Yeast response and tolerance to polyamine toxicity involving the drug : H\textsuperscript{+} antiporter Qdr3 and the transcription factors Yap1 and Gcn4

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The yeast QDR3 gene encodes a plasma membrane drug : H\textsuperscript{+} antiporter of the DHA1 family that was described as conferring resistance against the drugs quinidine, cisplatin and bleomycin and the herbicide barban, similar to its close homologue QDR2. In this work, a new physiological role for Qdr3 in polyamine homeostasis is proposed. QDR3 is shown to confer resistance to the polyamines spermine and spermidine, but, unlike Qdr2, also a determinant of resistance to polyamines, Qdr3 has no apparent role in K\textsuperscript{+} homeostasis. QDR3 transcription is upregulated in yeast cells exposed to spermine or spermidine dependent on the transcription factors Gcn4, which controls amino acid homeostasis, and Yap1, the main regulator of oxidative stress response. Yap1 was found to be a major determinant of polyamine stress resistance in yeast and is accumulated in the nucleus of yeast cells exposed to spermidine-induced stress. QDR3 transcript levels were also found to increase under nitrogen or amino acid limitation; this regulation is also dependent on Gcn4. Consistent with the concept that Qdr3 plays a role in polyamine homeostasis, QDR3 expression was found to decrease the intracellular accumulation of \textsuperscript{3}H]spermidine, playing a role in the maintenance of the plasma membrane potential in spermidine-stressed cells.

INTRODUCTION

Multidrug efflux transporters are found in all living cells and are thought to recognize a wide variety of structurally and pharmacologically unrelated drugs. They are proposed to actively extrude or compartmentalize drugs and other xenobiotics, thus providing protection from these compounds (Jungwirth & Kuchler, 2006; Paulsen, 2003; Prasad et al., 2002; Roepe et al., 1996; Sá-Correia & Tenreiro, 2002). The activity of these proteins underlies the manifestation of cellular drug resistance, seriously limiting the therapeutic potential of drugs (Hayes & Wolf, 1997). The \textit{in silico} analysis of the yeast genome revealed the existence of 23 putative drug: H\textsuperscript{+} antiporters of the multiple drug resistance (MDR) family of the major facilitator superfamily (MFS). Although many of these proteins have been shown to confer resistance to a wide variety of drugs and chemicals (Paulsen et al., 1998; Sá-Correia & Tenreiro, 2002; Sá-Correia et al., 2009), the molecular mechanisms behind their apparent promiscuity remain elusive and a topic of debate (Jungwirth & Kuchler, 2006; Paulsen, 2003; Prasad et al., 2002; Roepe et al., 1996; Sá-Correia & Tenreiro, 2002; Sá-Correia et al., 2009).

In the present work, we have further examined the biological function of the MFS–MDR transporter encoded by the QDR3 gene, which is present in the plasma membrane of \textit{Saccharomyces cerevisiae} (Huh et al., 2003; Tenreiro et al., 2005). Qdr3 belongs to cluster I of the DHA1 drug efflux family, including putative drug: H\textsuperscript{+} antiporters with 12 predicted membrane-spanning segments (Paulsen et al., 1998), and confers yeast resistance against the antiarrhythmic and antimalarial drug quinidine, the herbicide barban and the antitumour agents bleomycin and cisplatin (Tenreiro et al., 2005). Through genome-wide screenings, QDR3 gene deletion was also found to increase yeast susceptibility towards magnesium dichloride (Rieger et al., 1999), selenomethionine (Bockhorn et al., 2008), the antiarrhythmic drug amiodarone (Yadav et al., 2007) and the antifungal drug fluconazole (Giaever et al., 2004). Remarkably, at least six other members of the DHA1 family of \textit{S. cerevisiae} drug: H\textsuperscript{+} antiporters are individually required for quinidine resistance (do Valle Matta et al., 2001; Felder et al., 2009).
2002; Nunes et al., 2001; Tenreiro et al., 2002, 2005). The role of these yeast MFS–MDR transporters in alleviating the deleterious effects of quinidine and other compounds that are not usually present in the yeast cell’s natural environment appears to suggest that their physiological function may have nothing to do with broad chemoprotection. They may transport a still unidentified specific physiological substrate and drugs may be transported only fortuitously or opportunistically. Alternatively, the transport of their natural substrate may indirectly affect the partitioning of drugs across the plasma membrane. Experimental evidence indicates that Qdr3 is involved, directly or indirectly, in the active expulsion of quinidine from preloaded yeast cells (Tenreiro et al., 2005). Interestingly, Qdr3 shares most of its putative substrates with its closest homologue, Qdr2. Recently, Qdr2 was implicated in potassium uptake, presumably being able to couple K⁺ movement with substrate(s) export, in particular with quinidine, thus providing a physiological advantage to cells challenged with this drug (Vargas et al., 2007). However, the physiological role of the Qdr3 MDR transporter remained elusive.

This paper presents experimental evidence relating QDR3 expression to yeast resistance to the polyamines spermine and spermidine. These polyamines are essential organic cations that have multiple functions in the cell, including the regulation of nucleic acid and protein synthesis, as well as the gating of several ion channels (Cohen, 1998). Polyamine concentration is under tight regulation at the level of biosynthesis, catabolism and transport (Cohen, 1998). In particular, membrane transport of polyamines is receiving increasing attention, especially within the scope of cancer therapy based on anti-polyamine strategies. These strategies are based on the fact that specific oncogenes and tumour suppressor genes regulate polyamine metabolism (Gerner & Meyskens, 2004). Although little is known about polyamine transport mechanisms in higher eukaryotes, in the yeast S. cerevisiae, polyamine influx and efflux transporters have been characterized in the past few years (Igarashi & Kashiwagi, 2010). Among these transporters, four members of the DHA1 family of MFS–MDR transporters, Tpo1, Tpo2, Tpo3 and Tpo4, have been described as polyamine exporters (Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005). The best characterized of these four polyamine resistance transporters, Tpo1, has also been related to the extrusion of the antimalarial drugs quinidine (do Valle Matta et al., 2001) and artesunate (Alenquer et al., 2006) and the herbicide 2,4-dichlorophenoxyacetic acid (Cabrito et al., 2009; Teixeira & Sá-Correia, 2002).

