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Decreased cellular permeability to H_2O_2 protects *Saccharomyces cerevisiae* cells in stationary phase against oxidative stress

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Abstract The higher resistance of stationary-phase *Saccharomyces cerevisiae* to H_2O_2 when compared with exponential phase is well characterized, but the molecular mechanisms underlying it remain mostly unknown. By applying the steady-state H_2O_2-delivery model, we show that (a) cellular permeability to H_2O_2 is five times lower in stationary – than in exponential phase; (b) cell survival to H_2O_2 correlates with H_2O_2 cellular gradients for a variety of cells; and, (c) cells in stationary phase are predicted to be more susceptible to intracellular H_2O_2 than in exponential phase. In conclusion, limiting H_2O_2 diffusion into cells is a key protective mechanism against extracellular H_2O_2.
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Keywords: H_2O_2 compartmentalization; H_2O_2 gradients; Catalase; Enzyme latency; Exponential phase; Cell survival

1. Introduction

Hydrogen peroxide (H_2O_2) is the most abundant reactive oxygen species in vivo, being continuously produced intracellularly as a by-product of the aerobic metabolism, or extracellularly by stimulated host phagocytes [1]. It is usually assumed that H_2O_2 diffuses freely across biomembranes [1]. However, it has been recently shown in several cell types, such as Jurkat T-cells, a mammalian cell line [2], *E. coli* [3], and *Saccharomyces cerevisiae* (*S. cerevisiae*) [4], that H_2O_2 does not permeate biomembranes freely. So, upon exposure to external H_2O_2, the intracellular consumption of H_2O_2 catalyzed by antioxidant enzymes is able to generate a gradient of H_2O_2 across the plasma membrane, which makes the intracellular H_2O_2 concentration lower than the external one. The magnitude of this gradient is dependent on the extent of the intracellular consumption of H_2O_2 and on the permeability properties of cell barriers to H_2O_2 [2]. In addition, recent results obtained in our laboratory showed that the permeability of the plasma membrane towards H_2O_2 is under active regulation in *S. cerevisiae* cells during adaptation to H_2O_2, being part of the cellular mechanisms of response to oxidative stress [4].

Yeast has three growth stages, beginning with exponential fermentative growth when glucose is available and where cells actively replicate. When glucose is consumed, cells undergo a diauxic shift to respiratory growth and then enter the stationary phase when nutrients are exhausted becoming quiescent. It has been known for a number of years that the onset of the stationary phase increases yeast resistance towards oxidants, such as H_2O_2 [5,6]. The precise mechanisms of resistance to oxidants are not understood, but it has been suggested that this increased stress tolerance might be accounted, at least in part, by a derepression of the negative regulatory effect exerted by the RAS-cAMP-PKA pathway on the expression of stress genes [7], and also by an elevated expression of genes encoding antioxidant enzymes, including those responsible for H_2O_2 removal [8].

In this work, in view of the capacity of *S. cerevisiae* cells to regulate the H_2O_2 permeability of the plasma membrane, we tested the hypothesis that one of the factors that causes a higher resistance of stationary-phase *S. cerevisiae* cells to H_2O_2, when compared with exponential-phase *S. cerevisiae* cells is a lower cellular permeability to H_2O_2. If the hypothesis is correct then it would be expected that upon incubation with the same external H_2O_2 concentration, the actual concentration endured by the cells in stationary phase would be much lower than that endured by the cells in exponential phase, because of the formation of a steeper gradient of H_2O_2. To test this hypothesis, we measured both the H_2O_2 gradient and the permeability constant to H_2O_2 in stationary phase, applying the principle of enzyme latency.

2. Materials and methods

2.1. Materials

Saccharomyces cerevisiae strains used in this work are Y00000 (wild type, genotype BY4741 *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), Y05982 (*ccp1*Δ, isogenic to BY4741 with YKR066c::kanMX4), Y04718 (*ctt1*Δ, isogenic to BY4741 with YGR088w::kanMX4), Y02667 (*erg3*Δ, isogenic to BY4741 with *YLR056w::kanMX4*), and Y00568 (*erg6*Δ, isogenic to BY4741 with *YML008c::kanMX4*) and were obtained from EUROSCARF, Frankfurt, Germany.

