Resistance and Adaptation to Quinidine in *Saccharomyces cerevisiae*: Role of *QDR1* (*YIL120w*), Encoding a Plasma Membrane Transporter of the Major Facilitator Superfamily Required for Multidrug Resistance

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As predicted based on structural considerations, we show results indicating that the member of the major facilitator superfamily encoded by *Saccharomyces cerevisiae* open reading frame *YIL120w* is a multidrug resistance determinant. *Yil120wp* was implicated in yeast resistance to ketoconazole and quinidine, but not to the stereoisomer quinine; the gene was thus named *QDR1*. *Qdr1p* was proved to alleviate the deleterious effects of quinidine, revealed by the loss of cell viability following sudden exposure of the unadapted yeast population to the drug, and to allow the earlier eventual resumption of exponential growth under quinidine stress. However, *QDR1* gene expression had no detectable effect on the susceptibility of yeast cells previously adapted to quinidine. Fluorescence microscopy observation of the distribution of the *Qdr1*-green fluorescent protein fusion protein in living yeast cells indicated that *Qdr1p* is a plasma membrane protein. We also show experimental evidence indicating that yeast adaptation to growth with quinidine involves the induction of active expulsion of the drug from preloaded cells, despite the fact that this antiarrhythmic and antimalarial quinoline ring-containing drug is not present in the yeast natural environment. However, we were not able to prove that *Qdr1p* is directly implicated in this export. Results clearly suggest that there are other unidentified quinidine resistance mechanisms that can be used in the absence of *QDR1*.

Several transport systems play an important role in conferring multiple drug resistance (MDR), presumably due to the catalysis of energy-dependent extrusion of a large number of structurally and functionally unrelated compounds out of the cells (3, 4, 25). In *Saccharomyces cerevisiae*, the proton motive force-dependent multidrug efflux systems belong to the major facilitator superfamily (MFS) (21, 22). Other known determinants associated with MDR are other membrane transporters belonging to the ATP binding cassette (ABC) superfamily, which utilize ATP hydrolysis to drive drug extrusion, and factors for transcriptional regulation of all these putative multidrug transporters (2). On the basis of the complete yeast genome sequence, the MFS-MDR homologues comprise a large number of proteins that have escaped identification by classical approaches (16, 19, 22). However, the involvement of the vast majority as MDR determinants remains unknown.

Within the context of the European Functional Analysis Network (EUROFAN), we have identified open reading frame (ORF) *YIL120w*, encoding an MFS-MDR homologue, as a determinant of resistance to quinidine. The gene, named *QDR1*, also confers resistance to (at least) ketoconazole. These conclusions were based on the higher susceptibility to these compounds of the deletion mutant $\Delta qdr1$ compared with the wild-type strain and on the increased resistance of both strains upon increased expression of *QDR1* from a centromeric plasmid clone. A distinct MFS-MDR homologue encoded by *S. cerevi-*

siae ORF *YOR273c* was previously demonstrated to confer resistance to quinidine in yeast cells by using a different approach (11). The strategy used was based on *S. cerevisiae* transformation with a yeast genomic library and selection for resistance to elevated levels of quinidine. The only ORF encoding a member of the MFS-MDR identified was *YOR273c*, therefore failing the identification of ORF *YIL120w*.

The emergence of drug-resistant strains of *Plasmodium falciparum* is an obstacle in malaria control (10, 23). The antimalarial effects of quinoline ring-containing drugs are exerted in *Plasmodium* cells via physiological mechanisms that do not exist in yeast cells (10, 15), and the mechanisms of resistance in *S. cerevisiae* may not apply to the malaria parasite. However, the mechanisms of drug resistance have been conserved among phylogenetically distant organisms (4, 6, 10, 22), and experimental evidence suggests that the mechanisms of action of quinoline ring-containing drugs in *P. falciparum* are independent of the mechanisms of resistance (10). During the present study, we tried to gain some understanding of how *Yil120wp* confers resistance to quinidine, used for the treatment of life-threatening infections with *P. falciparum* and as an antiarrhythmic agent (<http://www.rxmed.com/monographs/quinidin2.html>). The role of *QDR1* in alleviating the deleterious effects of quinidine on unadapted yeast cells suddenly exposed to the drug was examined. Although the adaptation of yeast cells to growth in the presence of quinidine involves induction of active efflux of quinidine out of the cell, we were not able to prove that *Qdr1p* is directly implicated in this transport across the plasma membrane, where this protein was localized by fluorescence microscopy.

