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Antioxidant Functions Required for Insusceptibility of *Saccharomyces cerevisiae* to Tetracycline Antibiotics

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Cu,Zn superoxide dismutase (Sod1) is required for insusceptibility of *Saccharomyces cerevisiae* to oxytetracycline (OTC). Here we report that Sod1 is also required for insusceptibility to doxycycline (DOX). Furthermore, among a range of antioxidant and redox balance mutants, *mac1* and *ctr1* deletion strains exhibited marked sensitization to OTC and DOX. Certain mutants exhibited a slight sensitivity to methacycline and minocycline. Addition of copper suppressed antibiotic sensitivity. Thus, intracellular copper as well as superoxide dismutase can be critical for eukaryotic tolerance of several tetracycline antibiotics.

The tetracyclines are broad-spectrum antibiotics that block bacterial protein synthesis by inhibition of aminoacyl-tRNA binding to the ribosomal A site (5). As with any useful prokaryote-specific antibiotics, eukaryotic insusceptibility to tetracyclines is a prerequisite for successful chemotherapy. However, adverse reactions to antibiotics are common, arising in around 5 to 10% of patients to whom they are prescribed (11). Reported side effects of tetracyclines include hypersensitivity, photosensitivity, neurotoxicity, hepatotoxicity, teratogenicity, and nephrotoxicity (17). The underlying causes for these effects are unknown in most cases.

With the *Saccharomyces cerevisiae* yeast model, a single gene was recently identified that is required for eukaryotic insusceptibility to oxytetracycline (OTC) (3). Thus, *sod1*Δ cells (lacking Cu,Zn-superoxide dismutase) were sensitive to OTC, exhibiting a >95% reduction in colony-forming ability at OTC concentrations that had no effect on the wild type. Sod1p was required for protection against a novel oxidative mode of OTC action. This action was manifested as OTC-induced lipid peroxidation, protein oxidation, and cytotoxicity in *sod1*Δ cells only (3). Our main objective in the present study was to test whether these findings pertained specifically to Sod1p and OTC or whether they reflected a broader requirement for eukaryotic antioxidant functions in protection against a range of tetracyclines.

S. cerevisiae strains were derived from the parental background BY4741 and are available as the Y00000 series from Euroscarf (Frankfurt, Germany). The *gpx1/2/3*Δ triple mutant was constructed by short-flanking homology PCR (24) using the *URA3*, *His3MX6*, and *kanMX6* markers for gene disruption. Organisms were cultured for experimental purposes in liquid yeast extract-peptone-dextrose (YEPD) medium, as described previously (14). To test for antibiotic susceptibility, mid- to late-exponential-phase cultures were diluted to optical densities at 600 nm of ~2.5, 0.25, 0.025, 0.0025, and 0.00025 for each strain. Samples (4 μl) from each dilution were spotted on YEPD agar supplemented with filter-sterilized antibiotic where specified (filter-sterilized antibiotic stocks were dissolved in water). All antibiotics (as hydrochlorides) were obtained from Sigma, except methacycline-HCl, which was from US Pharmacopoeial Convention (Rockville, Md.).