Yeast extract, bactopeptone, yeast nitrogen base and agar were from Difco, Detroit, MI, USA. Digitonin was from Aldrich, Steinheim, Germany. L-α-amino acid oxidase (*Crotalus atrox*) and bovine liver

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Abbreviations: H_2O_2, hydrogen peroxide; OD_{600}, optical density at 600 nm; ROS, reactive oxygen species; *S. cerevisiae*, *Saccharomyces cerevisiae*

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catalase were from Sigma Chemical Company, St. Louis, MO, USA. Hydrogen peroxide was obtained from Merck & Co., Inc., Whitehouse Station, NJ, USA.

2.2. Media and growth conditions

For all experiments, *S. cerevisiae* cells were inoculated at an OD_{600} of 0.05 and cultured during 7 days in synthetic complete medium [9] at 30 °C and with shaking at 160 rpm. Before experiments, cells were washed and resuspended in an amino acid solution (0.1 M phosphate buffer, pH 7.4, containing amino acids with the same concentrations as in synthetic complete medium) at 0.5 OD_{600}/ml unless otherwise specified.

2.3. Exposure to H_2O_2 and cell survival

Cells were exposed to steady-state H_2O_2 concentrations at 30 °C and with shaking at 160 rpm for 1 h as described in [10] except that L-α-amino acid oxidase was used instead of glucose oxidase. Briefly, an initial concentration of H_2O_2 together with L-α-amino acid oxidase was added at such an activity that compensated for the rapid consumption of H_2O_2 by the cells for the given initial H_2O_2 concentration, thus keeping H_2O_2 concentration constant (steady-state) during the assay. Cell survival after exposure to steady-state concentrations of H_2O_2 was monitored by plating diluted sample aliquots on YPD plates [9] and counting colonies after 48 h. Cell survival was normalized using the number of colonies in control samples not exposed to H_2O_2.

2.4. Cell permeabilization

Cell membrane permeabilization was achieved by incubating cells in 0.1 M potassium phosphate buffer, pH 6.5, with 0.1% (w/v) digitonin, dissolved in dimethylsulfoxide, for 15 min at 30 °C with shaking.

2.5. Determination of H_2O_2 consumption and cellular H_2O_2 gradient

The consumption of H_2O_2 was measured in intact and permeabilized cells. Cells were suspended in 0.1 M potassium phosphate buffer, pH 6.5, containing amino acids with the same concentrations as in synthetic complete medium, at 30 °C with shaking and H_2O_2 consumption was measured by following O_2 release with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) after the addition of catalase [2]. H_2O_2 consumption is reported as a first order rate constant.

The H_2O_2 gradients were determined based on the latency of catalase [2], using the ratio between consumption rate in intact- and in permeabilized cells [4] (see Eq. (1) and Section 3). This enzymatic latency is due to an apparent lower activity shown by enzymes entrapped in compartments when compared with enzymes free in solution [11,12]. This lower activity is a consequence of the permeability barrier imposed by the compartment entrapping the enzyme, which limits the diffusion of the substrate to the enzyme, and not some other factor, such as inhibition of the enzyme when trapped in the compartment [11]. In addition to the wild-type cells, *ctt1*Δ and *ccp1*Δ *S. cerevisiae* strains were also used. Since cytosolic catalase and cytochrome *c* peroxidase are major enzymes involved in H_2O_2 removal in *S. cerevisiae* [6,13], they provide important controls for the application of the latency principle in the determination of H_2O_2 gradients (see Section 3).

2.6. Statistical analysis

Results presented are the means ± standard deviation of independent experiments. Statistical analysis was undertaken using a two-tailed Student's *t* test for comparison between means of two different groups.

3. Results

3.1. *S. cerevisiae* cells in stationary phase are more resistant to external H_2O_2

To measure the susceptibility of *S. cerevisiae* wild-type cells to H_2O_2, cell survival in stationary- and exponential phases was compared in cells treated with steady-state concentrations of H_2O_2. Fig. 1 shows that, as observed in studies where cells were treated with bolus additions of H_2O_2 [5,6], wild-type stationary-phase cells have a notorious higher resistance to H_2O_2 when compared with exponentially growing cells.

