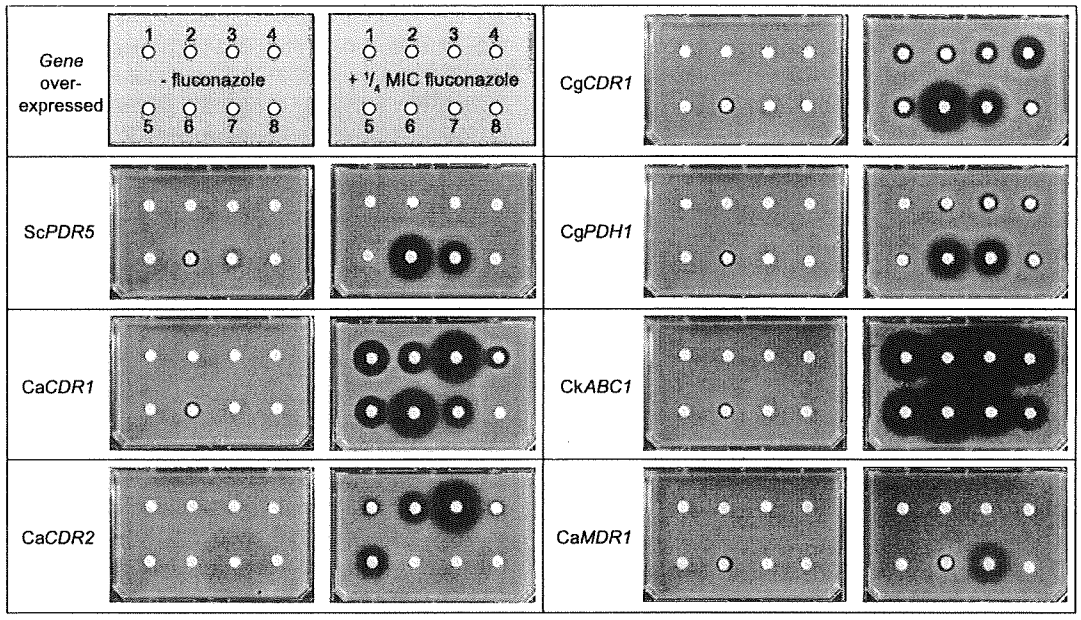

FIG. 8. Effects of ABC drug pump inhibitors. AD1-8u$^-$ cells overexpressing fungal ABC drug pumps ScPdr5p, CaCdr1Ap, CaCdr2Ap, CgCdr1p, CgPdh1p or CkAbc1p, or the control strain overexpressing the MFS drug pump CaMdr1Ap, were analyzed by agarose diffusion assays using CSM solidified with 0.6% agarose and containing either no FLC (left hand plates; control) or FLC at 0.25× MIC_{FLC} for each test strain, as described in Materials and Methods. Whatman 3MM paper disks containing the indicated amounts of drug pump inhibitors (1, 5 μg milbemycin α11; 2, 5 μg milbemycin α20; 3, 5 μg milbemycin α25; 4, 5 μg milbemycin β9; 5, 5 μg milbemycin β11; 6, 5 μg enniatin; 7, 25 μg FK506; 8, 50 nmol oligomycin) were placed on each plate.

pressing drug efflux pumps was determined using agarose diffusion assays (Fig. 8). Toxic effects of the test compounds on cells were identified using assays carried out in the absence of FLC. A strain overexpressing the structurally unrelated, membrane potential-dependent MFS drug efflux pump CaMdr1Ap provided a counterscreen to identify growth-inhibitory effects of a compound on cells in the presence of FLC that were independent of the ABC transporters. Although the ScPdr5p, CaCdr1Ap, CaCdr2Ap, CgCdr1p, and CkAbc1p transporters have highly similar primary sequences (≥50% similarity) and appear to produce efflux of a comparable range of substrates (Fig. 3), each overexpressing strain showed a unique pattern of responses to the panel of eight compounds (Fig. 8). Of the compounds tested, only enniatin and FK506 gave detectable toxicity with *S. cerevisiae* cells in the absence of FLC (Fig. 8). Enniatin weakly inhibited the growth of all the overexpressing strains tested except AD/CaCDR2A. In the presence of FLC, enniatin dramatically enhanced the growth inhibition of all the overexpressing strains except AD/CaCDR2A and AD/CaMDR1A. This indicated specific inhibition by enniatin of the in vivo activities of all the ABC transporters tested except CaCdr2Ap. In the absence of FLC, FK506 caused barely detectable toxicity with all the strains except AD/CaMDR1A. In the presence of FLC, FK506 appeared to chemosensitize cell growth in all strains except AD/CaCDR2A. The lack of inhibition of AD/CaMDR1A in the absence of FLC and its growth inhibition in the presence of FLC indicate that the effects of FK506 are more complex and are consistent with known effects of FK506 (34, 53), including its interaction with the calcineurin stress response pathway (31), its known inhibitory effects on CaCdr1p (19) and ScPdr5p (17), and its poor inhibition of CaCdr2p in vitro (Table 4). In the absence of FLC, the five milbemycins tested were not toxic to the overexpressing cells, and none of them chemosensitized AD/CaMDR1A to FLC. In the presence of FLC, each strain overexpressing an ABC transporter responded differently to the milbemycins. Both α and β milbemycins significantly inhibited the ABC transporters CaCdr1Ap, CaCdr2Ap, CgCdr1p, CgPdh1p, and CkAbc1p but not ScPdr5p. Of the affected ABC transporters, CkAbc1p was most strongly inhibited by all milbemycins tested. CaCdr1Ap was strongly inhibited by only milbemycin α25 and was more modestly inhibited by the remaining milbemycins, with β9 showing the weakest effect. CaCdr1Ap and CaCdr2Ap showed comparable inhibition profiles for the α25, α20, and β11 milbemycins, but α11 or β9 was ineffective against CaCdr2Ap. In contrast, the FLC efflux activity of CgCdr1p was modestly inhibited by milbemycin β9 and weakly by the remaining milbemycins. Its homolog, CgPdh1p, was weakly chemosensitized by only three (α20, α25, and β9) of the five milbemycins tested. Oligomycin affected the in vivo pump function of only three of the seven ABC transporters. It strongly inhibited FLC efflux by AD/CkABC1 and, as expected, weakly inhibited AD/CgCDR1 and AD/CgPDH1 (61). With the exception of CaCdr2Ap, which is resistant to oligomycin, this result is in contrast with the in vitro inhibition at submicromolar concentrations of all the efflux pumps, including ScPdr5p and CaCdr1Ap (Table 4).

DISCUSSION

This study investigated the functions of membrane proteins involved in fungal drug resistance. It used a membrane protein hyperexpression system that, unlike plasmid-based overexpres-

sion systems, drives the constitutive overexpression of heterologous membrane proteins from the chromosomal *PDR5* gene promoter with the help of the gain-of-function mutant transcription factor Pdr1-3p. Pdr1p and Pdr3p are two transcription factors regulating the expression of a set of genes involved in membrane protein biosynthesis and play a key role in the pleiotropic drug resistance (PDR) network (3, 14, 23, 32, 66). The *pdr1-3* mutation in AD1-8u$^-$, together with the disruption of *pdr3*, lead to the constitutive hyperexpression of *PDR5* and the coordinated overexpression of other members of the PDR gene network (10, 14). The *S. cerevisiae* host strain AD1-8u$^-$ has been deleted in seven of its major ABC transporters. This makes it exquisitely sensitive to a wide range of xenobiotics and reduces the background of large proteins in the PM fractions that might interfere with the biochemical analysis and purification of the similarly sized overexpressed heterologous ABC drug pumps (13, 36, 38, 40). These features facilitated our study of proteins conferring drug resistance on *S. cerevisiae*. It is possible that the genetic backgrounds of AD1-8u$^-$ and ADΔ or the overexpression of certain efflux pumps affects PM composition, and this may in turn affect membrane protein function, but this still permits comparison of the properties of heterologous membrane proteins. In addition, our data are consistent with reports of known drug pump substrates, including those for the human MDR pump HsAbcb1p (22). The fact that we found additional pump substrates, for example, KTZ for CaMdr1p, is likely due to the increased sensitivity of the expression system.

In applying this hyperexpression system to the analysis of fungal drug resistance, a panel of strains that overexpress ABC- and MFS-type transporters and the azole drug target Erg11p from a range of fungal pathogens has been created. This panel of strains was used to search for broad-spectrum chemosensitizers and will be an invaluable tool for a variety of other purposes. The ability to determine the substrate specificities of multidrug efflux pumps involved in resistance to azole drugs (Fig. 3 and 8) (61) and to characterize these pumps in vitro (Fig. 6; Table 4) (62) will help elucidate structure-function relationships of proteins that are normally difficult to distinguish from a background of related molecules. The ability to rapidly clone and express individual alleles at equivalent levels enables the study of the effects of single-nucleotide polymorphisms in ABC transporters, such as CaCdr1p, CaCdr2p, or HsAbcb1p, on pump function and substrate specificity (26, 27). The panel of pump-expressing strains provides a powerful counterscreen to identify drug candidates that are potential pump substrates and would quickly lead to the development of MDR (41). These drug candidates can then be eliminated from the drug discovery pipeline. The same panel of pump-expressing strains can be used to identify and characterize new classes of antifungal chemosensitizers that specifically target the pumps responsible for the MDR phenotype of fungal pathogens (Fig. 8) (37, 40). The panel of overexpressing strains is currently being extended to include drug pumps and azole targets from other human fungal pathogens, such as *Aspergillus fumigatus*.

Stable functional overexpression of membrane proteins can be used to determine the effectiveness of new drugs against targets such as the azole drug target Erg11p. For example, the overexpression of CaErg11Ap gave moderate resistance to ITC and MCZ but a high level of resistance to KTZ. This result indicates MCZ and ITC are much more effective inhibitors of CaErg11Ap than KTZ. Finally, the ability to express functionally active His-tagged membrane proteins and their ease of affinity purification will facilitate their structural analysis. A similar expression and affinity purification strategy was recently combined with anion exchange chromatography to isolate enzymatically active CaCdr1p-His from a crude membrane fraction (54).