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MATERIALS AND METHODS

Strains, media, and general methods. Disruption of ORF *YIL120w* was carried out in two *S. cerevisiae* strains: FY73 (*MAT α ura3-52 his3 Δ 200 GAL2⁺*), closely related to the sequenced strain FY1679, and W303.1b (*MAT α ura3-1 leu2-3,112, his3-11,15,15 trp1-1 ade1-2 can1-100*), another EUROFAN strain, used extensively in our laboratory to assess drug susceptibility phenotypes (7, 31). *Escherichia coli* strains and the routine growth media used were described before (7, 31). Cloning procedures were carried out by standard methods (29). Transformation of yeast cells was performed by the method of Gietz et al. (14), slightly modified.

Disruption and cloning of ORF *YIL120w*. Disruption of ORF *YIL120w* in *S. cerevisiae* FY73 was performed using a disruption cassette, consisting of the dominant resistance marker *loxP-kanMX4-loxP* flanked by short flanking homology regions to the target ORF. This was prepared by DNA amplification by PCR, using plasmid pUG6 (17, 33) as a template and the following primers: 5'-ATG ACCAAACAACAACTTCTGTAAATGCGTAACGCATCTATAGCCAAGG CGGCCGCTTCGTACGCTGCAGGTCGAC-3' and 5'-GTTATAAAATATA GATATCTGTTTCTGGAAGTGGGGGCAGAGACGCGGCCGCGCATA GGCACATAGTGGATCTG-3', where the sequence complementary to pUG6 is underlined. These primers included at the 5' end 48 and 45 nucleotides, respectively, homologous to the flanking region of the ORF followed by the *NotI* site and, at the 3' end, 20 and 22 nucleotides, respectively, homologous to pUG6. The PCR product of 1,690 bp, generated using the amplification conditions described before (7), was purified and used to transform strain FY73. Transformants were selected on YPD plates with geneticin (200 mg/liter), and the correct replacement of the gene was confirmed by two independent PCRs (7).

A replacement cassette was also prepared to be used for systematic inactivation of ORF *YIL120w* in any *S. cerevisiae* strain, in particular strain W303.1b, by creating longer homologous sequences on both sides of the *loxP-kanMX4-loxP* module. This *YIL120w* replacement cassette (long flanking homology) was obtained by PCR amplification with *Pwo* using genomic DNA isolated from the deletant strain and the following primers: 5'-GGCGCATGCGACATCTACGG CTACGGGAT-3' and 5'-GCGGATGCTGGATACCAAAGGAGAACCA-3', designed to be located 765 and 872 bp upstream and downstream of the start and the stop codon, respectively. The PCR product was cloned in the *SphI* site of pFL38 (5), generating plasmid pYORC_*YIL120w*.

ORF *YIL120w* was cloned by the gap repair technique (27) into pFL38, using plasmid pYORC_*YIL120w*, as described before (7), giving rise to plasmid pFL38_*YIL120w*.

