Biochemical assays

Inorganic phosphate (largely orthophosphate) levels were measured by molybdate reactivity [24,43]. Cultures were grown in SC medium without shaking to $O.D._{600nm}$ of ~2.0. 2×10^8 cells were harvested and washed twice with deionized H_2O and resuspended in 500 μL 0.1% Triton X-100. Cells were lysed by glass bead homogenization and lysates were clarified by

centrifugation for 10 minutes at 16,000×g. 100 μg protein from each sample or phosphoric acid standards were diluted in 3 mL deionized H_2O followed by the sequential addition of 1 mL 0.1 M NaCl, 0.5 mL 0.5 M H_2SO_4, 0.5 mL 50 mM $(NH_4)_6Mo_7O_{24}$-$4H_2O$, and 3 mL 1-butanol with vortexing between each addition. 2 mL of the upper organic phase was added to 1 mL of 9 mM $SnCl_2$-H_2O and mixed well by vortexing. The absorbance of the upper phase was measured at $O.D_{700}$ nm and the inorganic phosphate levels were calculated based on standard curves generated from the phosphoric acid standards.

Polyphosphate was detected by gel electrophoresis and toluidine blue staining [44]. Cells isolated as above for orthophosphate analysis were resuspended in 250 μL 20 mM Tris-HCl pH 7.4, 20 mM KCl, 1 mM EDTA and 250 μL of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Cells were subject to glass bead homogenization, followed by clarification through centrifugation for 5 minutes at 16,000×g. The upper phase was extracted with 250 μL chloroform, and total RNA concentration in the aqueous phase determined by absorbance at A_{260} nm. The equivalent of 80 μg of RNA was applied to 20% polyacrylamide gels buffered with 0.5X Tris-borate (45 mM Tris, 110 mM boric acid, pH 8). Gels were fixed with 10% acetic acid and 10% methanol followed by staining for polyphosphate using 0.5% Toluidine Blue O in 25% methanol, 5% glycerol and 5% acetic acid.

Atomic absorption spectroscopy (AAS) of total cellular manganese was carried as described [41]. Manganese SOD2 activity analysis was carried out by native gel electrophoresis and nitroblue tetrazolium (NBT) staining [45,46], and SOD2 protein levels were monitored by immunoblot [46]; in both cases, 35 μg of total cell lysate protein was analyzed. For ICP-MS (Inductively coupled plasma mass spectrometry) analysis of manganese and iron, cells were cultured in SC medium to an $O.D._{600}$ of 3.0–4.0, harvested and washed in cold TE buffer (10 mM Tris-hydrochloride and 1mM EDTA, pH 8.0). 20 OD_{600} units of cells were digested overnight at 95°C in 1ml 20% nitric acid and subsequently diluted to 1% nitric acid with deionized water. Metal analysis was performed on an Agilent 7500ce ICP-MS according to manufacturer specification and values were converted to nmoles metal/10^9 cells.

RESULTS

The Nramp transporters and resistance to oxidative stress in yeast sod1Δ mutants

In bacteria, resistance to radiation and oxidative stress has been associated with accumulation of high manganese and low iron [1,19,20]. Elevated manganese can suppress oxidative damage in the eukaryotes *C. elegans* and *S. cerevisiae* [21–24] but potential effects of iron have not been examined. We examined whole cell metals in cases where oxidative damage in yeast *sod1*Δ mutants is suppressed by mutations in either *pmr1* or *bsd2* [23,26]. As seen in Fig. 1A, suppression of oxidative damage in these strains correlated with increased manganese accumulation, even in strains lacking manganese containing SOD2, consistent with accumulation of a non-SOD manganese based anti-oxidant. Yet in all cases, iron levels were not lowered in the oxygen resistant strains. This was true not only of the genetic suppressors of *sod1*Δ (Fig. 1B), but also of *sod1*Δ mutants suppressed by treatment with mM concentrations of manganese salts (see ahead Fig. 6B). Suppression of oxidative damage in yeast cells is associated with elevated intracellular manganese without lowering iron.

The effects of manganese but not iron on oxidative stress resistance led us to examine the role of the manganese Nramp transporters Smf1p and Smf2p. We first tested whether Smf1p and Smf2p are required for suppression of *sod1*Δ deficiency by mM manganese. Due to oxidative damage to the lysine biosynthetic pathway, *sod1*Δ mutants cannot grow in air without exogenous lysine [13,25], and this defect was suppressed by supplementing the growth medium with ≈2–3 mM manganese (Fig. 2A). Manganese could still suppress oxidative damage in double *sod1*Δ *smf1*Δ and *sod1*Δ *smf2*Δ mutants. However, the *sod1*Δ *smf1*Δ mutant

consistently required higher concentrations of the metal (Fig. 2A), even though intracellular manganese levels were not greatly affected by the *smf1*Δ mutation (Fig. 2B). This was the first indication that Smf1p is needed for efficient manganese suppression of *sod1*Δ.