Application of the yeast expression system to the analysis of different classes of membrane protein involved in drug resistance has highlighted features that allow consistent expression. Unlike the case with other expression systems, such as that of *Pichia pastoris*, nearly all the transformants we obtained with the same construct showed identical phenotypes. This made the cloning process much more rapid and robust. The integration of a single copy of the expression cassette into the chromosomal *PDR5* locus was sufficient to obtain high levels of protein expression, probably because the induction of multiple genes by the Pdr1-3 transcription factor conditions the system at the transcriptional, translational and posttranslational levels (14, 39) for efficient delivery of membrane proteins to the PM. Other hyperexpression systems based on a yeast multicopy plasmid (19) may overload the posttranslational secretory machinery and lead to protein turnover or sequestration of proteins in internal membranes, analogous to the inclusion bodies found with the overexpression of certain proteins in *E. coli*. Often expression of membrane proteins encoded on multicopy plasmids can result in relatively low levels of functional protein expression in the PM that confer only modest phenotypes (27). Expression of CaCdr1p in our system increased ITC resistance 1,000-fold. In some cases we see GFP-tagged material in structures reminiscent of stacks of smooth PM or karmellae that are induced when membrane proteins are overexpressed. While incorrect trafficking appeared to be enhanced for the ABC transporters that are most phylogenetically distant from *S. cerevisiae*, it has not yet been possible to determine whether this reflects the normal distribution of the untagged recombinant protein or is an adventitious consequence of the GFP tag (56).

In an initial attempt to extend our analysis to membrane proteins from higher eukaryotes involved in drug pumping, we functionally overexpressed human HsAbcb1p at 3.3% of PM protein. This made the AD1-8u$^-$ host strain 40 times more resistant to ITC and 16-fold more resistant to the anticancer drug daunorubicin, a more significant change in phenotype than the 2-fold increase seen with another *S. cerevisiae* expression system (27). If genetic distance does reduce the yield of heterologously expressed membrane protein because of differences in biosynthetic, quality control, and trafficking machineries, codon optimization strategies could be used to overcome limitations for transcription and translation that may also affect protein function (30). In addition, the genetic tractability of *S. cerevisiae* opens many avenues for the improved expression and correct delivery of functional membrane proteins. For example, hosts selected for the expression of high levels of an endogenous membrane protein from a particular functional class may provide optimal vehicles for the heterologous expression of related membrane proteins. Improved hosts could be generated by directed modification of genes, such as those in-

volved in the unfolded protein response and protein turnover pathways, or by selecting extragenic mutations that enhance the relevant phenotypes of targets currently expressed at modest levels. Our system for constitutive protein overexpression in a background depleted of efflux pumps provides extra dimensions for chemogenomic screens that aim to identify both the on- and off-target effects of drugs (12). If the system was further modified to allow regulated expression of the heterologous protein, then it should be possible to overexpress lethal targets and proteins involved in cell cycle check points (58). Finally, the expression of regulated membrane proteins within defined physiological windows could be used to minimize protein microheterogeneity and make them more amenable to structural analysis by X-ray crystallography. We expect this expression system, therefore, to be a valuable addition to current technologies that will enable a more systematic study of a variety of fungal, plant, and human membrane proteins, including targets for drug discovery.

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NOTE

Is *Histoplasma capsulatum* a native inhabitant of Japan?

Ken Kikuchi[1,2], Takashi Sugita[3], Koichi Makimura[4], Kensaku Urata[5], Takashi Someya[6], Takashi Sasaki[1], Katsuhiko Kamei[7], Masakazu Niimi[8], Keiichi Hiramatsu[1] and Yoshimasa Uehara[8,*]

[1]Department of Infection Control Science, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, [2]Department of Infectious Diseases, Tokyo Women's Medical University School of Medicine, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 160-8666, [3]Department of Microbiology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, [4]Teikyo University Institute of Medical Mycology, 359 Ohtsuka, Hachioji, Tokyo 192-0395, [5]The Institute of Science and Technology, Osaka University of Economics and Law, 6-10 Gakuonji, Yao, Osaka 581-8511, [6]Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, 1 Honjo-machi, Saga, Saga 840-8502, [7]Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chu-o-ku, Chiba 260-8673, [8]Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Tokyo 162-8640, Japan

ABSTRACT

Histoplasmosis is an infectious disease caused by inhaling spores of the fungal pathogen *H. capsulatum* and in Japan is considered an imported mycosis. However, some patients in Japan with histoplasmosis have no history of traveling overseas nor of risk of occupational exposure to *Histoplasma*. To investigate the possibility of native distribution of *Histoplasma* in Japan, 187 bat guano samples from 67 bat-inhabited caves in 17 prefectures were collected. These were examined for *H. capsulatum* by culture and *Histoplasma*-specific PCR in three independent laboratories. No *H. capsulatum* was detected by either method, therefore *H. capsulatum* is unlikely to be present in bat guano in Japanese caves.

Key words bat guano, bat-inhabited caves, *Histoplasma capsulatum*

Histoplasmosis, a systemic mycosis caused by the dimorphic fungus *H. capsulatum*, is distributed worldwide and is endemic to North, Central and South America (1, 2). In Japan, twenty-eight (82.4%) of thirty-four cases of histoplasmosis have been reported among travelers returning from histoplasmosis-endemic areas (3). However, the remaining six of these patients (17.6%) were native-born Japanese who had not been outside Japan, and had not been subject to any risk of occupational exposure to *Histoplasma*-contaminated materials, such as imported soil or clay (3). These six cases occurred in Niigata, Gunma, Osaka (three cases), and Okayama, but the addresses at which these patients lived could not be ascertained.

Although *H. capsulatum* has never been isolated from the natural environment in Japan, there have been no systematic ecological studies for this fungus. The most common source of *H. capsulatum* in nature is bat guano. Indeed among speleologists acute pulmonary histoplasmosis has been termed a "cave sickness" because it occurs after exposure to *Histoplasma*-contaminated cave environments (4–6). Several recent outbreaks of cave-associated histoplasmosis among cavers have been reported (1, 4, 6, 7). In Japan, the first histoplasmosis case confirmed by positive culture of *H. capsulatum* occurred in the rural area of Okayama city (8), which is near to Atetsudai karst where there are many bat-inhabited limestone caves

***Present address**
Department of Microbial Chemical Biology and Drug Discovery, Iwate Medical University School of Pharmacy, 2-1-1 Nishitokuda, Yahabacho, Shiwa-gun, Iwate 028-3694, Japan

Correspondence
Ken Kikuchi, Department of Infection Control Science, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel: +81 3 3813 3111; fax: +81 3 5684 7830; e-mail: kikuti@med.juntendo.ac.jp

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List of Abbreviations: DNA, deoxyribonucleic acid; *H. capsulatum*, *Histoplasma capsulatum*; PCR, polymerase chain reaction

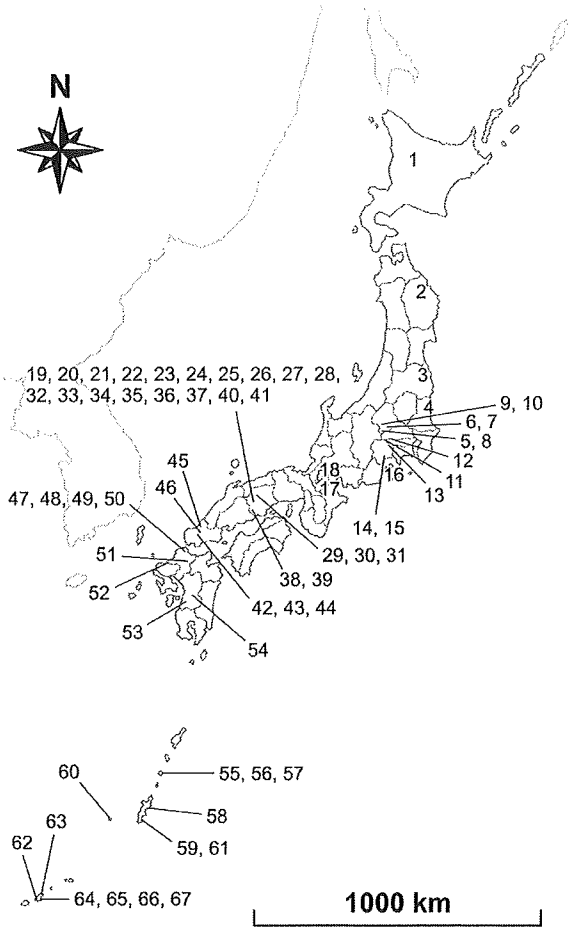

Fig. 1. Locations of the 67 caves investigated in Japan. Information on each numbered cave is shown in Table 1.

(Fig. 1, No. 19~41 caves). The aim of this study was to investigate whether *H. capsulatum* could be isolated from bat guano from Japanese caves.

Between October 2003 and June 2007, 187 bat guano samples were collected from 67 bat-inhabited caves located in 17 Japanese prefectures (Table 1, Fig. 1). Each sample was divided into three portions and analyzed in three independent laboratories (laboratory A: Kikuchi K, Tokyo Women's Medical University between October 2003 and March 2006, thereafter at Juntendo University with Sasaki T, and Hiramatsu K; laboratory B: Sugita T at Meiji Pharmaceutical University; laboratory C: Makimura K at Teikyo University).

