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QUARTERLY

Anaerobic growth of *Saccharomyces cerevisiae* alleviates the lethal effect of phosphotyrosyl phosphatase activators depletion

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Saccharomyces cerevisiae **homologues of phosphotyrosyl phosphatase activator (PTPA) are encoded by *RRD1* and *RRD2*, genes whose combined deletion is synthetic lethal. Previously we have shown that the lethality of *rrd1,2*Δ can be suppressed by increasing the osmolarity of the medium. Here we show that the lethality of *rrd1,2*Δ is also suppressed under oxygen-limited conditions. The absence of respiration *per se* is not responsible for the suppression since elimination of the mitochondrial genome or a block in heme biosynthesis fail to rescue the *rrd1,2*Δ double mutation.**

Phosphotyrosyl phosphatase activator, PTPA, stimulates phosphotyrosyl phosphatase (PTPase) activity of the dimeric form of protein phosphatase 2A (PP2A) *in vitro* [1]. Whether the stimulation of PTPase activity of PP2A is the cellular function of PTPA is not yet clear. However, conservation of PTPA from yeast to human, its wide distribution in different tissues, and micromolar concentration in the cell indicate an important physiological role.

In *Saccharomyces cerevisiae* PTPA is encoded by two genes, *RRD1* and *RRD2* [2, 3]. Van Hoof *et al.* [2] demonstrated that purified Rrd1p stimulates PTPase activity of PP2A *in vitro*, whereas no PTPA activity was detected for Rrd2p. Consequently, it can be concluded that *S.cerevisiae* has at least one functional homologue of human PTPA. Also functional analysis revealed differences between the *RRD1* and *RRD2* genes [3–6]. *rrd1*Δ cells display a spontaneous mutator phenotype [4], ab-

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Abbreviations: PTPA, phosphotyrosyl phosphatase activator; PP2A, protein phosphatase 2A.

errant bud morphology, shorter lag phase and faster entrance to the stationary phase [5, 6]. Such diverse effects of inactivation indicate a role of Rrd1p in multiple cellular processes. Ramotar *et al.* [4] reported on the involvement of *RRD1* in repair of oxidative DNA damage. Rrd1p has also been implicated in the regulation of *S. cerevisiae* cell cycle as a negative regulator of the G1/S transition [5]. Mitchell and Sprague [7] reported that Rrd1p, together with Cla4p, is involved in regulating the G2/M transition. Using coimmunoprecipitation, the authors showed a physical interaction between the Rrd proteins and Sit4p (PP2A-like catalytic subunit) both *in vivo* and *in vitro*. Those results point at Sit4p as a target of the Rrd proteins, at least in mediating in the function shared by *RRD1* and *CLA4* in the G2/M transition.

On the other hand, null mutants for either *RRD* gene exhibit also some common phenotypes. Their resistance to the antifungal and immunosuppressive drug rapamycin suggests that Rrd1p and Rrd2p may be involved in the TOR signalling pathway [3]. Abnormal actin distribution and sensitivity to the microtubule-destabilizing agent nocodazole indicate a role of the Rrd proteins in morphogenesis and mitosis [6]. The conditional lethal effect of a double *RRD1* and *RRD2* deletion is the strongest argument for the cooperation of the Rrd proteins, which, apart from their unique functions, also share functions essential for cell viability [3]. Growth of the *rrd1,2*Δ mutant can be restored by increased medium osmolarity. This phenotype parallels the finding that the *rrd1,2*Δ mutation is partially suppressed by compensatory inactivation of the *HOG1* or *SLT2* genes. *HOG1* encodes MAP kinase of the HOG (high osmolarity glycerol) pathway which mediates the response of yeast cells to hyperosmotic stress [8]. *SLT2/MPK1* codes for MAP kinase of the Pkc1p-activated Slt2p/Mpk1p cascade involved in heat shock response, osmotic stability and cell wall biogenesis [9].

In this work we report that anaerobiosis, in addition to high osmolarity, restores viability of cells devoid of the Rrd1 and Rrd2 proteins. In contrast to oxygen limitation, respiratory incompetence caused by inactivation of the mitochondrial genome or by a lack of heme failed to rescue the *rrd1,2*Δ double mutant.