We also tested the role of Smf1p and Smf2p in suppression of oxidative damage when manganese levels are elevated without manganese supplements to the growth medium, i.e., in *bsd2*Δ and *pmr1*Δ mutants. Mutations in *BSD2* are associated with a 2–3 fold increase in intracellular manganese and this was reversed by mutations in either *smf1* or *smf2* (Fig. 3A). Yet of the two Nramp transporters, Smf1p seems more critical for *bsd2* suppression of oxidative damage. Mutations in *smf1* greatly diminished the ability of *bsd2* mutations to suppress the aerobic methionine deficiency of a *sod1*Δ mutant (Fig. 3B) [26]. The growth tests of Figs. 2A and 3B were conducted under "microaerobic" conditions (liquid cultures not aerated by shaking), where *sod1*Δ cells generally grow well in complete minimal medium supplemented with methionine and lysine (e.g., see Fig. 3B, right). A more severe oxidative stress is imposed when cells are directly exposed to atmospheric oxygen by growth on solid medium (as in Fig. 3C) or are well aerated in liquid cultures (Fig. 3D). In both cases, the strong aerobic growth of the *sod1*Δ *bsd2*Δ strain was drastically impaired by a *smf1*Δ mutation; the effects of *smf2*Δ mutations were less pronounced.

PMR1 encodes a Golgi pump for manganese [47,48] and in yeast *pmr1*Δ mutants, the manganese that cannot enter the Golgi hyper-accumulates in the cytosol and other cellular locales [31,49]. The elevated manganese in *pmr1*Δ mutants reverses the aerobic lysine deficiency of *sod1*Δ mutants [49], however this oxygen resistance was abrogated in the triple *sod1*Δ *pmr1*Δ *smf1*Δ mutant (Fig. 4A). Effects of the *smf2*Δ mutation were minor by comparison (Fig. 4A).

Notably, the *smf1*Δ mutation had a profound effect on aerobic viability of the *sod1*Δ *pmr1*Δ strain. The triple *sod1*Δ *pmr1*Δ *smf1*Δ mutant failed to grow in complete medium under all aerobic conditions tested, including direct exposure to atmospheric oxygen (Fig. 4B), in well aerated liquid cultures (Fig. 4C) and under microaerobic conditions (Fig. 4A). The strong growth defect of the *sod1*Δ *pmr1*Δ *smf1*Δ strain was reversed by anaerobic conditions (Fig. 4B) or by treatments with micromolar levels of manganese (Fig. 4D). This rescue by low supplements of manganese would suggest that manganese is limiting in the *sod1*Δ *pmr1*Δ *smf1*Δ strain. The *sod1*Δ *pmr1*Δ *smf1*Δ strain does accumulate lower levels of manganese than the *sod1*Δ *pmr1*Δ parent, but this level approximates that of the *sod1*Δ parent and is still higher than the *sod1*Δ *smf1*Δ and *sod1*Δ *smf2*Δ strains that are all viable in air (Fig. 4E). As an additional marker of manganese, we tested for activity of manganese containing SOD2 in the mitochondria. As seen in Fig. 4F, SOD2 activity was not affected in the *sod1*Δ *pmr1*Δ *smf1*Δ strain. Hence a specialized pool of manganese distinct from that utilized by mitochondrial SOD2 supports aerobic life of *sod1*Δ cells and this pool becomes limiting with loss of Smf1p and Pmr1p. The data of Fig. 4F also addresses the non-SOD nature of the manganese antioxidant. The presence of robust SOD2 activity in the *sod1*Δ *pmr1*Δ *smf1*Δ strain underscores the notion that manganese promotes aerobic life in ways that are independent of the MnSOD enzyme.

The effects of phosphate on manganese suppression of oxidative damage

Manganese-phosphate complexes can scavenge superoxide *in vitro* [2], raising the intriguing possibility that manganese-phosphate is the anti-oxidant back up for Cu/Zn SOD1 *in vivo*. To address this, we introduced mutations in *sod1*Δ cells that disrupt phosphate uptake and storage and monitored effects on manganese suppression of oxidative damage.

Mutations in *vph1*Δ and *vtc4*Δ mutants impair phosphate storage [50,51], and both polyphosphate (Fig. 5A left) and orthophosphate (Fig. 5B left) were dramatically lowered in

these mutants. Mutations in *pho84*Δ encoding the high affinity metal-phosphate transporter [41,52] also reduce phosphate when grown in a defined medium containing 1 mM phosphate (normal synthetic medium contains close to 10 mM phosphate) (Fig. 5A, 5B, right). In spite of vastly different levels of intracellular phosphate, the *sod1*Δ, *sod1*Δ *vph1*Δ and *sod1*Δ *pho84*Δ strains all exhibited suppression of oxidative damage at ≥3.0 mM manganese, as monitored by reversal of the lysine biosynthetic defect (Fig. 5C, 5D). However, manganese suppression of the *sod1*Δ *vtc4*Δ strain was severely compromised and required at least 6–7 mM concentrations of the metal (Fig. 5C). This result was unexpected since *vtc4*Δ and *vph1*Δ mutations similarly lower phosphate even in cells grown in 3 mM manganese (Fig. 5A, 5B). We observed that *vtc4* mutants specifically exhibit a resistance to manganese toxicity (Fig. 6A) that correlates with a dramatic lowering of intracellular manganese (Fig. 6B). When treated with 3 mM manganese, *sod1*Δ *vtc4*Δ cells accumulate approximately half the level of manganese seen in the parental *sod1*Δ strain (left), while iron levels are not affected (right). At 6 mM manganese where the *sod1*Δ *vtc4*Δ strain begins to exhibit suppression of oxidative damage (Fig. 5B), intracellular manganese levels more closely approximate that of the *sod1*Δ parent grown at 3 mM manganese. Manganese suppression of oxidative damage is clearly correlating with intracellular manganese, not phosphate.

