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Multidrug Resistance in *Candida albicans*: Disruption of the *BEN*r Gene

MARTIN GOLDWAY,[1]† DINAH TEFF,[1] RUTH SCHMIDT,[2] AMOS B. OPPENHEIM,[1] AND YIGAL KOLTIN[2,3]*

Department of Molecular Genetics, The Hebrew University Hadassah Medical School, Jerusalem 91010,[1] and Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Ramat Aviv 69978,[3] Israel, and Myco Pharmaceuticals, Inc., Cambridge, Massachusetts 02139[2]

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The *BEN*r gene of *Candida albicans*, which confers resistance on susceptible strains of *Saccharomyces cerevisiae* to six structurally and functionally unrelated drugs, was described recently (R. Ben-Yaacov, S. Knoller, G. Caldwell, J. M. Becker, and Y. Koltin, Antimicrob. Agents Chemother. 38:648–652, 1994). This gene bears similarity to membrane proteins encoding antibiotic resistance in prokaryotes and eukaryotes. The effect of disruption of this gene on viability and drug susceptibility was determined. The results indicate that the gene is not essential but its inactivation leads to susceptibility to three of the four drugs tested. Inactivation of this gene did not increase the susceptibility of the mutant to benomyl, suggesting that *C. albicans* has other mechanisms of resistance, some of which may be additional efflux pumps that confer resistance to this tubulin-destabilizing agent.

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Candida albicans is the major fungal pathogen of immunocompromised patients (21). The pursuit of drugs effective against this pathogen has been hindered by the resistance of this organism to many drugs and metabolic inhibitors. The nature of the resistance to a few drugs has been identified as related to altered transport, modification of an enzyme, and a change in membrane composition (19, 20, 25). However, a broad-spectrum resistance, identified initially as combined resistance to benomyl and methotrexate and later as resistance to four additional agents, appears to be an inherent characteristic of the species (4, 9). This resistance was shown to be determined by a single gene encoding a polypeptide of 564 amino acids with a series of features typical of prokaryotic and eukaryotic transporter proteins and multidrug resistance efflux pumps (12, 27). This sequence, which is found in every isolate of *C. albicans* and *C. stellatoidea* examined, was identified by its ability to confer on *Saccharomyces cerevisiae* strains resistance to a series of six structurally and functionally unrelated drugs. This gene was shown to be related to *S. cerevisiae ATR1*, which encodes a transmembrane protein conferring resistance to aminotriazole (18) and 4-nitroquinoline-*N*-oxide (11) and imparts resistance to these agents to susceptible strains of *S. cerevisiae* (4). It is also related to the recently discovered *car1* gene of *Schizosaccharomyces pombe* (conferring amiloride resistance) (17), which appears to encode a transmembrane protein with structural characteristics similar to those of the *C. albicans* gene. A gene conferring resistance to cycloheximide with 76% amino acid similarity (57% identity) to the *C. albicans* gene was found in *C. maltosa* (29).

The fact that the multidrug resistance is an inherent characteristic of the species prompted an investigation of the role of *BEN*r in the cellular biology of *C. albicans* and of whether this is the sole component imparting multidrug resistance. To resolve these questions, efforts were focused on (i) characterization of the epistatic effects between mutations in genes affecting benomyl sensitivity and *BEN*r and (ii) the phenotypic expression of multidrug resistance in *BEN*r transformants and *BEN*r disruptants. The results obtained indicate that *BEN*r imparts three- to fivefold-increased benomyl resistance to wild-type *S. cerevisiae* and to supersusceptible mutants of *S. cerevisiae* when expressed from either a high-copy-number or a low-copy-number plasmid. Disruption of *BEN*r is not detrimental to *C. albicans* but leads to increased susceptibility to three of the four drugs tested. The *C. albicans* homozygous disruptants do not display reduced resistance to benomyl, suggesting that resistance to this drug can be imparted by additional genes, some of which may encode other efflux pumps. Since *BEN*r confers resistance to a number of structurally and functionally unrelated drugs, including benomyl, and disruption of this gene leads to susceptibility to a subset of these drugs, we propose that *BEN*r be renamed *CaMDR1* (for *C. albicans* multidrug resistance).