MATERIALS AND METHODS

Strains, plasmids and genetic methods. The *S. cerevisiae* diploid strain BR2 (MATa/α; *ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11, 15/his3-11,15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100, rrd1::kanMX4/+ rrd2::HIS3/+*), a derivative of W303, was from our collection [3]. Cells were made respiratory deficient (rho$^-$) through treatment with ethidium bromide [10]. To obtain a *hem1*Δ strain the genomic copy of the *HEM1* gene was removed with a single-step disruption technique by using the *hem1*Δ::*LEU2* cassette provided by B. Guiard (CGM, Gif-sur-Yvette, France) [11]. The two isoenzymes for yeast NAD$^+$-dependent glycerol 3-phosphate dehydrogenase were overexpressed from the YEplac181-GPD1 and YEplac181-GPD2 plasmids (kindly supplied by Dr. L. Adler) [12]. Transformations of yeast cells were done by the one-step lithium acetate method [13]. Standard media and procedures were used for crossing, sporulation and tetrad analysis [10].

Growth conditions. Standard complete YPD, minimal SD and SC-drop-out media were used [10]. The media for growing heme-deficient strains and for anaerobiosis were supplemented with 0.3% (v/v) Tween-80 and 0.003% ergosterol (YPDTE). Anaerobic cultures were grown on YPDTE plates in an anaerobic jar (Anaerocult) from Merck. Anaerobic conditions were generated with the use of the Anaerocult A system from Merck.

RESULTS AND DISCUSSION

Anaerobiosis suppresses the growth defect of the *rrd1,2*Δ mutant

Rrd1p and Rrd2p are required for an essential cellular function as cells depleted of both Rrd proteins do not survive in standard growth conditions (glucose-rich medium at 28°C). Our previous results showed that supplementation of the growth medium with an osmotic stabilizer, i.e. sorbitol, sufficed to partially restore viability of the *rrd1,2*Δ mutant [3]. In response to osmotic stress yeast cells accumulate the osmolyte glycerol, thereby maintaining the osmotic gradient at a level necessary to take up water from the medium. Since elevated synthesis of glycerol occurs also in anaerobiosis [12], we tested whether anaerobic conditions may compensate for the loss of Rrd1p and Rrd2p functions. Tetrad analysis of the heterozygous diploid BR2 (*rrd1*Δ *rrd2*Δ/++) showed that all four haploid segregants were viable when dissected on YPDTE plates and germinated in anaerobic conditions (Fig. 1A). When transferred into aerobic conditions, the *rrd1,2*Δ spores ceased growth unless the medium was supplemented with an osmotic stabilizer. Anaerobiosis not only restores growth of the double mutant, but it also suppresses the common phenotypes of the *rrd1*Δ and *rrd2*Δ strains, i.e. caffeine and rapamycin resistance (Fig. 2).

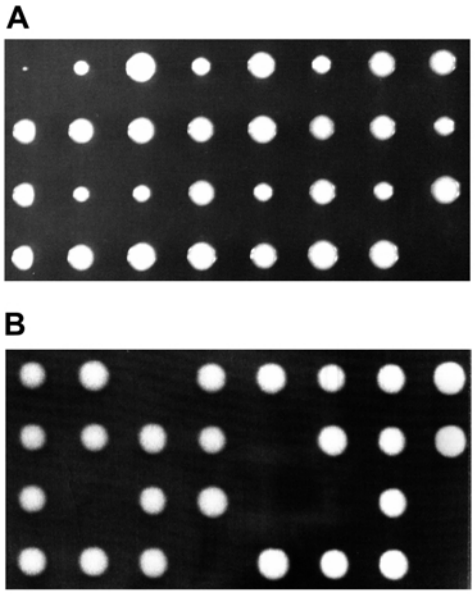

Figure 1. Germination of *rrd1,2*Δ spores.

Panel A, tetrads of BR2 diploid were dissected on YPDTE medium and incubated anaerobically for 5 days at 28°C. Panel B, tetrads of BR2(YEplac181-*GPD1*) were dissected on YPD plates and incubated aerobically for 5 days at 28°C.