The effect of *vtc4*Δ mutations on lowering manganese is only seen with manganese salts added to the growth medium; *sod1*Δ *vtc4*Δ cells accumulate normal levels of the metal without manganese supplements (Fig. 6B left, see legend). As such, *vtc4*Δ mutants provided a good background to test the effects of lowering phosphate in the *pmr1*Δ suppression of oxidative damage that does not require manganese supplements. As seen in Fig. 6C, the triple *sod1*Δ *pmr1*Δ *vtc4*Δ mutant accumulates very low orthophosphate. However, there was no loss in *pmr1*Δ suppression of oxidative damage (Fig. 6D). Together the studies of Figs. 5–6 demonstrate that a lowering of intracellular phosphate does not preclude manganese suppression of oxidative damage by either mM manganese supplements or by the *pmr1* suppressor of *sod1*Δ.

We also tested the effects of elevating intracellular phosphate using mutants of *pho80* encoding a negative regulator of phosphate uptake and storage [52–s55]. As seen in Fig. 7A, *pho80* mutants accumulate very high levels of polyphosphate (left) and orthophosphate (right). Since manganese orthophosphate complexes are good mimics of SOD [2], *pho80* was expected to increase oxygen resistance in *sod1*Δ strains. Surprisingly however, the double *sod1*Δ *pho80*Δ exhibited a profound oxygen sensitivity (Fig. 7B, 7C). The strain failed to grow aerobically under all conditions tested, similar to what we observed with the *sod1*Δ *pmr1*Δ *smf1*Δ strain (see Fig. 4). The *sod1*Δ *pho80*Δ strain did exhibit growth under anaerobic conditions (Fig. 7B) or when manganese was supplemented to aerobic cultures (Fig. 7C). Yet compared to the *sod1*Δ *pmr1*Δ *smf1*Δ strain, rescue of *sod1*Δ *pho80*Δ required much higher concentrations of the metal (millimolar as opposed to micromolar in minimal medium) and manganese was only mildly effective in restoring aerobic growth (Fig. 7C).

The poor rescue of oxidative damage by manganese in *pho80* mutants cannot be explained by a general limitation in manganese ion bioavailability. The manganese containing SOD2 of the mitochondria was active (Fig. 7D). Moreover there was no deficiency in manganese accumulation (Fig. 7E). In fact with manganese supplements, the *sod1*Δ *pho80* mutant accumulated 5–10 fold higher levels of the metal compared to the *sod1*Δ parent (Fig. 7E). Nevertheless, this very high intracellular manganese poorly rescues aerobic growth. Loss of phosphate control in *pho80* cells correlates with a drastic reduction in the efficacy of manganese as an anti-oxidant. Overall, these findings do not support a role for manganese-phosphate compounds in oxidative stress protection.

DISCUSSION

High intracellular manganese has long been known to suppress oxidative stress [1,14,22–24], but the requisite cellular factors were unknown. Using a yeast model system, we now show that the Nramp metal transporters together with the Golgi transporter for manganese are critical for manganese suppression of oxidative damage. We also provide evidence for phosphate compounds altering the efficacy of manganese as an anti-oxidant. Disruptions in manganese homeostasis or elevations in cellular phosphate can cause aerobic lethality to cells lacking Cu/Zn SOD1 in spite of abundant manganese SOD2 activity. Independent of its role as a co-factor for SOD2, manganese serves as an important anti-oxidant backup for Cu/Zn SOD1.

Of the two Nramp manganese transporters in yeast, we observed that Smf1p is most critical for manganese suppression of oxidative damage. This represents the first described function for this Nramp transporter. Most of the manganese requirements of the cell, e.g., manganese activation of SOD2 in the mitochondria and of sugar transferasese in the Golgi, involve the other less abundant Nramp of yeast, Smf2p [31]. Evidently, the increased demand for manganese during oxidative stress cannot be met by Smf2p and the more abundant Smf1p Nramp comes into play.

When combined with mutations in *pmr1* encoding the Golgi pump for manganese, loss of Smf1p is lethal to cells lacking SOD1. A fraction of Smf1p resides in the secretory pathway [33,36], and Pmr1p and Smf1p are predicted to transport the metal in opposite directions. An intriguing possibility is that some of the manganese transported into the secretory pathway by Pmr1p is re-claimed by Smf1p transport of manganese back into the cytosol. This re-cycling of manganese between the secretory pathway and cytoplasm together with the Smf1p-cell surface uptake of manganese may be critical for accumulating manganese as an anti-oxidant.

Nramp transporters are well-conserved throughout nature and Smf1p-like molecules from other organisms are likely to be used for oxidative stress protection. The oxygen tolerant *Lactobaccillus planarum* expresses a number of Nramp transporters that may support the strong manganese anti-oxidant activity of this organism [56], and a loss of the Smf-3 Nramp transporter of *C. elegans* has been associated with superoxide sensitivity [57]. Mammals express two Nramp transporters that have the capacity to transport manganese [58–60] and either one may help support oxidative stress resistance.

Studies have shown that certain manganese-phosphate complexes can serve as excellent mimics for SOD *in vitro* [2,15]. Yet in our genetic studies, we observed no requirement for cellular phosphate in manganese suppression of oxidative damage. If anything, high intracellular phosphate correlated with *increased* oxidative stress, and *sod1*Δ *pho80*Δ mutants that accumulate very high phosphate are inviable in air. In this manner, the *sod1*Δ *pho80*Δ cells phenocopy the aforementioned *sod1*Δ *pmr1*Δ *smf1*Δ mutants. Both are defective in their capacity to provide manganese anti-oxidant protection, but apparently through different mechanisms. The *sod1*Δ *pmr1*Δ *smf1*Δ mutants are limited for manganese, as the oxidative stress is fully reversed by supplementing the cells with low (micro molar in minimal medium) levels of the metal. By comparison, the *sod1*Δ *pho80*Δ strain is poorly rescued by even very high levels of manganese. Total cellular manganese is not limiting in *pho80* strains, rather the metal appears non-reactive as an anti-oxidant backup for SOD1.