During this work, we identified that Qdr3 is a determinant of resistance to spermine and spermidine, but not to putrescine. The effect of polyamine stress exposure or nitrogen limitation on the increase of QDR3 transcript levels was assessed, and Yap1 and Gcn4 were identified as the transcriptional regulators controlling QDR3 up-shift. Yap1 was found to be activated and accumulated in the nucleus of yeast cells under spermidine-induced stress, and it confers resistance to toxic concentrations of this metabolite. Finally, the effect of Qdr3 expression in spermidine intracellular accumulation and in the maintenance of the plasma membrane potential in yeast cells pre-exposed to a toxic concentration of this metabolite was confirmed, consistent with a role in spermidine homeostasis.

METHODS

Strains, plasmids and growth media. S. cerevisiae strain BY4741 (MATα, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) and the derived single deletion mutants BY4741_Δqdr3, BY4741_Aqdr2, BY4741_Δqdr1, BY4741_Atpo1, BY4741_Atpo2, BY4741_Atpo3, BY4741_Atpo4, BY4741_Atpo5, BY4741_Agtn1, BY4741_Agtn3, BY4741_Agcn4 and BY4741_Δyap1 were obtained from the Euroscarf collection. The amino acid prototrophic strain 23344Δ (MATα, urs3) was provided by B. André (Université Libre de Bruxelles, Belgium). The plasmids pYCpG_QDR3 and pFL38 (Tenreiro et al., 2002), pAH152_QDR2-lacZ (Vargas et al., 2004), pYCpG_YAP1 (Tenreiro et al., 2001), pYAP1-GFP (Coleman et al., 1999), pYEP354_QDR3-lacZ, pYEP354_QDR3(700)-lacZ and pYEP354_QDR3(350)-lacZ (this study) were used in this work.

Cells were batch-cultured at 30 °C, with orbital agitation (250 r.p.m.) in YPD growth medium, with the following composition (l⁻¹): 20 g glucose (Merck), 20 g yeast extract (Difco) and 20 g peptone (Difco). For some of the experiments, minimal medium was used, which consisted of the basal medium (BM) supplemented with different nutrients or polyamines (l⁻¹): 1.7 g yeast nitrogen base without amino acids or NH₄Cl (Difco), 20 g glucose (Merck) and 2.65 g (NH₄)₂SO₄ (Merck). Strain BY4741 was grown in MM4 medium, which was BM supplemented with 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil (l⁻¹); all from Sigma. For the amino acid prototrophic strain 23344Δ, the growth medium used (MM5) was BM supplemented with 20 mg uracil l⁻¹. Ammonium phosphate-derived KNA medium was also used to test BY4741 and derived Δqdr3 under K⁺ limitation. Ammonium phosphate basal medium contained (l⁻¹) a mixture of 0.492 g MgSO₄·7H₂O (Merck), 0.02 g CaCl₂ (anhydrous) (Panreac), 1.056 g (NH₄)₂HPO₄ (Merck), 3.96 g (NH₄)₂SO₄, 20 g glucose, 2 mg niacin, 2 mg pyridoxine, 2 mg thiamine, 2 mg pantothenate, 0.02 mg biotin and the desired concentration of KCl (all from Sigma). For BY4741 growth, this medium was supplemented with 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil (l⁻¹); all from Sigma. To maintain selective pressure over the recombinant strains, the addition of uracil to this medium was only carried out to grow the host yeast cells. Agar (20 g l⁻¹; Iberagar) was added to media to obtain solid media.

Polyamine susceptibility assays. The susceptibility of the parental strain BY4741 towards toxic concentrations of polyamines was compared with that of the deletion mutants BY4741_Δqdr3, BY4741_Aqdr2, BY4741_Aqdr1, BY4741_Atpo1, BY4741_Atpo2, BY4741_Atpo3, BY4741_Atpo4, BY4741_Agcn4 and BY4741_Δyap1 by spot assays. The ability of QDR3 gene expression to increase wild-type resistance to spermine and spermidine and to complement the susceptibility phenotype exhibited by the single deletion mutants was also examined, using the pYCpG_QDR3 centromeric plasmid in which QDR3 is expressed under its own promoter. The effect of YAP1 gene expression in wild-type and Δqdr3 susceptibility towards spermidine-induced stress was also analysed by using the pYCpG_YAP1 centromeric plasmid in which YAP1 is expressed under its own promoter.
Cell suspensions used to inoculate the agar plates were mid-exponential cells grown in basal YPD medium to OD_{600} 0.4 ± 0.02 and then diluted in sterile water to obtain suspensions of OD_{600} 0.05 ± 0.005. These cell suspensions and subsequent dilutions (1:5, 1:25) were applied as 4 μl spots onto the surface of solid YPD medium, supplemented with adequate polyamine concentrations. The concentrations of spermine, spermidine and putrescine (all Sigma) used in these tests ranged from 3 to 5 mM, 2 to 10 mM and 8 to 50 mM, respectively.