Overexpression of *GPD1* and *GPD2* does not rescue the lethality of the *rrd1,2*Δ mutant

What apparently connects the two conditions restoring growth of *rrd1,2*Δ cells, high osmolarity and anaerobiosis, is that both induce a similar adaptive response in yeast cells: elevated glycerol production, in each case for a different purpose. At high osmolarity, accumulation of glycerol inside the cell increases internal osmolarity; in anaerobiosis, glycerol synthesis plays the role of a sink for reducing equivalents allowing oxidized NAD^+ nucleotides to regenerate, when it is not possible through the mitochondrial respiratory chain [12]. The increased glycerol production results from an enhanced synthesis of the isoenzymes of glycerol-3-phosphate dehydrogenase encoded by the *GPD1* and *GPD2* genes, induced by osmotic stress or anoxia, respectively [12]. As it was shown previously [3], deletion of *GPD1* in the *rrd1,2*Δ background did not improve growth of the mutant. Therefore, a constitutive overproduction of glycerol is not responsible for the *rrd1,2*Δ lethality. If in aerobic conditions the *rrd1,2*Δ mutant suffers from a defect in maintaining proper redox balance, one could expect that enhanced synthesis of Gpdp, like in high osmolarity or anaerobiosis, could restore the viability of *rrd1,2*Δ cells in standard growth conditions. To test this hypothesis we transformed the BR2 (*rrd1*Δ *rrd2*Δ/++) strain with multicopy plasmids carrying *GPD1* or

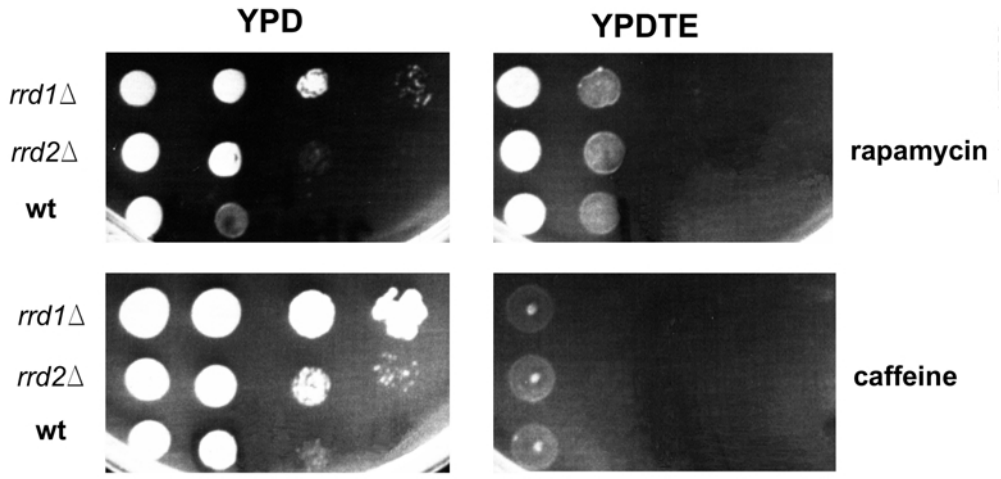

Figure 2. Anaerobiosis suppresses rapamycin and caffeine resistance of *rrd1*Δ and *rrd2*Δ mutants.

Saturated overnight cultures in YPD medium were diluted and 5 μl drops of undiluted and serial 33-fold dilutions were spotted onto indicated media supplemented with rapamycin (0.1 μg/ml) or caffeine (10 mM). Plates were incubated for 5 days at 28°C in aerobic (YPD) or anaerobic (YPDTE) conditions.

GPD2. After sporulation the resulting tetrads were dissected and allowed to grow aerobically on YPD medium. The phenotype segregation pattern was similar to that characteristic for a nontransformed strain (Fig. 1B, shown only for strain BR2 transformed with plasmid carrying *GPD1*), so overexpression of *GPD1* or *GPD2* did not rescue the double mutant *rrd1,2*Δ.

Nonfunctional respiratory chain causes temperature sensitive growth defect of the *rrd1*Δ strain

S. cerevisiae cells grown in anaerobiosis are incapable of respiratory metabolism similar to the respiratory incompetent *rho*$^-$ and *rho*^{o} mutants with extensively or totally deleted mtDNA, respectively. To separate the effect of the absence of oxygen from respiratory deficiency we induced the *rho*^{o} mutation in the single mutants *rrd1*Δ and *rrd2*Δ, the double mutant *rrd1,2*Δ and in parental *RRD1 RRD2* strains and analysed the phenotypes of the isolated *rho*^{o} strains.