The *pho80* mutants hyperaccumulate a wide array of phosphate compounds, and *in vitro* studies have shown that manganese binding to pyro and polyphosphate can inhibit superoxide scavenging activity of the metal [2,14,18]. In addition, inorganic phosphate and polyphosphate can inhibit manganese reactivity with H_2O_2 in the presence of bicarbonate [61]. It is possible that in *pho80* mutants, manganese binding to one or more of these phosphate complexes or to another cellular metabolite interferes with the metal's ability to guard against oxidative stress.

While we cannot totally exclude the possibility that a certain manganese-phosphate complex acts as an SOD-mimic *in vivo*, our genetic studies of phosphate control suggest that other possibilities should be considered such as carboxylates [20].

Lastly, these studies address the enigma of whether Cu/Zn SOD1 is actually essential for aerobic life. In spite of the widespread indices of oxidative damage noted with various eukaryotic models of SOD1 deficiency, the organisms for the most part are viable in atmospheric oxygen [6,7,13]. Based on the studies presented here, we propose that the physiological levels of manganese that accumulate in all cells serves as a backup to Cu/Zn SOD1. Although SOD is the only enzyme known to scavenge superoxide, non-SOD complexes of manganese can act as a secondary means of removing reactive oxygen species.

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LIST OF ABBREVIATIONS

SOD
: superoxide dismutase

PCR
: polymerase chain reaction

YPD
: yeast extract, peptone, dextrose medium

SC
: synthetic complete medium

AAS
: Atomic absorption spectroscopy

NBT
: nitroblue tetrazolium

ICP-MS
: Inductively coupled plasma mass spectrometry

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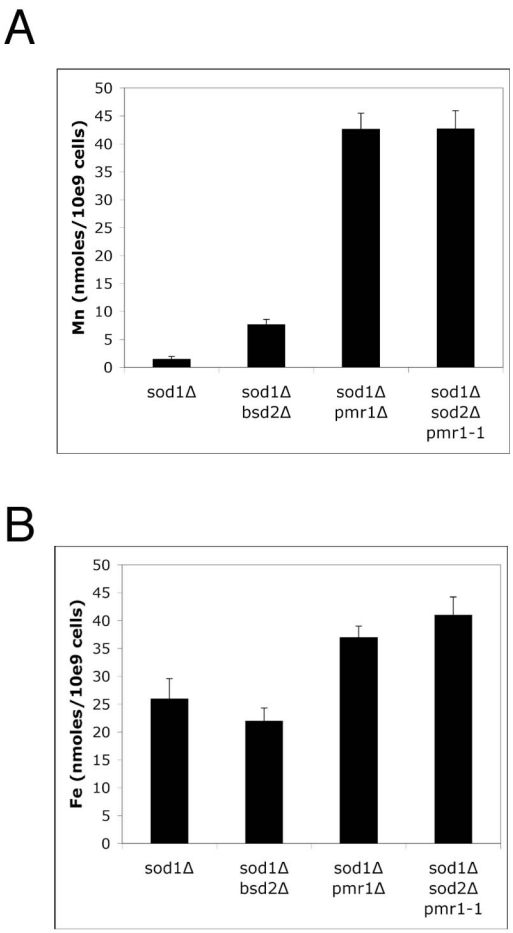

Fig. 1.

Suppression of *sod1*3 deficiency by changes in manganese, not iron.

The indicated strains were grown to an $O.D._{600}$ nm of 3.0 in SC medium. Whole cell manganese (A) and iron (B) was analyzed by ICP-MS as described in *Materials and Methods*. Values represent the average of three-four independent cultures with error bars representing standard deviation. Strains employed: *sod1* Δ, KS107; *sod1* Δ *pmr1* Δ, KS111; *sod1* Δ *bsd2* Δ, XL110; *sod1* Δ *sod2* Δ *pmr1-1*, VCSUP1.

