Saccharomyces cerevisiae Multidrug Resistance Transporter Qdr2 Is Implicated in Potassium Uptake, Providing a Physiological Advantage to Quinidine-Stressed Cells[▽]

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The *QDR2* gene of *Saccharomyces cerevisiae* encodes a putative plasma membrane drug:H^+ antiporter that confers resistance against quinidine, barban, bleomycin, and cisplatin. This work provides experimental evidence of defective K^+ (Rb^+) uptake in the absence of *QDR2*. The direct involvement of Qdr2p in K^+ uptake is reinforced by the fact that increased K^+ (Rb^+) uptake due to *QDR2* expression is independent of the Trk1p/Trk2p system. *QDR2* expression confers a physiological advantage for the yeast cell during the onset of K^+ limited growth, due either to a limiting level of K^+ in the growth medium or to the presence of quinidine. This drug decreases the K^+ uptake rate and K^+ accumulation in the yeast cell, especially in the Δ*qdr2* mutant. Qdr2p also helps to sustain the decrease of intracellular pH in quinidine-stressed cells in growth medium at pH 5.5 by indirectly promoting H^+ extrusion affected by the drug. The results are consistent with the hypothesis that Qdr2p may also couple K^+ movement with substrate export, presumably with quinidine. Other clues to the biological role of *QDR2* in the yeast cell come from two additional lines of experimental evidence. First, *QDR2* transcription is activated under nitrogen (NH_4^+) limitation or when the auxotrophic strain examined enters stationary phase due to leucine limitation, this regulation being dependent on general amino acid control by Gcn4p. Second, the amino acid pool is higher in Δ*qdr2* cells than in wild-type cells, indicating that *QDR2* expression is, directly or indirectly, involved in amino acid homeostasis.

The therapeutic potential of drugs is seriously limited by the manifestation of cellular drug resistance (14). The multidrug efflux transporters found in the plasma membrane of all living cells apparently recognize a wide variety of structurally and pharmacologically unrelated drugs and actively extrude them from the cytoplasm to the outer medium, providing protection from these compounds (1, 4, 14, 17, 29, 32, 37, 40). However, the molecular mechanisms behind the apparent promiscuity of these multidrug efflux pumps remain elusive and a topic of debate (17, 29, 32, 36, 37, 40). Moreover, the inspection of the genome sequences becoming available is revealing the intriguing presence of a large number of predicted multidrug transporters. In particular, this is the case for *Saccharomyces cerevisiae* (26, 30).

In the present work, we have further examined the biological function of the multidrug resistance (MDR) transporter encoded by the *QDR2* gene, present in the plasma membrane of *S. cerevisiae* (47). According to the classification of Nelissen et al. (26), Qdr2p belongs to cluster I of the so-called DHA12 drug efflux family (EC 2.1.3) (30), including putative drug:H^+ antiporters with 12 predicted membrane-spanning segments. Qdr2p confers yeast resistance against the antiarrhythmic and antimalarial drug quinidine, the herbicide barban, and the antitumour agents bleomycin and cisplatin (45, 47). Remarkably, at least six other members of the DHA12 family of *S. cerevisiae* drug:H^+ antiporters, belonging to the same cluster I or to cluster II, are individually required for quinidine resistance (8, 9, 10, 27, 44, 45). Together with the stereoisomer quinine, the cinchona alkaloid quinidine is the antimalarial agent most widely used intravenously (46, 49). As an antiarrhythmic agent, quinidine blocks cardiac Na^+ channels to alter action potential duration and slow conduction. It also blocks repolarizing cardiac K^+ channels to prolong action potential duration (6). The role of all of the referred yeast MDR transporters of the major facilitator superfamily in alleviating the deleterious effects of quinidine and other compounds that are not usually present in the yeast cell 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. However, experimental evidences indicated that Qdr2p is involved, directly or indirectly, in the active expulsion of quinidine from preloaded yeast cells (47).

In the present work, we show results supporting the involvement of Qdr2p in K^+ uptake. *QDR2* expression was found to confer a physiological advantage for the yeast cells under conditions leading to K^+-limited growth, due either to a limiting level of K^+ in the growth medium or to the presence of quinidine. Potassium uptake in *S. cerevisiae* occurs essentially through the plasma membrane proteins Trk1p and Trk2p, re-

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sponsible for high- and moderate-affinity K$^+$ transport, respectively (11, 21, 34). *TRK1* appears to be the most important component of potassium uptake, as mutations of this gene result in a significant defect in potassium transport and the inability to grow under limiting concentrations of this cation (11, 21, 33, 34). Mutation of *TRK2* aggravates the requirement for potassium of a $\Delta trk1$ mutant. However, the viability of $\Delta trk1$ $\Delta trk2$ cells has revealed the existence of additional forms to transport potassium. Although the nature of this transport remains unclear, the activities of a nonselective cation channel and different nonspecific transporters have been postulated (3, 23, 38, 50). Here, we provide evidence suggesting that Qdr2p may be one of these predicted low-affinity uptake systems for K$^+$. In a previous study, Qdr2p expression was found to reduce the decrease of intracellular pH registered in quinidine-stressed yeast cells suspended in slightly acidified medium (pH 5.5) optimal for yeast growth (47). Having this in mind, we examined the hypothesized role of quinidine in the dissipation of the H$^+$ gradient across the plasma membrane and the involvement of *QDR2* in this process.

In this work, we also searched for environmental conditions leading to the activation of *QDR2* transcription in the hope that this information might contribute to the elucidation of the physiological role of this gene. *QDR2* transcription, which is not stimulated in quinidine-stressed cells (47), is here demonstrated to be activated under nitrogen (NH$_4^+$) limitation or when the auxotrophic strain examined enters stationary phase due to leucine limitation in a Gcn4p-dependent way. Having in mind these expression results and the fact that *S. cerevisiae* Aqr1p, a drug:H$^+$ antiporter highly homologous to Qdr2p (26, 30), was proposed to be involved in the excretion of amino acids (48), we also tested the effect of *QDR2* expression on amino acid partition between the cell interior and the surrounding medium. The results suggest the involvement of Qdr2p in amino acid homeostasis, directly or indirectly promoting amino acid excretion.

MATERIALS AND METHODS

Strains, plasmids, and growth media. *Saccharomyces cerevisiae* strain BY4741 (*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) and the derived single deletion mutants from the Euroscarf collection, the BY4741 *Δqdr2* and BY4741 *Δgcn4* strains, were used in this study, as well as the amino acid prototrophic strain 23344c (*MAT*α *ura3*) (24), provided by B. André (Université Livre de Bruxelles, Belgium). The DBY746 *Δtrk1 Δtrk2* double mutant (*trk1::LEU2 trk2::HIS3*) was a gift from Joaquín Ariño and Amparo Ruiz (Departament de Bioquímica i Biologia Molecular, Universitat Autónoma de Barcelona, Barcelona, Spain). This double mutant was constructed in the DBY746 background (*MAT*α *ura3 leu2 his3 trp1*) (39). The DBY746 *Δtrk1 Δtrk2 Δqdr2* strain was constructed in the present work, as described below.