To determine whether antioxidant proteins other than Sod1p might be required for insusceptibility to OTC, we examined a range of *S. cerevisiae* mutants deficient in the following components of the oxidative stress response and/or maintenance of cellular redox balance: Sod2p (mitochondrial Mn-superoxide dismutase), Ctt1p (cytosolic catalase), Gsh1p (γ-glutamyl cysteine synthetase), Ogg1p (8-oxoguanine DNA glycosylase), PHGpx1p, PHGpx2p, PHGpx3p (phospholipid hydroperoxide glutathione peroxidases), Mxr1p (methionine sulfoxide reductase), Yap1p (oxidative stress response transcription factor), and Mac1p (copper metalloregulatory transcription factor). Of these strains, only *mac1*Δ *S. cerevisiae* was found to exhibit a growth defect on OTC similar to that in *sod1*Δ cells (Fig. 1). The growth of *mac1*Δ and *sod1*Δ cells was inhibited at an OTC concentration of 100 μg ml^{-1}, and this effect was accentuated at an OTC concentration of 500 μg ml^{-1}, where growth was almost fully abolished; the same concentrations had no effect on growth of the wild type or the other mutants tested (not shown) (note that some inhibition of *sod1*Δ *S. cerevisiae* is detectable at OTC concentrations as low as 10 μg ml^{-1} [3]). Two of the key genes under Mac1p-control in *S. cerevisiae* are *CTR1*, encoding a high-affinity Cu(I) transporter, and *FRE1*, encoding a cell surface Cu(II)/Fe(III) reductase. Ctr1p is limiting for cellular Cu uptake (7), whereas Fre1p activity affects copper uptake by ~50% (13) but is limiting for ferric iron uptake (6). To help establish the downstream determinant of susceptibility to OTC in *mac1*Δ *S. cerevisiae*, *ctr1*Δ and *fre1*Δ mutants were examined for inhibition by OTC. Whereas the growth of *fre1*Δ *S. cerevisiae* cells was unaffected by OTC (not shown), *ctr1*Δ cells exhibited a marked sensitivity to OTC, similar to that of *mac1*Δ and *sod1*Δ cells (Fig. 1). Therefore, in addition to Sod1p, Mac1p and Ctr1p are required for yeast insusceptibility to OTC.

Diminished Ctr1p-dependent Cu uptake in *mac1*Δ or *ctr1*Δ strains could exacerbate OTC-dependent oxidative damage in two principal ways: (i) via diminished Sod1p activity, since free copper is strictly limited in *S. cerevisiae* (22) and active Sod1p requires Cu (8, 18), or (ii) via diminished direct antioxidant

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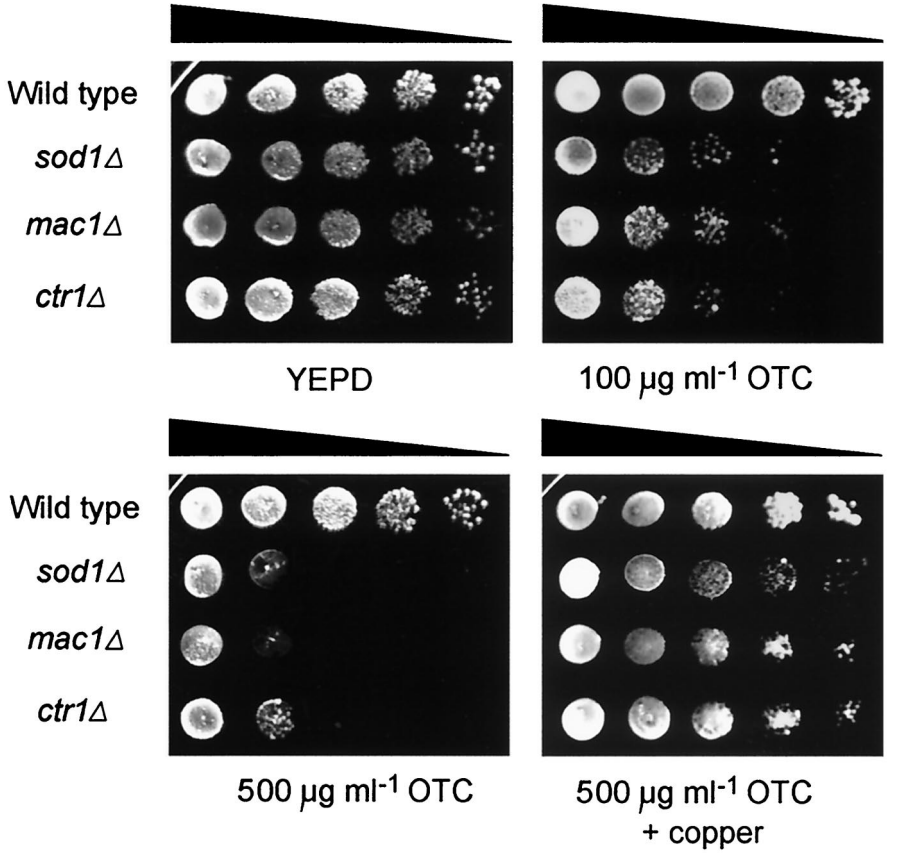

FIG. 1. Susceptibilities of antioxidant-deficient and redox balance-deficient *S. cerevisiae* mutants to OTC. Dilutions of decreasing cell concentration were spotted from left to right on each plate. *S. cerevisiae sod2*Δ, *ctt1*Δ, *gsh1*Δ, *ogg1*Δ, *gpx1/2/3*Δ, *mxr1*Δ, *yap1*Δ, and *fre1*Δ mutants were unaffected by OTC (not shown). Typical results from one of three independent experiments are shown.