In laboratory A, 1 g of each sample was suspended in 5 ml of sterile saline and mixed vigorously. After the suspension had stood at room temperature for 1 hr, the supernatant was collected gently and diluted 100-fold with sterile saline. An aliquot of 100 μl was inoculated onto Mycosel agar (BD, Sparks, MD, USA) containing 10 μg/ml of gentamicin, 10 μg/ml of polymyxin B, and 20 μg/ml of aztreonam. The plates were incubated at 25°C for up to eight weeks. Colorless colonies that became visible after at least one week of incubation were identified morphologically. Genomic DNA was extracted from each bat guano specimen directly using ISOIL for Beads Beating kit (Nippon Gene, Tokyo, Japan). *H. capsulatum*-specific target sequences were PCR-amplified using primer sets (Table 2) as described previously (9–11). Genomic DNA from *H. capsulatum* CBS 137.72 was used as a positive control. All positive PCR-products were directly sequenced and confirmed.

In laboratory B, guano samples were cultured as described previously (12). For DNA extraction, 1 g of the guano sample was incubated in 10 ml of Brain Heart Infusion Medium (EIKEN Chemical, Tokyo, Japan) including 50 μg/ml of streptomycin and penicillin at 27°C for one day. Fungal DNA was extracted using ISOPLANT (Nippon Gene). *H. capsulatum* was detected by a real-time PCR assay with TaqMan probe (Applied Biosystems, Foster City, CA, USA) (Table 2). The primer/probe set is capable of detecting plasmid DNA derived from the *H. capsulatum* ITS region linearly for dilution from 10^1 to 10^9 copies ($r^2 = 0.999$).

In laboratory C, 1 g of guano sample was suspended in 20 ml of distilled water, mixed vigorously for 2 min, and then left at room temperature for 1 hr. Supernatant was transferred to a fresh tube and centrifuged. The 250 μl of pellet suspension was inoculated onto Mycosel agar (BD) and incubated at room temperature for up to eight weeks. Genomic DNA was extracted by the methods of Makimura (13) from white and colorless conidia colonies, and subjected to *H. capsulatum*-specific PCR with primer-pair sets (Table 2).

None of the laboratories detected any *H. capsulatum* by either culture or PCR techniques. In laboratory A, one positive Hc-100 PCR product was recovered from a bat guano specimen collected from Kara-do B cave. DNA sequences of this PCR product were 99% identical to the positive control (198 of 200 bases). However, no other *H. capsulatum*-specific PCR products were detected from the same specimen by laboratory A, B, or C. This PCR product may have been amplified from another environmental fungus which fortuitously had similar sequences to Hc-100 in the nucleotide region tested.

These results suggest that bat guano is not likely to be the native environmental source of *H. capsulatum* in Japan. Many different species of the genus *Chioptera* have been reported to harbor *H. capsulatum* (5, 14). In this study, we examined guano specimens from the following bat species: *Rhinolophus ferrumequinum, Rhinolophus cornutus, Rhinolophus pumilus, Rhinolophus perditus, Plecotus auritus, Hipposideros turpis, Myotis macrodactylus,*

Table 1 List of bat guano collection sites and bat species inhabiting those caves.

No.*	Cave	Geological background	Location	Month/Year sampled	No. of samples	Bat species inhabiting the cave
1	Touma-shonyuudo	limestone	Hokkaido	10/2004	1	Unknown
2	Uchimagi-do	limestone	Iwate	5/2004	2	*R. cornutus, R. ferrumequinum*
3	Tateishi-no-ooana	limestone	Fukushima	12/2003	2	*R. cornutus*
4	Ookubo-no-kazaana	limestone	Ibaragi	10/2003	1	*R. cornutus*
5	Koumori-ana	limestone	Gunma	11/2003	1	Unknown
6	Medama-ana	limestone	Gunma	2/2004	1	*R. cornutus*
7	Nuke-ana	limestone	Gunma	2/2004	1	*R. cornutus*
8	Yakyusawa-do	limestone	Gunma	9/2004	2	*R. ferrumequinum*
9	Shimogou-shonyuudo No.1	limestone	Gunma	10/2004	1	*R. cornutus*
10	Shimogou-shonyuudo No.2	limestone	Gunma	10/2004	1	*R. cornutus*
11	Bussekisan-shonyuudo	limestone	Saitama	1/2004	3	*R. ferrumequinum, R. cornutus, M. macrodactylus*
12	Nippara-shonyuudo	limestone	Tokyo	10/2003	3	*R. ferrumequinum*
13	Aoiwa-shonyuudo	limestone	Yamanashi	11/2003	1	*R. ferrumequinum*
14	Jinza-fuketsu Kawagoe	volcano	Yamanashi	11/2003	1	*R. cornutus, R. ferrumequinum*
15	Jinza-fuketsu No. 10	volcano	Yamanashi	11/2003	1	*R. cornutus, R. ferrumequinum*
16	Muroiwa-do	abandoned mine	Shizuoka	9/2004	2	*R. cornutus, R. ferrumequinum*
17	Kiri-ana	limestone	Mie	12/2003	1	Unknown
18	Kawachi-fuketsu	limestone	Shiga	5/2004	1	*R. cornutus, R. ferrumequinum, M. macrodactylus, M. leucogaster, M. fuliginosus*
19	Himesaka-kamachi-ana	limestone	Okayama	9/2006	5	*R. ferrumequinum*
20	Iwaya-no-ana	limestone	Okayama	9/2006	4	Unknown
21	Ikura-do	limestone	Okayama	9/2006	3	*R. ferrumequinum*
22	Gonbouzone-no-ana	limestone	Okayama	9/2006	2	Unknown
23	Futatsuki-no-ana	limestone	Okayama	9/2006	7	*R. ferrumequinum*
24	Kimen-do	limestone	Okayama	9/2006	4	*M. fuliginosus*
25	Karamatsu-koumori-ana	limestone	Okayama	9/2006	5	*R. ferrumequinum, R. cornutus, M. fuliginosus*
26	Shogo-no-ana	limestone	Okayama	9/2006	1	Unknown
27	Tsuchihashi-no-ana	limestone	Okayama	9/2006	8	*R. ferrumequinum*
28	Maki-do	limestone	Okayama	9/2006	2	*R. ferrumequinum*
29	Kanba-no-oni-no-ana	limestone	Okayama	9/2006	4	*R. ferrumequinum, M. fuliginosus*
30	Koujiro-no-oni-no-ana	limestone	Okayama	9/2006	3	*R. ferrumequinum, R. cornutus, M. leucogaster*
31	Koujiro-no-oni-no-ana No.2	limestone	Okayama	9/2006	1	Unknown
32	Kazato-no-ana	limestone	Okayama	9/2006	4	*R. cornutus*
33	Iama-no-ana	limestone	Okayama	9/2006	3	Unknown
34	Mitsugi-no-ana	limestone	Okayama	9/2006	5	*R. ferrumequinum*
35	Bicchu-kanachi-ana	limestone	Okayama	9/2006	3	*R. ferrumequinum, R. cornutus*
36	Iya-no-ana	limestone	Okayama	9/2006	2	Unknown
37	Uyama-do	limestone	Okayama	9/2006	4	*R. ferrumequinum*
38	Diamond cave	limestone	Okayama	9/2006	4	*R. ferrumequinum, P. auritus, M. ussuriensis*
39	Ja-no-ana	limestone	Okayama	9/2006	3	*R. ferrumequinum, M. fuliginosus*
40	Hongoya-no-ana	limestone	Okayama	9/2006	6	Unknown
41	Ushioigoya-no-ana	limestone	Okayama	9/2006	5	Unknown
42	Akiyoshi-do	limestone	Yamaguchi	12/2003	11	*R. ferrumequinum, R. cornutus, M. nattereri, M. fuliginosus*
43	Koumori-ana	limestone	Yamaguchi	12/2003	5	*R. cornutus, R. ferrumequinum*
44	Ookubo-no-koana	limestone	Yamaguchi	12/2003	2	*R. cornutus, R. ferrumequinum*
45	Sazare-do	limestone	Yamaguchi	1,3/2003	4	*R. cornutus*
46	Naganobori mine	abandoned mine	Yamaguchi	2/2004	2	*R. cornutus, R. ferrumequinum*

Table 1 Continued.

No.*	Cave	Geological background	Location	Month/Year sampled	No. of samples	Bat species inhabiting the cave
47	Senbutsu-shonyudo	limestone	Fukuoka	12/2003	7	*R. ferrumequinum*
48	Mejiro-do	limestone	Fukuoka	12/2003	3	*R. ferrumequinum*
49	Seiryu-kutsu	limestone	Fukuoka	12/2003	6	*R. cornutus, R. ferrumequinum, M. fuliginosus*
50	Komusou-ana	limestone	Fukuoka	1/2004	1	*R. cornutus*
51	Goya-shonyudo No.1	limestone	Fukuoka	1/2004	1	*R. ferrumequinum*
52	Mizunashi-shonyudo No.2	limestone	Fukuoka	3,8/2004	4	*R. cornutus, R. ferrumequinum*
53	Kyuusen-do	limestone	Kumamoto	12/2003	1	*R. ferrumequinum*
54	Tsuzurase-do	limestone	Kumamoto	1/2004,2/2005	7	*R. cornutus, R. ferrumequinum, M. fuliginosus*
55	Shouryuu-do	limestone	Kagoshima	1/2004	1	*R. pumilus*
56	Ooyama-suikyo-do	limestone	Kagoshima	1/2004	1	Unknown
57	Ginsui-do	limestone	Kagoshima	9/2004	2	*R. pumilus*
58	Dosuikan tunnel	abandoned tunnel	Okinawa	2/2004	1	*M. fuscus, R. pumilus*
59	Hananta-gou	limestone	Okinawa	4/2004	1	*R. pumilus*
60	Yaja-gama	limestone	Okinawa	4/2004	1	*M. fuscus, R. pumilus*
61	Keimushomae-no-ana	limestone	Okinawa	4/2004	1	*R. pumilus*
62	Tabata-gama No.2	limestone	Okinawa	5/2004	1	*H. turpis*
63	Hirano-do	limestone	Okinawa	7/2004	1	*H. turpis*
64	Kara-do A	limestone	Okinawa	9/2007	2	*R. perditus, H. turpis, M. fuscus*
65	Kara-do B	limestone	Okinawa	9/2006,6/2007	4	*R. perditus, H. turpis, M. fuscus*
66	Kara-do D	limestone	Okinawa	6/2007	1	*R. perditus, H. turpis, M. fuscus*
67	Kara-do E	limestone	Okinawa	9/2006,6/2007	2	*R. perditus, H. turpis, M. fuscus*
Total no.					187	

*The location of each cave is shown on the map in Figure 1.