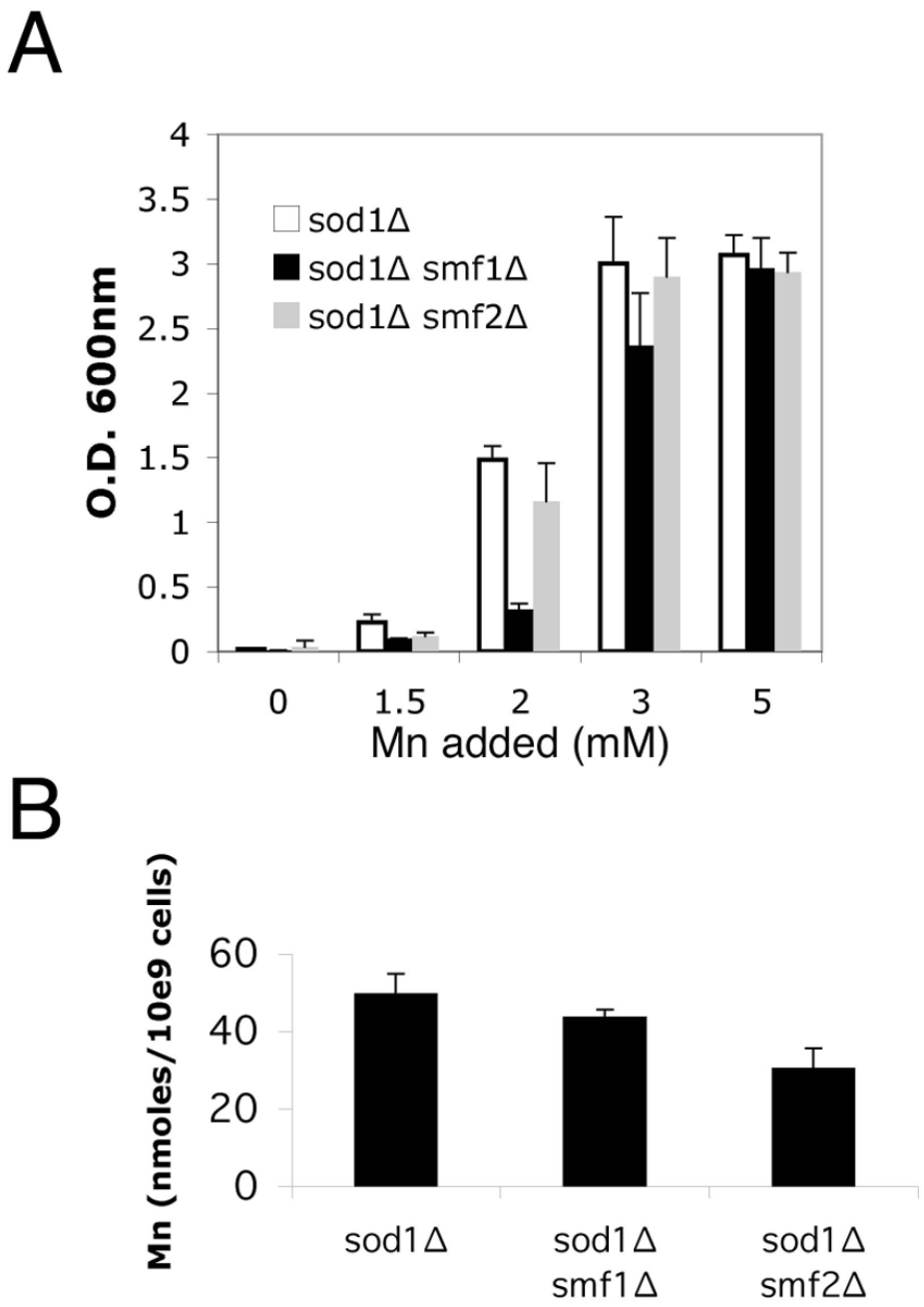

Fig. 2.

The role of Nramp metal transporters in suppression of oxidative damage by mM manganese. A) The indicated strains were grown in air without shaking in liquid SC medium lacking lysine and supplemented with the designated concentrations of $MnCl_2$. Following 16 hours incubation, total cell growth was monitored as a function of absorbance at 600 nm. B) The indicated strains were grown as above in (A), except medium was supplemented with lysine and 3 mM $MnCl_2$. Total cellular manganese was determined by AAS as described in *Materials and Methods*. Values represent the average of three independent cultures with error bars representing standard deviation. Strains employed *sod1* Δ, KS107; *sod1* Δ *smf1* Δ, SL109; *sod1* Δ *smf2* Δ, MC123.

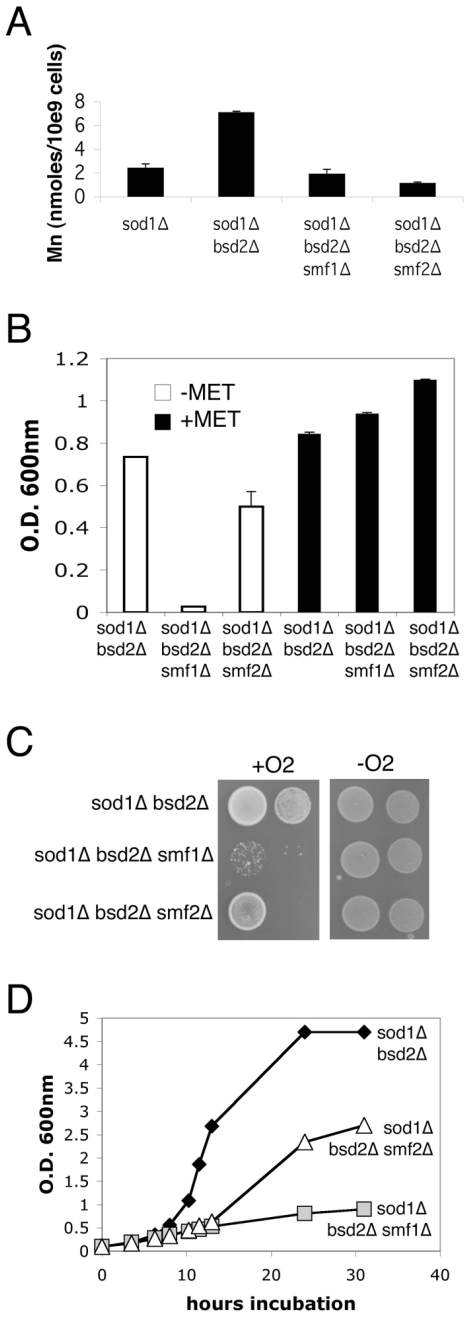

Fig. 3.

Smf1p and suppression of oxidative damage by *bsd2* Δ mutations.