* Corresponding author. Mailing address: Myco Pharmaceuticals, Inc., One Kendall Square, Bldg. 300, Cambridge, MA 02139.

† Present address: MIGAL South Industrial Zone, Kiryat Shmona 10200, Israel.

MATERIALS AND METHODS

Strains and plasmids. The *S. cerevisiae* and *C. albicans* strains used in this study are described in Table 1. Plasmid pGO140 was constructed by using the *S. cerevisiae* centromeric vector pRS316 (31) and a 5.7-kb *Bam*HI fragment from pBEN7-1 described by Fling et al. (9) containing the open reading frame (ORF) and the promoter from *C. albicans* encoding the gene that was reported to confer resistance to benomyl and methotrexate on a susceptible strain of *S. cerevisiae*. Plasmid pGO146 was constructed by inserting the 5.7-kb *Bam*HI fragment from pBEN7-1 into the *Bam*HI site of the *S. cerevisiae* integrative vector YIp5 (6). Plasmid pGO152 contains *CaMDR1*, into which a 1.4-kb *Sal*I-*Bgl*II deletion in the ORF was introduced and the *hisG-URA3-hisG* cassette replaced the deleted sequence. The plasmid was constructed as follows. pGO140 was digested with *Bam*HI and *Pst*I, and a 4.5-kb fragment containing the ORF of *CaMDR1* was ligated with *Bam*HI-*Pst*I-digested pUC18 to yield plasmid pGO149. The 4.4-kb *hisG-URA3-hisG* cassette was isolated from pCUB6 (10) and ligated with *Bgl*II-*Sal*I-digested pGO149. The resulting plasmid, pGO152, consists of pUC18 and the *CaMDR1* ORF with a deletion of 1.4 kb (82% of the ORF) replaced with the *hisG-URA3-hisG* cassette and retaining both the 5′ and 3′ sequences of *CaMDR1* sufficient for homologous recombination to allow gene transplacement.

Integration of *CaMDR1* into the chromosome of *S. cerevisiae* was performed with plasmid pGO146. The plasmid was digested at the *Stu*I site of the *URA3* gene, and the linear DNA was used to transform the appropriate strains of *S. cerevisiae* (see Table 2). Transformants were selected on medium devoid of uracil. The uracil prototrophs were inoculated into nonselective liquid medium (YEPD [28]). After 24 h at 30°C, the suspension was streaked onto YEPD solid medium. The colonies that grew on YEPD were replica plated to synthetic

TABLE 1. Strains used in this study

Strain	Genotype	Source
S. cerevisiae		
DBY1826	*Mat***a** *ura3-52 leu2-3,112 his3-200 ade2-101*	G. R. Fink[a]
DBY1828	*Mat***a** *tub1-1 ura3-52 leu2-3,112 his3-200 ade2-101*	Fink
DBY2304	*Mat***a** *tub2-304 ura3-52 his4-539 lys2-801*	Fink
DBY3393	*Mat***a** *cin1*::*HIS3 ura3-52 his3-200 leu2-3,112*	Fink
yGO641	DBY1828 with *ura3*::*URA3 CaMDR1*	This study
yGO642	DBY2305 with *ura3*::*URA3 CaMDR1*	This study
yGO643	DBY3384 with *ura3*::*URA3 CaMDR1*	This study
yGO644	DBY1826 with *ura3*::*URA3 CaMDR1*	This study
C. albicans		
792-WC3	*ura3/ura3 ade2/ade2 CaMDR1/CaMDR1*	Y. Koltin[b]
cMD716	*ADE2* spontaneous revertant of 792-WC3	This study
cMD731	cMD716 heterozygote; *Camdr1 Bgl*II-*Sal*I::*hisG-URA3-hisG/CaMDR1*	This study
cMD732	cMD731 after *URA3* loopout; *Camdr1 Bgl*II-*Sal*I::*hisG/CaMDR1*	This study
cMD734	cMD732 with homozygous *Camdr1 Bgl*II-*Sal*I::*hisG/Camdr1 Bgl*II-*Sal*I::*hisG-URA3-hisG*	This study
CAI4	*ura3/ura3 CaMDR1/CaMDR1*	W. A. Fonzi[c]

[a] Whitehead Institute for Biomedical Research, Cambridge, Mass.
[b] Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel.
[c] California College of Medicine, University of California, Irvine.

medium (28) devoid of uracil. Stable prototrophs were assumed to have integrated *CaMDR1* into the chromosome, and the integration was confirmed by Southern analysis.

