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Effects of Mancozeb and Other Dithiocarbamate Fungicides on *Saccharomyces cerevisiae*: the Role of Mitochondrial petite Mutants in Dithiocarbamate Tolerance

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ABSTRACT. *Saccharomyces cerevisiae* as model system was used to evaluate the occurrence of resistant mutants and adaptation mechanism to mancozeb (MZ), a widespread fungicide of the dithiocarbamate class with a broad spectrum of action and multiple cell targets. We were unable to isolate mutants resistant to inhibitory concentration of MZ but found an unusually large number of mitochondrial defective petite mutants among cells incubated in the presence of subinhibitory MZ concentration. Similar results were obtained with two other dithiocarbamate fungicides. Comparison of wild type and petite mutants showed that the latter were more resistant to toxic effects of MZ, highlighting the role of mitochondria in MZ-tolerance. The data suggest that petite cells, arising by exposure to sub-inhibitory MZ concentration, are not induced by fungicides but are spontaneous mutants already present in the population before the contact with the fungicide.

Abbreviations

DTC(s)	dithiocarbamate(s)	MZ	mancozeb
EtBr	ethidium bromide	PB	propineb
MIC	minimum inhibitory concentration	WT	wild type
MR	metiram	YPD	yeast peptone–dextrose medium
MTC(l)	mitochondria(l)	YPG	yeast peptone–glycerol medium

S. cerevisiae has been proposed as a useful eukaryotic model system to study cell interaction with toxic compounds such as fungicides (Ribeiro *et al*. 2000; Fai and Grant 2009). DTCs are a widely used class of fungicides that, for their metal-chelating ability and high affinity for –SH groups, are efficient enzyme inhibitors with a broad spectrum of action and multiple cell targets (Santos *et al*. 2009). MZ, one of the most important DTC, is widely used in crop and vineyard to control phytopathogenic fungi. As a side effect, MZ significantly affects biodiversity of yeast populations on leaves of different plants (Southwell *et al*. 1999), and fungicide residues, still present at grape harvest, can pass into the wine-must and affect yeast activity during the fermentation (Cabras and Angioni 2000). Prolonged and repeated uses of different fungicides may result in the build up of fungicide resistance in target fungi populations (Parnell *et al*. 2006) and also in non-target yeasts (Buck and Burpee 2002). However, resistance to MZ has never been reported in economically important pathogenic fungi (Kato *et al*. 1997; Miles *et al*. 2006; Hollomon and Brent 2009), and it still maintains its efficacy against a broad spectrum of phytopathogenic fungi (Maroni *et al*. 2000). Of major concern are the possible human health risks associated with the use of MZ and other DTCs (Calviello *et al*. 2006; Domico *et al*. 2007).

MZ acts as a pro-oxidant agent in rat cells (Calviello *et al*. 2005; Domico *et al*. 2007), mainly through its thiol-reactivity (Dias *et al*. 2010). Similarly, thiram (another DTC) has been reported to cause a rapid decrease in glutathione content and oxidative stress in *S. cerevisiae* (Elskens and Penninckx 1997). Recent studies by gene expression analysis (Teixeira *et al*. 2008), toxiproteomics (Santos *et al*. 2009), and chemogenomic (Dias *et al*. 2010) approaches, showing the induction of a battery of oxidant defences and heat shock genes in *S. cerevisiae*, highlight the importance of oxidative stress response to MZ toxicity. A small network of transcription factors (*YAP1* and *RPN4*) and plasma membrane multidrug transporters of the major facilitator superfamily (*FLR1* and *TPO1*) seems to play an important role in MZ stress response (Texeira *et al*. 2008; Dias *et al*. 2010).

Different data suggest an interaction between MZ and MTC. MZ and other DTCs inhibit MTCl functions in human, rat and yeast cell cultures (Domico *et al*. 2007; Zhang *et al*. 2003; Diala *et al*. 1980); MZ

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was found to induce MTCl dysfunction (Domico *et al*. 2006). In addition, the transcription factor Pdr3p, monitoring the functional levels of the F_o subunit of the MTCl ATPase (Zhang and Moye-Rowley 2001), should regulate, by retrograde response (Liu and Butow 2006), the expression of the *FLR1* gene (Broco *et al*. 1999).