[^3]H]Spermidine accumulation assays. Accumulation assays of [^3]H]spermidine assessed as described previously (Vargas et al., 2004). To estimate the accumulation of spermidine (intra- and extracellular [^3]H]spermidine) from yeast cells, the parental strain BY4741 and the mutant strains Δqdr3 and Δqdr2 were grown in YPD medium till mid-exponential phase, reincubated into fresh YPD medium supplemented with 15 mM spermidine for 1.5 h and harvested by filtration. Cells were washed and resuspended in TM buffer (0.1 M MES (Sigma), 41 mM Tris (Sigma) adjusted to pH 5.5 with HCl), with 2% glucose, to obtain dense cell suspensions (OD_{600} 5.0 ± 0.2, equivalent to approximately 2.2 (mg dry weight) mL^{-1}). After 5 min incubation at 30°C, with agitation (150 r.p.m.), 0.1 μM [^3]H]spermidine (ICN; 37 MBq ml^{-1}) and 100 μM unlabelled spermidine were added to the cell suspensions, which were incubated for 2 h. During this period of incubation, the intracellular accumulation of labelled spermidine was followed by filtering 200 μl cell suspension, at adequate time intervals, through pre-wetted glass microfiltration filters (Whatman GF/C). The filters were washed with ice-cold TM and the radioactivity was measured in a Beckman LS 5000TD scintillation counter. Extracellular [^3]H]spermidine was estimated by radioactivity assessment of 50 μl supernatant.

Non-specific [^3]H]spermidine adsorption to the filters and the cells (less than 5% of the total radioactivity) was assessed and taken into consideration. To calculate the intracellular concentration of labelled spermidine, the internal cell volume (V_i) of the exponential cells, grown in the absence of drug and used for accumulation assays, was considered constant and equal to 2.5 μl (mg dry weight)^{-1} (Rosa & Sá-Correia, 1996).

Methylamine uptake experiments. Methylamine uptake was assessed as described previously (Hoeberichts et al., 2010). To estimate the uptake of methylamine in yeast cells, the parental strain BY4741 and the mutant strain Δqdr3 were grown in YPD medium till mid-exponential phase, reincubated into fresh YPD medium and grown until OD_{600} 0.5 ± 0.05. Cells were washed by centrifugation (15,000 r.p.m., 1 min) three times with water and resuspended in water at the same density. Cell suspension (0.1 ml) was added to 0.9 ml of a 2% glucose solution with 0, 0.5 or 5 mM spermidine and incubated with rotation at 30°C. After 30 min, 3 μl [^4]C]methylamine (Biotrend) was added to a final concentration of 55 μM, and at 0, 20, 40 and 60 min, samples (200 μl) were diluted in 10 ml cold water, filtered through pre-wetted glass microfilters (Whatman, GF/C) and washed four times with 5 ml cold water. After addition of 7 ml scintillation liquid (Beckman) and overnight incubation, the radioactivity was measured in a Beckman LS 5000TD scintillation counter. Non-specific [^4]C]methylamine adsorption to the filters and to the cells (less than 5% of the total bound radioactivity) was assessed and taken into consideration. To calculate the intracellular concentration of [^4]C]methylamine, the volume (V_i) of the different strains was considered constant and equal to 2.5 μl (mg dry weight)^{-1} (Rosa & Sá-Correia, 1996).

QDR3 expression measurements. The levels of QDR3 gene in nutrient-limited conditions were examined based on Northern blot experiments, while the transcript levels following yeast exposure to 3 mM spermidine or 18 mM spermidine were assessed by real-time RT-PCR. RNA extraction from yeast cells and Northern blot hybridizations were carried out as described previously (Teixeira & Sá-Correia, 2002). The total RNA in each sample used was kept constant (20 μg). The DNA probe for QDR3 gene was prepared by performing a Clai restriction of the pYCG, QDR3 plasmid (Teneiro et al., 2005). The specificity of the probe was confirmed by using RNA extracts from Δqdr3 deletion mutant cells. The ACT1 mRNA level was used as an internal control; the ACT1 probe was prepared as described previously (Teixeira & Sá-Correia, 2002). High performance MP (Amersham Biosciences) films were exposed to the nitrocellulose membranes and incubated with an intensifying screen at -70°C. The relative intensities of the hybridization signals were quantified by densitometry, using an Image Scanner and the Image Master 1D Elite software (both from Amersham Biosciences). Synthesis of cDNA for real-time RT-PCR experiments, from total RNA samples, was performed using the Multiscribe reverse transcriptase kit (Applied Biosystems) and the 7500 RT-PCR thermal cycler block (Applied Biosystems), following the manufacturer’s instructions. The quantity of cDNA for the following reactions was kept around 10 ng. The subsequent RT-PCR step was carried out using SYBR Green reagents. Primers for the amplification of the QDR3 and ACT1 cDNA were designed using Primer Express Software (Applied Biosystems): 5'-TCTGACACATCTCAGCGCAACATC-3', 5'-GGGTCGGCGTCTT-3' and 5'-CTCCACAGGCGGCGGAAGAA-3', 5'-CCAGAGGCGTACACAGTTT-3', respectively. The RT-PCR was carried out using a thermal cycler block (7500 Real-Time PCR System; Applied Biosystems). Default parameters established by the manufacturer were used and fluorescence was detected by the instrument and registered in an amplification plot (7500 System SDS Software; Applied Biosystems). The ACT1 mRNA level was used as an internal control. The relative values obtained for the wild-type strain in control conditions were set as 1 and the remaining values are presented relative to that control. To avoid false-positive signals, the