The plasmid pYCG_*QDR2*, constructed during a previous study (47), using pFL38 as the cloning vector, was used in this work for complementation assays. Transformation of yeast cells was performed by the method of Gietz et al. (12).

Cells were batch cultured at 30°C, with orbital agitation (250 rpm) in growth media resulting from different nutrient or drug supplementation of the basal medium (BM) with the following composition (per liter): 1.7 g yeast nitrogen base without amino acids or NH$_4^+$ (Difco), 20 g glucose, and 2.65 g (NH$_4$)$_2$SO$_4$ (Merck). Strain BY4741 was grown in MM4 medium that resulted from BM supplementation with 20 mg methionine, 20 mg histidine, 60 mg leucine, and 20 mg uracil (all from Sigma). For the amino acid prototrophic strain 23344c, the growth medium used (MM5) resulted from BM supplementation with 20 mg uracil.

Ammonium phosphate-derived media were also used to test BY4741, DBY746 *Δtrk1 Δtrk2*, and derived strains' growth under K$^+$ limitation (5). Ammonium phosphate basal medium contains, per liter, a mixture of 0.492 g MgSO$_4$7H$_2$O

(Merck), 0.02 g anhydrous CaCl$_2$ (Panreac), 1.056 g (NH$_4$)$_2$HPO$_4$ (Merck), 3.96 g (NH$_4$)$_2$SO$_4$, 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 (all from Sigma), while for the DBY746 *Δtrk1 Δtrk2* strain, this medium was supplemented with 20 mg histidine, 60 mg leucine, 20 mg tryptophan, and 20 mg uracil (all from Sigma). To maintain selective pressure over the recombinant strains, the addition of uracil to this medium was carried out only to grow the host yeast cells.

Growth medium supplementation with quinidine (Sigma) was carried out as described below. Quinidine was dissolved in 1.4% ethanol, and the control assays were prepared with identical concentrations of the solvent.

Agarized solid media contained, besides the above-indicated ingredients, 20 g/liter agar (Iberagar) or 20 g/liter bacteriological agar no. 1 (Oxoid), used to meet the purity requirements of the ammonium phosphate medium.

***QDR2* disruption in the *S. cerevisiae* DBY746 *Δtrk1 Δtrk2* strain.** Disruption of *QDR2* (*YIL121w*) in the *S. cerevisiae* DBY746 *Δtrk1 Δtrk2* strain was performed as described previously, using a replacement cassette prepared before (47) to be used for the systematic inactivation of this gene in any *S. cerevisiae* strain, by creating longer homologous sequences on both sides of the *loxP-kanMX4-loxP* module.

Spot growth assays under K$^+$ limitation and/or quinidine-induced stress. Spot growth assays using BY4741 and BY4741*Δqdr2* cells under K$^+$ limitation were compared on agarized medium derived from ammonium phosphate supplemented with different KCl concentrations (0.4 to 20 mM) (5), in the presence or absence of quinidine (2.4 mM; Sigma). The cells used to inoculate the agar plates were mid-exponential cells grown in the absence of chemical stress. These cells were resuspended in sterile H$_2$O to obtain suspensions with an optical density at 600 nm (OD$_{600}$) of 0.05 ± 0.005 (mean ± standard deviation), and these and diluted cell suspensions were applied as spots (4 µl) onto the surface of the agarized media and incubated at 30°C for 3 to 5 days, depending on the cell growth rate.

K$^+$ content of the yeast cells. In order to determine the effect of quinidine on the K$^+$ content of yeast cells, yeast cells were grown overnight in liquid MM4 medium. When cultures reached an OD$_{600}$ of 0.5 to 0.6, cells were collected and resuspended in fresh medium supplemented or not supplemented with quinidine (4.1 mM). After overnight incubation, the K$^+$ content of cells was determined by collecting them on Millipore filters, which were rapidly washed with 20 mM MgCl$_2$. The cells were then extracted with acid and the extracts analyzed by atomic emission spectrophotometry (35). The experiments were repeated three to six times and the standard deviations calculated.

To test the K$^+$ content of yeast cells incubated with different KCl concentrations, yeast cells were grown overnight in liquid ammonium phosphate medium supplemented with 10 mM KCl until the culture reached an OD$_{600}$ of 0.5 to 0.6. Cells were collected and resuspended in fresh ammonium phosphate medium containing different KCl concentrations, either supplemented or not supplemented with quinidine (2.4 mM).

Rb$^+$ (K$^+$) uptake and K$^+$ efflux experiments. Rb$^+$ was used as a K$^+$ transport analogue (35). The time course of Rb$^+$ uptake in cells grown in ammonium phosphate medium supplemented with 10 mM KCl and in the absence or presence of quinidine (2.4 mM) was studied. When the OD$_{600}$ of the culture reached 0.3, the cells were centrifuged, washed, and suspended in the buffer, consisting of 10 mM MES [0.1 mM Mg$_2$Cl, 2 mM Ca$_2$Cl, and 2% (wt/vol) glucose, brought to pH 5.8 with Ca(OH)$_2$]. When required, the uptake buffer was supplemented with quinidine (2.4 mM). Immediately, RbCl (35, 50, or 100 mM) was added to the medium (time zero) and cell samples were withdrawn at various times thereafter (35). The cells were then extracted with acid and the extracts analyzed for Rb$^+$ by atomic emission spectrometry and treated as described above.

K$^+$ efflux was determined in cells grown in MM4 medium up to an OD$_{600}$ of 0.3. Cells were recovered by centrifugation, washed, and resuspended in MES buffer containing different quinidine concentrations. At different times, samples of cells were taken and treated as described before to determine the K$^+$ content of the cells.

The experiments were repeated three times and the standard deviations calculated.