Growth media and drug susceptibility tests. The susceptibility tests for several metabolic inhibitors were carried out first by comparing the susceptibilities of the wild-type strain (W303.1b) and the mutant Δ *yil120w* strain (W303.1b Δ *yil120w*). Whenever a consistent phenotype was detected, these tests were followed by comparison of the susceptibility of the wild-type (W303.1b) and the mutant Δ *yil120w* strain transformed with pFL38_*YIL120w* or the cloning vector. Cells were grown on minimal medium (MM2 or MM2 lacking uracil [MM2-U] for plasmid maintenance) agar plates supplemented with suitable concentrations of the different compounds. MM2 medium contained (per liter): 1.7 g of yeast nitrogen base without amino acids or NH₄⁺ (Difco), 20 g of glucose, 2.65 g of (NH₄)₂SO₄, 80 mg of adenine, 10 mg of histidine, 10 mg of leucine, 20 mg of tryptophan, and 20 mg of uracil. The ranges of drug concentrations tested in agar plates were 0.11 to 0.18 μ M for cycloheximide, 52 to 69 μ M for benomyl, 22 to 35 μ M for methotrexate, and 0.9 to 1.1 μ M for 4-nitroquinoline-*N*-oxide (all from Sigma, stock dissolved in dimethyl sulfoxide [DMSO]); 3.7 to 6.1 μ M for crystal violet and 3.2 to 5.4 μ M for malachite green (obtained from Merck, dissolved in water); 2.3 to 3.0 μ M for fluconazole (Diflucan, in saline solution); 1.4 to 2.8 μ M for itraconazole and 1.9 to 2.9 μ M for ketoconazole (kindly supplied by Janssen Research Foundation, dissolved in DMSO); 24 to 100 nM for miconazole (Sigma, stock in DMSO); 3.0 to 4.0 mM for quinine and 3.0 to 3.8 mM for quinidine (sulfate salt dehydrate) (both from Sigma, dissolved in 70% ethanol). DMSO and ethanol concentrations in the growth media (including the control medium lacking the growth inhibitors) were kept below 0.1% (wt/vol) and 1.4% (vol/vol), respectively; these concentrations had no detectable effect on yeast growth kinetics. Cells used to inoculate the agar plates were mid-exponential-phase cells grown without drugs until a culture optical density at 600 nm (OD₆₀₀) of 0.2 $\pm$ 0.02 was reached and resuspended in sterile H₂O to obtain cell suspensions with an OD₆₀₀ of 0.05 $\pm$ 0.005. These and diluted (1:2 and 1:4) cell suspensions were applied as spots (4 μ l) onto the surface of the agar media and incubated at 30°C for 3 to 5 days, depending on the severity of growth inhibition. Conclusions on drug susceptibility were based on consistent results from several independent spot assay experiments carried out with recently transformed yeast cells.

The effect of quinidine on growth kinetics was additionally assessed using minimal liquid medium supplemented with quinidine. A volume of 500 ml of this medium in 1-liter Erlenmeyer flasks was inoculated with cells that were either unadapted or previously adapted to growth with quinidine (3 mM). Cells in the inocula were exponential-phase cells harvested from quinidine-supplemented or unsupplemented growth medium at the standardized culture OD₆₀₀ of 0.2 $\pm$ 0.01. Cultures were grown at 30°C with orbital agitation (250 rpm), and growth was monitored by measuring culture OD₆₀₀. The concentration of viable cells during yeast cultivation was assessed as the number of CFU on minimal medium agar plates (triplicate) incubated at 30°C for 3 days. Under the standardized conditions indicated above, the many independent growth experiments carried out gave rise to identical growth curves.

Subcellular localization of Yil120w-GFP fusion protein. The subcellular localization of Yil120wp was based on the observation, by fluorescence microscopy, of the distribution of Yil120w-green fluorescent protein (GFP) fusion protein in *S. cerevisiae* living cells. The *YIL120w-GFP* fusion plasmid was prepared by using the multicopy expression vector pMET25_GFP, kindly provided by E. Boles (University of Düsseldorf, Düsseldorf, Germany), and the protocol described before (31). The two primers used in the present work (5'-GATACATAGATA CAATTCTATTACCCCATCCATACTCTAGAAAATGACCAAACAACAA ACTTC-3' and 5'-GTGAAAAGTCTCTCTCTTACTCATACCAGCACCA GCGGCCGCGCTCGAAACTTTTCTGAATTTT-3') were designed to have the first 44 bp complementary to the end of the MET25 promoter (sequence underlined) followed by the first 20 bp of ORF *YIL120w*, starting at the ATG. The 3' primer was designed to have the first 44 bp complementary to the beginning of the *GFP* gene (sequence underlined) followed by the last 23 bp of ORF *YIL120w* before the stop codon, which was not included.