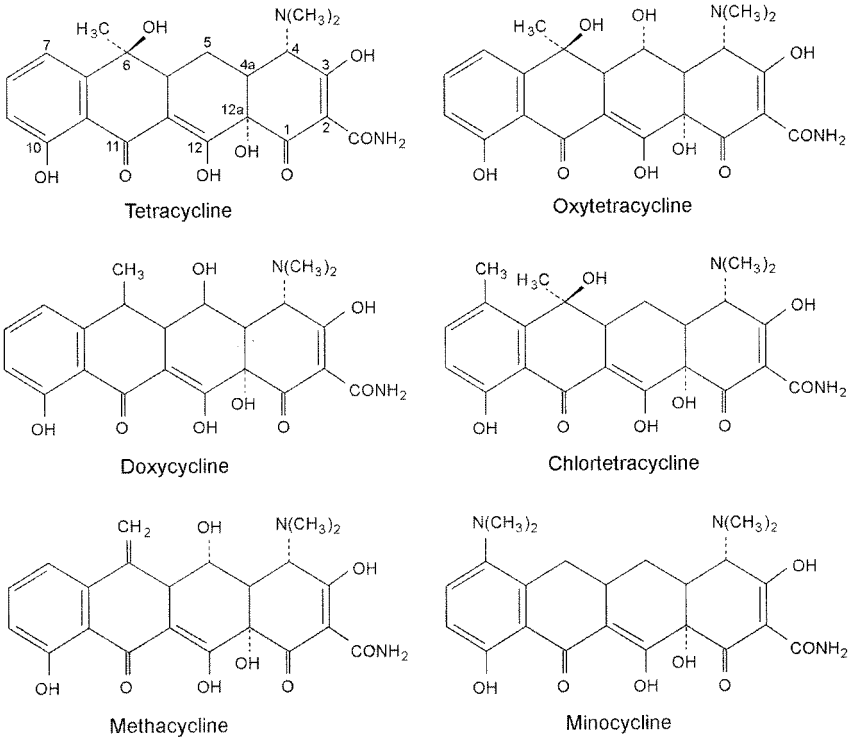

FIG. 2. Structures of the tetracyclines.