A) Total cellular manganese from three independent cultures was analyzed in the indicated strains grown in complete SC medium as described in Fig. 2B. B) The indicated strains were grown 15 hours non-shaking in SC medium containing lysine, but lacking or supplemented with methionine as designated. Total cell growth was monitored as a function of absorbance at 600 nm. Values represent the average of three independent cultures with error bars representing standard deviation. C) 10^4 and 10^3 cells of the indicated strains were spotted onto enriched YPD medium and incubated for 3 days either in air or in anaerobic culture jars. D) Strains were pre-grown overnight in complete SC medium under anaerobic conditions; cultures

were then diluted to $O.D._{600nm}$ = 0.1 in the same medium and monitored for aerobic growth under well-aerated conditions for the indicated time points. Strains utilized: *sod1*Δ, KS107; *sod1* Δ *bsd2* Δ, XL110; *sod1* Δ *bsd2* Δ *smf1* Δ, XL111; *sod1* Δ *bsd2* Δ *smf2* Δ, MC127.

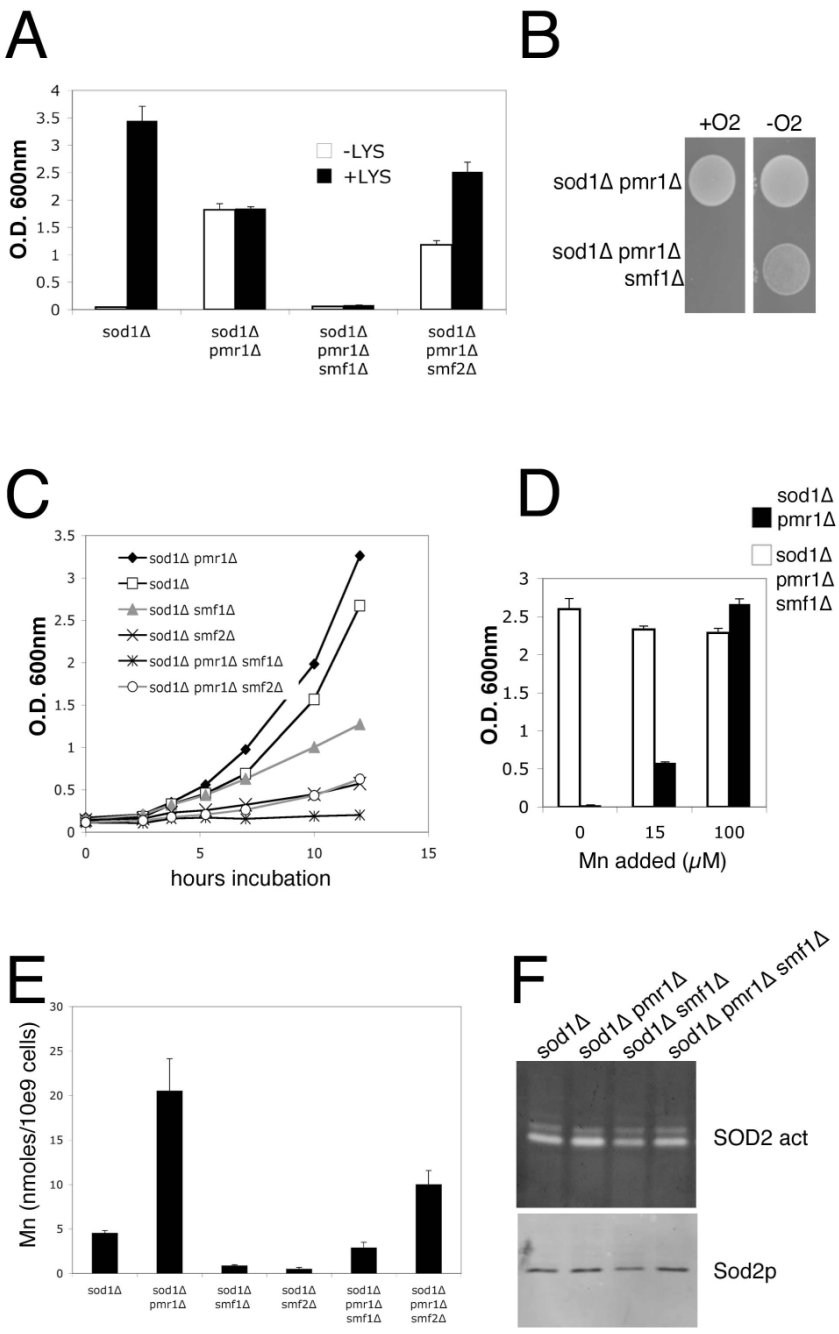

Fig. 4.

A combination of *smf1* Δ and *pmr1* Δ mutations causes severe oxidative stress

A) Yeast strains were grown in triplicate cultures as in Fig. 3B in medium supplemented with methionine and containing or lacking lysine as indicated. B) 10^4 cells of the indicated strains were grown as in Fig. 3C. C) Aerobic growth test in enriched YPD medium was conducted as described in Fig. 3D. D) Yeast strains were grown in triplicate cultures as in Fig. 3B in medium supplemented with both lysine and methionine and also with the indicated concentrations of $MnCl_2$. E) The indicated cells pre-grown in enriched YPD medium under anaerobic conditions and switched to air for 1.5 hours were analyzed for total manganese content by AAS. Values represent averages of four independent cultures analyzed in two experimental trials; error bars

represent standard deviation. F) The indicated strains grown as in (E) were lysed and assayed for mitochondrial manganese SOD2 activity by native gel electrophoresis and NBT staining (top) and for SOD2 polypeptide levels by immunoblotting (bottom). Strains utilized include: *sod1* Δ, KS107; *sod1* Δ *pmr1* Δ, KS111; *sod1* Δ *smf1* Δ, SL109; *sod1* Δ *pmr1* Δ *smf1* Δ, SL113; *sod1* Δ *pmr1* Δ *smf2* Δ, AR001; *sod1* Δ *smf2* Δ, MC123.