Accumulation assays and energy-dependent efflux of [9-³H]quinidine. To estimate quinidine accumulation and its eventual active export from yeast cells, cells of the wild type or the Δ *yil120w* mutant were grown in minimal medium to an OD₆₀₀ of 0.7 $\pm$ 0.05 and then introduced into the same medium supplemented with 3 mM quinidine or unsupplemented (initial pH of the growth medium was adjusted to pH 5.5 with NaOH to consider the alkalizing effect of quinidine supplementation) and harvested, during cultivation with orbital agitation at 30°C, at time zero (immediately before inoculation), after 2 h of incubation or during exponential growth, at the standardized culture OD₆₀₀ of 0.2 $\pm$ 0.01. These cells were washed twice with ice-cold water and resuspended in TM buffer (0.1 M MES [morpholineethanesulfonic acid; Sigma], 41 mM Tris [Sigma] adjusted to pH 5.5 with HCl) to obtain dense cell suspensions (OD₆₀₀, 5.0 $\pm$ 0.1, equivalent to approximately 2.2 mg [dry weight] ml⁻¹). After 5 min of incubation in buffer at 30°C with agitation (150 rpm), 0.2 μ M [9-³H]quinidine (ICN; 37 MBq/ml) was added to the cell suspensions, and incubation proceeded for an additional 15 min, found to be enough for reaching equilibrium. To follow intracellular accumulation of labeled quinidine in the absence of glucose, 200 μ l of that cell suspension was taken at adequate time intervals, filtered through prewetted glass microfiber filters (Whatman GF/C), and washed with ice-cold TM buffer, and the radioactivity was measured in a Beckman LS 5000TD scintillation counter. The eventual active efflux of the labeled quinidine, accumulated beforehand, was followed, after the addition of a pulse of glucose (1%, wt/vol), by measuring cell radioactivity for an additional period of 45 min. The effect on quinidine-induced active efflux of the addition of 0.35 mM cycloheximide to the quinidine-supplemented growth medium was also examined. Nonspecific [9-³H] quinidine adsorption to the filters and to the cells (less than 5% of the total radioactivity) was assessed and taken into consideration. The extracellular concentration of labeled quinidine was estimated by the radioactivity of 100 μ l of the supernatant. To calculate the intracellular concentration of labeled quinidine, the internal cell volume (*V_i*) was considered constant and equal to 2.5 μ l (mg [dry weight])⁻¹ (26). Transport assays were repeated at least twice using cells from at least two independent growth experiments. Results are means ($\pm$ standard deviation) of these repeats or are values from selected representative and complete experiments.

Intracellular pH. The comparison of the average intracellular pH (pH_i) of wild-type and Δ *yil120w* exponential-phase cell populations grown in the absence or presence of 3 mM quinidine and used to calculate intracellular/extracellular quinidine concentrations was based on the relative distribution of [2-¹⁴C]propionic acid between the cytoplasm and the extracellular medium (26, 31) using the same *V_i* used to estimate quinidine intracellular concentration.

RESULTS

ORF *YIL120w* is an MDR determinant. *S. cerevisiae* W303.1b deleted for ORF *YIL120w* did not display any evident

FIG. 1. ORF *YIL120w* is required for resistance to quinidine and ketoconazole. Comparison of susceptibility to the drugs, at the indicated concentrations, of *S. cerevisiae* W303.1b (wild type, WT) and the deletion mutant $\Delta yil120w$ harboring either recombinant plasmid pFL38_ *YIL120w* (ORF *YIL120w* inserted into pFL38) or the cloning vector. The cell suspensions used to prepare the spots in lanes b and c were 1:2 and 1:4 dilutions of the cell suspension used in lanes a, respectively.

growth phenotype on minimal medium. Also, we could not find any evidence for the involvement of *Yil120wp* in yeast resistance to benomyl, methotrexate, 4-nitroquinoline-*N*-oxide, crystal violet, malachite green, cycloheximide, miconazole, or itraconazole, based on the supplementation of minimal medium with inhibitory concentrations of these compounds within the ranges of interest, as determined before (7, 31). Susceptibility assays revealed, however, the involvement of ORF *YIL120w* in yeast resistance to quinidine (Fig. 1) but not to quinine, and to ketoconazole and fluconazole, although the phenotypic differences with fluconazole were more subtle (Fig. 1 and results not shown). These conclusions were based on the increased resistance to the referred compounds upon increased expression of this ORF in plasmid pFL38, either in the deletion mutant or in the wild-type strain (Fig. 1). Based on the resistance phenotype observed with quinidine, ORF *YIL120w* was named *QDR1*.