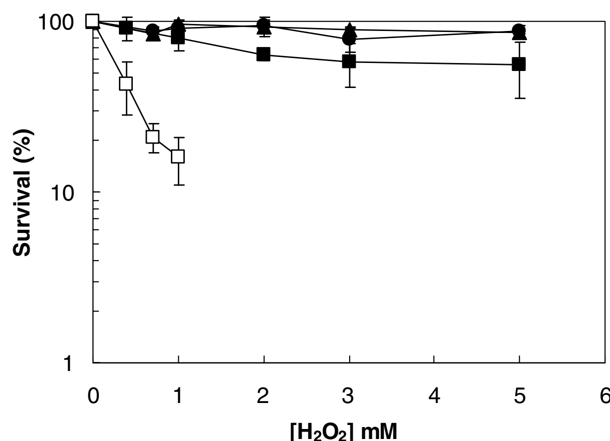

Fig. 1. Cell survival for *S. cerevisiae* cells challenged with H_2O_2 is higher in stationary phase than in exponential phase. Stationary phase wild-type (■), *erg3*Δ (▲) and *erg6*Δ (●) cells; exponential phase wild-type cells (□). Cells were treated with external steady-state concentrations of H_2O_2 for 1 h. Data for stationary-phase cells are the averages of a minimum of three independent experiments and data for exponential phase cells are taken from [4]. For H_2O_2 doses higher than 5 mM, it was not possible to maintain the steady-state, probably due to the inhibition of the enzyme L-α-amino acid oxidase.

3.2. The cellular permeability to H_2O_2 is decreased in stationary phase

To test the hypothesis that a decrease in cellular permeability is correlated with a higher resistance to H_2O_2, we measured the cellular permeability to H_2O_2 in stationary-phase *S. cerevisiae* cells. To determine this constant, we used the principle of enzyme latency, from which the following equation can be derived [2,11,12],

$$\frac{[H_2O_2]_{in}}{[H_2O_2]_{out}} = \frac{k_{perm}}{k_{perm} + k_{catabolism}} = R = \frac{k_{intact\ cells}}{k_{catabolism}} \quad (1)$$

in which k_{perm} refers to the first order rate constant for the permeation of H_2O_2 into the cell, $k_{catabolism}$ to the intracellular catabolism of H_2O_2, and R to the ratio between the overall H_2O_2 consumption rate constant in intact cells over the H_2O_2 consumption rate constant in permeabilized cells. Therefore, the determination of the gradient can be based on the experimental measurement of R, while the determination of k_{perm} can be based on the knowledge of R and $k_{catabolism}$ (i.e. H_2O_2 consumption in permeabilized cells) [2]. A key issue when applying Eq. (1) is that H_2O_2 removing enzymes operating in intact and in permeabilized cells must be the same, otherwise the ratio R obtained experimentally is not due only to cell permeabilization and consequently the determination of k_{perm} from R is erroneous. It is known that for catalase, the latency is caused by the limitation of H_2O_2 diffusion and not by some other factor, such as inhibition of the enzyme when trapped in the compartment [11]. Also, permeabilization using digitonin is a technique that allows the assay of enzyme activities in conditions similar to those prevailing in intact cells with regard to concentration and interaction with macromolecules, which are kept inside the permeabilized cell [14].

Table 1
The H_2O_2 gradient across the plasma membrane and the permeability constant are changed in stationary-phase *S. cerevisiae* cells when compared with exponential phase *S. cerevisiae* cells

Phase of growth/strain	$k_{intact\ cells}$ ($min^{-1}\ OD_{600}^{-1}$)	$k_{catabolism}$ ($min^{-1}\ OD_{600}^{-1}$)	R	k_{perm} ($min^{-1}\ OD_{600}^{-1}$)
Stationary				
wt	0.016 ± 0.003*	0.31 ± 0.10*	0.056 ± 0.020*	0.017 ± 0.004**
*ctt1*Δ	0.011 ± 0.01	0.027 ± 0.006	nd[a]	nd[a]
*ccp1*Δ	0.012 ± 0.002	0.22 ± 0.04	0.057 ± 0.017	0.013 ± 0.002
Exponential				
wt	0.030 ± 0.003	0.048 ± 0.004	0.64 ± 0.09	0.083 ± 0.028

k, H_2O_2 consumption rate constant; R, H_2O_2 gradient across the plasma membrane; k_{perm}, permeability constant. ($14 \geqslant n \geqslant 4$); $^*P < 0.0001$, $^{**}P < 0.01$ versus exponential phase cells. Results for exponential phase cells are taken from [4].
[a]Eq. (1) was not applied because cytochrome *c* peroxidase activity is limited in permeabilized cells by the availability of reduced cytochrome *c*, and therefore, the ratio R obtained experimentally is not only the result of the permeabilization of the membrane (see main text).