Extracellular acidification curves promoted by yeast cells in the presence of increasing concentrations of quinidine. In order to compare the in vivo active exports of protons from energized cells of the BY4741 parental strain and the BY4741 *Δqdr2* mutant strain, in the presence of increasing concentrations of quinidine, the external medium pH was followed for 10 min of incubation. Cells of both strains were grown in liquid MM4 medium, collected by centrifugation (8,000 rpm, 5 min, 4°C) when the culture reached an OD$_{600}$ of 0.5 to 0.6, washed twice with distilled water, and incubated in sorbitol (20 g/liter) for 30 min to
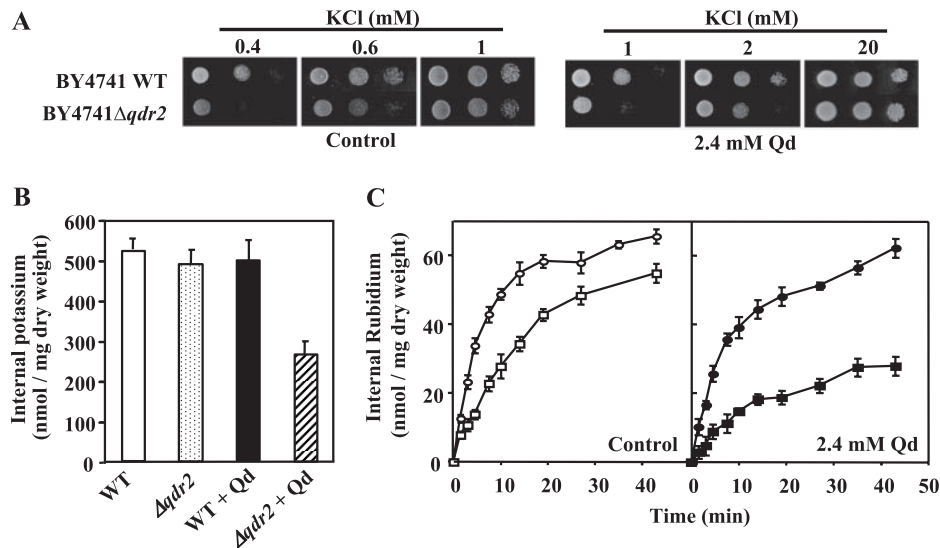
FIG. 1. *QDR2* deletion increases the requirement for K^+ by reducing K^+ (Rb^+) uptake, this effect being aggravated under quinidine stress. (A) Growth of *S. cerevisiae* BY4741 (parental strain) and of the Δ*qdr2* deletion mutant in agar plates with ammonium phosphate medium supplemented with increasing K^+ concentrations, in the presence or absence of quinidine. Serial dilutions (1:1, 1:5, and 1:10, from left to right) of a cell suspension were spotted in agar plates, and growth was observed after 3 to 5 days of incubation, depending on growth kinetics. (B) Effect of quinidine on the K^+ content of the parental strain BY4741 (white and black bars) and the Δ*qdr2* mutant (dotted and striped bars). Cells were grown as described in Materials and Methods and incubated in fresh medium, either in the absence (white and dotted bars) or in the presence (black and striped bars) of quinidine (4.1 mM). Data are means ± standard deviations (SD) for three independent experiments. (C) Rb^+ uptake in the BY4741 parental strain (○ and ●) and in the Δ*qdr2* mutant (□ and ■), in the absence (control) or in the presence of 2.4 mM quinidine. Cells of the wild type and the mutant were grown and treated as described in Materials and Methods. At time zero, RbCl (35 mM) was added to the uptake buffer. Then, cell samples were taken and internal Rb^+ was determined. Data are means ± SD for three independent experiments. WT, wild type; Qd, quinidine.

deenergize the cells and deactivate plasma membrane H^+-ATPase. These cells were washed with water and resuspended in distilled water to obtain a dense cell suspension (OD_{600} of 20.0 ± 2.0). Acidification experiments were carried out in a water-jacketed cell of 10-ml capacity, at 30°C, containing 3.5 ml of water or quinidine solutions (from 0.3 to 4.1 mM) and 1 ml of the cellular suspension described above. The pH of the resulting suspension was adjusted to 5.50 ± 0.05, and 1 ml of 100 g/liter glucose (at pH 5.5) was added (to obtain a final concentration of 20 g/liter). The variation of extracellular medium pH was followed by potentiometry using a pH microelectrode (Metrohm 6.0204.000) attached to a pH meter (Metrohm 605) for 10 min.

The increase of the acidification ability of the wild-type or Δ*qdr2* strain by the expression of *QDR2* from plasmid pYCG_QDR2 was also tested. Recombinant cells were grown in MM4 minimal medium without uracil.

Northern blot analysis. *QDR2* mRNA levels under different growth conditions were assessed by Northern blot analysis. RNA extraction from yeast cells and Northern experiments were carried out as described before (47). The total RNA in each sample used for Northern blotting was approximately constant (20 μg, assessed by the measurement of A_{260}). The specific DNA probe used to detect *QDR2* transcripts was prepared by PCR amplification using the same primers as those used before (47), and the *ACT1* mRNA level was used as an internal control. The probe was radiolabeled with a random-primed DNA labeling kit (Roche), using [α^{32}P]dCTP. Kodak BioMax MS (Amersham) films were exposed to nitrocellulose membranes and incubated with an intensifying screen at −70°C for approximately 1 to 4 days to obtain the hybridization signals. Their relative intensities in the autoradiograms were quantified by densitometry (Molecular Dynamics computing densitometer, ImageQuant software version).

Intracellular and extracellular concentrations of amino acids. The intracellular concentration of amino acids was determined, as described by Klasson et al. (19), in BY4741 and BY4141 Δ*qdr2* cells harvested in mid-exponential growth phase and in early stationary phase of growth. The concentration of amino acids in the supernatants of the same culture samples was also determined. To assess intracellular amino acid pools, cells were harvested by centrifugation and the cell pellets were washed twice with 1.5 ml of water and resuspended in 1.5 ml of AA buffer (2.5 mM K_2HPO_4-KH_2PO_4 buffer at pH 6.0, 0.6 M sorbitol) with 10 mM glucose and 0.2 mM $CuCl_2$. These cell suspensions were incubated for 10 min at 30°C with gentle shaking to allow Cu^{2+}-induced plasma membrane permeabili-

zation, followed by filtration (Whatman GF/F filters) of 1-ml aliquots. The filters were washed four times with the basal AA buffer without glucose or $CuCl_2$, and the filtrates were combined and considered the total extracts. The concentrations of amino acids in total extracts and the corresponding supernatants were determined by high-performance liquid chromatography at the Amino Acid and Sequencing Service of ITQB, Oeiras, Portugal.

The values of the cell content for each amino acid are expressed as μmol of amino acid per unit of dry biomass.

RESULTS

QDR2 **deletion increases the requirement for potassium, and this defective growth phenotype is aggravated under quinidine stress.** When cells of BY4741 or the respective mutant with the *QDR2* gene deleted (Δ*qdr2*) were spotted onto minimal agar medium with a saturating K^+ concentration (20 mM), similar growth patterns were observed for both strains, either in the absence or in the presence of 2.4 mM of quinidine (Fig. 1A and data not shown). However, the Δ*qdr2* mutant showed a defective growth phenotype, either in the presence or in the absence of quinidine, at limiting K^+ concentrations (Fig. 1A). This effect was observed in the presence of quinidine at 2 mM of K^+ but became more evident with the decrease of K^+ concentration in the growth medium, being maximal for the lowest concentration tested (0.4 mM). These results indicate that the presence of quinidine increases potassium requirements in both the wild type and the Δ*qdr2* mutant, this effect being severely aggravated in the Δ*qdr2* mutant.