Effects of *QDR1* expression level on quinidine-stressed cultivation. The role of *QDR1* as a determinant of resistance to quinidine in yeast cells was confirmed in minimal liquid medium. The comparison of cell viability during cultivation of unadapted wild-type and $\Delta qdr1$ cells under mild stress imposed by 3 mM quinidine indicated that *QDR1* expression reduced the period of adaptation to exponential growth with quinidine by decreasing quinidine-induced death following sudden exposure of these unadapted cells to the drug (Fig. 2). However, after this initial period of adaptation, the exponential growth of both strains in the presence of 3 mM quinidine resumed, exhibiting indistinguishable kinetics independent of *QDR1* expression (Fig. 2). The increased expression of *QDR1* in a plasmid in the wild-type strain exerted additional protection for growth under high quinidine stress (Fig. 3). At 3.8 mM quinidine supplementation and after 30 h of cultivation, the strain expressing wild-type levels of *QDR1* did not recover from the initial period of viability loss to resume exponential growth in the presence of the drug (Fig. 3). This contrasted with the behavior of the recombinant strain, expressing increased levels of *QDR1*, which resumed growth after approximately 12 h of adaptation (Fig. 3).

The role of *QDR1* expression in alleviating the deleterious effects of quinidine in unadapted yeast cells was not detectable in cells previously adapted to the drug (Fig. 4). Indeed, when cells used as the inoculum were previously adapted to growth

FIG. 2. Effect of *QDR1* gene expression on yeast viability and growth under quinidine stress. Growth curves were followed by (A) culture OD_{600nm} and (B) concentration of viable cells. The growth curves of the wild-type ($\square$, $\blacksquare$) and the deletion mutant $\Delta qdr1$ ($\circ$, $\bullet$) in the absence ($\circ$, $\square$) or presence of 3 mM quinidine ($\blacksquare$, $\bullet$) are compared. Cells used as inoculum were exponential-phase cells cultivated in the absence of quinidine. Viable cell values are the means of at least two independent growth experiments done in triplicate.

with 3 mM quinidine by their cultivation in the presence of the drug to the standardized culture OD_{600} of 0.2, the growth curves of the wild-type and the mutant strain devoid of the *QDR1* gene in the presence of either 3 or 3.2 mM quinidine were absolutely coincident (Fig. 4).

Localization of Qdr1p-GFP fusion protein in plasma membrane. The fluorescence of exponential-phase cells of *S. cerevisiae* W303.1b expressing Qdr1-GFP fusion protein from plasmid pMET25_ *YIL120w* _GFP was predominantly localized to the cell periphery, while control cells, expressing GFP alone from plasmid pMET25_ *GFP*, exhibited a slight and uniform distribution of green fluorescence throughout the cell (Fig. 5), similar to the autofluorescence of the host cells. The strong ring-like fluorescence staining around the cell was observed for the majority of the cells from an exponential-phase culture of transformants expressing Qdr1p-GFP protein (Fig. 5B and C) but was absent from the control cells (Fig. 5A). As observed before (31), some heterogeneity in the signal intensity in the periphery of cells expressing Qdr1-GFP protein was detected, possibly due to plasmid copy number differences. Since ORF *YIL120w* was predicted to code for an integral membrane protein (1, 19, 20, 22), these results strongly suggest that Qdr1p is a plasma membrane protein.

Quinidine-induced active efflux of drug: role of *QDR1* gene expression. Since Qdr1p was localized in the plasma membrane, we examined its possible involvement in the reduction

FIG. 3. Effect of increased expression of *QDR1* on quinidine-stressed yeast growth. Comparison of the growth curves in MM2-U medium supplemented with 3.5 mM (A) or 3.8 mM (B) quinidine of the wild-type strain W303.lb transformed with the cloning vector pFL38 (□) or the recombinant plasmid pFL38_*QDR1*, with the *QDR1* gene inserted (■). Cells used as inoculum were exponential-phase cells grown in the absence of quinidine supplementation.