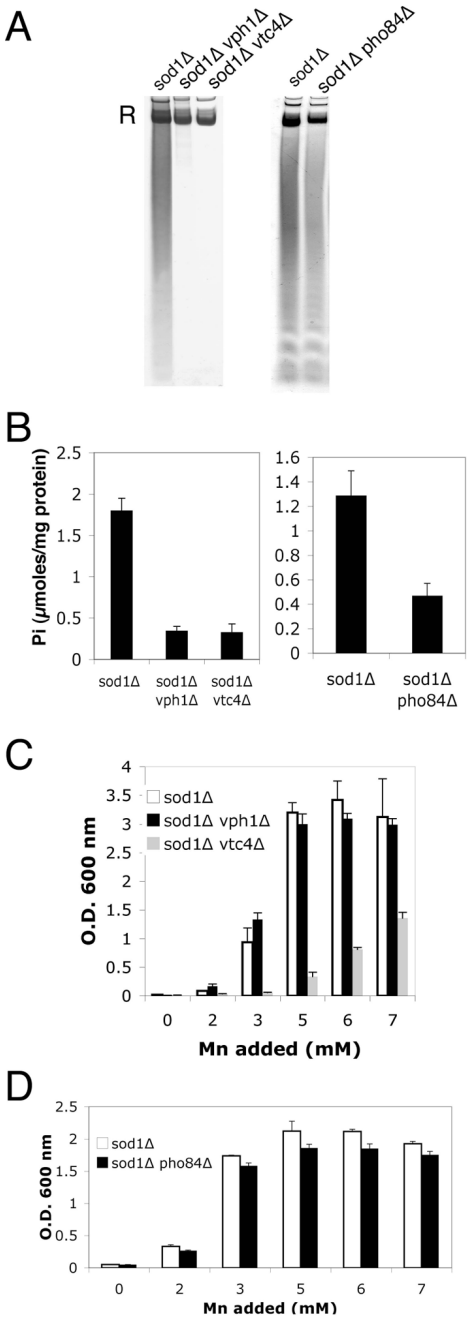

Fig. 5.

Phosphate deficient mutants of *S. cerevisiae* and manganese suppression of oxidative damage. A–B) The indicated cultures were grown to early stationary phase in minimal medium containing methionine and lysine and supplemented with 3 mM $MnCl_2$ (manganese levels that typically suppress *sod1* Δ deficiency). Cell lysates were prepared for analysis of polyphosphate (A) by polyacrylamide gel electrophoresis and toluidene blue staining, or for analysis of ortho phosphate (B) by molybdate reactivity as described in *Materials and Methods*. "R" = RNA staining by toluidene blue. Values of orthophosphate (Pi) are the averages of three independent cultures with error bars representing standard deviation. C,D) Suppression of the *sod1* Δ lysine biosynthetic defect by mM manganese was examined in triplicate cultures of the indicated

strains as described in Fig. 2A. Strains used: *sod1* Δ, LJ284; *sod1* Δ *pho84* Δ, RS001; *sod1* Δ *vph1* Δ, MC130; *sod1* Δ *vtc4* Δ, LJ286. Standard SC medium containing ≈7 mM phosphate was used for A, B left panels and for C. A,B right panels and D employed a minimal medium containing 1 mM phosphate to maximize effects of the *pho84* mutation (see *Materials and Methods*).

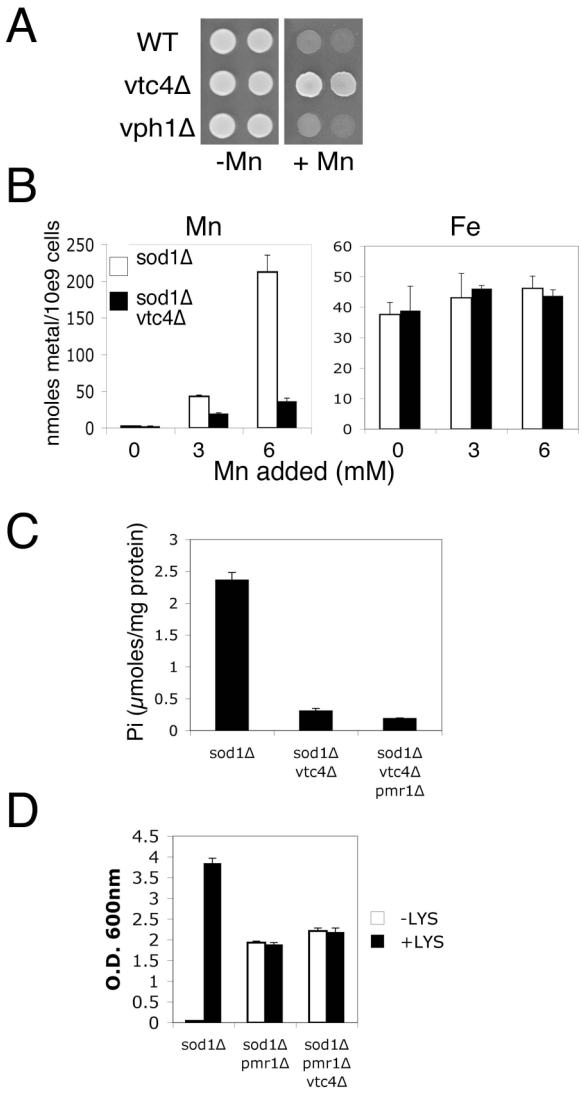

Fig. 6.