Table 2 List of the primers used for PCR experiments

PCR		Primer name	Sequence	Target	Laboratory performed	Reference
nested PCR	1st PCR	fungus-I fungus-II	GTTAAAAAGCTCGTAGTT TCCCTAGTCGGCATAGTTTA	18S rRNA gene	A	(9)
	nested PCR	Histo-I Histo-II	GGCCGGACCTTTCCTCCTGGGGAGC CAAGAATTTCACCTCTGACAGCCGA			
Hc100 (nested PCR)	1st PCR	Hc-I Hc-II	GCGTTCCGAGCCTTCCACCTCAAC ATGTCCCATCGGGCGCCGTGTAGT	100 kDa protein gene	A	(9)
	nested PCR	Hc-III Hc-IV	GAGATCTTAGTCGCGGCCAGGTTCA AGGAGAGAACTGTATCGGTGGCTTG			
H-antigen (hemi-nested PCR)	1st PCR	Hc2 Hc3	GCGGGGTTGGCTCTGCTCT TTGGAAACCCCGGGCTTG	H-antigen gene	A	(10)
	hemi-nested PCR	Hc2 Hc1	GCGGGGTTGGCTCTGCTCT TCATAGTAGGCTGTTCACCCCCG			
M-antigen (2 different PCR)	PCR1	Msp1F Msp1R	ACAAGAGACGACGGTAGCTTCACG GCGTTGGGGATCAAGCGATGAGCC	M-antigen gene	A	(11)
	PCR2	Msp2F Msp2R	CGGGCCGCGTTTAACAGCGCC ACCAGCGGCCATAAGGACGTC	M-antigen gene	A	(11)
Real-time PCR		H1F H1R TaqManH	GGCCACCCTTGTCTACCGG GCCAATCGTTCACCGACG AGCTTCTCCTCCCCGGG	ITS	B	this study
PCR		Hs1F Hs1R	GAGAGACGGCTACCACATCT CAGTGAAGGCCATGAGGCTC	18S rRNA gene	C	this study

Myotis nattereri, *Murina leucogaster*, *Murina ussuriensis*, *Miniopterus fuscus*, and *Miniopterus fuliginosus*, and *H. capsulatum* was not detected. It may be that the above bat species are not the native hosts of *H. capsulatum*. The carriage of *H. capsulatum* by a different species of bat or a different environmental niche for *H. capsulatum* in Japan cannot be excluded.

As the incidence of histoplasmosis has increased in Japan (3), further work is needed to determine the native source of *H. capsulatum* in order to improve control of the disease.

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Identification of the Putative Protein Phosphatase Gene *PTC1* as a Virulence-Related Gene Using a Silkworm Model of *Candida albicans* Infection▽†

Nozomu Hanaoka,[1,2] Yukie Takano,[1] Kazutoshi Shibuya,[3] Hajime Fugo,[2] Yoshimasa Uehara,[1]§ and Masakazu Niimi[1]*

Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640,[1] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-City, Tokyo 183-8509,[2] and Department of Pathology, Toho University School of Medicine, 6-11-1 Omori Nishi, Ota-ku, Tokyo,[3] Japan

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Protein phosphatases are critical for the regulation of many cellular processes. Null mutants of 21 putative protein phosphatases of *Candida albicans* were constructed by consecutive allele replacement using the *URA3* and *ARG4* marker genes. A simple silkworm model of *C. albicans* infection was used to screen the panel of mutants. Four null mutant (*cmp1*Δ, *yvh1*Δ, *sit4*Δ, and *ptc1*Δ) strains showed attenuated virulence in the silkworm model relative to that of control and parental strains. Three of the mutants, the *cmp1*Δ, *yvh1*Δ, and *sit4*Δ mutants, had previously been identified as affecting virulence in a conventional mouse model, indicating the validity of the silkworm model screen. Disruption of the putative protein phosphatase gene *PTC1* of *C. albicans*, which has 52% identity to the *Saccharomyces cerevisiae* type 2C protein phosphatase *PTC1*, significantly reduced virulence in the silkworm model. The mutant was also avirulent in a mouse model of disseminated candidiasis. Reintroducing either of the *C. albicans PTC1* alleles into the disruptant strain, using a cassette containing either allele under the control of a constitutive *ACT1* promoter, restored virulence in both infection models. Characterization of *ptc1*Δ revealed other phenotypic traits, including reduced hyphal growth in vitro and in vivo, and reduced extracellular proteolytic activity. We conclude that *PTC1* may contribute to pathogenicity in *C. albicans*.

The opportunistic fungal pathogen *Candida albicans*, a member of the normal human microflora, can cause superficial or life-threatening systemic infections, particularly in immunocompromised patients. Virulence determinants of *C. albicans* include the yeast-to-hypha transition, adherence to host receptors, and the ability to produce a variety of secreted hydrolytic enzymes, such as aspartic proteinases, phospholipases, and lipases. In addition, genes encoding metabolism and stress response proteins of this pathogen are important for pathogenicity (9, 16, 22).

It would be of interest to determine whether other, previously uncharacterized *C. albicans* genes have a role in virulence. However, screening of whole families of *C. albicans* genes for virulence-related properties using a mammalian model of infection, such as the mouse model, would pose issues both of ethics and of practical costs. A number of substitute, invertebrate models of microbial virulence more suitable for screening experiments have been developed, including the use of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Galleria mellonella* (10, 18, 35, 41), silkworms (20), and locusts (32). The silkworm model of *C. albicans* infection has been used for the quantitative evaluation of antifungal agents, with results equivalent to those in a mouse model. In addition, the silkworm infection model has been used successfully to identify and evaluate uncharacterized genes required for the virulence of *Staphylococcus aureus* (28, 29).

The fungus must adapt to stresses encountered in vivo, such as various host defense mechanisms and/or microenvironmental changes in pH, oxygen concentration, or nutritional status during the progress of an infection (5, 11). One of the major mechanisms governing the regulation of a wide variety of cellular processes is reversible protein phosphorylation, catalyzed by protein kinases and phosphatases. Protein phosphatases are a family of enzymes that catalyze the phosphate hydrolysis of phosphoproteins and can be classified, based on sequence similarity and substrate specificity, into three groups: phosphoserine/phosphothreonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases. The group of *Saccharomyces cerevisiae* protein phosphatases comprises more than 30 members, which have been extensively studied (26, 38, 56). Seven *C. albicans* genes have been identified as protein phosphatases, including the well-characterized type 2B-related serine/threonine protein phosphatase calcineurin complex *CMP1* (also known as *CNA1*), which plays key roles in virulence (2, 3, 6, 50), sensitivity to antifungal drugs, and control of morphogenesis (50). Furthermore, a deletion mutant of *cnb1*, which encodes a regulatory subunit of calcineurin, has significantly attenuated virulence in a mouse model of *C. albicans* infection

* Corresponding author. Mailing address: Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1272. E-mail: niimi@nih.go.jp.

§ Present address: Department of Microbial Chemical Biology and Drug Discovery, Iwate Medical University School of Pharmacy, 2-1-1 Nishitokuda, Yahabacho, Shiwa-gun, Iwate 028-3694, Japan.

† Supplemental material for this article may be found at http://ec.asm.org/.

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Reference
CAI4	SC5314 (clinical isolate)	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	17
TUA4	CAI4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200*	30
TUA5	TUA4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*ARG4*	21
TUA6	TUA5	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*ARG4 RP10*::p3HA-ACT1(*URA3*)	21
PTC101	TUA4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200–URA3–hph200*/*PTC1*	This study
PTC102	PTC101	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200–URA3–hph200*/*ptc1*Δ::*ARG4*	This study
PTC103	PTC102	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200*/*ptc1*Δ::*ARG4*	This study
PTC1A	PTC103	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200*/*ptc1*Δ::*ARG4 RP10*::pCaPTC1A(*URA3*)	This study
PTC1B	PTC103	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200*/*ptc1*Δ::*ARG4 RP10*::pCaPTC1B(*URA3*)	This study
PTC1C	PTC103	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 arg4*Δ::*hisG200*/*arg4*Δ::*hisG200 ptc1*Δ::*hph200*/*ptc1*Δ::*ARG4 RP10*::p3HA-ACT1(*URA3*)	This study

(6). Other *C. albicans* genes identified as protein phosphatases include *CDC14* (13), *CYR1* (24, 36), *CPP1* (14), *PTC7* (25), *SIT4* (34), and *YVH1* (21). *SIT4* and *YVH1* have been implicated in the control of virulence-related genes.