of quinidine accumulation in the cell by active transport of the drug out of the cell. After a pulse of glucose, both the $\Delta qdr1$ mutant and wild-type cells grown in the absence of quinidine supplementation were unable to export the labeled quinidine that rapidly entered the cell beforehand and reached an intracellular concentration ranging from 1- to 1.6-fold the concentration in the surrounding medium (Fig. 6A, E, and F). However, after 2 h of cultivation in medium supplemented with 3 mM quinidine, wild-type cells became capable of actively reducing the concentration of quinidine accumulated in the cell before a glucose pulse (Fig. 6B). This rapid induction of quinidine active export detected in wild-type cells was impaired by the addition of cycloheximide to the growth medium with the drug (Fig. 6D), indicating that the observed phenomenon depends on de novo protein synthesis induced by the drug. Evidence for the induction of a putative quinidine transporter(s) were, however, not obtained using cells devoid of *QDR1* gene after the same 2 h of incubation with the drug, being the values of quinidine accumulation, in the absence or presence of glucose, above the corresponding values in the wild-type cells (Fig. 6B and C). These results were consistent with our first expectations that *QDR1* might encode an H^+ -dependent exporter for quinidine, thus modulating the concentration of the drug accumulated in the cell. However, after a more extended period of adaptation to quinidine, the more susceptible $\Delta qdr1$ culture also resumed exponential growth (Fig. 2), and the quinidine-adapted exponential-phase cells devoid of *QDR1*,

harvested at the standardized culture OD₆₀₀ of 0.2, were also capable of active expulsion of the quinidine accumulated beforehand (Fig. 6H). Nevertheless, the amount of quinidine accumulated in $\Delta qdr1$ cells in the absence of glucose was significantly above that accumulated in wild-type cells (threefold compared with twofold), although, at the equilibrium following the glucose pulse, the concentration of labeled quinidine accumulated in the deletion mutant reached a value closer to the corresponding wild-type value (Fig. 6F and H).

The cells used in the accumulation-active efflux assays were harvested at the same standardized culture OD₆₀₀ of 0.2. In spite of the attempt to standardize the cell populations examined, it is clear from the viability curves in Fig. 2 that these populations exhibit a slightly different number of viable cells. This value was minimal for the quinidine-susceptible $\Delta qdr1$ population grown with quinidine and maximal for both the wild-type and the $\Delta qdr1$ populations grown in the absence of stress and shows an intermediate value for the quinidine-stressed wild-type population. Moreover, the average pH_i values for quinidine-stressed exponential-phase populations (6.4 ± 0.02 and 6.2 ± 0.02 for the wild type and mutant, respectively) were below the value in unstressed cell populations (6.6 ± 0.02 for both the wild type and mutant), reaching the lowest value in the stressed $\Delta qdr1$ mutant. We conclude that the stress imposed by quinidine on yeast cells led to a slight global acidification of the cell interior, even though the accumulation of this weak base is more compatible with alkalization of the cell interior. Since yeast growth medium is acidic (pH 5.5), these results strongly suggest that quinidine led to plasma membrane permeabilization with a consequent increase in the H^+ influx rate. The relative average pH_i estimates are consistent with lower susceptibility to the deleterious effects of quinidine of the unadapted wild-type population compared with the deletion mutant $\Delta qdr1$ population. They are also consistent with the passive accumulation of labeled quinidine being minimal for both the wild-type and $\Delta qdr1$ cell populations grown in the absence of quinidine and maximal for the $\Delta qdr1$ mutant cell population grown with the drug, as it would be expected from the higher protonation of this weak base in the more acidic cell interior.

DISCUSSION

As predicted from structural considerations (1, 16, 19, 20, 22), we show results indicating that the MFS-MDR homologue encoded by ORF *YIL120w*, here named *QDR1*, is an MDR determinant. Indeed, the expression of *QDR1* was implicated in yeast resistance to ketoconazole, fluconazole, and quinidine, but not its stereoisomer quinine. Like *Yil120wp*, the first MFS-MDR homologue implicated in quinidine resistance, *Yor273cp*, belongs to the 12-spanner family 1, although to a different cluster of proteins (16, 19, 22). ORF *YOR273c* was identified based on functional overexpression in a hypersensitive *S. cerevisiae* strain of genes from a yeast genomic library and selection of transformants that grew in the presence of elevated levels of quinidine (11). However, the authors failed to isolate ORF *YIL120w* in this screening; among other reasons, it is possible that the conditions used for their screen might have been too stringent. Interestingly, *Yor273cp* is also very specific for quinidine, displaying no cross-resistance to any of the other

