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NOTES

Reduced Virulence of *Candida albicans* Mutants Affected in Multidrug Resistance

JEFFREY M. BECKER,[1]* L. KEITH HENRY,[1] WEIDONG JIANG,[2] AND YIGAL KOLTIN[2]

Department of Microbiology and Program in Cellular, Molecular, and Developmental Biology, University of Tennessee, Knoxville, Tennessee 37996-0845,[1] and Myco Pharmaceuticals Inc., Cambridge, Massachusetts 02139[2]

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Disruption of a multidrug resistance gene (*CaMDR1*) in *Candida albicans* resulted in mutant strains that colonized mouse kidneys to very high levels but were markedly reduced in their virulence. No obvious differences in several properties related to colonization and dissemination were noted among *MDR*$^+$ or *mdr*$^-$ strains. These results suggest that specific fungal efflux pumps play a role in fungal pathogenicity.

Multidrug resistance (MDR) may arise from the export of structurally and functionally unrelated drugs and antibiotics used against human cancer cells, protozoa, and bacteria (5, 12). Several superfamilies of membrane transport proteins, including the ABC transporters (13) and the major facilitators (19), are associated with multidrug resistance. Members of these superfamilies are typified by six transmembrane-spanning domains that are duplicated in the physiologically active transporter and are separated by a hydrophilic region.

Saccharomyces cerevisiae multidrug resistance, which is referred to as pleiotropic drug resistance, is the best characterized of these systems among the fungi (1). Genes of *S. cerevisiae* such as *ATR1*/*SNQ1* and *PDR5*, which encode polypeptides similar to members of the major facilitators (11, 15) and the ABC superfamily (2), respectively, have been identified. Ste6p, an *S. cerevisiae* protein mediating the export of the lipopeptide pheromone **a**-factor, is structurally related to the ABC family and can be functionally complemented by specific mammalian and rodent MDR proteins (17, 23–25). The large number of potential membrane transport proteins which currently have no assigned biological function, as indicated in the ongoing analysis of the *S. cerevisiae* genome sequence (9), indicates that additional multiple drug resistance proteins may be extant in this organism.

The normal physiological function of MDR gene products in eucaryotic cells may be to translocate lipids and hydrophobic compounds across membranes (14, 27), although the role of these proteins in the fungi has not been established. For *Candida albicans*, the most prevalent fungal pathogen of humans (28), we have reported the characterization of an MDR gene, termed *CaMDR1*, which had been previously referred to as *BEN*^{r} (3, 7). The 564-amino-acid polypeptide encoded by this gene appears to act as an efflux pump and is structurally related to the major facilitator superfamily. This gene is not a part of the repertoire of recently reported genes involved in the emergence of resistance resulting from selection pressures imposed by the increasing use of antifungal agents (20). *CaMDR1* may be part of a mechanism that makes this pathogen intractable to many antifungal compounds; the disruption of this gene leads to sensitivity to a number of drugs toward which a parental strain is not susceptible (3, 7, 10). In the present study, we address the question of whether *CaMDR1* has a role in the virulence and pathogenesis of *C. albicans.*

A clinical isolate of *C. albicans* was engineered to generate a stepwise disruption of both copies of *CaMDR1* in this diploid fungus. Homozygous disruptants are viable; thus, the gene is not essential for survival. The strains in the present study were derived from *C. albicans* CAI4 (8) according to published methods (10). The disruption was performed with a DNA fragment in which 84% of the *CaMDR1* open reading frame was deleted and replaced with the *CaURA3* gene flanked by *hisG* from the disruption vector pCUB6 developed previously (8). The disruption was performed in two steps to disrupt the two alleles of the diploid. The first step led to a heterozygote *CaMDR1*/*Camdr1 CaURA3*/*Caura3*; the second round of transformation was preceded by selection of *CaURA3* loop outs by using 5-fluoroorotic acid selection (4). The DNA fragment that was used in the first round of gene transplacement was also used in the second round. Each phase of the transplacement was verified by Southern analysis to identify that the correct transplacements occurred. For all of the virulence tests, uracil prototrophs were used, since uracil auxotrophy leads to loss of virulence (16).