As can be observed in Table 1, whereas the consumption of H_2O_2 in permeabilized wild-type cells is almost entirely (92%) due to cytosolic catalase ($k_{catabolism}$ is 0.31 and 0.027 $min^{-1}\ OD_{600}^{-1}$ for the wild-type cells and the catalase mutant, *ctt1*Δ, respectively), the consumption of H_2O_2 in intact wild-type cells is due to both cytosolic catalase and cytochrome *c* peroxidase and possibly other enzymes, as judged from the H_2O_2 consumption rate constants in the mutant strains for these enzymes (0.016, 0.011 and 0.012 $min^{-1}\ OD_{600}^{-1}$ for the wild-type cells, *ctt1*Δ and *ccp1*Δ, respectively). Therefore, for wild-type stationary-phase *S. cerevisiae* cells, the values reported for R (imposed by catalase) and k_{perm} represent upper limits. In spite of this, an important conclusion can be drawn: for stationary-phase cells, the permeability constant is at least 5-fold lower (<0.017 ± 0.004 $min^{-1}\ OD_{600}^{-1}$) than the published value for exponential cells (0.083 ± 0.028 $min^{-1}\ OD_{600}^{-1}$) [4], indicating that stationary-phase cells have a lower permeability to H_2O_2 than exponential-phase cells. It should be stressed that k_{perm} for exponential phase cells reported in Table 1 is an accurate value, as catalase is the only enzyme contributing to H_2O_2 removal both in intact and in permeabilized cells, because both these consumptions are absent in the *ctt1*Δ strain [4]. If we were able to obtain the accurate value for k_{perm} in stationary phase by taking in account the other H_2O_2 removing enzyme activities, the difference between k_{perm} in exponential and stationary phase would be even higher.

3.3. S. cerevisiae *cells consume less H_2O_2 in stationary- than in exponential phase*

One important implication of the decreased cellular permeability found in stationary-phase cells is that it helps to understand apparently kinetic measurements. On the one hand, catalase and cytochrome *c* peroxidase, which are major enzymes involved in H_2O_2 removal in *S. cerevisiae* [6,13], have higher activities in stationary phase than in exponential phase (see $k_{catabolism}$ in Table 1, which is a measure of cellular catalase activity, and [6]). On the other hand, the overall H_2O_2 consumption rate constant, i.e., consumption measured with synthetic complete medium, is about 3–4-fold lower (0.017 ± 0.005 $min^{-1}\ OD_{600}^{-1}$, $n = 10$) in intact stationary-phase cells than in intact exponential-phase cells (0.059 ± 0.009 $min^{-1}\ OD_{600}^{-1}$, $n = 6$) [4]. In order to understand these results, it is helpful to derive the following expression for the H_2O_2 consumption ($v_{H_2O_2}$):

$$v_{H_2O_2} = \frac{k_{catabolism} k_{perm}}{k_{perm} + k_{catabolism}} [H_2O_2]_{out} \quad (2)$$

As can be observed from Eq. (2), it is possible to have a decrease of the overall H_2O_2 consumption rate even if $k_{catabolism}$ is increased, provided k_{perm} is decreased. This scenario, where k_{perm} is decreased, has a significant advantage over a scenario in which the H_2O_2-removing activities are increased but k_{perm} is kept constant. In the latter case, a higher extracellular–intracellular gradient would be formed (because catalase activity is increased, see Eq. (1)), thus protecting cells, but this would be achieved at the expense of a higher consumption rate of H_2O_2 (see Eq. (2)). As a consequence, a higher oxidative load would be inflicted upon *S. cerevisiae* cells due to a higher rate of oxidation of reducing equivalents – cytochrome *c* and NADPH – necessary for H_2O_2 reduction by cytochrome *c* peroxidase and catalase [15], respectively. The decrease in cellular permeability to H_2O_2 allows the cell to maintain low H_2O_2 intracellular concentrations without the need for an increased external H_2O_2 removal, thus sparing endogenous reserves that are essential for the survival of stationary-phase cells, living in a medium deprived of nutrients.