Quinidine decreases internal K^+ levels and inhibits K^+ (Rb^+) uptake, these effects being aggravated in the Δ*qdr2* mutant. The growth differences registered between the paren-

tal strain and Δ*qdr2* cells under K$^+$-limiting conditions (Fig. 1A) suggested that the capacity to transport K$^+$ may be altered in the mutant strain, this defect being aggravated by the presence of quinidine. To check this possibility, we determined the internal K$^+$ content, the time course of Rb$^+$ (a K$^+$ analogue) uptake, and the K$^+$ loss process in wild-type and Δ*qdr2* cells either in the absence or in the presence of the drug. The lack of *QDR2* slightly reduced the K$^+$ content of the yeast cell compared with the parental strain. However, in quinidine-stressed cells, the values of internal K$^+$ measured in the Δ*qdr2* mutant were about one-half of those determined for the wild-type strain (Fig. 1B).

A reduction of KCl concentration in the incubation medium resulted in a decrease of the internal concentration in both strains but led essentially to the same conclusions (data not shown). Results from Rb$^+$ transport assays indicate that the K$^+$ (Rb$^+$) uptake rate in the Δ*qdr2* mutant is below the value determined for the wild-type strain (Fig. 1C) and that this defect is significantly increased by quinidine (Fig. 1C). These observations suggest that quinidine affects potassium uptake into the yeast cells and that Qdr2p plays an important role in the maintenance of physiological levels of K$^+$, especially in quinidine-stressed cells, by increasing K$^+$ uptake. This idea was reinforced by results from K$^+$ efflux experiments, since they showed that neither the lack of *QDR2* nor the addition of quinidine to the buffer has a significant effect on K$^+$ loss (data not shown).

Increased K$^+$ uptake due to *QDR2* expression and quinidine-induced inhibition of K$^+$ uptake are independent of the Trk1p/Trk2p system. Since the most important K$^+$ uptake system in yeast, involving Trk1p and Trk2p, is active in the BY4741 Δ*qdr2* mutant, we have deleted the *QDR2* gene in a DBY746 double mutant with the *TRK1* and *TRK2* genes deleted. This triple mutant exhibited defective growth at limiting K$^+$ concentrations compared with the Δ*trk1* Δ*trk2* strain, the growth phenotype becoming clearer as the K$^+$ concentration in the growth medium decreased to 0.5 mM (Fig. 2A). Based on K$^+$ (Rb$^+$) transport assays, these results can be explained on the basis of the impaired K$^+$ (Rb$^+$) transport displayed by Δ*trk1* Δ*trk2* Δ*qdr2* cells compared with that displayed by the cells of the double mutant parental strain, this difference being more important at the lowest concentration of RbCl tested (50 mM) (Fig. 2B and C). The results indicate that increased growth at limiting K$^+$ concentrations and increased K$^+$ (Rb$^+$) influx observed in yeast strains expressing *QDR2* are independent of the main K$^+$ transport system encoded by *TRK1* and *TRK2* genes. Having in mind the important effect of quinidine in reducing K$^+$ uptake and K$^+$ accumulation in yeast, we also tested the effect of this drug on the Δ*trk1* Δ*trk2* Δ*qdr2* mutant strain (Fig. 2C). The results indicate that quinidine is not essentially acting via an inhibition of the Trk1p/Trk2p system (Fig. 1C). Indeed, the dose-dependent effect of quinidine in reducing K$^+$ uptake was also observed in the mutant lacking the main transport systems for K$^+$ uptake, Trk1p and Trk2p, the quinidine-induced inhibition of K$^+$ (Rb$^+$) uptake being more drastic for the mutant with the *QDR2* gene deleted (Fig. 2C).

Quinidine reduces the rate of extracellular acidification by yeast cells, and this effect is diminished by *QDR2* expression. Results from a previous study indicated that intracellular pH
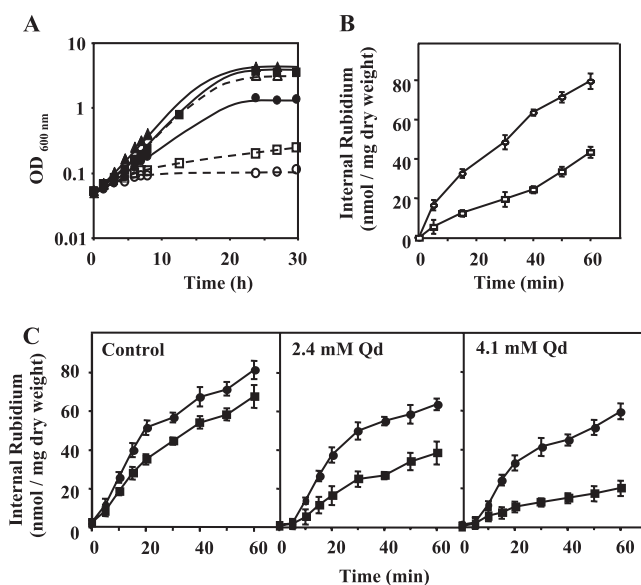

FIG. 2. Increased K$^+$ (Rb$^+$) uptake due to *QDR2* expression and inhibition of K$^+$ (Rb$^+$) uptake by quinidine are independent of the Trk1p/Trk2p system. (A) Effect of *QDR2* expression on growth curves of the *S. cerevisiae* DBY746 Δ*trk1* Δ*trk2* mutant (full line and black symbols) and the corresponding DBY746 Δ*trk1* Δ*trk2* Δ*qdr2* mutant (dotted line and white symbols) under potassium limitation. The growth media were prepared using ammonium phosphate medium supplemented with 0.5 mM (● and ○), 5 mM (□ and ■), and 100 mM (▲ and △) of KCl. The cells used as inoculum were exponential-phase cells cultivated in the presence of 100 mM of KCl. (B and C) Effect of *QDR2* expression on the time course of Rb$^+$ uptake in the DBY746 Δ*trk1* Δ*trk2* mutant (○ and ●) and in the respective Δ*trk1* Δ*trk2* Δ*qdr2* mutant (□ and ■), in the presence or absence of quinidine. At time zero, (B) 50 mM RbCl or (C) 100 mM RbCl was added to the uptake buffer, in the absence (B and C, control) or presence of quinidine (C, 2.1 mM and 4.2 mM). Cell samples were taken during incubation and internal Rb$^+$ was determined, as described in Materials and Methods. Data are means ± standard deviations for three independent experiments. Qd, quinidine.