To better evaluate the role of MTC in MZ-tolerance, we have used the petite positive *S. cerevisiae* (Chen and Clark-Walker 2000), able to survive loss of mtDNA and to grow in strictly fermentative conditions, as a model system. The occurrence and nature of DTC resistance and the possible onset and persistence of adaptation mechanisms in yeast cells have also been investigated.

MATERIALS AND METHODS

Fungicides used are listed in Table I.

Table I. Fungicides used, their chemical class and inhibitory activity on *S. cerevisiae* 1014 on YPD plates

Fungicide	Commercial name	Trade name	MIC, mg/L
MZ	Mancozeb[a]	Dithane DG Neotec	8
MR	Metiram[a]	Polyram DF	4
PB	Propineb[b]	Antracol	16

[a]Alkylene-bis-dithiocarbamate. [b]Alkyl-dithiocarbamate.

Yeasts and culture media. The wine yeast *S. cerevisiae* 1014 (*Industrial Yeasts Collection* DBVPG, Perugia, Italy) was used. YPD and YPG were prepared according to Rose *et al.* (1990). Growth was performed at 28 °C.

Assay of activity of fungicides. To determine the activity under fermentative and respiratory conditions, ≈200 cells from fresh YPD cultures were plated in triplicates on YPD and YPG with different concentrations of fungicides. After convenient incubation time, the plates were inspected for growth and MIC, expressed as the lowest concentration of fungicide that completely inhibits colony formation, was determined (Table I).

Selection of mutants resistant to MZ. Aliquots of $\approx 2 \times 10^7$ cells were seeded on a series of YPD plates containing 16 or 20 mg/L of fungicide. The plates were incubated for 7 d.

Adaptation to MZ. To assess ability of the cells to adapt to a subinhibitory concentration of MZ, $\approx 10^7$ cells were inoculated in liquid YPD with 0, 2 or 4 mg/L MZ; after 8 h of incubation, $\approx 2 \times 10^6$ cells were plated on YPD with 8 mg/L MZ. Furthermore, $\approx 2 \times 10^4$ cells of the same cultures were streaked on YPD plates (diameter 150 mm) along a preformed 0–16 mg/L MZ linear gradient. Plates were incubated for 2 d before inspection.

To assess the duration of the adaptation state, cells were inoculated at $\approx 5 \times 10^6$ cells per mL in liquid YPD with 0 or 4 mg/L MZ and incubated for 8 h; afterwards, MZ concentration was lowered by diluting the culture 1:10 in YPD (time 0 h); diluted cultures were incubated for further 16 h (time 16 h), then the same dilution procedure was repeated and cells were incubated for further 8 h (time 24 h). At 0, 16 and 24 h of incubation, 2 mL culture was streaked onto YPD plates along a preformed 0–16 mg/L MZ linear gradient and incubated for 2 d before inspection.

Determination of the frequency of petite mutants in cells exposed to DTCs. Aliquots of 200 cells from cultures incubated for 8 h of in liquid YPD with 4 mg/L MZ or 8 mg/L PB were plated on YPD; alternatively, aliquots of 10^4 cells, from cultures not previously exposed to fungicides, were plated on YPD plates containing 4 mg/L MZ, 2 mg/L MR or 8 mg/L PB. Colonies from these plates were tested for the petite phenotype by replica-plating on YPG.

Isolation of ET-induced petite *mutants.* These mutants were obtained by EtBr-treatment (Fox *et al*. 1991) and were selected as those colonies that failed to growth after replica-plating from YPD to YPG plates.

Characterization of WT and petite mutants for sensitivity to MZ. About 5×10^6 cells from overnight YPD cultures of WT and petite mutants were plated on YPD supplemented with 8 and 16 mg/L MZ; moreover, 2 μL suspensions, corresponding to $\approx 10^6$ cells, from the same cultures were streaked on YPD plates along a preformed 0–16 mg/L MZ linear gradient; after 3 d, plates were checked for yeast growth.

In addition, WT and petite cells, grown overnight in the presence of 2 mg/L MZ, were inoculated in 1-L flasks with 100 mL YPD with different concentrations of MZ; the initial absorbance A_{630} was adjusted to 0.05. Growth with orbital shaking was monitored for 1 d by determining the A_{630} at time intervals.