In this study, 21 null mutants of putative protein phosphatases were screened using the silkworm model of *C. albicans* infection. Four of these mutants (*cmp1*Δ, *sit4*Δ, *yvh1*Δ, and *ptc1*Δ) possessed significantly attenuated virulence. The *C. albicans PTC1* gene has not been described previously. In *S. cerevisiae*, the *PTC1* gene encodes a type 2C Ser/Thr-specific phosphatase, which acts by dephosphorylating the mitogen-activated protein kinase Hog1p (19). Here we have analyzed the phenotypes of *C. albicans PTC1* disruptant and revertant strains, with particular reference to two known virulence-related properties of *C. albicans*: the yeast-to-hypha transition and the production of secreted hydrolytic enzymes. We have also examined the effect of disrupting *C. albicans PTC1* on susceptibility to antifungal drugs and environmental stressors. Our results suggest that *PTC1* may contribute to pathogenicity in *C. albicans*, and we also propose that the silkworm infection model provides a simple technique for screening virulence-related genes of this pathogenic fungus.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used or developed during cloning and disruption of the *C. albicans PTC1* gene are listed in Table 1. Disruption of 20 other putative phosphatase genes of *C. albicans* (strains not listed) was attempted using the methods described below for *C. albicans PTC1*. *Escherichia coli* XL-1 Blue and cloning vector pUC19 were used for DNA manipulation. SD-URA medium (synthetic dextrose medium without uracil) containing 0.67% yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI), 2% dextrose, and 0.072% CSM-URA (complete synthetic mixture without uracil) (QBiogene, Irvine, CA) was used for *C. albicans* transformation. For examination of *C. albicans* morphology, yeast or hyphal cells were produced by manipulation of the medium composition and temperature as follows. Yeast cells were produced in liquid medium by growth in 1% yeast extract–2% peptone–2% dextrose (YPD, pH 5.6; QBiogene) or SD-AU (0.67% YNB without amino acids–2% dextrose–0.072% CSM-ARG-URA [complete synthetic mixture without arginine and uracil] [QBiogene]) at 30°C with shaking. Yeast growth was assessed by measuring the optical densities at 600 nm (OD_{600}) of cultures. Hyphal cells were produced in liquid medium from a yeast cell inoculum by growth in YPD (pH 7.2) plus 10% filtered, heat-inactivated bovine serum (GIBCO Invitrogen [Carlsbad, CA] cell culture) at 37°C with shaking. Hyphal growth was assessed by measuring the length of hyphae for 100 cells in three separate experiments. Filamentous growth on solid medium was obtained by inoculating agar plates (2%) containing 10% filtered, heat-inactivated bovine serum and incubating at 37°C for 7 days. Microscopic observation was performed by using a conventional fluorescence microscope (IX81; Olympus, Tokyo, Japan) equipped with a DP70 digital camera (Olympus).

Disruption of *PTC1* in *C. albicans* and plasmid construction for the generation of revertant strains expressing either *PTC1* allele. The primers used for *PTC1* are listed in Table S1 in the supplemental material. The primers used for the attempted disruption of 25 other putative phosphatase genes are listed in Table S2 in the supplemental material. The nucleotide sequences of cloned fragments were confirmed using an Applied Biosystems (Foster City, CA) genetic analyzer, model 3130. *C. albicans* was transformed as described by Umeyama et al. (53). *C. albicans PTC1* was disrupted in the *ura*$^-$ *arg*$^-$ strain TUA4 by using a strategy described previously (21). Briefly, two auxotrophic markers, *URA3* and *ARG4*, were used to disrupt both alleles of the *PTC1* gene, producing the null mutant strain PTC103 (see Fig. S1 in the supplemental material). Primers ΔcheckPTC1-3′ and Δcheck-5′ were designed for PCRs to confirm that the expected constructs were obtained (see Fig. S1 in the supplemental material). At least three independently constructed null mutants were produced for each gene, and the consistency of phenotypes (growth rate, colony morphology and yeast-to-hypha transition properties) was confirmed before one of each construct was selected for further analyses. To produce revertant *C. albicans* strains containing either allele (A or B) of *PTC1*, plasmids p3HA-PTC1A and p3HA-PTC1B were constructed as follows. DNA fragments containing either allele A or allele B of *PTC1* were PCR amplified from TUA4 genomic DNA using two primers, PTC1-N and PTC1-C. Following digestion with BamHI and SphI, the fragments were cloned into the BamHI and SphI sites of plasmid p3HA-ACT1 (53) to generate p3HA-PTC1A or p3HA-PTC1B. Then *PTC1* alleles derived either from StuI-digested p3HA-PTC1A or from StuI-digested p3HA-PTC1B were reintegrated at the high-expression *RP10* locus (40) of the null mutant PTC103, generating strain PTC1A or PTC1B. The StuI-digested empty vector p3HA-ACT1 was integrated into strain PTC103 as a null mutant control strain (PTC1C). The reason for choosing the integrative plasmid p3HA-ACT1 containing the *ACT1* promoter, rather than a homologous promoter, was that it provided consistent expression of each of the alleles introduced, allowing direct comparison of the phenotypes of the recombinant strains.

Virulence studies. Animal experiments were performed by following the provisions of the Principles of Morality for animal experiments of the National Institute of Infectious Diseases, Japan. For the mouse model of disseminated *C. albicans* infection, groups of six CD-1 (ICR) mice were inoculated intravenously with 1×10^6 CFU of the indicated *C. albicans* strain (53). Silkworm infection experiments were performed as described previously (20) with slight modifications. Briefly, larvae after the fourth molt, purchased from Ehime Sansyu (Ehime, Japan), were fed with Silkmate (Nihon Nosan Kogyo Corporation, Yokohama, Japan) at 27°C until they developed to fifth-instar larvae. On the second day of the fifth-instar stage, silkworms were anesthetized by contact with ice-cold water prior to inoculation. Late-log-phase *C. albicans* yeast cells grown in YPD were washed and resuspended in phosphate-buffered saline. Portions (0.05 ml) of the cell suspensions containing 1×10^6 CFU were injected into the

hemolymph through the dorsal surface (abdominal segment) of 10 silkworms for each *C. albicans* strain by using a 30-gauge needle. Silkworms were not fed after the injections, and mortality was checked at 24-h intervals. Silkworms were kept at 27°C throughout the experiments. The experiments were repeated twice more (total, 30 silkworms). To determine the morphology of *C. albicans* cells of either wild-type, null mutant, or revertant strains during the progress of silkworm infections, transverse sections of infected silkworms (12 h postinoculation) were examined by microscopy. Silkworms ($n = 5$) were fixed in 10% phosphate-buffered formaldehyde before embedding, transverse sectioning, and staining with periodic acid-Schiff stain.

RNA isolation and real-time PCR. The cells were collected by centrifugation, washed twice with ice-cold water, frozen with liquid nitrogen, and stored at −80°C until use. Total RNA was extracted from the cells resuspended in TES buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% sodium dodecyl sulfate) with acid phenol (Sigma-Aldrich, St. Louis, MO) at 65°C for 45 min. The aqueous-phase solution was purified with acid phenol and then chloroform and was precipitated with ethanol. cDNA was synthesized with SuperScript III reverse transcriptase in a SuperScript III Platinum two-step qRT-PCR kit with Sybr green (Invitrogen, Carlsbad, CA). mRNA expression levels were examined by quantitative real-time PCR (QRT-PCR) using ABI Prism 7000 (Applied Biosystems) and Sybr Premix ExTaq (Takara, Otsu, Japan) and were standardized with *ACT1* mRNA. All primers used for QRT-PCR are listed in Table S1 in the supplemental material.

Measurement of secreted hydrolytic enzyme activities of *C. albicans*. *C. albicans* secreted hydrolytic enzymes were induced in modified medium as previously described by Bramono et al. (7) and Tsuboi et al. (52). Proteinases were induced on a 2% agar plate containing 1.2% yeast carbon base (YCB; Difco) and 0.5% bovine serum albumin at 30°C for 3 days. Lipases were induced on a 2% agar plate containing 0.67% YNB without amino acids (Difco) and 2.5% Tween 80 at 30°C for 3 days. Hydrolytic enzyme activities were measured as the ratio of the diameter of the colony to the total diameter of the colony plus the zone of precipitation according to the method of Price et al. (45).

Susceptibilities of *C. albicans PTC1* mutants to antifungals and chemical and environmental stressors. The antifungals used in this study were terbinafine (Wako Pure Chemical Industries, Osaka, Japan), micafungin (Astellas Pharma Inc., Osaka, Japan), fluconazole (Pfizer), and amphotericin B (Sigma-Aldrich). Chemical stressors included incubation of cultures with the indicated concentrations of NaCl, H_2O_2, and Congo red. Environmental stressors included growth at a high temperature (42°C) and in a medium with a low or a high pH (pH 2.5 or 9.0, respectively). The effects of antifungal drugs or stressors on the yeast cell growth of each *C. albicans* strain were determined using an agar plate dilution assay (43) and a broth microdilution assay adapted from the standard CLSI (formally NCCLS) document M27-A2 (42), in which the medium was inoculated with *C. albicans* yeast cells from a 16-h culture (YPD; pH 5.6; 30°C). For the agar plate dilution assay, the inoculum cells were washed and resuspended in sterile distilled water to an OD_{600} of 0.1 before the suspension was serially diluted 10 times. Five microliters of cell suspensions (containing 10^4, 10^3, 10^2, and 10 CFU, respectively) was spotted onto YPD agar plates containing antifungals or stressors at the indicated concentrations or under the indicated conditions. Cell growth was monitored after incubation at 30°C for 48 h or at the indicated temperature for the indicated period. For the broth microdilution assay, 4×10^3 washed yeast cells were resuspended in YPD (100 μl) and inoculated into YPD (100 μl) containing an antifungal drug or chemical stressor at the concentrations indicated in flat-bottom microtiter plates. Plates were incubated at 30°C for 48 h, and the OD_{595} of the cells was measured using a Beckman Coulter (Fullerton, CA) DTX880 multimode detector.

Statistics. The log rank test was used to analyze survival rates for silkworm and mouse model infections, and the *t* test was used to analyze fungal burdens in the kidneys of infected mice as well as proteinase activities among wild-type, revertant, and null mutant strains.

RESULTS

Identification of 28 putative protein phosphatase genes in *C. albicans* and disruption of 21 of these genes. The presence of at least 28 putative protein phosphatases in *C. albicans* (Table 2) was determined using sequence-searching tools and analysis of the *C. albicans* genome sequence (1) by homology to annotated *S. cerevisiae* protein phosphatases (http://www.yeastgenome.org/), as well as domain searches (consensus patterns) of protein phosphatases using PROSITE (http://au.expasy.org/prosite).