As shown in Fig. 1, the growth rates of the disruptants are indistinguishable from that of the parental strain (Fig. 1A). Likewise, germination and hyphal development rates of the disruptants and the parental strain were also indistinguishable (Fig. 1B and C). More than 90% of blastoconidia of both the parental strain and the disruptants germinated within 30 min at 37°C. The lengths of the germ tubes were virtually the same in all strains, indicating that the rate of nuclear division in the germ tubes is not affected by the disruption of *CaMDR1*. No effect on adherence to endothelial or epithelial cells was detected in the disruptants as well (data not shown). Therefore, three factors associated with virulence in *C. albicans* (21)—growth rate, germ tube formation, and adherence—were not affected by disruption of *CaMDR1.*

* Corresponding author. Mailing address: Microbiology Department, University of Tennessee, Knoxville, TN 37996-0845. Phone: 615-974-3006. Fax: 615-974-4007. Electronic mail address: in%"jbecker@utkvx.utk.edu".

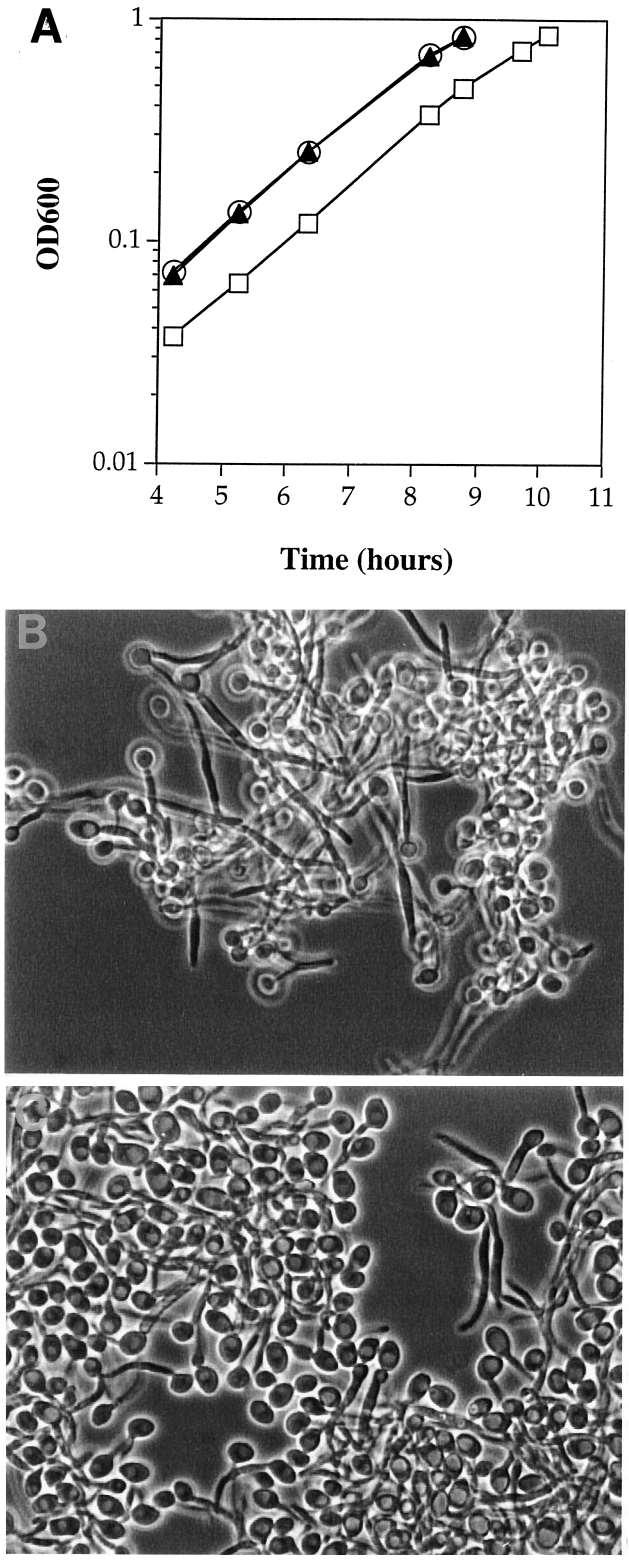

FIG. 1. Physiological characteristics of the *Camdr1* mutants. (A) Growth rates of the wild-type strain (□), the heterozygote disruptant (▲), and the homozygote disruptant (○) in YPD medium grown at 30°C. OD, optical density. (B) Germination of blastoconidia of the wild type (top panel) and the homozygote disruptant (lower panel) in 50% serum after 60 min of incubation at 37°C.