DNA manipulations were performed as described by Ben-Yaacov et al. (4) and by following the manufacturer's instructions. Southern analysis was performed as described in *Current Protocols in Molecular Biology* (2).

Northern (RNA) analysis. Cultures of *C. albicans* were grown in YEPD (28) to the mid-log phase. Total RNA was extracted by using glass beads essentially as described in *Current Protocols in Molecular Biology* (2), with the addition of a wash step using 3 M Na acetate to remove DNA from the nucleic acid precipitate. Samples of 10 μg were prepared and run on a 1% agarose gel by using the glyoxal-dimethyl sulfoxide procedure described in *Current Protocols in Molecular Biology* (2). The RNA was transferred to Amersham Hybond-N membranes with the Pharmacia LKB VacuGene XL apparatus by following the manufacturer's instructions. The portion of the membrane containing the molecular weight markers was stained with methylene blue as described by Wilkinson et al. (36). The blots were probed with the 2,161-bp *Eco*RV fragment of *CaMDR1* (9). An 858-bp PCR fragment of the *S. cerevisiae* actin gene (provided as a gift by J. Becker, University of Tennessee) was used as a control. The nucleotide sequence of the *C. albicans* actin gene (*CaACT1*) is 87% identical to that of the actin gene of *S. cerevisiae* (13, 23). The 5% sodium dodecyl sulfate hybridization method was used (35).

Media. Both YEPD and synthetic medium were prepared as described by Rose et al. (28). Synthetic medium was supplemented as described by Rose et al. (28), but *C. albicans* uracil auxotrophs were supplemented with uridine (50 μg/ml) instead of uracil. For selection of uracil auxotrophs, the 5-fluoroorotic acid selection procedure described by Boeke et al. (5) was used.

The drugs used in this study were as follows. Benomyl was a gift from the E. I. du Pont de Nemours & Co. Agricultural Products Division, Wilmington, Del. Cycloheximide, methotrexate, and 4-nitroquinoline-*N*-oxide were obtained from Sigma. Benomyl was dissolved in dimethyl sulfoxide as a stock solution of 10 mg/ml and stored at −20°C. All other agents were dissolved in water. Methotrexate susceptibility tests were conducted after addition of 200 μg of sulfanilamide (Sigma) per ml to deplete the folate pool.

Drug susceptibility assays. Two types of assays were used, one with solid medium on gradient plates as described by Fling et al. (9) and one with liquid medium in 96-well microtiter plates. The latter test was performed with cells grown initially in YEPD medium, with the exception of methotrexate susceptibility tests, in which efforts were directed to reduction of the folate pool as much as possible prior to the assay. Some 10^4 cells were added to each well and suspended in 50 μl of medium. Each dilution series was a twofold dilution of the drug. Cell growth after a 24-h period at 30°C was recorded with an enzyme-linked immunosorbent assay plate reader. In tests conducted with gradient plates, the results were recorded after 48 h of incubation.

Transformation of *S. cerevisiae* and *C. albicans*. Cells of both *S. cerevisiae* and *C. albicans* were transformed by the Li acetate procedure of Ito et al. (16) by using single-stranded DNA as the carrier as described by Schiestl and Gietz (30).