BY4741_Agap4 and BY4741_Agap1 deletion mutants. Cells transformed with the QDR3-lacZ fusion plasmid were cultivated in unsupplemented or YPD medium supplemented with 3 mM spermidine or 18 mM spermidine. pYEP354_QDR3-lacZ fusion plasmids were obtained as described previously (Teixeira et al., 2010) using the pYEP354 expression vector and three PCR amplification fragments overlapping the QDR3 promoter region from positions -1000 to 0 (pYEP354_QDR3-lacZ), -700 to 0 (pYEP354_QDR3(700)-lacZ) and -350 to 0 (pYEP354_QDR3(350)-lacZ). These PCR fragments were generated using the following primers: 3'-AAAAACTGAGACGACGCCTGGCCTGATG-5' (which includes a protective AAAA sequence, a CTGCAG restriction sequence and an extra nucleotide so that the start codon is in phase with the lacZ fusion), 5'-CGGGGGATCCAAAGGCGACGACGGAGG-3', 5'-CGGGATCCGGTACGAGCTATGCTTTT-3' and 5'-CGGGGGATCCGATTTCAGCCACCGCGTGCGC-5'. The absence of mutations inside the selected amplified fragments was verified by DNA sequencing. Cells transformed with pQDR3-lacZ fusion plasmids were cultivated in YPD liquid medium supplemented with 18 mM spermidine. Growth was followed by measuring OD_{600} and samples were harvested after 3 and 5 h cultivation in the presence or absence of the polynamine. Viability of the transformed cells at the same time intervals was determined in both YPD and MM4 liquid medium without uracil to account for plasmid losses. β-Galactosidase activity assays were based on the method of Miller, as described previously (Bröco et al., 1999). The specific enzyme activity units (U) were defined as the increase in A_{420} min^{-1} (OD_{600})^{-1} × 1000. The lacZ expression profiles are the means±SD of at least three independent growth experiments for each condition.

The changes in the transcript levels from the QDR3 gene in nutrient-limited conditions were examined based on Northern blot experiments, while the transcript levels following yeast exposure to 3 mM spermidine or 18 mM spermidine were assessed by real-time RT-PCR. RNA extraction from yeast cells and Northern blot hybridizations were carried out as described previously (Teixeira & Sá-Correia, 2002). The total RNA in each sample used was kept constant (20 μg). The DNA probe for QDR3 transcripts was prepared by performing a Clai restriction of the pYCG, QDR3 plasmid (Teneiro et al., 2005). The specificity of the probe was confirmed by using RNA extracts from Δqdr3 deletion mutant cells. The ACT1 mRNA level was used as an internal control; the ACT1 probe was prepared as described previously (Teixeira & Sá-Correia, 2002). High performance MP (Amersham Biosciences) films were exposed to the nitrocellulose membranes and incubated with an intensifying screen at -70°C. The relative intensities of the hybridization signals were quantified by densitometry, using an Image Scanner and the Image Master 1D Elite software (both from Amersham Biosciences). Synthesis of cDNA for real-time RT-PCR experiments, from total RNA samples, was performed using the Multiscribe reverse transcriptase kit (Applied Biosystems) and the 7500 RT-PCR thermal cycler block (Applied Biosystems), following the manufacturer’s instructions. The quantity of cDNA for the following reactions was kept around 10 ng. The subsequent RT-PCR step was carried out using SYBR Green reagents. Primers for the amplification of the QDR3 and ACT1 cDNA were designed using Primer Express Software (Applied Biosystems): 5'-TCTGACACATCTCAGCGCAACATC-3', 5'-GGGTCGGCGTCTT-3' and 5'-CTCCACAGGCGGCGGAAGAA-3', 5'-CCAGAGGCGTACACAGTTT-3', respectively. The RT-PCR was carried out using a thermal cycler block (7500 Real-Time PCR System; Applied Biosystems). Default parameters established by the manufacturer were used and fluorescence was detected by the instrument and registered in an amplification plot (7500 System SDS Software; Applied Biosystems). The ACT1 mRNA level was used as an internal control. The relative values obtained for the wild-type strain in control conditions were set as 1 and the remaining values are presented relative to that control. To avoid false-positive signals, the

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absence of non-specific amplification with the chosen primers was confirmed by the generation of a dissociation curve for each pair of primers.

**Yap1 subcellular localization.** The subcellular localization of Yap1 under spermidine-imposed stress was assessed in BY4741 cells harbouring the pYAP1-GFP fusion plasmid (Coleman et al., 1999), kindly provided by Scott Moye-Rowley (Department of Physiology and Biophysics, University of Iowa, Iowa City, USA). Cells transformed with pYAP1-GFP were cultivated in MM4 liquid medium until OD600 0.5 ± 0.05. Then spermidine was added to a final concentration of 18 mM. The distribution of the Yap1–GFP fusion protein in living *S. cerevisiae* cells was detected at appropriate time intervals by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelengths of 395 and 509 nm, respectively.

**RESULTS**

**QDR3 is a determinant of yeast resistance to spermine and spermidine but is not involved in yeast tolerance of limited potassium**

The susceptibility to inhibitory concentrations of the polyamines spermine, spermidine and putrescine of *S. cerevisiae* BY4741 parental cells and mutant strains with deletions in QDR1, QDR2 or QDR3 was compared by spot assays. QDR2 and QDR3 were found to confer resistance to spermine and spermidine (Fig. 1). QDR2 also confers resistance to putrescine, while QDR3 does not (Fig. 1). Δqdr1 cells showed no increased susceptibility towards polyamine stress. To compare the relative importance of Qdr2 and Qdr3 in yeast resistance towards spermidine stress with that of the previously identified polyamine transporters Tpo1–5, the susceptibilities of deletion mutants devoid of each of these transporters towards spermidine challenge were evaluated. QDR3 was shown to play a less significant role than TPO1 in spermidine resistance (Fig. 2a). However, QDR3 gene expression seems to be as important as that of TPO3 or TPO4 and more relevant than that of TPO2 and TPO5 in this context (Fig. 2a). Finally, QDR3 gene expression from a recombinant plasmid was found to increase yeast resistance to spermidine-induced stress, complementing the susceptibility phenotype exhibited by the Δqdr3 and Δtpo1 cells (Fig. 2b), but not that exhibited by the other deletion mutants devoid of QDR2 or TPO2–5.