Studies with *vtc4* Δ show a role for intracellular manganese but not phosphate in suppression of oxidative damage.

A) 10^5 and 10^4 cells of the indicated strains were spotted onto YPD plates supplemented where indicated with 5 mM $MnCl_2$. Cells were allowed to grow for three days. B) Strains were grown in SC complete medium supplemented where indicated with the designated levels of $MnCl_2$ prior to analysis of total cellular manganese (left) and iron (right) by AAS. In the absence of added manganese, the total manganese accumulation in the *sod1* Δ and *sod1* Δ *vtc4* Δ mutants is 2.54 and 2.25 nmoles/10^9 cells respectively. C) Orthophosphate analysis of the indicated strains grown in SC medium was carried out as in Fig. 5B. D) Test for lysine independent growth as in Fig. 2A was conducted in the designated strains supplemented with the indicated levels of $MnCl_2$. Strains employed include: WT, BY4741; *vtc4* Δ and *vph1* Δ, the corresponding kanMX4 derivates of BY4741; *sod1* Δ, LJ284; *sod1* Δ *vtc4* Δ, LJ286; *sod1* Δ *vtc4* Δ *pmr1* Δ, LJ285; *sod1* Δ *pmr1* Δ, LJ283. (B–D) results represent the averages of 3–4 independent cultures and error bars represent standard deviation.

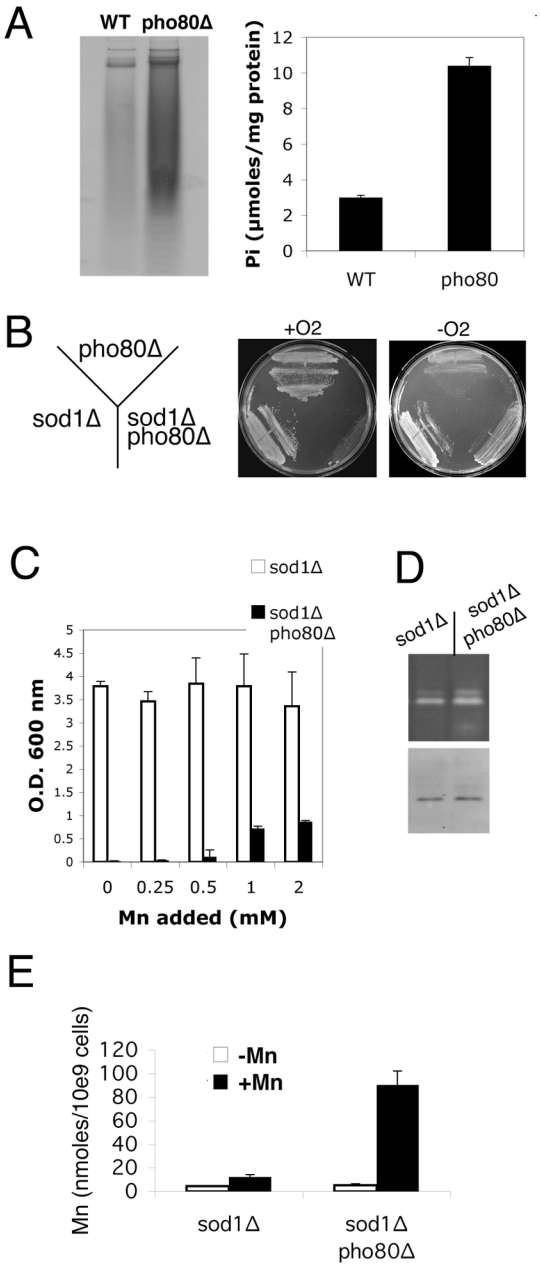

Fig. 7.

Deleterious effects of *pho80* mutations on cells lacking Cu/Zn SOD1.

A) The indicated strains were grown in YPD medium to an O.D. ≈ 6.0 and analyzed for polyphosphate and ortho phosphate as in Fig. 5A,B. Orthophosphate measurements were taking in three independent cultures with error bars representing standard deviation. B) Strains were plated onto YPD medium and allowed to grow in air or in anaerobic culture jars for three days. C) The indicated strains were grown in SC complete medium supplemented with the indicated concentrations of $MnCl_2$ as in Fig. 4C. Results were averaged over three independent cultures, error bars represent standard deviation. D) The indicated strains were grown as in Fig. 4E and tested for SOD2 activity and protein levels as in Fig. 4F. E) Total cellular manganese was monitored in the indicated cells grown in enriched YPD medium as in Fig. 4E.

Where indicated, cells were supplemented or not supplemented with 100 μM $MnCl_2$, the concentration of manganese in enriched medium that supports maximal aerobic growth of the *sod1* Δ *pho80* Δ mutant (not shown). Results represent the averages of four cultures in two independent trials; error bars represent standard deviation. Strains employed: WT, BY4741; *pho80*, *pho80* Δ*::kanMX4* derivative of BY4741; *sod1* Δ, LJ284; *sod1* Δ *pho80* Δ LR156C.