(pH$_i$) is fairly constant and close to neutrality in exponential yeast cells either expressing or not expressing the *QDR2* gene (47). However, in that same study, we found that the presence of quinidine (4.1 mM) leads to an internal acidification (down to pH 6.3) of yeast cells suspended in the acidic growth medium (initial pH of 5.5) suitable for fungal growth. Moreover, the lack of *QDR2* implicated a more drastic internal acidification (down to pH 5.2) of cells challenged with identical quinidine concentrations.

The intracellular pH regulation is essentially explained by the action of the plasma membrane H$^+$-pumping ATPase Pma1p (41). Considering that the result of the in vivo activity of this H$^+$ pump and of the passive H$^+$ influx through the plasma membrane can be assessed by the acidification of the external medium (31), the acidification curves promoted by the wild-type strain BY4741 and the Δ*qdr2* mutant, in the presence of increasing concentrations of quinidine, were compared (Fig. 3A). This drug was found to reduce the rate of extracellular acidification by yeast cells suspended in an aqueous glucose solution supplemented with quinidine. The inhibition of the rate of H$^+$ active efflux was dose dependent, and for concentrations equal to or above 1.0 mM of quinidine, no net
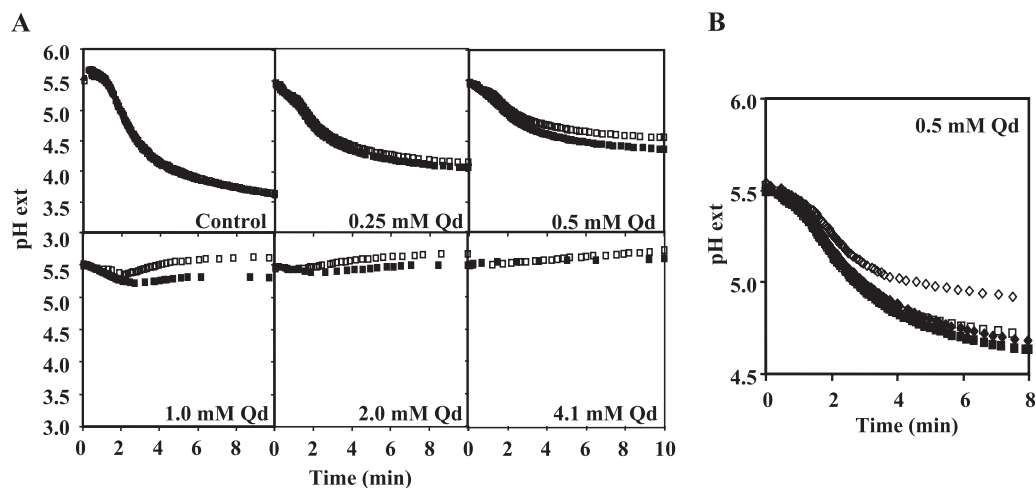
FIG. 3. *QDR2* expression increases external acidification rate affected by quinidine. (A) Dose-dependent inhibition by quinidine of external medium acidification promoted by energized cells of the BY4741 parental strain (■) and the BY4741 Δ*qdr2* mutant strain (□). (B) Complementation of the reduced ability of energized cells of the BY4741 Δ*qdr2* deletion mutant (◇ and ◆) to promote extracellular acidification in the presence of quinidine stress compared with that of the BY4741 parental strain (□ and ■) by expressing the *QDR2* gene from plasmid pYCG_*QDR2*. Mutant cells with *QDR2* deleted or the respective wild-type strain was transformed either with the recombinant plasmid pYCG_*QDR2* (■ and ◆) or with the cloning vector pFL38 (□ and ◇), and experimental assays were carried out under conditions identical to those used for panel A. Values are representative of two independent experiments that gave rise to similar results. Qd, quinidine; pH ext, extracellular pH.

efflux was detected for the Δ*qdr2* cells while the wild-type cells were still able to acidify the extracellular medium, although with severely inhibited kinetics. Although inhibited, a net proton extrusion was registered at 0.25 and 0.5 mM of quinidine for wild-type and Δ*qdr2* strains, but the H^+ efflux in the presence of the drug by yeast cells expressing *QDR2* was higher. In the absence of quinidine, the extracellular H^+ acidification curves registered for the wild type and the Δ*qdr2* mutant cells were, however, apparently coincident. The expression of the recombinant plasmid pYCG_*QDR2* in BY4741 Δ*qdr2* cells restored the rate of H^+ efflux in the presence of quinidine to values similar to those observed for BY4741 transformed with the cloning vector pFL38 (Fig. 3B). Moreover, Qdr2p-increased expression was also able to increase the parental strain H^+ efflux rate under identical quinidine stress (Fig. 3B). These observations reinforce the idea that the *QDR2* gene is implicated in the maintenance of pH homeostasis across the plasma membrane in the presence of quinidine.

QDR2 transcription is activated when the auxotrophic strain BY4741 enters stationary phase due to leucine limitation. *QDR2* transcription was highly activated (18-fold) when cells of *S. cerevisiae* BY4741 entered the stationary phase of growth in MM4 medium, due to leucine limitation (Fig. 4A). 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/liter) is growth limiting (data not shown). Moreover, the high levels of *QDR2* mRNA present in leucine-limited cells returned to basal levels when fresh leucine was added to the culture with leucine-starved cells (Fig. 4B).

The strong activation of *QDR2* transcription registered in BY4741 cells approaching the stationary phase of growth, due to leucine limitation, was not detected in the respective mutant with the *GCN4* gene deleted (Fig. 4B). This gene encodes the transcription factor Gcn4p, which has been implicated in the activation of several genes responsive to amino acid starvation

(16, 25). These results indicate that *QDR2* transcription is activated in response to leucine limitation, this activation being dependent on the presence of Gcn4p.

QDR2 transcription is activated under NH$_4^+$ limitation but not under carbon limitation. The activation of *QDR2* expression in yeast cells under nitrogen source (NH$_4^+$) limitation was also registered in the auxotrophic yeast strain examined in the present work (data not shown) and in strain 23344c (Fig. 5). This prototrophic strain was used to avoid the presence of amino acids in the growth medium which are necessary for the auxotrophic strain BY4741. Results from Northern blotting experiments indicate that the levels of *QDR2* transcripts increased fivefold when the 23344c cell population approached the stationary phase of growth due to ammonium limitation (initial concentration of ammonium sulfate, 0.0265 g/liter), compared with the values for cells grown with a saturating ammonium concentration (Fig. 5).