FIG. 4. Quinidine effect on the growth curve of yeast cells previously adapted to the drug is independent of *QDR1* gene expression. Comparison of the growth curves in minimal medium supplemented with 3 mM (A and B) or 3.2 mM (C and D) quinidine of *S. cerevisiae* W303.lb (wild type) (■) and $\Delta qdr1$ mutant (□). Cells used as inoculum were exponential-phase cells (at culture OD_{600} of 0.2 ± 0.01) cultivated in the absence of quinidine (A and C) or in the presence of 3 mM quinidine (B and D).

quinoline-containing antimalarial drugs tested, including quinine and chloroquine (11), which could not be tested in the present work due to very high concentrations of chloroquine necessary for growth inhibition of the yeast strains examined. On the basis of high sequence homology of Yil120wp with other putative drug transporters of the MFS and of its subcellular localization, *QDR1* was expected to encode an H^+ -dependent exporter for quinidine through the plasma membrane. However, we show results that do not confirm this mode of action. Indeed, the differences registered in the active efflux of quinidine from cells of the wild type and the mutant devoid of the *QDR1* gene were essentially detected during the initial period of adaptation to the drug, while exponential-phase cells of both strains adapted to the drug were able to actively pulse

quinidine out of the cell. The detection of an active quinidine efflux in the wild-type population but not in the population devoid of *QDR1* after the same 2 h of incubation following the sudden exposure of cells to quinidine may be the result of the higher susceptibility of the unadapted $\Delta qdr1$ population, revealed by the higher quinidine-induced viability loss during this adaptation period. In fact, after surpassing a shorter or longer initial period of adaptation, exponential growth with quinidine was resumed in both populations, and these adapted cells became capable of active expulsion of the drug independent of *QDR1* expression. It is intriguing why quinidine, which is not present in the yeast natural environment, may induce its active expulsion from cells which are actively dividing in its presence. *QDR1* gene transcription levels do not increase in cells cultivated with quinidine (P. Nunes, M. Teixeira, and I. Sá-Correia, unpublished results), consistent with other indications suggesting that this gene is not involved in the induction of quinidine export by the drug. Nevertheless, the level of expression of *QDR1* is critical to surpass the viability loss during the initial period of adaptation to quinidine and to eventually resume exponential growth, which depends on the remaining viable population. Results also suggest that there are other resistance mechanisms that can be used when *QDR1* is absent, possibly involving other MFS-MDR and/or ABC transporters.

Quinidine accumulation assays were carried out at an external pH of 5.5, and the uptake of labeled quinidine into the yeast cell was extremely rapid. However, at this pH quinidine, which is a weak base with two protonation sites with pK_a s of 5.4 and 10.0 (8), is basically in the singly protonated form and not

FIG. 5. Fluorescence of exponential-phase cells of *S. cerevisiae* W303.lb (A) harboring the expression vector pMET25_GFP (control cells) or (B and C) transformed with the multicopy plasmid pMET25_YIL120w_GFP, indicating that Qdr1-GFP fusion protein is found in the plasma membrane.

FIG. 6. Time course of [9-³H]quinidine accumulation in the absence of glucose (○, □, △) and its eventual subsequent expulsion after a glucose (Glc) pulse (■, ●, ▲). Quinidine accumulation values are means or representative values of at least two independent experiments using cells of wild-type (wt) (□, △, ▲, ■) or $\Delta qdr1$ (○, ●) strains that had been grown in the absence of quinidine (QD), used to inoculate quinidine-supplemented or unsupplemented media, and harvested during the growth curves documented in Fig. 2 at time zero (A), after 2 h of cultivation in quinidine-supplemented medium (B and C), and at the exponential phase (exp) (culture OD₆₀₀ of 0.2) of cultivation in unsupplemented medium (E and G) or quinidine-supplemented medium (F and H). In panel D we show the results obtained using wild-type cells incubated for 2 h in the absence (□, ■) (as in B) or presence (△, ▲) of 0.35 mM cycloheximide (CYH).