RESULTS

Yeast sensitivity to DTCs. For MIC values of DTCs on YPD *see* Table I. MZ MIC value on YPG was identical to that on YPD (*not shown*). However, when cells were grown with a subinhibitory dose of MZ (2–4 mg/L) and without MZ (control), the colony growth rate was substantially lower on YPG relative to YPD (6–7 d compared to 3–4 d, respectively), suggesting that MZ acts more strongly on respiring than on fermenting cells.

Selection of mutants resistant to MZ. When ≈2×10^9 cells were seeded, at aliquots of 2×10^7 cells per plate, on YPD plates containing 8 or 12 mg/L MZ, no colony growth was observed after 4–5 d, but a slight cell lawn appeared later (*not shown*) that has been interpreted as an adaptation process, facilitated by high cell density.

Adaptation of cells to MZ. To better understand the process of MZ-adaptation, cells incubated in the absence (non-adapted cells) or presence (adapted cells) of a subinhibitory concentration (4 mg/L) of MZ were streaked on YPD plates containing a 0–16 mg/L MZ gradient. Adapted cells grew better than the non-adapted control (Fig. 1A). The adaptation state persisted for at least 24 h after MZ exposure (Fig. 1B). When cells from the upper ends of the 0 h streaks were tested for the petite phenotype, all the cells (475/475) from adapted (streak +) and only 24 % (126/489) of the cells from non-adapted (streak –) cultures showed a petite phenotype (Fig. 1C).

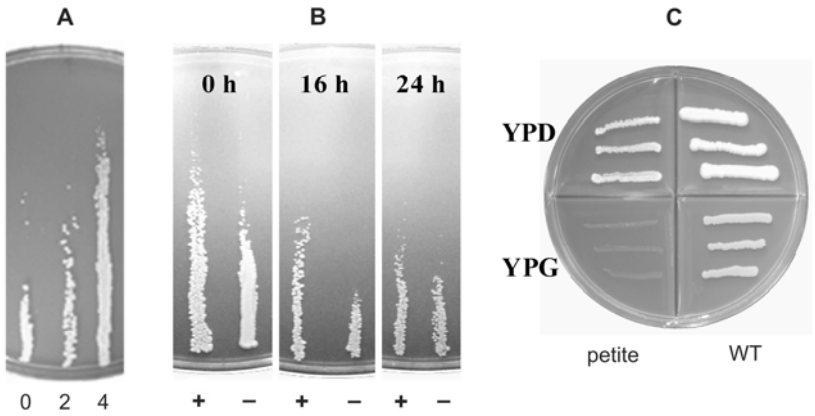

Fig. 1. Adaptation of *S. cerevisiae* 1014 strain to MZ; **A** – cells, pre-incubated 8 h in liquid YPD with no fungicide (0), 2 mg/L MZ, or 4 mg/L MZ, were streaked on YPD plate with 0–16 mg/L gradient of MZ; **B** – duration of the adaptation state in the cells grown in liquid YPD in presence (+) of 4 mg/L MZ and in the absence (–) of MZ. At 0, 16 and 24 h MZ was removed, the cultures were plated on YPD with a 0–16 mg/L MZ linear gradient; **C** – growth phenotype of colonies from cells from the upper ends of the 0 h streaks in Fig. 1B, plated at low density on YPD, and streaked on YPD and YPG; inspected for growth after 3 d at 28 °C.

Frequency of petite mutants in cells exposed to DTCs. To better understand the occurrence of petite mutants in a population exposed to DTCs, we have quantified the phenomenon. Frequency of these mutants among cells adapted to a subinhibitory concentration of MZ (4 mg/L), MR (2 mg/L) and PB (8 mg/L) was ≈10^{-3}, compared to a value of 10^{-5} for non-adapted cells (Table II). When 10^4 non-adapted cells were see-

Table II. Frequency of petite mutants from 1014 cells grown overnight in liquid YPD with subinhibitory concentration of DTCs, plated on YPD without fungicides and replicated on YPG

Fungicide	mg/L	Petite mutants		
		number	per plated cells[a]	frequency, $\times 10^{-3}$
None	0	2	3.6×10^4	0.06
MZ	4	12	4×10^3	3.00
MR	2	33	4×10^3	8.25
PB	8	16	4×10^3	4.00

[a]Total number of seeded cells at aliquots of 2×10^2 cells per plate; virtually all seeded cells formed colonies on YPD.

ded on YPD plates containing a subinhibitory concentration of MZ (4 mg/L), MR (2 mg/L) and PB (8 mg/L), the frequency of petite in the total number of survived cells was 67, 34 and 7 %, respectively (Table III).