Gene disruption using the split-Ura-blaster technique (17), also used previously for disruption of the *C. albicans YVH1* gene (21), was attempted for 25 of the 28 putative protein phosphatase genes identified. The *CYR1/CDC35*, *CDC14*, and *CPP1* genes were not included in the study, because they have been previously annotated in *C. albicans* (13, 14, 24, 36) and did not have reported effects on virulence. For four genes (orthologues of *S. cerevisiae* genes *GLC7*, *PPG1*, *PPH21*, and *TEP1*), one allele was deleted but homozygous null mutants could not be obtained. However, 21 genes were successfully disrupted in both alleles (Table 2). The collection of 21 disruptant strains included 4 (the *yvh1*Δ, *cmp1*Δ, *sit4*Δ, and *ptc7*Δ strains) derived from genes already annotated in the *C. albicans* genome database; 3 of these (the *yvh1*Δ, *cmp1*Δ, and *sit4*Δ disruptants) have been reported as showing attenuated virulence in a mouse model of systemic infection (2, 3, 21, 34).

Virulence of null mutants of putative protein phosphatase genes in the silkworm model of *C. albicans* infection. Twenty-one protein phosphatase disruptants were evaluated for their abilities to kill silkworms in comparison with the wild-type parental *C. albicans* strain TUA6. The survival of silkworms was evaluated at day 1 following inoculation into the hemolymph. The parental wild-type strain TUA6 killed all 30 silkworms within 24 h, a result consistent with the findings of a previous report of silkworm infection with *C. albicans* (20). Control silkworms inoculated with the diluent only (phosphate-buffered saline) showed 100% survival. Decreasing the inoculum of the wild-type strain 10-fold (1×10^5 cells) increased the survival rate of the silkworms (data not shown). Six mutant strains (orthologues of *S. cerevisiae* genes *PPZ1*, *PTC7*, *MIH1*, *LTP1*, *PTP1*, and *PPS1* [7033, one of the two orthologues]) possessed wild-type virulence properties (all silkworms died within 24 h), and 11 mutant strains (orthologues of *S. cerevisiae* genes *SAL6*, *PPT1*, *PTC2/PTC3*, *PTC6*, *PTC5*, *PTC4*, *OCA1*, *SIW14*, *OCA6*, *PTP3*, and *PPS1* [4405, the other orthologue]) showed slight to moderate (up to 50% survival) reductions in virulence properties. However, these findings were preliminary, because we have not demonstrated that full virulence of the mutants is restored by complementation. Table 3 shows virulence data for four strains (*sit4*, *yvh1*, *cmp1*, and *ptc1* null mutants) for which virulence in the silkworm model was considerably attenuated (>63% survival). These results revealed that the *C. albicans PTC1* gene is involved in virulence for the silkworm model, as well as confirming the virulence-related nature of three genes (*YVH1*, *CMP1*, and *SIT4*) previously identified using a mouse model of systemic infection (2, 3, 21, 34).

Comparison of the virulence of the parental strain TUA6 with that of the *ptc1*Δ null mutant and heterozygous revertant strains in silkworm and murine infection models. To substantiate the observation that the *C. albicans PTC1* gene is involved in virulence, the heterozygous revertant strains PTC1A and PTC1B were constructed. Either one of the alleles of *PTC1* was reintroduced, under the control of the *ACT1* promoter, into the *RP10* locus of the *C. albicans ptc1*Δ null mutant as described in Materials and Methods. All strains were *URA*$^+$ and *ARG*$^+$. The loss and gain of *PTC1* mRNA in the null mutant and revertant strains, respectively, were confirmed by

TABLE 2. Identification and disruption of putative protein phosphatase genes of *C. albicans*

Protein phosphatase family	*C. albicans* gene name and/or assembly 19 ORF no.[a]	*S. cerevisiae* annotation[b]	Gene disruption in this study[c]	Reference[d]
PPP family				
Type 1 serine/threonine	726	*PPZ1*	−/−	
	5758	*SAL6*	−/−	
	6285	*GLC7*	−/+	
Type 2A-related serine/threonine	1683	*PPH21*	−/+	
	3774	*PPG1*	−/+	
	SIT4, 5200	*SIT4*	−/−	34
Type 2B-related serine/threonine	*CMP1*, 6033	*CMP1/CNA1*	−/−	2, 3, 50
Other	1673	*PPT1*	−/−	
PPM family, type 2C-related serine/threonine	2538	*PTC2/PTC3*	−/−	
	3705	*PTC6*	−/−	
	4785	*PTC1*	−/−	
	CYR1/CDC35, 5148	*CYR1/CDC35*	ND	24, 36
	PTC7, 5661	*PTC7*	−/−	25
	6376	*PTC5*	−/−	
	6638	*PTC4*	−/−	
PTP family, tyrosine specific	1762	*OCA1*	−/−	
	1850	*SIW14*	−/−	
	3071	*MIH1*	−/−	
	3699	*TEP1*	−/+	
	5104	*LTP1*	−/−	
	6365	*PTP1*	−/−	
	7206	*OCA6*	−/−	
	7610	*PTP3*	−/−	
Dual specific	*CDC14*, 4192	*CDC14*	ND	13
	YVH1, 4401	*YVH1*	−/−	21
	4405	*PPS1*	−/−	
	CPP1, 4866	*CPP1*	ND	14
	7033	*PPS1*	−/−	

[a] From the *Candida* Genome Database (http://www.candidagenome.org/). ORF, open reading frame.
[b] From the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[c] −/−, both alleles were disrupted; −/+, homozygous null mutants could not be obtained; ND; not determined.
[d] For published protein phosphatase genes of *C. albicans*.

TABLE 3. Virulence in the silkworm model of infection of the *C. albicans* wild-type strain TUA6 and derivative mutant strains in each of which a single putative protein phosphatase gene has been disrupted

C. albicans putative[a] protein phosphatase disruptant	Survival rate (%)[c] in the silkworm model at day 1 after injection
Wild-type TUA6	0
*sit4*Δ[b]	100
*cmp1/cna1*Δ[b]	76.7 ± 11.5
*ptc1*Δ	63.3 ± 20.8
*yvh1*Δ	96.7 ± 5.8

[a] Identified as putative phosphatase genes in the *C. albicans* genome database (1) or by sequence homology searches as described in the text.
[b] Gene for which a protein dephosphorylation function has been confirmed.
[c] For each *C. albicans* strain, 30 silkworms were infected with yeast cells (1×10^6 CFU) injected into the hemolymph as described in Materials and Methods. Groups infected with the wild-type strain TUA6 all died within 24 h of infection.

QRT-PCR (Fig. 1A). The two revertant strains, the parent strain TUA6, and the null mutant strain PTC1C were compared in further infection experiments. As shown in Fig. 1B, all the silkworms in groups infected with either PTC1A, PTC1B, or the parental strain TUA6 were killed within 30 h of inoculation. In contrast, it took more than 70 h to kill all the silkworms in the group infected with the null mutant strain. Silkworms infected with either TUA6, PTC1A, PTC1B, or PTC1C were examined by microscopy of transverse sections at 12 h postinoculation as described in Materials and Methods. Hyphal growth morphology predominated in the silkworms inoculated with either the wild-type or the revertant strains, whereas hyphal growth morphology was significantly reduced in silkworms infected with strain PTC1C (Fig. 2).

The mutant strains were also tested in a mouse model of systemic infection. Mice that were injected with the revertant strains or wild-type strain were all killed within 15 days postinfection, whereas 60% of mice infected with the null mutant survived more than 30 days (Fig. 1C). The revertant strains

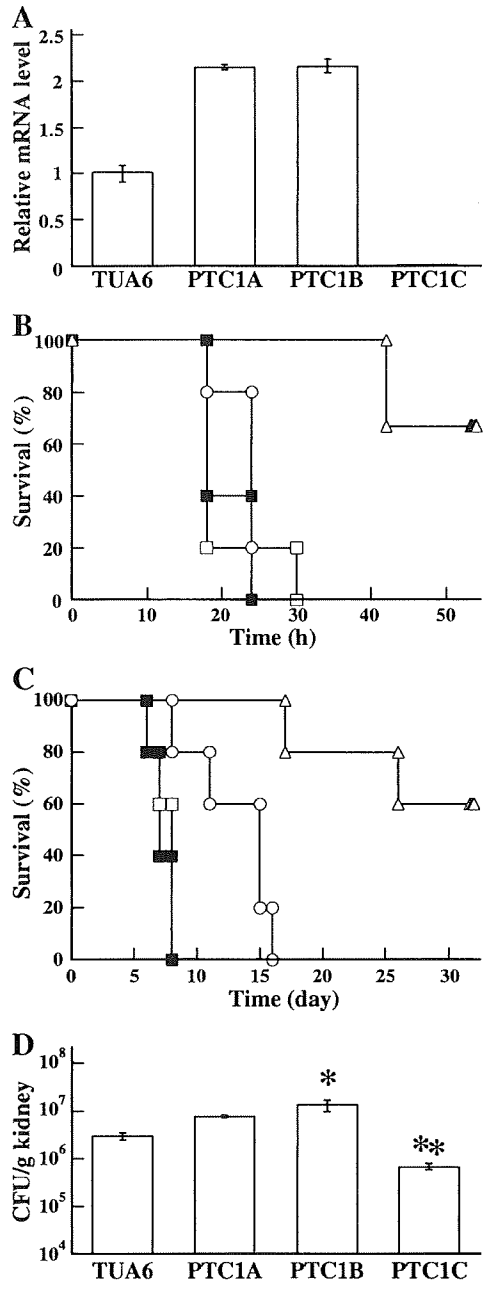

FIG. 1. Expression levels of *PTC1* mRNA in *C. albicans* wild-type and mutant strains and the virulence of these strains in silkworm and mouse infection models. (A) Relative expression levels of the *PTC1* transcript were determined by QRT-PCR and standardized with *ACT1* mRNA. mRNAs were extracted from exponentially growing TUA6 (wild-type), PTC1A and PTC1B (revertant), and PTC1C (null mutant) cells grown in YPD (pH 5.6) at 30°C. (B) *C. albicans* cells (1×10^6 CFU), either TUA6 (○), PTC1A (□), PTC1B (■), or PTC1C (△), were inoculated into silkworms ($n = 10$), and the survival of the silkworms was monitored. Significant differences between the survival rates with TUA6 versus PTC1C ($P < 0.001$) were observed. (C) *C. albicans* strains (1×10^6 CFU) (symbols as explained for panel B) were each injected into mice ($n = 6$) via the tail vein, and survival was monitored. Significant differences between the survival rates with TUA6 versus PTC1C ($P < 0.005$) and with TUA6 versus PTC1B ($P < 0.01$) were observed. (D) Fungal burdens in the kidneys of infected mice ($n = 3$) were determined at day 5 after inoculation. Asterisks indicate statistically significant differences between TUA6 and PTC1B (*, $P < 0.01$) and between TUA6 and PTC1C (**, $P < 0.005$).