3.4. *Role of plasma membrane sterol composition in the protection against H_2O_2 in stationary phase*

In exponential phase, it has been shown that changes in plasma membrane sterol composition alter membrane permeability to H_2O_2. In fact, *erg3*Δ and *erg6*Δ cells in exponential phase, which have a defective ergosterol biosynthesis pathway, have a increased plasma membrane permeability to H_2O_2 and a decreased resistance to H_2O_2 [4]. So, to test if the same occurred in stationary phase, we determined the survival fraction after H_2O_2 exposure and H_2O_2 gradients in *erg3*Δ and *erg6*Δ cells. Contrary to exponential phase, *erg3*Δ and *erg6*Δ cells in stationary phase have slightly higher resistance to H_2O_2 (Fig. 1) and higher or similar H_2O_2 cellular gradients than wild-type cells ($1/R$ is 58 ± 19 for *erg3*Δ, 23 ± 6 for *erg6*Δ and 20 ± 6 for wild-type cells).

3.5. *Correlation between cell survival in the presence of H_2O_2 and the magnitude of H_2O_2 gradients*

To further evaluate the role of the gradients in cell survival in the presence of H_2O_2, we analyzed cell survival for cells in a variety of conditions that form different H_2O_2 gradients when subjected to external H_2O_2 (Fig. 2). For cells subjected to 0.7 mM H_2O_2 steady state for 1 h, cell survival correlates with the value of R indicating that the extracellular–intracellular H_2O_2 gradient determines the resistance of cells to H_2O_2. The results for the *erg3*Δ and *erg6*Δ strains are particularly relevant, since, while in exponential phase they show a higher sus-

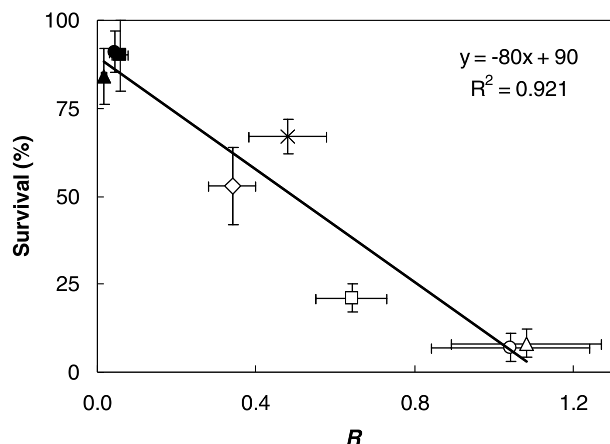

Fig. 2. Cell survival for *S. cerevisiae* cells challenged with H_2O_2 correlates with H_2O_2 gradients. *S. cerevisiae* cells were treated with 0.7 mM steady-state H_2O_2 for 1 h. Stationary phase, closed symbols; exponential phase, open symbols. Wild-type cells, (■,□); cells adapted with 150 μM H_2O_2 for 90 min, (◇); exponential-phase cells pre-exposed with cycloheximide (15 μg/ml) for 90 min (×); *erg3*Δ cells, (▲,△); *erg6*Δ cells, (●,○). Data for exponential phase are taken from [4].

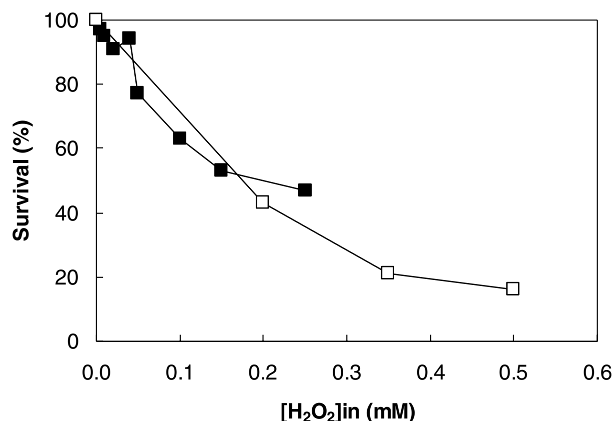

Fig. 3. Cell survival for *S. cerevisiae* cells in stationary phase (■) and in exponential phase (□) as a function of H_2O_2 intracellular concentration. Data from Fig. 1 were reploted, but H_2O_2 intracellular concentrations were used instead of using H_2O_2 extracellular concentrations. The former were estimated from H_2O_2 external concentrations by using Eq. (1) and the experimental value of R (see Table 1).

ceptibility to H_2O_2 than the wild-type cells, in stationary phase the opposite happens (see also Fig. 1). This apparent paradox is explained by the different effects of the mutations on H_2O_2 gradients for the exponential and stationary phases: in the former the gradients are decreased, while in the latter they are similar or higher than in wild-type cells.