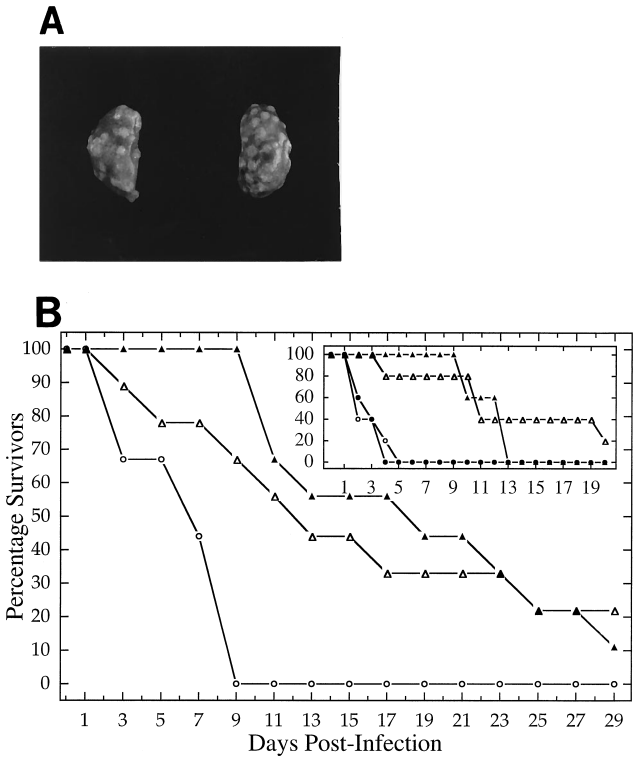

FIG. 2. (A) Appearance of kidneys taken from neutropenic mice at 30 days postinfection of the homozygote *Camdr1*/*Camdr1* disruptant strain. (B) Survival of mice infected with strains carrying various alleles of the *CaMDR1* gene. The larger graph plots survival of immunocompetent mice, and the inset represents immunocompromised mice. ▲, CAI4-116 (*camdr1*/*camdr1*); △, CAI4-01 (*CaMDR1*/*Camdr1*); ○, CAI4-2312 (*CaMDR1*/*CaMDR1*); ●, SC5314 (clinical isolate [*CaMDR1*/*CaMDR1*]).

To test the effect of *CaMDR1* on virulence in an animal model, both normal and neutropenic mice were infected with the parental strain, the heterozygous *CaMDR1*/*Camdr1* disruptant, and the homozygous disruptant *Camdr1*/*Camdr1*. The survival rates of the mice and the CFU recoverable from the kidneys were monitored. ICR 4-week-old male mice (Harlan-Sprague-Dawley) were used in virulence studies. Five mice were housed in each cage, with food and water given ad libitum according to NIH guidelines for ethical treatment of animals. For preparation of the fungal inocula, the *C. albicans* strains were grown in YPD medium (1% yeast extract, 2% Bacto Peptone [Difco Co., Detroit, Mich.], 2% dextrose) or Sabouraud dextrose medium (1% dextrose and 2% Neopeptone [Difco Co.]) overnight at 37°C, washed twice in sterile distilled water or saline, and resuspended to a concentration of 10^7 cells per ml as determined by hemocytometer counting. One-tenth milliliter of this suspension was inoculated into mice via the lateral tail veins. Neutropenia was induced by intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) 4 days and 1 day prior to infection and once every 4 days postinfection. Neutrophil counts indicated that severe neutropenia was induced and maintained. For quantitation of CFU of *C. albicans* from kidney tissues, the mice were anesthetized with methoxyflurane and were killed by cervical dislocation. The kidneys were excised, suspended in 1 ml of sterile water in a sterile plastic whirl-pak bag, and homogenized by rolling of a metal rod over the bag until the tissue was thoroughly homogenized. Serial dilutions of the suspensions were plated onto

TABLE 1. CFU of *C. albicans* recovered from homogenized kidney tissue

Strain inoculated	Day of necropsy	CFU/g of kidneys[a]	5-FOA resistance[b]	
			No. of cells plated	No. of 5-FOA-resistant cells (%)[c]
CAI4-2312 (*CaMDR*/*CaMDR*)	3	3.3×10^4	4.2×10^3	1 (0.02)
	6	8.6×10^4	1.1×10^4	10 (0.09)
	9	1.5×10^6, 2.2×10^3[d]	1.2×10^5	46 (0.04)
CAI4-116 (*Camdr*/*Camdr*)	3	4.3×10^3	6.2×10^2	0
	6	3.1×10^4	1.9×10^3	0
	9	2.7×10^5	1.5×10^4	3 (0.02)
	30	0[e]	NA[f]	NA
CAI4-01 (*CaMDR*/*Camdr*)	3	2.9×10^3, 8.6×10^1[d]	2.5×10^2	0
	6	1.1×10^4, 6.9×10^1[d]	9.9×10^2	2 (0.20)
	9	1.6×10^5, 1.3×10^3[d]	1.3×10^4	5 (0.04)
	30	9.9×10^3, 0[g]	1.0×10^3	1 (0.10)