showed slightly increased virulence compared to that of the parental strain. The reduced virulence of the null mutant compared to that of the parental or revertant strains in the mouse model of infection was also reflected in reduced fungal burdens in the kidneys of infected mice (Fig. 1D). Recovery of *C. albicans* from the kidneys of groups of infected mice was determined 2 days after inoculation. Significant differences in the tissue burden among the four *C. albicans* strains were observed (Fig. 1D); the number of *C. albicans* cells recovered from the kidneys of mice infected with the null mutant was an order of magnitude lower than that from those infected with the wild-type or revertant strains. However, kidneys were not examined by microscopy to determine whether the CFU recovery could have been affected by different levels of hyphal formation in kidneys infected with different strains. As noted below, the mutant strain showed a reduced ability to form hyphae, and this may have affected the CFU yield from mice infected with this strain.

Effects of *PTC1* disruption on yeast and hyphal growth. The effect of *PTC1* gene disruption on the growth of *C. albicans* in the yeast morphology was determined by measuring the mass doubling times of the wild-type, PTC1C disruptant, and PTC1A and PTC1B revertant strains grown in YPD (pH 5.6) at 30°C. Mean mass doubling times were 1.65, 1.51, 1.40, and 1.41 h, respectively. There were no significant statistical differences among these strains.

In order to investigate the effect of *PTC1* disruption on the yeast-to-hypha transition in *C. albicans*, cells were incubated in either liquid or agar-solidified YPD medium supplemented with 10% serum (pH 7.2) at 37°C. In liquid medium, there was a difference in hyphal formation in liquid serum-YPD between the wild-type and null mutant strains (Fig. 3A). The lengths of hyphae of the wild-type and *ptc1*Δ strains in liquid YPD containing 10% serum after 3 h at 37°C were 49.0 ± 7.2 μm and 33.3 ± 12.1 μm, respectively. For the revertant strains, the mean hyphal length was 55.8 ± 9.4 μm. The *ptc1*Δ disruptant strain (PTC1C) was more clearly defective in hyphal formation on serum agar (Fig. 3B). The revertant strains PTC1A and PTC1B rescued the defect of hyphal formation of the null mutant on serum agar, although hyphae were longer than those of the wild-type strain on serum agar (Fig. 3B). These in vitro results of cell morphology were consistent with the observed difference in hyphal growth in vivo (Fig. 2). The expression of the *PTC1* transcript was assessed by QRT-PCR of mRNA extracted from wild-type TUA6 cells grown under conditions inducing yeast or hyphal growth in liquid media. The expression level of *PTC1* mRNA in cells growing as budding yeasts was higher than that in cells displaying hyphal morphology (Fig. 3C).

The *PTC1* gene is required for proteolytic activity. Hydrolytic enzymes such as secreted aspartic proteinases and phospholipases are well-known virulence factors in *C. albicans*. We determined the secreted proteolytic, lypolytic, and hemolytic activities of the *ptc1*Δ strain relative to those of the parental and revertant strains by using agar plate assays. No significant differences were observed between the strains in the assay of phospholipase or hemolysin activity (data not shown). However, the null mutant had decreased proteolytic activity compared to that of the wild-type parental strain or the revertant

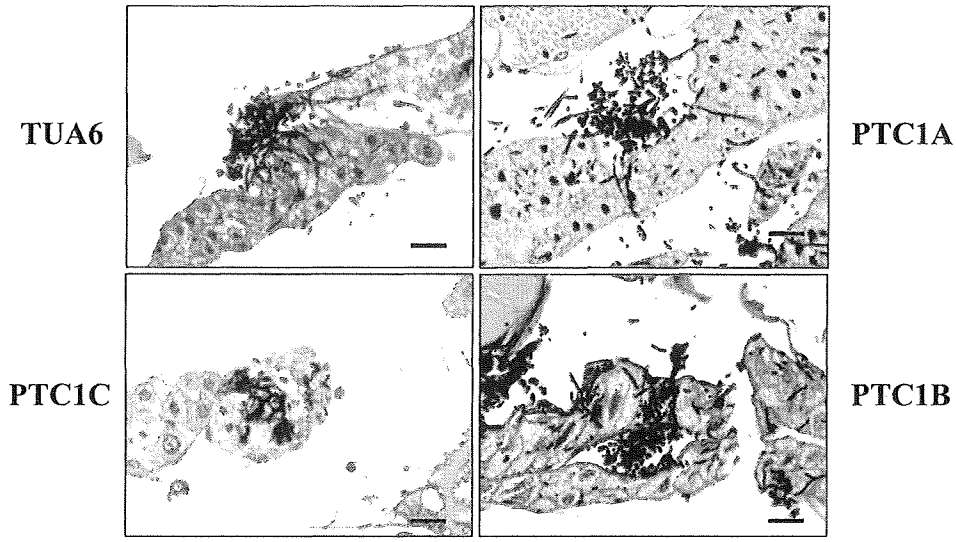

FIG. 2. Morphology of *C. albicans* cells in the infected silkworm. *C. albicans* strains (1×10^6 CFU) were each inoculated into silkworms ($n = 5$). Silkworms infected with each strain were obtained 12 h after inoculation, stained with periodic acid-Schiff stain, cross-sectioned, and examined by microscope. Representative silkworm cross-sections were photographed with an object lens of ×40. Bar, 10 μm.

strains PTC1A and PTC1B ($P < 0.005$ for TUA6 versus PTC1C) (Fig. 4).

Susceptibilities to antifungals and stressors. The *S. cerevisiae PTC1* gene has several specific cellular functions, including a regulatory role in the osmotically activated HOG pathway (19, 47–49, 54), and deletion of *PTC1* in *S. cerevisiae* resulted in cells with pleiotropic stress phenotypes (19, 37, 48). Hence, we examined the susceptibilities of the *C. albicans ptc1*Δ mutant in comparison with those of the parent and revertant strains to a wide range of stress conditions by using plate dilution assays (Fig. 5A). Stressors such as high temperatures, high pH, high salt concentrations, and oxidative stress, to all of which the *S. cerevisiae ptc1*Δ strain was highly sensitive (19), did not affect the *C. albicans ptc1*Δ strain to any greater extent than the wild-type or revertant strains. However, the *ptc1*Δ strain showed greater resistance than the parental or revertant strains to the β-1,3-glucan-binding dye Congo red and to the squalene epoxidase inhibitor terbinafine. In contrast, the *ptc1*Δ strain was more susceptible to the β-1,3-glucan synthase inhibitor micafungin than the wild type. The differential responses of the parental strain TUA6, the revertants, and the null mutant to terbinafine and micafungin were confirmed by microdilution assays (Fig. 5B).

DISCUSSION

This study investigated the virulence-related properties of 21 putative or confirmed protein phosphatase genes of *C. albicans* by comparing the derived null mutants with the parent strain in a silkworm infection model. Using the silkworm model, *PTC1*

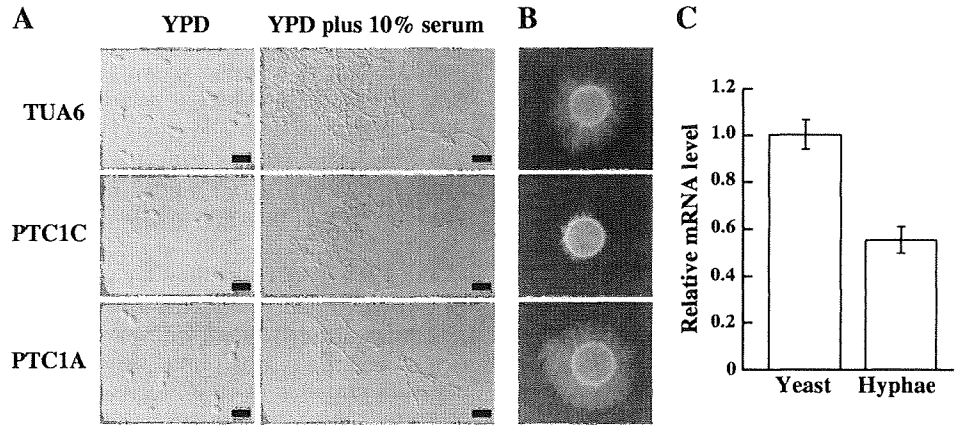

FIG. 3. Cell and colony morphology of *C. albicans* strains TUA6 (wild type), PTC1A (revertant), and PTC1C (null mutant). (A) (Left) Yeast growth in fresh YPD (pH 5.6) with shaking at 30°C for 3 h. Bar, 10 μm. (Right) Hyphal growth in fresh YPD (pH 7.2) containing 10% serum with shaking at 37°C for 3 h. Bar, 20 μm. (B) Cells (1×10^4 CFU) were spotted onto the surface of 10% serum agar and incubated at 37°C for 7 days. (C) Relative expression levels of the *PTC1* transcript were determined by QRT-PCR and standardized against *ACT1* mRNA. mRNAs were extracted from wild-type cells grown under different morphological growth conditions as described for panel A.