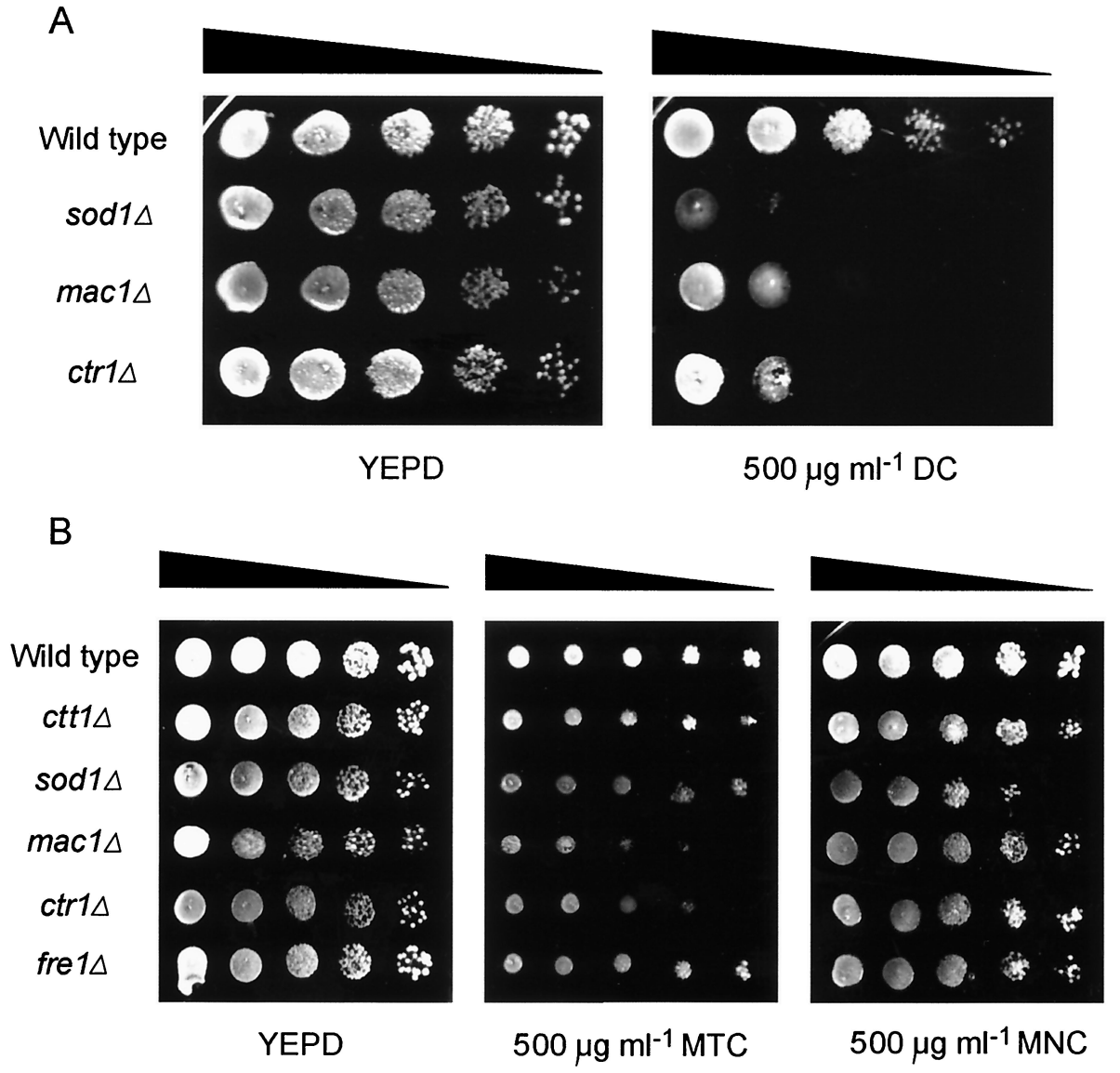

FIG. 3. Susceptibilities of antioxidant-deficient and redox balance-deficient *S. cerevisiae* mutants to a range of tetracyclines. Dilutions of decreasing cell concentration were spotted from left to right on each plate. Results are shown for doxycycline (DC) (A) and for methacycline (MTC) and minocycline (MNC) (B). Note that spots were smaller with MTC due to the surface tension imparted by the antibiotic. The mutants were not affected by TET or CTC (not shown). Typical results from one of three independent experiments are shown.

activity of Cu (19) (note that although loss of Ctr1p function also affects iron accumulation [7], a role for Fe seems unlikely here considering that *fre1*Δ cells were not sensitized to OTC). We sought to test the first possibility by introducing the *SOD1*-bearing multicopy plasmid YEp600, described by Nishida et al. (20), to *mac1*Δ *S. cerevisiae*. We observed partial suppression of the OTC susceptibility phenotype in these *SOD1*-overexpressing cells (data not shown) (copper limitation would preclude full suppression with excess Sod1p), indicating that diminished Sod1p activity may at least partly account for the mutant's OTC sensitivity. To test whether cellular Cu could contribute to OTC insusceptibility independently of Sod1p (ii), we examined the OTC susceptibility of *sod1*Δ *S. cerevisiae* treated with 50 μM $Cu(NO_3)_2$ (note that exogenous complexation with cationic metals would not adversely affect cellular uptake of tetracyclines [23]). Inhibition of growth of the *sod1*Δ strain (as well as the *mac1*Δ and *ctr1*Δ strains) by OTC was suppressed in the presence of copper (Fig. 1), confirming that Cu can act independently of Sod1p in conferring insusceptibility to OTC. Therefore, a combination of the two mechanisms listed above likely accounts for the sensitivity of the *mac1*Δ and *ctr1*Δ *S. cerevisiae* strains to OTC.