RESULTS AND DISCUSSION

Epistatic relation between *CaMDR1* and mutations of *S. cerevisiae* imparting benomyl susceptibility. *CaMDR1* was shown previously to impart benomyl resistance to wild-type *S. cerevisiae* when expressed from a high-copy-number or low-copy-number plasmid (4, 9). To determine the efficiency of the resistance conferred by this gene, we integrated *CaMDR1* by directed integration into the chromosomes of benomyl-susceptible *S. cerevisiae* mutants and wild-type *S. cerevisiae*. The resistance of the integrants to benomyl was tested. The strains used in these experiments included yGO641, carrying an α-tubulin mutation (*tub1-1*); yGO642, carrying a β-tubulin mutation (*tub2-403*); and yGO643, carrying a mutation in a gene encoding a putative microtubule-associated protein (*cin1*) (7, 14, 15, 33). Strain yGO644 was used as the wild-type control with the wild-type alleles of both *TUB* and *CIN1*. Resistance to benomyl was determined by plating suspensions with equal cell densities on solid medium containing a gradient of benomyl as described by Fling et al. (9) and Ben-Yaacov et al. (4). The results were scored after incubation for 48 h at 26°C. The results shown in Table 2 indicate that a single copy of *CaMDR1* integrated into the chromosome of *S. cerevisiae* conferred three- to fivefold resistance to benomyl. This increase in resistance is similar to the level reported earlier for transformants with high-copy-number plasmids pBEN4-3 and pBEN13-2 and low-copy-number plasmid pBEN7-1 described by Fling et al. (9). Furthermore, the increment of resistance in the susceptible *tub1-1, tub2-403*, and *cin1* mutants was the same as that of the wild type. These results suggest that there are some limitations in the expression of *CaMDR1* in *S. cerevisiae* and the same amount of the gene product can be accommodated by the wild type and the mutants, leading to similar increments in the level of resistance. One model may suggest that the product of *CaMDR1* can occupy a predetermined number of sites, which still allows some uptake of the drug. An experiment supporting this view was reported by Fling et al. (9), who used a mutant of *S. cerevisiae* with β-tubulin instability which requires benomyl for growth (34). Transformants of this mutant with *CaMDR1* grow on benomyl and retain their dependence on the drug.

Disruption of *CaMDR1* in *C. albicans*. To determine whether *CaMDR1* is an essential gene in *C. albicans* and to determine if *CaMDR1* is the only multidrug resistance gene expressed in this species, disruption of this gene was attempted. Disruption of a gene in *C. albicans*, which is a diploid organism with no sexual cycle, requires sequential disruption of the two alleles of

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TABLE 2. Resistance to benomyl of isogenic strains of *S. cerevisiae* with integrated *CaMDR1*[a]

Strain	Relevant genotype	Growth[b] at benomyl concn (μg/ml) of:				
		2	5	10	20	30
DBY1828	*tub1-1*	−	−	−	−	−
yGO641	*tub1-1 CaMDR1*	+	+	−	−	−
DBY2304	*tub2-304*	+	−	−	−	−
yGO642	*tub2-304 CaMDR1*	+	+	±	−	−
DBY3393	*cin1*	−	−	−	−	−
yGO643	*cin1 CaMDR1*	+	+	±	−	−
DBY1826	*TUB1 TUB2 CIN1*	+	+	±	−	−
yGO644	*TUB1 TUB2 CIN1 CaMDR1*	+	+	+	+	+

[a] A 3-μl cell suspension volume (10^3 cells) was spotted onto gradient plates containing benomyl at the concentrations indicated. The plates were scored after incubation at 26°C for 48 h.

[b] +, growth; −, no growth; ±, slight growth.

the host. This can be achieved through homologous recombination with a modified truncated allele by using the procedure developed by Alani et al. (1) and modified by Fonzi and Irwin (10) for *C. albicans*. To disrupt *CaMDR1*, we constructed plasmid pGO152, in which 84% of the ORF of *CaMDR1* was deleted and replaced with selectable marker *CaURA3* flanked by *hisG* from the disruption vector constructed by Fonzi and Irwin (10). To disrupt *CaMDR1*, pGO152 was digested with *Pst*I and *Kpn*I, providing a fragment containing the *hisG-URA3-hisG* cassette flanked by sequences of *CaMDR1* that was expected to allow transplacement of *CaMDR1* because of the homology of the flanking sequences. The digested DNA was used to transform a *Caura3* auxotroph (cMD716) with selection for prototrophs on medium devoid of uridine. The expected results of the integration are depicted in Fig. 1. As shown, a diagnostic *Kpn*I restriction site should be eliminated by the integration (*CaURA3* does not have a *Kpn*I site) and a new *Bam*HI site (contained in *CaURA3*) should be introduced into the transplaced allele of *CaMDR1.*