4. Discussion

A decreased cellular permeability to H_2O_2 during stationary phase is not surprising because: (a) the same has been previously found for other compounds such as ethyl methane sulfonate, nitrous acid and mitomycin C [16,17]; (b) the plasma membrane order parameter, which correlates with a decreased membrane permeability to several compounds [18], including H_2O_2 [19], is increased in stationary phase [20] and, (c) the cell wall is thicker and less permeable in stationary phase [21–23]. In exponential phase, published data indicate that the plasma membrane has a major role in determining cellular permeability to H_2O_2, because H_2O_2 gradients are similar in intact cells and in spheroplasts [4]. Also, deletion of a single gene of the ergosterol biosynthetic pathway (*ERG3* or *ERG6*) changes plasma membrane biophysical properties, resulting in increased permeability to lipophilic compounds [24] including H_2O_2 [4]. For both exponential and stationary phase, it is usually assumed that H_2O_2 diffuses freely across the cell wall because this wall has pores that are permeable to low molecular weight molecules like H_2O_2 [21–23]. However, the fact that knocking-out *ERG3* and *ERG6* genes causes a lower H_2O_2 gradient in exponential phase [4], but similar or higher H_2O_2 gradients in stationary phase (this work) argues in favor of the possibility that for cells in stationary phase the cell wall may also contribute to limiting H_2O_2 diffusion into the cell.

The limitation in H_2O_2 consumption in intact stationary-phase cells imposed by the plasma membrane and/or cell wall implies that, upon exposure to external H_2O_2, a steep gradient between extracellular and intracellular H_2O_2 concentrations is formed. As can be seen in Table 1, the R value imposed by catalase is at least ten times lower in stationary-phase cells than in exponential cells, meaning that in the stationary phase of growth, *S. cerevisiae* cells have a 10-fold higher H_2O_2 gradient. This higher gradient is not only a consequence of a lower k_{perm} but it is also due to a higher catalase activity in stationary-phase cells [6], when compared with exponential-phase cells. Therefore, stationary-phase *S. cerevisiae* cells will endure a lower H_2O_2 intracellular concentration than exponential-phase cells when exposed to the same external H_2O_2 concentration. In Fig. 3, we represent the survival fraction versus an estimated upper limit for H_2O_2 intracellular concentration when cells in both stationary- and exponential phases of growth were treated with the external steady-state concentrations of H_2O_2 shown in Fig. 1. As can be seen, *S. cerevisiae* cells' susceptibility to intracellular steady-state H_2O_2 concentrations is similar in stationary- and exponential phase. In the calculation of the intracellular concentrations in Fig. 3, it was not possible to account for the gradient induced by cytochrome *c* peroxidase and other H_2O_2 removing enzymes. However, in view of the 4-fold higher activity of cytochrome *c* peroxidase [6] and the 5-fold lower k_{perm} (this work) in stationary phase compared with exponential phase, it can be predicted that if the overall gradient could be calculated, susceptibility to intracellular steady-state H_2O_2 concentrations would be higher for cells in stationary- than in exponential phase.

5. Final remarks

According to our results, cell survival to extracellular H_2O_2 correlates with the magnitude of H_2O_2 gradients and this justifies why stationary-phase *S. cerevisiae* cells are more resistant to extracellular H_2O_2 exposure than exponential phase cells, but the opposite is predicted when considering resistance to intracellular H_2O_2 concentrations. These findings are in agreement with the higher sensitivity of stationary-phase cells to stresses that do not involve cellular barriers, such as UV

penetrating radiation, when compared with exponential-phase cells [16,25], as opposed to what is observed for drugs like ethyl methane sulfonate, nitrous acid and mitomycin C, which involve uptake through the plasma membrane [16,17].

Altogether these results confirm that the formation of H_2O_2 gradients has a fundamental role in the protection against H_2O_2 and that cells have the ability of altering these gradients, through changes in the plasma membrane and/or the cell wall, both during adaptation, where they are able to rapidly decrease (within 1 h) the plasma membrane permeability to H_2O_2 [4], and during growth, where upon transition from the replicative to the quiescent phase cell permeability to H_2O_2 decreases 5-fold.

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