The eventual transcription activation of *QDR2* under carbon source limitation was also investigated. Under glucose-limiting conditions (initial concentration of 0.05%, wt/vol), *S. cerevisiae* BY4741 growth proceeded with a specific growth rate below the value possible at a glucose-saturating concentration (2%, wt/vol) until the exhaustion of the carbon source. However, no modification of the mRNA level from *QDR2* was observed when cells entered the stationary phase due to carbon limitation (data not shown). Altogether, the results indicate that the activation of *QDR2* transcription in cells entering the stationary phase of growth cannot be attributed to nutrient limitation, in general, but is specific for nitrogen source limitation.

Intracellular amino acid pool size depends on QDR2 expression. In light of the expression patterns registered for *QDR2* under nitrogen source/leucine limitation and of the role in the excretion of amino acids postulated for the *AQR1* gene (48), we compared the amino acid concentrations in cells of BY4741 and the Δ*qdr2* mutant, grown in the absence of quinidine. The
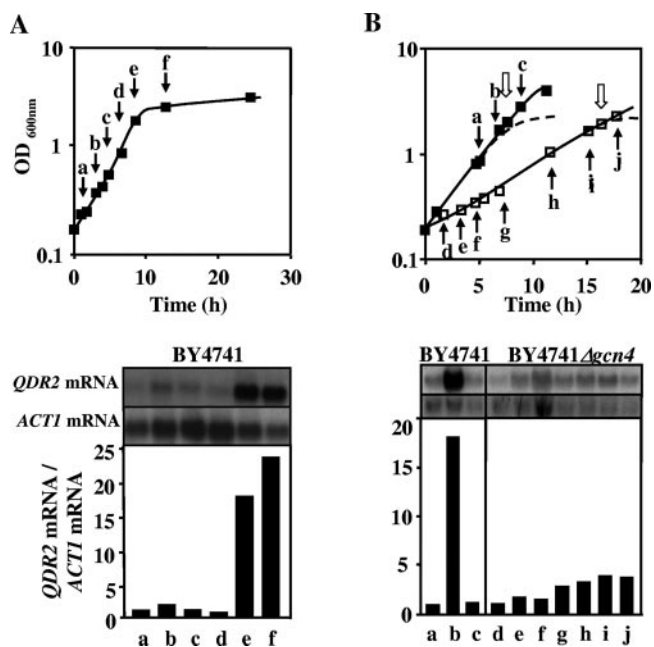
FIG. 4. Gcn4-dependent activation of *QDR2* transcription when the auxotrophic strain BY4741 enters stationary phase due to leucine limitation. (A) Relative levels of *QDR2* mRNA in *S. cerevisiae* BY4741 cells during growth in MM4 liquid medium. Cell samples were harvested at the times indicated by the letters in the figure. Relative ratios of *QDR2* mRNA to *ACT1* mRNA were obtained by densitometry of the autoradiograms. The basal *QDR2* mRNA relative value of exponential cells at an OD_{600} of 0.3 ± 0.05 was set as 1. (B) Relative levels of *QDR2* mRNA in *S. cerevisiae* BY4741 (■) and BY4741 Δ*gcn4* (□) cells during growth in MM4 medium. Cell samples were harvested at different times of cultivation, as indicated by the black arrows and the letters, during exponential growth in early stationary phase and 1 hour following the addition of fresh leucine to starved cells, as indicated by the white arrows. The growth curves in medium without extra leucine supplementation are represented by dotted lines. Relative ratios of *QDR2* mRNA to *ACT1* mRNA were obtained by densitometry of the autoradiograms. The basal *QDR2* mRNA relative value of exponential cells at an OD_{600} of 0.3 ± 0.05 was set as 1. Values are representative of two independent experiments.

FIG. 5. *QDR2* transcription is activated under NH$_4^+$ limitation. Relative levels of *QDR2* mRNA in cells of the amino acid prototrophic strain *S. cerevisiae* 23344c during growth in MM5 medium where the concentration of (NH$_4$)$_2$SO$_4$ was saturating (2.65 g/liter) (◆) or limiting (0.0265 g/liter) (■). Cell samples were harvested at different incubation times as indicated. Relative *QDR2* mRNA/*ACT1* mRNA ratios were obtained by densitometry of the autoradiograms. Values are representative of two independent experiments.

DISCUSSION

The mechanisms behind the apparent promiscuity of the multidrug efflux pumps in different organisms and the presence of a multitude of different efflux pumps that may provide protection against compounds that are not usually present in their natural environment remain elusive and a topic of debate. In the present work, we show evidence supporting the notion that the expression of the yeast multidrug resistance transporter Qdr2p plays an important role in the maintenance of physiological levels of K$^+$ in the cell. This biological function becomes crucial under environmental conditions leading to K$^+$-

amino acid pool sizes were determined in cells harvested in the middle of the exponential growth phase and when the culture reached an OD_{600} of 2, corresponding to entrance in the stationary phase of growth due to leucine limitation and to the consequent activation of *QDR2* transcription (Table 1 and Fig. 4). The intracellular concentrations of 18 amino acids were higher in the mutant with *QDR2* deleted than in the parental strain (Table 1). The registered differences were, in general, more evident in cells entering the stationary phase of growth due to leucine limitation, when *QDR2* is overexpressed. Exceptions were registered only for exponential cells and essentially for the three amino acids that were added to the culture medium due to the auxotrophies of the tested yeast strain. The amino acids levels determined in the supernatants of the two cultures tested, at concentrations within the sensitivity levels of the method used, are consistent with the notion that *QDR2* expression alters amino acid partition between the yeast cell interior and the extracellular medium, leading to the increase of their concentrations in Δ*qdr2* cells (data not shown).
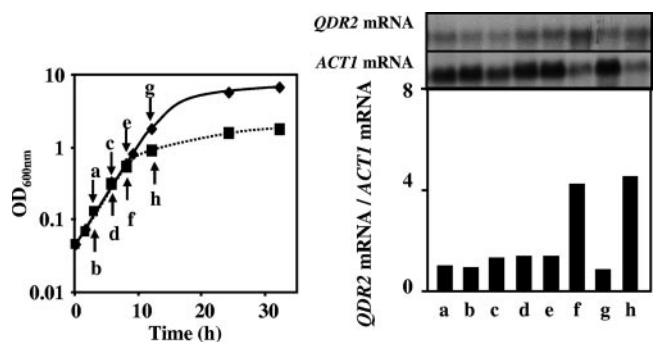
TABLE 1. Concentrations of amino acids in *Saccharomyces cerevisiae* BY4741 and BY4741 Δ*qdr2* cells[a]