To verify that the integration transplaced *CaMDR1* and was not ectopic integration, *Bam*HI- and *Kpn*I-digested DNA from the uracil prototrophs was subjected to Southern analysis, probed with the 5.7-kb *Pst*I-*Bam*HI fragment from the original *CaMDR1*-containing insert described by Fling et al. (9). As expected, the sizes of the fragments identified by the probe changed from 1.9 and 3.8 kb in the wild-type *CaMDR1* allele to 3.2 and 5.3 kb in the allele containing the inserted *hisG-URA3-hisG* cassette (Fig. 1b, compare lanes 1 and 2). To derive homozygous disruptants, the uracil transformants were grown on a non-selective medium to allow unrestricted recombination between the *hisG* flanking repeats, leading to the loss of *URA3* by a loopout of the internal *hisG-URA3-hisG* cassette. The uracil auxotrophs resulting from the loopout of *URA3* were selected on 5-fluoro-orotic acid-containing medium (5). To verify that in fact the uracil auxotrophy resulted from the looping out of *URA3*, DNA was extracted from *Caura3* auxotrophs and subjected to Southern analysis as described above. The fragments detected with the probe were, as expected, the 2.6- and 3.2-kb fragments (Fig. 1b, lane 3).

To derive homozygous strains with a disrupted *CaMDR1* gene, the *Caura3* auxotrophs, verified as being heterozygous *Camdr1*/*CaMDR1* mutants, were retransformed with the *Pst*I-*Kpn*I fragment of pGO152 containing the *hisG-URA3-hisG* cassette flanked by sequences from *CaMDR1*. Uracil prototrophic transformants were selected, and the DNAs from some of these transformants were subjected to Southern analysis. Two