Since the increased susceptibility of the Δqdr2 mutant towards polyamines is consistent with its increased susceptibility towards other cationic compounds, used as markers for cell depolarization, and its role in K+ homeostasis (Vargas et al., 2007), a role in K+ homeostasis was also hypothesized for Qdr3. However, when exponentially growing wild-type and Δqdr3 cells were inoculated into fresh KNA medium with a limiting K+ concentration (0.2 mM), similar growth patterns were observed for both strains (results not shown). This is consistent with the observation that QDR3 expression is unable to rescue the polyamine susceptibility phenotype exhibited by Δqdr2 cells and suggests a different role for these transporters in conferring polyamine stress tolerance.

**QDR3 plays a role in reducing the intracellular accumulation of [3H]spermidine**

In view of Qdr3 localization at the plasma membrane (Tenreiro et al., 2005), we examined the possible involvement of QDR3 in the reduction of spermidine accumulation in yeast cells. In the absence of glucose there is no detectable uptake of [3H]spermidine (results not shown). This is in agreement with the notion that polyamines, being polycationic molecules, are unable to cross the plasma membrane by passive diffusion. In the presence of glucose, they enter the cell by active influx, mediated by Sam3, Dur3 (Uemura et al., 2007) and Agp2 (Aouida et al.,

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![Fig. 1. QDR3 is a determinant of yeast resistance to spermine and spermidine, like its close homologue QDR2, which also provides resistance to putrescine, and different from its other close homologue QDR1. The susceptibility of the parental strain BY4741 and Δqdr1, Δqdr2 and Δqdr3 mutant strains to the indicated concentrations of spermine, spermidine and putrescine was compared based on spot assays. The cell suspensions used to prepare the spots in lanes (ii) and (iii) were 1:5 and 1:25 serial dilutions, respectively, of the suspension with an OD600 0.05 ± 0.005 in (i). Some parts of this figure were composed from different images in order to make comparison easier; this is shown by gaps between the images.](https://www.microbiologyresearch.org/)

948
At the same time, energy-dependent efflux mechanisms are activated, including those presumably catalysed by the Tpo1–5 proteins (Tachihara et al., 2005; Tomitori et al., 2001). To determine whether Qdr3 contributes to the differential accumulation of $[^{3}H]spermidine$, its overall accumulation was assessed in wild-type and Δqdr3 cells suspended in growth medium supplemented with 7 mM cold spermidine.

The differences registered in $[^{3}H]spermidine$ intracellular concentration at the equilibrium, together with all the other indications obtained so far, support the idea that Qdr3 is, directly or indirectly, involved in the active export of spermidine. Indeed, the spermidine level exhibited by the parental strain, in the presence of glucose, was below that registered for the Δqdr3 deletion mutants (Fig. 3). Interestingly, this difference was mostly observed in cells pre-exposed for 1.5 h to 15 mM spermidine, suggesting that Qdr3 activity increases in the presence of this metabolite.

The same experimental procedure was used to examine an eventual role of Qdr2 in polyamine homeostasis, but no differences in $[^{3}H]spermidine$ intracellular accumulation were registered between the wild-type strain and the Δqdr2 deletion mutant, in either stressed or non-stressed conditions (results not shown).

**QDR3 expression decreases spermidine-induced disruption of the plasma membrane potential**

Given that the toxicity of polyamine is mainly due to its effect as a disruptor of plasma membrane potential, the role of QDR3 expression in the maintenance of this physiological trait was analysed. Yeast plasma membrane potential was estimated based on the uptake of methylammonium, a non-metabolizable ammonium analogue, whose influx is strongly dependent on the maintenance of the transmembrane potential (van de Mortel et al., 1988). The deletion of QDR3 did not exert an effect upon the transmembrane potential in control conditions compared with the parental strain (Fig. 4). Furthermore, exposure to moderately toxic concentrations of spermidine is enough to completely abrogate methylammonium transport (Fig. 4).

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**Fig. 2.** (a) Comparison, by spot assays, of the susceptibility of the Δqdr2 and Δqdr3 deletion mutants with that of the Δtpo1–5 strains towards spermidine stress, in which each of the five previously described polyamine transporters was individually deleted. (b) Comparison, by spot assays, of the effect of QDR3 expression from a recombinant plasmid (pYCG_QDR3, derived from the cloning vector pFL38) on the resistance of wild-type, Δqdr3 and Δtpo1 cells towards spermidine stress. Cells used to prepare the spots were grown in the absence of polyamine stress, until OD<sub>600</sub> 0.5 ± 0.05 was attained (exponential phase). The cell suspensions used to prepare the spots in lanes (ii) and (iii) were 1 : 5 and 1 : 25 serial dilutions, respectively, of the suspension with an OD<sub>600</sub> 0.05 ± 0.005 in (i). Some parts of this figure were composed from different images in order to make comparison easier; this is shown by gaps between the images.

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**Fig. 3.** QDR3 gene expression is important to reduce spermidine intracellular accumulation in yeast cells exposed to toxic concentrations of this metabolite. Time-course accumulation of $[^{3}H]spermidine$ was followed during incubation of wild-type (●) or Δqdr3 (■) yeast cells in the presence of the radiolabelled polyamine. Relative levels of spermidine, assessed as described in Methods, are means ± SD of at least three independent growth experiments. Cells used to prepare the inocula for the accumulation assays were grown for 1.5 h in the presence of 7 mM spermidine.
GLN3 deletion could be detected on D950

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observed increased accumulation of spermidine in of the of methylammonium uptake (by 50 %) in yeast cells devoid of the QDR3 gene (Fig. 4). These results correlate with the observed increased accumulation of spermidine in Δqdr3 cells compared with the parental strain and show that QDR3 gene expression contributes to maintain the plasma membrane potential in polyamine-stressed cells.