[a] CFU of *C. albicans* per gram of total kidney tissue recovered from homogenized kidneys from two mice. The CFU values for each mouse necropsied were determined separately, and the values given are averages of the values for the two mice. Each value was within 0.7 log units unless otherwise stated.
[b] Kidney homogenates were diluted 1:20 and plated on yeast minimal medium (plus 0.45 mM uridine), with and without 0.1% 5-fluoroorotic acid (5-FOA).
[c] Numbers in parentheses are percentages of cells plated that were resistant to 5-fluoorotic acid (5-FOA).
[d] Numbers of CFU obtained from each of two mice, since the number of CFU obtained from each mouse varied by more than 0.7 log units.
[e] No *C. albicans* was recovered from the only surviving mouse available for necropsy.
[f] NA, not applicable.
[g] Two surviving mice were necropsied. One yielded no *C. albicans.*

petri plates containing YPD medium with 2% agar, the plates were incubated for 1 or 2 days, and the CFU were counted.

The results indicate that *CaMDR1* inactivation leads to dramatic attenuation of virulence in both immunocompetent and immunocompromised mice (Fig. 2B). There were no survivors after 9 days among mice infected with the parental strain, whereas some mice survived infection with the *Camdr1*/*Camdr1* strain for at least 29 days. A two- to threefold increase in the length of survival of 50% of the animals was noticed in tests with normal mice, and a more striking effect, that of a sixfold increase in survival, was seen in tests in which neutropenic mice infected with *Camdr1*/*Camdr1* were compared with parental strains. Infection with the heterozygous disruptant resulted in an intermediate level of survival. Reconstitution of *CaMDR1* in the homozygous disruptant restored virulence (data not shown). Similar effects were noted previously regarding the drug sensitivity of heterozygous versus homozygous *CaMDR1* disruptants, in that homozygous disruptants were sensitive to drugs, heterozygous disruptants were partially sensitive to drugs, and parental strains were drug resistant (3, 7, 10).

Remarkably, a large number of CFU was recoverable from the kidneys of mice infected with *CaMDR1*/*Camdr1* and *Camdr1*/*Camdr1* strains before the mice were moribund, although variability in CFU recovered was noted (Table 1). *C. albicans* cells recovered from kidneys were identical to the infecting strain, as indicated by the maintenance of 5-fluoroorotic acid sensitivity (Table 1). The wild-type strain caused 100% death of neutropenic mice by 4 days after infection, whereas the disruptant-infected mice survived for more than 14 days in repeated experiments (Fig. 2B). However, the survival rate of the host did not reflect the clearance of the pathogen or a significant decrease in the CFU in the kidneys of the infected host. One explanation for this phenomenon is that the host responds in a unique way by containing the infectious particles of the disruptants in nodules in the kidneys (Fig. 2A). Homogenization of the tissue for counting of CFU released large numbers of viable organisms entrapped in the nodules. A similar containment phenomenon in lung tissue has been observed for aspergilloma and in mycobacterial infections (18, 26).

There are several plausible ways to explain the reduced virulence of *C. albicans Camdr1* mutants. Perhaps a specific protective host response is elicited against *C. albicans* impaired in *CaMDR1*, as indicated by the nodular containment of the mutants from the host. In addition, it is possible that *Camdr1* mutants lack the ability to elicit host responses that are necessary for the pathogenic process. For example, pathology may be engendered by the interaction of host factors with specific substances secreted by *C. albicans* through an MDR-mediated pathway (6). The inability to secrete these substances in a *Camdr1* mutant would reduce the pathogenicity of these organisms. In this model, the host response would actually contribute to the pathological process, as indicated by the observation that *Camdr1* mutants colonize but are deficient in causing mortality.

The emphasis of many studies on MDR is currently focused on drugs to impair the MDR in order to overcome MDR-mediated resistance of cancer cells, procaryotes, and protozoans (22). The results of our studies suggest another therapeutic approach to fungal infections, i.e., the potential for inhibiting specific fungal efflux pumps to render the pathogen less virulent.

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