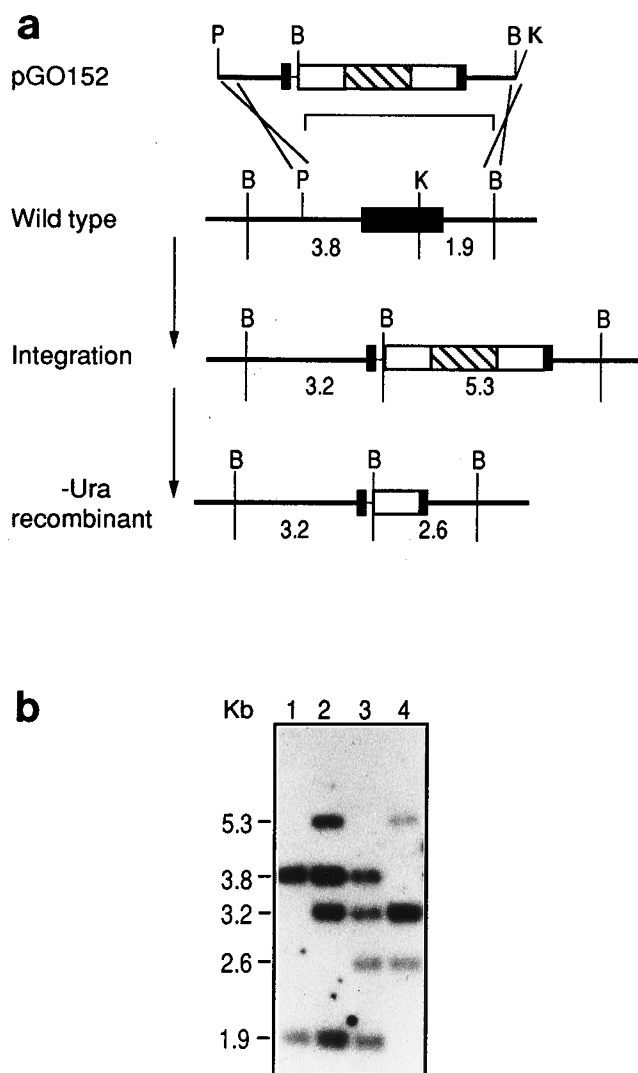

FIG. 1. Disruption of *CaMDR1* and confirmation of the disruption by Southern analysis. (a) Sequence of disruption of *CaMDR1* and description of the expected fragment sizes in each step of the disruption process. The top line shows plasmid pGO152, carrying the *hisG-URA3-hisG* cassette inserted in place of the disrupted *CaMDR1* gene as described in Materials and Methods. Restriction sites relevant to the construction of the vector and analysis of the results are abbreviated as follows: *Pst*I, P; *Bam*HI, B; *Kpn*I, K. The black boxes represent the *CaMDR1* sequence, the open boxes represent the *hisG* sequence, the hatched boxes represent the *URA3* sequence, the thin line represents the pBR322 sequence, and the wide line represents *C. albicans* genomic DNA. The second line shows the chromosomal region in the host containing the active *CaMDR1* gene. The third line shows the disrupted *CaMDR1* gene after transplacement of the wild-type allele. The bottom line describes the disrupted *CaMDR1* gene after looping out of *URA3* by homologous recombination between the *hisG* repeats. (b) Southern blot analysis of the genomic DNA of parental strain cMD716 (lane 1), heterozygous strain cMD731 after the first round of transformation (lane 2), heterozygous strain cMD732 after looping out of *URA3* (lane 3), and strain cMD734, the homozygous *CaMDR1* disruptant, after the second round of transformation in which one allele is disrupted as shown for strain cMD732 and the *hisG-URA3-hisG* cassette is inserted into the second allele (lane 4). Southern analysis was performed with the probe represented in panel a by the bracket above the diagram of the wild-type allele (second line). The DNA was digested with *Bam*HI and *Kpn*I.

different integration events were detected; one was an integration into the disrupted allele (data not shown), and the second was an integration into the intact wild-type allele in the *CaMDR1*/*Camdr1* heterozygote, creating a homozygous dis-

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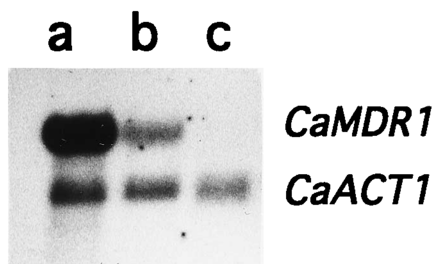

FIG. 2. Northern analysis of the mRNA transcribed by *CaMDR1*. Lanes (from left to right): a, wild-type strain cMD716; b, heterozygote *CaMDR1* disruptant strain cMD731; c, homozygote *CaMDR1* disruptant strain cMD734. The upper mRNA bands hybridized to a probe consisting of an *Eco*RV fragment containing the entire *CaMDR1* ORF. The lower bands hybridized to a probe consisting of the *S. cerevisiae* actin gene, a control showing that equal amounts of mRNA were loaded in each lane.

ruptant of the multidrug resistance gene (Fig. 1b, lane 4). These results suggest that *CaMDR1* is a nonessential gene and the homozygous disruptant can grow and divide indistinguishably from its wild-type progenitor.

The expression of *CaMDR1* in the wild-type and disruptant strains was determined by Northern analysis with the entire *CaMDR1* ORF as a probe. The results shown in Fig. 2 indicate that an approximately 1.8-kb hybridizing mRNA species (corresponding to the *CaMDR1* transcript) was present in the wild-type and heterozygous disruptant strains but absent in the homozygous disruptant. There was no evidence of a truncated message in either of the disruptant strains which might retain partial function and hence interfere with the drug susceptibility assays. The level of transcription in the heterozygous disruptant was reduced compared with that of the wild type but significantly more so than expected on the basis of gene dosage, suggesting a more complicated manner of regulation. Nonetheless, it is clear that the homozygous *CaMDR1* disruption abolished the expression of this gene. Furthermore, as RNA was isolated from mid-log-phase cells growing on rich medium in the absence of any drugs, the *CaMDR1* gene appears to be expressed under normal growth conditions.