QDR3 expression is activated in response to spermine or spermidine challenge in a Gcn4- and Yap1-dependent manner

The low expression level of the QDR3 gene during S. cerevisiae BY4741 growth in YPD medium is activated following yeast cell exposure to spermine or spermidine. Preliminary evidence was based on the comparison of β-galactosidase activity values of yeast cells transformed with a plasmid containing a QDR3 promoter–lacZ fusion. Under the experimental conditions used in this work, QDR3 expression reached maximal levels during spermine- or spermidine-induced latency. The expression of Qdr2, assessed from a QDR2–lacZ fusion plasmid, was also analysed in yeast cells exposed to inhibitory concentrations of spermine and spermidine. QDR2 expression remained unaltered in these conditions (results not shown).

Based on the analysis of the QDR3 promoter (Fig. 5a) using the YEASTRACT database (www.yeastact.com; Monteiro et al., 2008; Teixeira et al., 2006), four transcription factors were inspected for a role in QDR3 regulation under polyamine stress: Yap1, the major regulator of oxidative stress response, and Gcn4, Gln3 and Gat1, involved in the control of nitrogen metabolism. The transcriptional upregulation of QDR3 taking place during the period of yeast adaptation to spermine or spermidine was found to be reduced in mutants with deletions of either GCN4 or, especially, YAP1 (Fig. 5b). No significant effect of GAT1 or GLN3 deletion could be detected on QDR3 expression. Interestingly, Δyap1 deletion mutant was found to be extremely sensitive to spermine, spermidine and putrescine compared with the parental strain (Fig. 5c). On the other hand, GCN4 expression does not seem to affect polyamine resistance, since the Δgcn4 mutant exhibited a level of susceptibility towards polyamines similar to that detected in wild-type cells, as assessed by spot assays (Fig. 5c).

Role of Yap1 in spermidine stress response

In order to gain further insights into the role of Yap1 in spermidine stress response, yeast cells harbouring a plasmid expressing a Yap1–GFP fusion protein were exposed to 18 mM spermidine. Yap1 subcellular localization was evaluated in the first hours of cultivation in the presence of toxic concentrations of this metabolite and Yap1 was seen to accumulate in the nucleus of these stressed yeast cells (Fig. 6a). Yap1 accumulation could be clearly seen after 30 min exposure to spermidine stress, but was no longer registered after 5 h adaptation to this stress. The transient nuclear accumulation of Yap1 correlates with the profile of QDR3 upregulation, which was also seen to be transient, as QDR3 expression returned to basal levels following 5 h of spermidine challenge (Fig. 6b).

To assess whether Yap1 could be regulating QDR3 transcription directly through the two potential Yap1-responsive elements (YRE) found in the QDR3 promoter region, the effect of the deletion of the promoter regions containing these YREs was examined. β-Galactosidase activity from yeast cells harbouring QDR3 promoter–lacZ fusion plasmids pYEP354_QDR3-lacZ, pYEP354_QDR3(700)-lacZ and pYEP354_QDR3(350)-lacZ was assessed (Fig. 7). β-Galactosidase activation profiles resulting from the expression of lacZ under the control of the full QDR3 promoter, considered to include the 1000 bp upstream of the QDR3 start codon, were found to be very similar to that resulting from the control of the first 700 bp of the QDR3 promoter, containing a single YRE at position −355 (Fig. 7). As expected, no increase in β-galactosidase activity was seen in yeast cells devoid of Yap1. However, when an even shorter promoter region was considered, containing only the first 350 bp of the QDR3

Fig. 4. QDR3 gene expression is important to maintain the plasma membrane potential in yeast cells exposed to toxic concentrations of spermidine. Time-course accumulation of [14C]methylammonium was followed during incubation of wild-type (left) or Δqdr3 (right) yeast cells in growth medium in the presence of the radiolabelled ammonium analogue. Relative levels of [14C]methylammonium, assessed as described in Methods, are means ± s.o of at least three independent growth experiments. Cells used to prepare the inocula for the accumulation assays were pre-cultivated for 0.5 h in the presence of 0 mM (diamonds), 0.5 mM (squares) or 5 mM (circles) spermidine.
promoter and excluding the second potential YRE, the wild-type increase in β-galactosidase activity was no longer observed in response to spermidine-induced stress. These results are consistent with the hypothesized role of Yap1 on the QDR3 promoter and indicate that the YRE located at position −355 bp is the active Yap1 binding site.

A possible epistatic relationship between YAP1 and QDR3 was also examined. The expression of YAP1 from a centromeric plasmid was seen to increase yeast resistance to spermidine stress, in both wild-type and Δqdr3 cells (Fig. 8). However, the expression of QDR3 from a centromeric plasmid, under the control of its natural promoter, was unable to rescue the Δyap1 susceptibility phenotype (Fig. 8). These results confirm that Yap1 appears upstream of Qdr3 in the signalling pathway leading to spermidine stress response and adaptation and that Yap1 is most likely controlling the expression of many other genes conferring spermidine stress tolerance in yeast.