Amino acid	Amino acid concn (μmol/g of cells [dry wt]) in:			
	Exponential phase		Early stationary phase	
	WT cells	Δ*qdr2* cells	WT cells	Δ*qdr2* cells
Alanine	37.0	49.0	123.9	200.1
Arginine	32.5	50.4	52.4	95.8
Asparagine	11.8	18.4	21.2	29.6
Aspartate	9.3	18.0	22.5	25.9
Cysteine	15.1	20.2	9.6	15.4
Glutamine	5.7	9.7	21.2	41.8
Glycine	4.3	6.5	8.1	9.2
Isoleucine	1.3	2.2	2.6	4.9
Lysine	11.3	17.1	29.8	31.0
Phenylalanine	1.7	1.6	2.7	4.9
Serine	4.4	7.9	11.5	12.3
Threonine	4.3	7.2	9.4	19.7
Tryptophan	11.3	11.6	5.7	8.4
Tyrosine	0.8	1.8	5.4	15.0
Valine	6.1	9.1	9.8	18.8
Histidine*	11.4	8.9	29.2	41.9
Leucine*	2.5	2.2	1.3	2.5
Methionine*	1.0	1.2	6.5	10.7

[a] Cells were harvested in mid-exponential growth phase or when the culture entered the stationary phase of growth (OD_{600} of 2) due to leucine limitation. The amino acids corresponding to BY4741 auxotrophies are indicated by an asterisk. WT, wild type.

limited growth, due either to the presence of limiting levels of K^+ in the growth medium or to the presence of quinidine.

The presence of quinidine was found to strongly affect the K^+ uptake rate and K^+ requirements in the yeast cells. This quinidine-induced inhibition of K^+ uptake was also observed in a strain with *TRK1* and *TRK2* genes deleted. Therefore, the major transport system for K^+ uptake is not the sole or the main direct target of the drug. The results also indicate that increased K^+ availability improves yeast growth in the presence of quinidine, in agreement with a physiological advantage of cells expressing the *QDR2* gene under quinidine stress. Indeed, *QDR2* expression leads to an increase of the K^+ (Rb^+) uptake rate, this biological activity being crucial to counteract the deleterious effect of quinidine.

The uptake of K^+ has been linked to a number of very important cellular processes, in particular the regulation of intracellular acidification. The activities of the major K^+ influx transporters, Trk1p and Trk2p, are important in setting the pH_i (51) since H^+ pumping by Pma1p is electrogenic and K^+ is the major return current in yeast (41). It is thus conceivable that the difficulty of the $\Delta qdr2$ mutant to accumulate K^+ in the presence of quinidine could impair this mutant's ability to counteract cytosolic acidification as the result of decreased H^+ extrusion. Consistent with this hypothesis, we demonstrated before that, for the same quinidine concentration, $\Delta qdr2$ exponential cells exhibit an average pH_i (5.2) significantly below the average pH_i of wild-type exponential cells (6.3) (47). The protective effect exerted by *QDR2* expression against quinidine may in part be related to its role in controlling pH_i and the internal K^+ concentration in the presence of the drug. The maintenance of pH_i within a permissive range for the functionality of sensitive cellular systems is crucial for cell physiology, due to its effects on proteins and biochemical reactions.

The data obtained in this study are consistent with quinidine acting as an uncoupler that dissipates the H^+ gradient across the plasma membrane. Consistent with this hypothesis, we found that the well-established uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has an effect that mimics the action of quinidine on the inhibition of extracellular medium acidification by yeast. Moreover, *QDR2* expression was also found to play a role in counteracting this effect of CCCP (unpublished results). However, although CCCP was used at low concentrations (5 to 20 μM), expected to lead to the dissipation of H^+ gradient across the plasma membrane without significantly affecting mitochondrial respiration, the anticipated effect of this uncoupler on the $\Delta qdr2$ phenotype under K^+ limitation could not be clearly demonstrated (unpublished results), presumably due to the much higher toxicity of CCCP which may have masked the observation of the clear phenotype registered for quinidine.

Other possible clues to the biological function of *QDR2* in the yeast cell may come from two lines of experimental evidence obtained during this work. First, it appears that the expression of *QDR2* not only increases the permeability of the yeast cell to K^+ but also increases cell permeability to the nonspecific efflux of amino acids. This conclusion is suggested by the larger amino acid pool in $\Delta qdr2$ cells, with the *QDR2* gene deleted, than in wild-type cells. This observation is also in agreement with the proposed role for the multidrug transporter Aqr1p, a Qdr2p homologue, in the excretion of amino acids (48). However, the effect of *QDR2* expression on amino acid homeostasis is not specific for a narrow range of amino acids but was registered for at least 18 different amino acids, raising doubts about the specific and direct role of Qdr2p as an amino acid exporter.

The second remarkable observation is that *QDR2* transcription was highly activated when yeast cells entered the stationary phase of growth, due either to the nitrogen source (NH_4^+) limitation or to leucine limitation, for the auxotrophic strain examined. Moreover, this regulation is dependent on general amino acid control by Gcn4p. Translation of *GCN4* mRNA is derepressed in amino acid-deprived cells, leading to transcriptional induction of nearly all genes encoding amino acid biosynthetic enzymes (16). Although the uptake and accumulation of amino acids from the external medium seem to be irreversible, amino acids are excreted into the medium whenever they are overproduced above a given threshold by yeast cells (13). This can occur when growth is arrested under conditions where amino acid synthesis can continue and amino acid efflux is mediated by mechanisms that are distinct from the characterized uptake systems (13). Interestingly, the first proposed outward-pumping system for amino acid excretion was the multidrug resistance transporter of the major facilitator superfamily, Aqr1p (48), belonging to the same cluster I of the 12-spanner drug:H^+ antiporter DHA12 family (26, 30, 40). However, this same role was hypothesized before for *ATR1* (13, 18), a member of the 14-spanner drug:H^+ antiporter DHA14 family (26, 30). *ATR1* is required for yeast resistance to aminotriazole (18), a competitive inhibitor of the *HIS3* gene product, an imidazoleglycerolphosphate dehydratase (20, 42), causing histidine starvation. Interestingly, Gcn4p also controls the expression of *ATR1* through a DNA element related to the yAP-1 recognition element (7). Also, in the case of the *QDR2* promoter region, there is no established DNA binding site for Gcn4p [the sequence TGA(G/G)TCA]. Using the YEASTRACT database (http://www.yeastract.com) (43), five *cis*-acting replication element-like sequences were found. Since Gcn4p was proven to compete with Sko1p for these binding sites in the *HAL1* promoter region (28), it is possible that they may also be used to bind Gcn4p to the *QDR2* promoter region. Moreover, there are three yAP-1 sequences in the *QDR2* promoter region also recognized by Gcn4p and Yap1p (7).