Disruption of *CaMDR1* and drug resistance. The effect of *CaMDR1* disruption on the resistance of the mutant to a series of agents was tested. This study included four of the six agents (because of the limited availability of two agents) to which *CaMDR1* imparted resistance when expressed in a susceptible strain of *S. cerevisiae* (4). The agents tested were methotrexate, 4-nitroquinoline-*N*-oxide, cycloheximide, and benomyl. As shown in Fig. 3, the *Camdr1*/*Camdr1* homozygous disruptant displayed increased susceptibility to methotrexate and 4-nitroquinoline-*N*-oxide and somewhat reduced resistance to cycloheximide compared with the parental strain. High cycloheximide concentrations were required to reach the level at which the susceptibility of *C. albicans* can be detected. The disruptant clearly displayed susceptibility to cycloheximide compared with the parental strain, but on the basis of the degree of susceptibility, the results may indicate that resistance to cycloheximide is imparted by more than one gene and perhaps in combination with other mechanisms, such as modified ribosomal proteins (8). In every case it appears that the resistance is a function of the gene dosage since the heterozygote cMD731 displayed increased susceptibility to the three agents, although somewhat less than the homozygous disruptant cMD734. The only unaffected resistance among the four agents tested was resistance to benomyl. This result suggests that the high level of resistance to benomyl displayed by *C. albicans* is the result of more

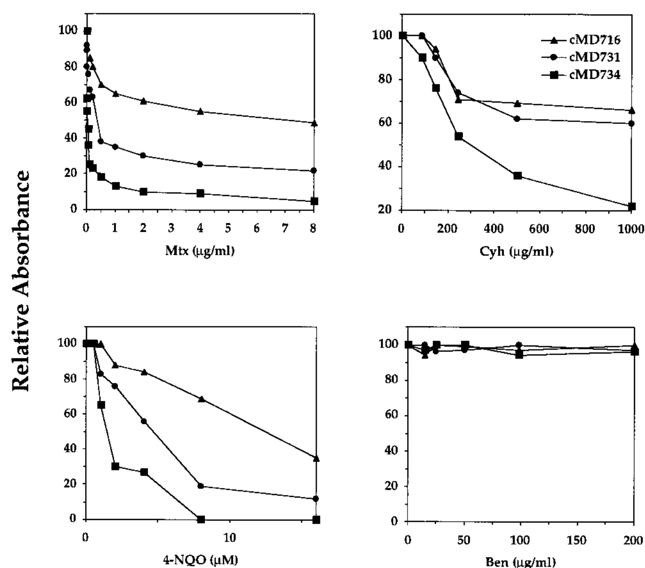

FIG. 3. Effect of disruption of *CaMDR1* on drug resistance. Drug resistance was determined in a 96-well microtiter dish with a twofold serial dilution of medium containing each drug. The same number of cells was added to each well, and after 24 h of incubation at 30°C the cell density was determined with an enzyme-linked immunosorbent assay plate reader (A_{600}). The strains tested were cMD716 (*CaMDR1*/*CaMDR1*; wild type), cMD731 (*Camdr1*/*CaMDR1*; heterozygous disruptant), and cMD734 (*Camdr1*/*Camdr1*; homozygous disruptant). Mtx, methotrexate; Cyh, cycloheximide; 4-NQO, 4-nitroquinoline-*N*-oxide; Ben, benomyl.

than one gene. Since another mechanism of resistance to benomyl is known, such as β-tubulin mutations (15, 26), an additional interpretation of the results may suggest that it is a combination of multidrug resistance and a tubulin mutation that leads to this exceptional degree of resistance. However, since the cloned β-tubulin gene of the strain used in this study was tested by functional expression in a benomyl-susceptible strain of *S. cerevisiae* and it did not confer resistance upon this strain (32), it does not appear that the resistance results from any contribution by the β-tubulin gene. It is more likely that other efflux mechanisms contribute to this type of resistance. This was further reinforced by results obtained with a second set of *CaMDR1* gene disruptions performed in strain CAI4, which has no relation to cMD716 (10). The disruptions followed the same protocol and involved the same vectors as those described for cMD716. The results of the drug susceptibility assays followed the same pattern as shown for the heterozygote cMD731 and the homozygote cMD734. In *S. cerevisiae*, a number of genes displaying pleiotropic drug resistance have been identified that include a number of efflux mechanisms that impart resistance to structurally dissimilar drugs (3, 22, 24). A similar situation may be anticipated in *C. albicans*, and current efforts are directed towards identification of the components that contribute to the resistance of this organism to many antifungal agents and antimetabolites.

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