**QDR3 transcription is activated when the auxotrophic strain BY4741 enters stationary phase due to leucine limitation or is under nitrogen limitation**

To determine the physiological role of Qdr3, we searched for physiological conditions leading to its transcriptional activation. Given that QDR3 expression depends on the major amino acid starvation response regulator Gcn4 (Hinnebusch, 2005), the physiological conditions selected included amino acid and nitrogen limitation. QDR3 transcription was seen to be highly activated (fivefold) in *S. cerevisiae* BY4741 entering the stationary phase of growth in MM4 medium due to leucine limitation (Fig. 9a). Leucine is one of the amino acids that have to be added to the growth medium due to BY4741 auxotrophies and the concentration added (60 mg l⁻¹) is growth limiting (Vargas et al., 2007). Interestingly, the high levels...
of QDR3 mRNA present in leucine-limited cells returned to basal levels when fresh leucine was added to the culture with leucine-starved cells (Fig. 9a). As seen for QDR2 (Vargas et al., 2007), the strong activation of QDR3 transcription registered in BY4741 cells approaching stationary phase, due to leucine limitation, was not detected in the respective mutant with the GCN4 gene deleted (Fig. 9a). These results indicate that QDR3 transcription is activated in response to leucine limitation, which is dependent on the presence of Gcn4.

Significantly, QDR3 expression was also found to be upregulated in yeast cells under NH₄⁺ limitation in strain 23344c (Fig. 9b). This prototrophic strain was used to avoid the need to add amino acids to the growth medium, which are necessary for the auxotrophic strain BY4741. Results from Northern blot experiments indicate that the levels of QDR3 transcripts increased sixfold when 23344c approached stationary phase due to ammonium limitation (initial concentration 0.0265 g ammonium sulphate l⁻¹), compared with the values in cells grown with a saturating ammonium concentration (Fig. 9b).

The eventual transcription activation of QDR3 under carbon source limitation was also investigated. Under glucose-limiting conditions (initial concentration 0.05 %, w/v), S. cerevisiae BY4741 growth proceeded with a specific growth rate below the value possible at a glucose-saturating concentration (2 % w/v) until the carbon source was exhausted. However, there was no

Fig. 6. (a) Subcellular localization of Yap1 under spermidine-induced stress, assessed in BY4741 cells harbouring pYAP1-GFP fusion plasmid and cultivated in MM4 liquid medium in the presence of 18 mM spermidine. The distribution of the Yap1–GFP fusion protein in living S. cerevisiae cells was detected by fluorescence microscopy in a Zeiss Axiosplan microscope. The selected images are representative of the majority of the yeast population. (b) Comparison of the relative values of QDR3 mRNA/ACT1 mRNA, as obtained through RT-PCR, in the wild-type BY4741, cultivated in the absence (●) or presence (■) of 18 mM spermidine. The relative value of QDR3 mRNA at time zero was set as 1 and the remaining values were calculated relative to this. Indicated expression levels are mean values of at least three independent experiments; bars, SD.

Fig. 7. QDR3 promoter analysis based on QDR3 promoter–lacZ fusions. The changes in β-galactosidase activation, expressed from three QDR3 promoter–lacZ fusions, in wild-type or Δyap1 yeast cells exposed to 18 mM spermidine. The strength of the full 1000 bp QDR3 promoter region (black bars) was compared with that of a 700 bp QDR3 subpromoter, devoid of the first YRE (grey bars), and to that of a 350 bp QDR3 subpromoter, devoid of both the first and second YREs (white bars), based on the corresponding spermidine-induced β-galactosidase activity, assessed at the indicated times. Values are the means ± SD of at least three independent experiments.
change in the mRNA level from QDR3 when cells entered the stationary phase due to carbon limitation (results not shown). Altogether, the results indicate that the activation of QDR3 transcription in cells entering the stationary phase of growth cannot be attributed to nutrient limitation, in general, but is specific for nitrogen source limitation.

**DISCUSSION**

This study is focused on the participation of QDR3 in polyamine resistance, given that QDR3 expression was found to increase yeast resistance to spermine and spermidine, but not to putrescine. Remarkably, QDR3 expression does not improve yeast growth under limiting potassium concentrations, indicating a physiological role distinct from that of its close homologue QDR2 (Vargas et al., 2007). This seems very intriguing since Qdr2 and Qdr3 share a role in the resistance to a number of chemical stress agents, including the antimalarial/antiarrythmic drug quinidine, the anticancer drugs cisplatin and bleomycin and the herbicide barban, indicating that QDR3 expression is able to complement, to some extent, the sensitivity phenotypes exhibited by the Δqdr2 deletion mutant (Tenreiro et al., 2005). However, the effect of QDR2 expression in K⁺ homeostasis (Vargas et al., 2007) indicates that the role of QDR2 in polyamine resistance may be indirect, whereas Qdr3 may be involved in polyamine homeostasis. In agreement with this notion, QDR3 gene expression was found not to complement the Δqdr2 susceptibility phenotype towards polyamine-induced stress. Furthermore, QDR3 was shown to play a role in reducing the intracellular accumulation of [¹⁴C]-spermidine. This fact may be linked with the participation of Qdr3 in maintaining yeast plasma membrane potential under spermidine stress observed in this study. Indeed, a higher intracellular concentration of this polycationic...
compound may lead to a higher imbalance of charges across the plasma membrane.