All the strains tested grew on YPD medium supplemented with sorbitol, both at 28 and 37°C, while on a medium without an osmotic stabilizer the *rrd1,2*Δ double mutant did not grow at either temperature. Interestingly, *rrd1*Δ *rho*^{o} cells exhibited slow grow phenotype on YPD medium without sorbitol at 37°C (Fig. 3). Other phenotypes of the *rrd1*Δ mutant: sensitivity to calcofluor white, congo red and vanadate, were the same in *rrd1*Δ *rho*^{o} as in *rrd1*Δ *rho*$^+$ cells (not shown). These results indicate that the suppressing effect of anoxia is not a result of respiratory incompetence.

Deletion of the *HEM1* gene does not rescue the *rrd1,2*Δ deficiency in aerobic growth conditions

The ability of *S. cerevisiae* to grow only by fermentation made it possible to isolate mutants deficient in heme synthesis. The phenotype of *Hem*$^-$ mutants resembles that of anarobically grown cells. They are respiratory incompetent due to a lack of mitochondrial cytochromes and require unsaturated fatty acids and ergosterol for growth owing to the absence of cytochromes b_5 and P450. In addition, heme plays a regulatory role as an oxygen sensor and the expression of a set of the so called hypoxic genes is repressed by heme in the presence of oxygen (for review see [14]).

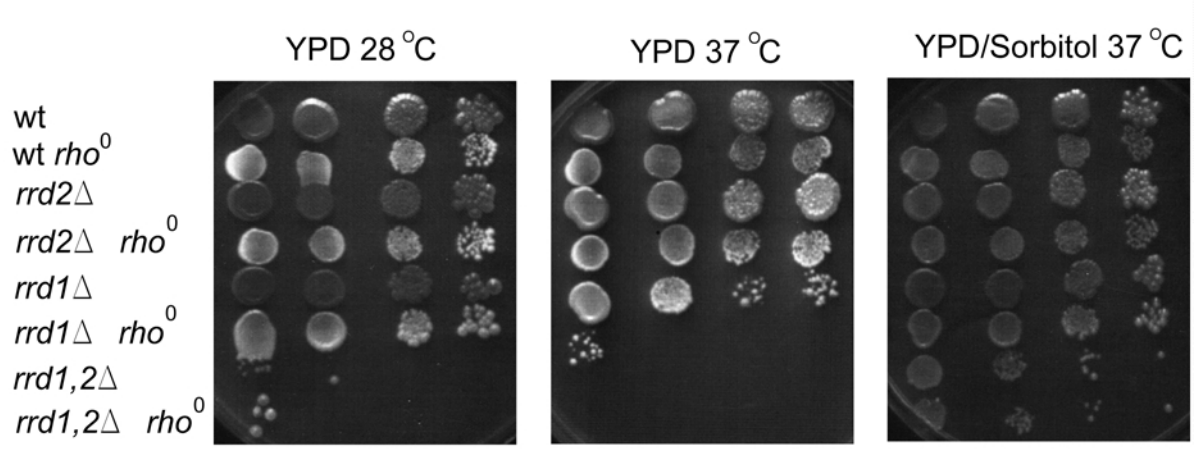

Figure 3. Effect of inactivation of the mitochondrial genome on the growth of *rrd*Δ mutants.

Before spotting on media tested, strains were cultivated overnight at 28°C in liquid YPD/0.8 M sorbitol. Plates were incubated at indicated temperature for 5 days.

Anaerobically grown cells are devoid of heme. Therefore, culturing *Hem*$^-$ mutants aerobically in the absence of heme leads to the expression of oxygen-independent, heme-repressed genes. One of the phenomena dependent on the heme status of the cell is aerobic sterol exclusion. Cells are impermeable to exogenous sterols in aerobiosis, unless they are *Hem*$^-$ [15], whereas in anaerobiosis cells became permeable to sterols.