Mitochondrial protein synthesis could be a target of tetracyclines, and it is known that deletion of *SOD1* and *MAC1* results in respiratory deficiency (9, 16). To test whether forced dependency on mitochondrial function might sensitize wild-type *S. cerevisiae* to tetracyclines, we examined the cells' ability to grow on YEPG medium supplemented with a 500-μg ml^{-1} concentration of OTC or tetracycline (TET); YEPG contains glycerol and ethanol as respiratory carbon sources (12). As on

YEPD, the growth of wild-type *S. cerevisiae* on YEPG was unaffected by these antibiotics (data not shown). Furthermore, some limited respiratory growth of the *sod1*Δ strain that was discernible on YEPG was abolished by OTC (not shown). Therefore, the results evident during forced respiratory growth were similar to our original findings using YEPD, suggesting that the observed effects are not directly linked to mitochondrial function.

Previously, Sod1p appeared to be required for insusceptibility to OTC specifically, since growth with TET was unaffected by *SOD1* deletion (3). The presence of a hydroxyl group at the C-5 position distinguishes OTC from TET (see Fig. 2). An -OH group occurs at the same position also in doxycycline (DOX). Therefore, we tested *sod1*Δ *S. cerevisiae* for growth in the presence of DOX (Fig. 3A). The mutant exhibited a marked sensitivity to DOX, similar to that for OTC; as with OTC, wild-type cells grew normally up to a DOX concentration of 500 μg ml^{-1}. The growth of the *mac1*Δ and *ctr1*Δ deletion strains, but not the *fre1*Δ strain (data not shown), was also strongly inhibited by DOX (Fig. 3A). Therefore, these single gene products establish the insusceptibility of yeast to DOX as well as OTC.

To test further the specificity for particular tetracycline antibiotics, the growth of some key mutants was examined in the presence of a range of tetracyclines: chlortetracycline (CTC), methacycline (MTC), minocycline (MIN), and TET (OTC and DOX served as routine positive controls). To ensure that any moderate susceptibility was not missed, 500 μg of antibiotic ml^{-1} was used for tests. All of the strains tested (wild type, *ctt1*Δ, *sod1*Δ, *mac1*Δ, *ctr1*Δ, and *fre1*Δ) exhibited full tolerance of TET and CTC. MTC was of particular interest, since it has an -OH group at the C-5 position (see above). However, the *sod1*Δ mutant grew normally in the presence of MTC (Fig. 3B), eroding the model in which the -OH functional group plays a key role in susceptibility. A very slight but consistent sensitization to MTC was apparent in the *mac1*Δ and *ctr1*Δ strains and also in *sod1*Δ *S. cerevisiae* grown in the presence of MIN. All of the other strains tested grew normally at a MIN concentration of 500 μg ml^{-1}.

One property that could account for oxidative stress generated by tetracyclines is the high metal-binding affinities of these antibiotics (10, 21); certain complexed metals can act as foci for redox cycling activity and/or free radical generation (2). Although the metal-binding affinities of OTC are very similar to those of TET and CTC (10), OTC and DOX are distinctive in having greater polarity than the other tetracyclines (4). Thus, one possibility could be that OTC and DOX complexes partition more readily into (polar) subcellular milieus that favor reactions to which Sod1- or copper-deficient cells might be susceptible. Note that total cellular OTC uptake is not affected by *SOD1* deletion (3).

This report underscores the potentially fragile nature of antibiotic insusceptibility in eukaryotes. Cellular copper homeostasis and superoxide dismutase activity are critical determinants of yeast insusceptibility to both OTC and DOX. Since the mechanisms for handling oxidative stress and regulating copper homeostasis are quite similar in higher eukaryotes and *S. cerevisiae* (1, 15), the insusceptibility of higher eukaryotes to tetracyclines may well also rely on these functions.

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