in the highly lipophilic unprotonated form which would pass freely through biological membranes by passive diffusion. A very rapid uptake rate of chloroquine into *Plasmodium* cells was also observed phase and a similar question was raised (10, 13, 15). The internal pH (pH_i) of exponential-phase yeast cells was estimated to be within the range from 6.2 to 6.6, depending on the presence or absence of the drug or of a functional *QDR1* gene; cells grown under quinidine stress exhibited an average pH_i slightly below the pH_i of cells grown in the absence of drug. This observation is consistent with the disturbing effect of quinidine on plasma membrane spatial organization and the consequent increase in H⁺ passive influx, with the acidification of the interior of cells incubated at pH 5.5, even though quinidine accumulation, presumably into the slightly acidic yeast vacuole (9, 10, 15, 18), is expected to lead to alkalinization. At high concentrations, chloroquine also has a demonstrable negative effect on *Plasmodium* membrane stability, and the protonated form may interact with various phospholipids by both hydrophobic and electrostatic forces (15). Therefore, the slightly lower pH_i exhibited by the exponential-phase $\Delta qdr1$ population compared with the wild-type population, both grown under identical quinidine stress, is consistent with the higher susceptibility of unadapted cells devoid of *QDR1* to the deleterious effects of quinidine during the initial period of adaptation to the drug. Since the average pH_i of the $\Delta qdr1$ population used in the accumulation-active efflux assays was slightly below the average pH_i of the wild-type population harvested at the same culture OD (pH_i 6.2 compared with 6.4), the higher accumulation of quinidine in the absence of glucose

into the more acidic interior of $\Delta qdr1$ cells may merely be a consequence of the weak base properties of this drug. This hypothesis is also consistent with the apparently unexpected lower intracellular accumulation of quinidine (around onefold) in exponential-phase cells of both strains grown in the absence of quinidine compared with the values calculated for quinidine-adapted cells with a more acidic interior (two- or three-fold).

Studies carried out before to gain some understanding of how another plasma membrane MFS-MDR homologue, encoded by ORF YGR224w (*AZRI* gene), facilitates yeast adaptation to inhibitory concentrations of acetic acid also failed to directly implicate this protein in the active export of acetate (31). It is possible that a number of the MFS-MDR homologues may play other roles in yeast cells than detoxification, such as the transport of a specific molecule unrelated to the drugs to which they confer resistance. This is apparently the case for polyamine transport by YII028cp (32) or the transport of dihydroxy precursors by Ybr180wp Dtr1p (12). It is possible that Qdr1p could alter ion fluxes that indirectly control drug accumulation in yeast cells by affecting pH and/or membrane potential (25). Further studies are required to find out the natural physiological role of Qdr1p as a plasma membrane transporter of the MFS and to establish the precise biochemical mechanisms whereby this putative transporter functions in alleviating the toxic effects of, at least, quinidine, ketoconazole, and fluconazole in *S. cerevisiae*. The elucidation of these mechanisms may provide useful information for the understanding of the physiological functions of the poorly characterized fam-

ily of MFS-MDR transporters that have largely escaped identification by classical approaches. The quinidine concentrations used in the present work to inhibit yeast growth were much higher than the one required for killing *Plasmodium* cells, consistent with the idea that the antimalarial effects of quinoline ring-containing drugs are exerted via physiological processes that do not exist in *S. cerevisiae* (10, 15). However, the expression in yeast cells of the *P. falciparum pfmdr1* gene, a member of the ABC superfamily of transporters that confers resistance to multiple antimalarials in the malaria parasite (24), was associated with decreased cellular accumulation and a concomitant increase in resistance to quinoline-containing drugs in yeast transformants (28). Nevertheless, it is clear that this gene cannot also be the sole cause of chloroquine resistance in *P. falciparum* (30). Since the mechanisms of multidrug resistance are apparently conserved among phylogenetically distant organisms (4, 6, 10, 22), the characterization of drug resistance determinants and of the mechanisms of multidrug resistance in yeast cells may contribute to the understanding of these mechanisms in more complex and less easily accessible eukaryotes, such as those underlying the resistance to quinoline-containing drugs in the malaria parasite.

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