The conditional rescue of the *rrd1,2*Δ mutant in anaerobiosis and the previously reported [3] sensitivity of the *rrd1*Δ strain to ketoconazole, an azole antibiotic which blocks sterol biosynthesis in yeast at the level of C-14 lanosterol demethylase [16], could also be interpreted as indications of sterol synthesis defects in the mutants. Sterol auxotrophic mutants can be grown on a medium supplemented with ergosterol in aerobic conditions when the heme synthesis pathway is inactivated [15]. To test if *rrd1,2*Δ would be suppressed in aerobic conditions in a similar way to mutants conferring sterol auxotrophy, we introduced the *hem1*Δ mutation into the double heterozygous strain BR2. The triple heterozygote was induced to sporulate and tetrads were dissected on YPDTE plates. Spores were germinated and grown in anaerobic conditions. All spores in the dissected tetrads formed colonies in these conditions (not shown). When, after 7 days of anaerobic growth, the spore clones were transferred to aerobic conditions for tetrad analysis, *rrd1,2*Δ *hem1*Δ segregants (identified by inferring their genotypes on the basis of sibling genotypes in the tetrads) were not able to grow on either YPDTE (Fig. 4) or on a medium selective for the *hem1*Δ allele (synthetic complete without leucine, supplemented with ergosterol/Tween-80 and methionine) (not shown). Other *hem1*Δ segregants in the analysed tetrads, *RRD1 RRD2 hem1*Δ, *rrd1*Δ *RRD2 hem1*Δ and *RRD rrd2*Δ *hem1*Δ, could grow on both media. Thus, inactivation of the heme biosynthesis pathway does not rescue the *rrd1,2*Δ deficiency in aerobic growth conditions. In addition, gas-chromatographic analysis of sterols extracted from the *rrd1*Δ and *rrd2*Δ mutants and wild-type reference strains has not revealed any significant changes in sterols profiles (not shown). Therefore, the possibility that it was the Tween/ergosterol supplement in the medium that protected anaerobically grown *rrd1,2*Δ cells from lysis can be ruled out.

Osmoremedial cell lysis defect of *rrd1,2*Δ cells together with calcofluor white sensitivity of the *rrd1*Δ single mutant indicate that the conditional lethality of the *rrd1,2*Δ strain re-

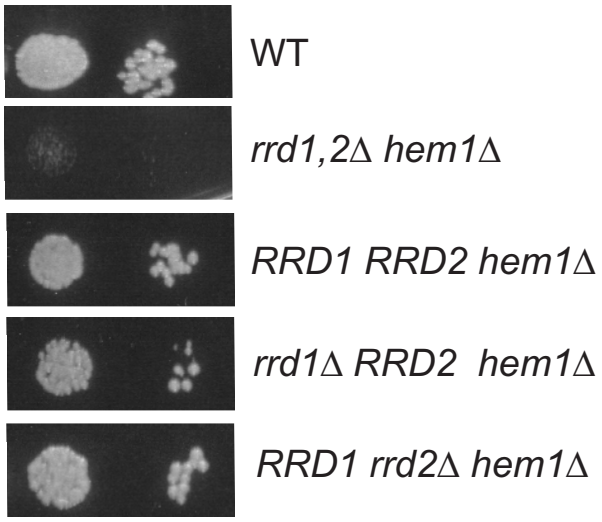

Figure 4. Introduction of *hem1*Δ into the genetic background of the BR2 strain does not suppresses *rrd1,2*Δ lethality.

Spore clones derivatives of the triple heterozygote *rrd1,2*Δ *hem1*Δ/+++ obtained after growth in anaerobic conditions were suspended and diluted in Ringer solutions and 5 μl drops were spotted on YPDTE medium. Plates were incubated in aerobic condition at 28°C for 5 days.

sults from a deficiency in the maintenance of cell wall structure and integrity [3]. Adaptation of yeast cells to anaerobiosis causes changes in the level of expression of a large and diverse set of genes [17]. This and the presented results, suggesting that processes other than respiration or sterol deficiency are responsible for aerobic lethality of the *rrd1,2*Δ mutant, prompt us to propose that oxygen-deprivation may induce alternative pathways for proper cell wall assembly resistant to osmotic challenge. Aerobically and anaerobically grown *S. cerevisiae* strains differ in the cell wall structure and composition. Cwp1 and Cwp2, major mannoproteins of the cell wall of yeast cultivated in normal conditions, are replaced by their anaerobic counterparts, the Dan/Tir proteins, after shift from aerobic to anaerobic conditions [18].

On the other hand, it can not be ruled out that the lethality of *rrd1,2*Δ in aerobiosis is caused by sensitivity of the mutant to oxidative stress and that anaerobiosis protects cells against action of reactive oxidants.

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