Table III. Frequency of petite mutants from overnight cultures of yeast in liquid YPD without fungicides, plated on YPD with subinhibitory concentration of DTCs and replicated on YPG[a]

Fungicide	mg/L	Number of cells[b]	Number of survived cells[b]	Frequency, %[c]
MZ	4	10^4	61	67.0
MR	2	10^6	212	34.0
PB	8	10^4	59	7.0

[a]Means of triplicates. [b]Per YPD plate. [c]Of petite mutants per survived cells.

Comparison of MZ-tolerance in WT and petite mutants. When WT and petite cells were plated on YPD with 8 and 16 mg/L MZ, only spontaneous and ET-induced petite mutants grew (*not shown*). In fact, the petite mutants have a MZ MIC of 20 mg/L (*not shown*), compared to 8 mg/L of the WT. Furthermore, when both types of cells adapted to 2 mg/L MZ were inoculated in YPD supplemented with increasing concentration of MZ, at 8 mg/L of fungicide only petite cells grew, whereas WT ones were completely inhibited (Fig. 2).

DISCUSSION

As all DTCs tested show similar results, the discussion will be focused on MZ only. In accordance with different papers reporting that MZ has never induced resistance in fungi (Kato *et al.* 1997; Miles *et al.* 2006; Hollomon and Brent 2009), we found no spontaneous MZ-resistant mutants in *S. cerevisiae*; instead, a time-persistent MZ-adaptation was observed in cells exposed to a subinhibitory concentration of MZ. The nature of MZ-adaptation has been recently dissected at molecular level (Teixeira *et al*. 2008; Santos *et al*. 2009; Dias *et al.* 2010). Here we report that the frequency of MTCl petite mutants in a yeast population exposed to subinhibitory concentration of MZ is higher relative to that measured in a numerically similar not-exposed population. This increase should be mainly attributed to the intrinsic MZ-resistance of petite cells and to their capability to outgrow WT cells during overnight incubation in media with subinhibitory concentration of MZ. MZ-tolerance in petite mutants could be explained by retrograde response from dysfunctional MTC to the nucleus, resulting in the activation of Pdr3p, responsible for the expression of multidrug resistance transport proteins such as Pdr5p (Zhang and Moye-Rowley 2001) and Flr1p (Texeira *et al.* 2008; Dias *et al.* 2010). Our data suggest that the greater MZ-tolerance of petite relative to WT cells could be due to a negative interaction of MZ with MTCl respiration, which is in accordance with the greater MZ inhibitory activity on respiring than on fermenting yeast.

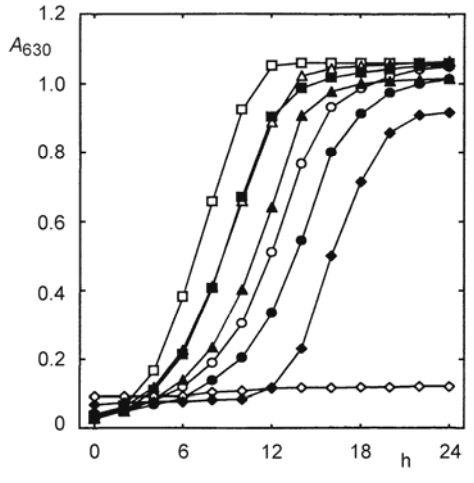

Fig. 2. Growth (A_{630}) of WT (*empty symbols*) and petite (*black symbols*) *S. cerevisiae* 1014, previously adapted to 2 mg/L MZ, in liquid YPD with none (*squares*), 2 mg/L (*triangles*), 4 mg/L (*circles*), and 8 mg/L (*diamonds*) MZ.

Of some interest here is the finding that the ability of *S. cerevisiae* to develop tolerance to DTC fungicides, besides well characterized molecular cellular adaptation mechanisms (Teixeira *et al*. 2008; Santos *et al*. 2009; Dias *et al*. 2010), relies also on the ratio of petite to the total number of cells. Modifications in this ratio could be seen as a mechanism of stress response, which acts at the level of the whole yeast population rather than of individual cells.

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