The presence of a number of MFS–MDR transporters providing yeast resistance to chemically and structurally unrelated drugs supports the notion that their physiological function may not be related to broad chemoprotection but rather to the transport of still unidentified substrates. This seems to be the case for Dtr1, Aqr1, Tpo1–4 and Qdr2, which are involved in the transport of the physiological substrates bisformyl dityrosine (Felder et al., 2002), amino acids [alanine, aspartate and glutamate (Velasco et al., 2004)], polyamines (Albertsen et al., 2003) and K⁺ (Vargas et al., 2007), respectively. In this work, the role in polyamine resistance attributed to four DHA1 proteins, Tpo1–4, is extended to Qdr3 and, apparently indirectly, to Qdr2. Although generally said to confer polyamine resistance, the role of each of these transporters reveals some degree of specificity: Tpo1, Tpo4 and Qdr2 confer resistance to spermine, spermidine and putrescine, Tpo2 mostly confers resistance to spermine, and Qdr3 and Tpo3 to spermine and spermidine. More work is required to gain a full understanding of the precise biological function(s) of polyamines and to extend the knowledge gathered in polyamine homeostasis of yeast to higher eukaryotes. Furthermore, whether the catalysis of polyamine extrusion may be involved in the ability of Qdr3 and its homologues to confer MDR remains to be established.

During the course of this work, QDR3 transcript levels were shown to be upregulated in yeast cells exposed to spermine or spermidine. This upregulation was shown to occur under the control of the basic leucine zipper transcription factors Gcn4 and Yap1. Consistent with our observations, a global analysis of transcription factor ability to interact in vitro with yeast promoter regions showed that Gcn4 is able to bind to the QDR3 promoter (Lee et al., 2002). However, Gcn4 was found not to confer resistance to polyamines in yeast. A similar phenomenon has been seen before, for instance, for the transcription factor Pdr3, a major regulator of the pleiotropic drug resistance network, which controls the upregulation of the FLR1 gene in response to the fungicides benomyl (Bróco et al., 1999; Tenreiro et al., 2001) and mancozeb (Teixeira et al., 2008, 2010). In this case, even though FLR1 is a clear determinant of resistance to these fungicides, PDR3 deletion has no apparent effect on yeast tolerance to benomyl (Bróco et al., 1999; Tenreiro et al., 2001) or mancozeb (Teixeira et al., 2008, 2010). The fact that GCN4 expression does not increase yeast tolerance to polyamines may indicate that its role in QDR3 upregulation is not crucial for polyamine stress response. It may further imply that the threefold induction of QDR3 transcript levels found to occur upon exposure to toxic polyamine concentrations is of limited consequence in terms of polyamine resistance, the basal levels of QDR3 being enough to confer yeast resistance to spermine and spermidine. On the other hand, we demonstrated that YAP1 is a major determinant of yeast resistance to spermine and spermidine, revealing a new pathway in the sensing and regulation of polyamine stress tolerance in yeast. Indeed, exposure to polyamine-induced stress was found to trigger the signalling for Yap1 activation and consequent nuclear accumulation. At least one of the potential Yap1 binding sites found in the QDR3 promoter is required to mediate its spermidine-induced upregulation. However, the strong effect of Yap1 deletion on polyamine susceptibility may result from not only its role in QDR3 regulation but also its effect in the transcription regulation of a number of other genes involved in polyamine detoxification (e.g. TPO1, TPO2 and TPO4; www.yeastact.com; Monteiro et al., 2008; Teixeira et al., 2006). This notion seems to be supported by the observation that YAP1 expression is able to complement the qdr3 susceptibility phenotype, while QDR3 is unable to do so for the yap1 deletion mutant. Since Yap1 is one of the major regulators of the oxidative stress response in S. cerevisiae, the strong sensitivity phenotype of yap1 towards polyamines seems to suggest that they may induce oxidative stress. Yap1 activation and accumulation in the nucleus is known to occur via two possible mechanisms, depending on the action of reactive oxygen species or of thiol-reactive compounds. Although the exact mechanism underlying a possible pro-oxidant action of polyamines is still unknown, a survey on the dual role of polyamines as anti-oxidant/pro-oxidant agents has verified that these compounds do act as pro-oxidant agents in the presence of free iron (Mozdzan et al., 2006). Yap1 also plays a role in the control of MDR, regulating the expression of at least two other MFS–MDR proteins, Flr1 (Bróco et al., 1999; Tenreiro et al., 2001) and Atr1 (Coleman et al., 1997). Atr1, a member of the 14 spanner drug:H⁺ antiporters DHA2 family, confers resistance to aminotriazole (Kanazawa et al., 1988), a competitive inhibitor of the HIS3 gene product, causing histidine starvation, and has been shown to be the main boron exporter in yeast (Kaya et al., 2009). Interestingly, Gcn4 also controls the expression of ATR1 through the Yap-1 recognition element (Coleman et al., 1997), and the upregulation of QDR2 (Vargas et al., 2007) and QDR3 (this work) under limitation of the nitrogen or amino acid source. Whether or not the upregulation of QDR3 in the presence of polyamine-induced stress and in nitrogen/amino acid limitation conditions is linked somehow remains to be clarified. It is possible that polyamine concentrations vary significantly in yeast cells reaching stationary phase due to lack of nitrogen source, since it is known that the activity of ornithine decarboxylase, which catalyses the first step of polyamine biosynthesis, is severely decreased in both circumstances (Kaye et al., 1980). Exposure to a toxic level of polyamines may also imbalance the intracellular amino acid pools, at least affecting membrane-potential-dependent amino acid transport, which could underlie the observed Gcn4-dependent response. A link between Qdr3 expression and amino acid homeostasis suggested by the results...
described herein is consistent with the fact that QDR3 was found to be a determinant of resistance to toxic concentrations of the amino acid selenomethionine (Bockhorn et al., 2008).

ACKNOWLEDGEMENTS

This work was supported by FEDER and Fundação para a Ciência e a Tecnologia (contract PTDC/BIA-MIC/72577/2006 and a PhD grant to T. R. C.). We acknowledge Scott Moye-Rowley, from the Department of Physiology and Biophysics, University of Iowa, Iowa City, USA, for kindly providing the pYAP1-GFP plasmid.

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M. C. Teixeira and others


Edited by: K. Kuchler