The interpretation of results of amino acid partition between the cell interior and the surrounding medium is complicated by the possible modification of processes involving amino acid synthesis and the degradation and mobilization of stored nitrogen sources under nitrogen starvation. Nevertheless, the results shown in this work are consistent with the presence of a larger amino acid pool in $\Delta qdr2$ cells. The indirect (or direct) role of Qdr2p in promoting this apparently nonspecific efflux of amino acids may become crucial to avoid abnormal large amounts of amino acids resulting from deregulated metabolism that may be deleterious to cell physiology, as is the case during growth arrest due to leucine limitation. Remarkably, a recently published work has established a link between K^+ limitation, ammonium toxicity, and amino acid excretion (15). According to the indication of this microarray analysis, complemented by metabolite profiling, under limiting potassium concentrations, toxic amounts of ammonium might enter yeast via potassium channels, as observed in other organisms. When
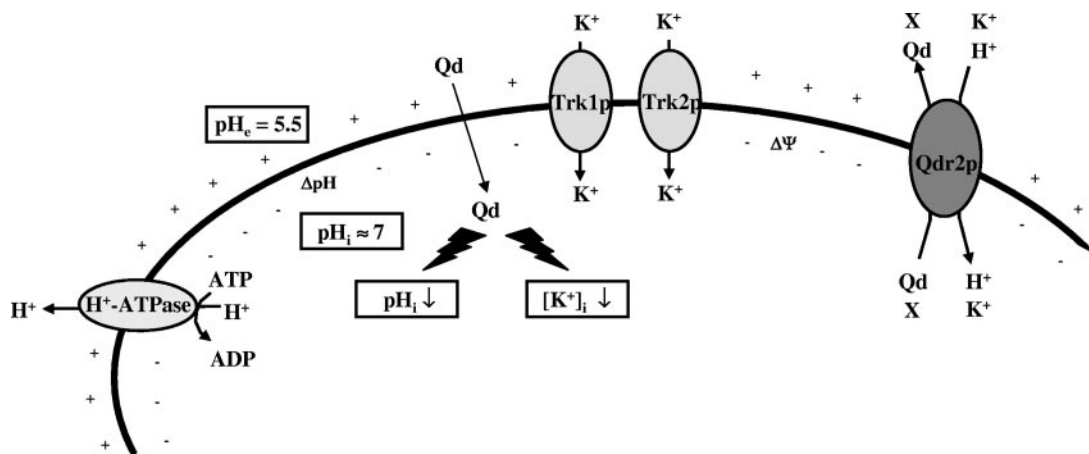
FIG. 6. A model for the possible relationship between Qdr2p activity, K$^+$ transport, and the effect of quinidine on the yeast cell. It is proposed that the drug:H$^+$ antiporter Qdr2p may also be capable of coupling K$^+$ ions to drive the export of its physiological substrate(s) (X). The presence of quinidine leads to impaired K$^+$ uptake and the acidification of the cell interior by reducing H$^+$ efflux from energized cells. In such a situation, the K$^+$ uptake coupled with *QDR2* expression would help the cell to counteract these deleterious effects. Qdr2p also reduces the internal concentration of quinidine by promoting, directly or indirectly, the active expulsion of the drug (47). Although the eventual antiport of quinidine with K$^+$/H$^+$ mediated by Qdr2p is considered fortuitous or opportunistic, the natural substrates(s) (X) for Qdr2p is (are) still unclear. Our model also involves Qdr2p, either directly or indirectly, in the extrusion of amino acids from the cell. See text for details. Qd, quinidine; $\Delta\Psi$, membrane potential.

experiencing ammonium toxicity, yeast responds by excreting amino acids which may constitute a rudimentary ammonia detoxification mechanism in the yeast cell (15).

Although the dominant model in MDR literature is still the drug pump model, the results of the present work support the altered partitioning model (36, 37). This model considers that the altered expression of an MDR transporter may lead to altered ion transport, indirectly affecting intracellular accumulation of the drug by perturbing plasma membrane potential and/or intracellular pH. According to the model postulated here (Fig. 6), the putative drug:H$^+$ antiporter Qdr2p may also be capable of coupling K$^+$ ions to drive the export of its physiological substrate(s), as hypothesized before for the amino acid:H$^+$ symport, by amino acid permeases (50). It is also possible that Qdr2p catalyzes directly the fortuitous export of specific drugs, in particular quinidine.

The viability of $\Delta trk1$ $\Delta trk2$ cells has indicated the existence of additional forms for transporting potassium into the yeast cell that do not require the Trk1p/Trk2p transport system. However, the nature of this transport remains to be elucidated, although the nonspecific uptake of potassium occurring through sugar and amino acid permeases has been postulated (22, 23, 50). Based on a number of evidences, Wright et al. (50) have considered that amino acid:H$^+$ symporters may not have an absolute requirement for H$^+$ as a cosubstrate and may be capable of coupling K$^+$ ions to drive amino acid uptake. The results obtained during this work are consistent with the hypothesis that the putative drug:H$^+$ antiporter Qdr2p may also be capable of coupling K$^+$ ions to drive the export of its physiological substrate and of drugs, in particular quinidine. It is, however, difficult to believe that the yeast cells have evolved a gene like *QDR2*, together with several others, whose normal physiological role is to export a compound not found in its natural environment. The hypothesis that Qdr2p may be directly involved in K$^+$ uptake is reinforced by the fact that,

when *QDR2* was deleted in the $\Delta trk1$ $\Delta trk2$ background, growth deficiency and decreased K$^+$ uptake in this $\Delta qdr2$ triple mutant under K$^+$ limitation were still observed, indicating that K$^+$ uptake due to *QDR2* expression is independent of the Trk1p/Trk2p system. The results also show that even after deletion of *TRK1*, *TRK2*, and *QDR2*, there is still K$^+$ transport, indicating that other transporters may be involved in K$^+$ uptake. It is usually accepted that several nonspecific transporters may be involved in the low-affinity transport of K$^+$ observed in the $\Delta trk1$ $\Delta trk2$ mutant (22). The existence of a yeast nonselective cation channel responsible for the low-affinity K$^+$ uptake was postulated before, although the identity of the protein(s) responsible for this inwardly rectifying pathway is not known (2, 3). Amino acid or sugar transporters could be involved in this process (22, 50) together with Qdr2p and, eventually, other plasma membrane multidrug resistance transporters. Since quinidine strongly decreases the uptake of K$^+$, presumably by acting as an uncoupler at the plasma membrane, the involvement of Qdr2p in the uptake of this essential ion confers a physiological advantage to quinidine-stressed cells. This biological activity may be the mechanism behind the role of Qdr2p as a determinant of resistance to the antiarrhythmic and antimalarial drug tested.

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