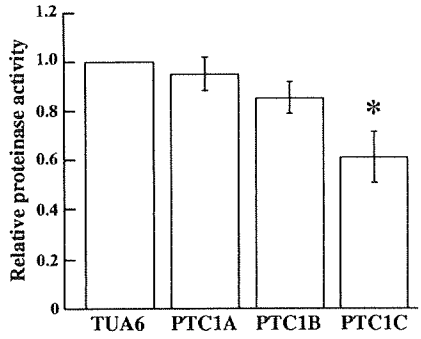

FIG. 4. Proteinase activities of *C. albicans* strains TUA6 (wild type), PTC1A and PTC1B (revertants), and PTC1C (null mutant). Proteinase activities were measured by spotting cells (1×10^4 CFU) onto a 1.2% yeast carbon base containing 0.5% bovine serum albumin at 30°C for 3 days. Relative activities were estimated as described in Materials and Methods. Data are means ± standard errors for three independent experiments. The asterisk indicates a statistically significant difference ($P < 0.005$) between TUA6 and PTC1C.

was identified as a novel virulence-related protein phosphatase gene (Table 3). This result was confirmed by testing in a conventional mouse infection model (Fig. 1). Mice infected with the *ptc1*Δ strain had significantly increased survival rates and significantly reduced kidney fungal burdens compared to mice infected with the parental or revertant strains. Null mutants of three protein phosphatase genes, *CMP1*, *SIT4*, and *YVH1*, previously reported to have attenuated virulence in a mouse infection model, also showed reduced virulence in the silkworm infection model (Table 3). Thus, the silkworm infection model was verified as a useful model system with which to identify uncharacterized genes affecting *C. albicans* virulence. This insect-based system is more amenable to screening studies than vertebrate models such as the mouse tail vein inoculation model. In addition, although rodent-based models mimic human physiology more closely than insect-based models, they are more expensive and labor-intensive, and they have ethical implications (32). The silkworm is also a good model organism because the whole-genome sequence has been completed (39, 55). In addition, silkworms have immunity factors and defense-related genes, including Toll-related genes (12, 23) and genes encoding antibacterial proteins such as the gloverin-like genes (31).

The markedly reduced virulence of the *C. albicans ptc1*Δ strain in the silkworm and mouse models of infection appeared to be due to defects in multiple regulatory functions, since the mutant strain possessed defective hyphal formation (Fig. 2 and 3) and reduced proteolytic activity. The involvement of the *C. albicans PTC1* gene in virulence and the control of phenotypic properties such as hyphal growth, proteolysis, and antifungal sensitivities was confirmed by the construction of two revertant strains expressing either allele of *PTC1*, which restored wild-type virulence and phenotypes. DNA sequence analysis of *PTC1* revealed that there were eight single-nucleotide polymorphisms between diploid *PTC1* alleles (data not shown), a reflection of the highly variable degree of heterozygosity of *C. albicans* (27). However, the revertant strains expressing either *PTC1* allele, constitutively regulated by the *ACT1* promoter, had very similar properties, indicating that the single-nucleotide polymorphisms in *PTC1* did not affect the function of *PTC1*. The elevated expression of *PTC1* mRNA in the revertant strains may be a result of the use of the strong *ACT1* promoter in our studies. Consequently, the changes in growth and virulence of the revertant strains relative to the parental

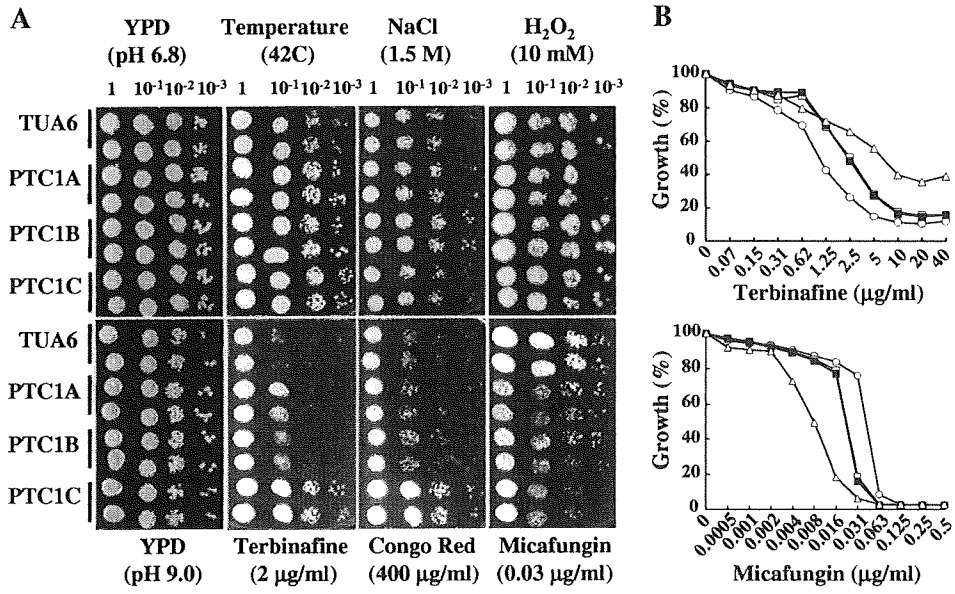

FIG. 5. Susceptibilities of *C. albicans* strains TUA6 (wild type), PTC1A and PTC1B (revertants), and PTC1C (null mutant) to antifungals and stressors. (A) Agar plate spotting assay. Tenfold serial dilutions of exponentially growing cells (5 μl) were spotted onto YPD agar plates containing antifungals or stressors at the indicated concentrations or under the indicated conditions. Cell growth was monitored after incubation at 30°C or the indicated temperature for 48 h. Representative results of an experiment repeated twice are shown. (B) Microdilution susceptibility assay. Susceptibilities to micafungin and terbinafine were determined for *C. albicans* TUA6 (○), PTC1A (□), PTC1B (■), and PTC1C (△) cells grown in YPD containing the indicated concentrations of antifungals at 30°C for 48 h.

wild-type strains possibly reflected this increased gene expression driven by the *ACT1* promoter. A position effect of the *URA3* marker (8, 33) was not responsible, because the *URA3* marker was also reintegrated at the *RP10* loci of the wild type and the null mutant PTC1C. Phenotypes more closely replicating the wild type may have been obtained for the revertant strains if the native gene promoter had been used in the constructs. However, the reversal of the disruptant strain phenotype confirmed the role of *PTC1* in the *C. albicans* virulence properties examined. In this study we used the split-Ura-blaster technique (17). Alternative methods for disrupting *C. albicans* genes (44, 46, 51) are available but were not used in this study. It is possible that the use of 5-fluoroorotic acid to generate mutants using the split-Ura-blaster technique may have introduced chromosomal aberrations during mutant construction, although the behavior of the revertants indicates that this was not responsible for the disruptant phenotype.

Our results showed that a number of properties of the *S. cerevisiae ptc1*Δ strain were not present in the *C. albicans* disruptant strain. Other studies have found that a number of phenotypic traits were not necessarily well matched between null mutants of *S. cerevisiae* and *C. albicans* (4). Lack of *PTC1* affects lithium tolerance in *S. cerevisiae* (48), and Ptc1p is considered important for cell wall integrity. The *S. cerevisiae* mutant was reported to be highly sensitive to cell wall-damaging conditions such as calcoflour white, Congo red, high pH, and high temperature (19). In contrast, the *C. albicans ptc1*Δ mutant was not affected by these stress conditions (Fig. 5). However, the increased sensitivity of the *C. albicans ptc1*Δ mutant to the echinocandin antifungals caspofungin and micafungin, which interfere with cell wall glucan synthesis, was consistent with the *S. cerevisiae ptc1*Δ phenotype (37). Although the precise nature of the defect in the *C. albicans ptc1*Δ mutant is unknown, the current studies showed limited similarities between the phenotype of the *C. albicans PTC1* gene and that of its orthologue in *S. cerevisiae*. Our results are consistent with the recent report of global transcriptional response to stress in *C. albicans*, which emphasized that the Hog1 signaling network in *C. albicans* has diverged significantly from the corresponding networks in the model yeast (15). Martín et al. also reported that removal of only one of these negative regulators does not usually produce an obvious phenotype (38). A direct comparison of the phenotype of a *C. albicans HOG1* mutant strain with that of the *PTC1* disruptant in assays such as those we applied would elucidate this but was not considered in the scope of this study.

In conclusion, we have shown the validity of the silkworm infection model for pathogenesis studies of *C. albicans* gene families. This study presented early findings on the regulatory function of the previously uncharacterized putative *C. albicans* protein dephosphorylation gene *PTC1*. The user-friendly, low-cost, and ethically acceptable silkworm infection model can be a valuable tool not only for the investigation of virulence factors in this fungus and other pathogenic fungi but also for the in vivo screening of new antifungal compounds.

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Inhibition of fungal ABC transporters by unnarmicin A and unnarmicin C, novel cyclic peptides from marine bacterium

Koichi Tanabe [a], Erwin Lamping [b], Kyoko Adachi [c], Yukie Takano [a], Kazutaka Kawabata [c], Yoshikazu Shizuri [c], Masakazu Niimi [a,*], Yoshimasa Uehara [a,1]

[a] *Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Oral Sciences, University of Otago, P.O. Box 647, Dunedin, New Zealand*
[c] *Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan*

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Abstract

Novel inhibitors of fungal ATP-binding cassette transporters were obtained by screening compounds and crude extracts from marine-derived fungi and bacteria using disk diffusion assays of *Saccharomyces cerevisiae* strains overexpressing a variety of fungal multi-drug efflux pumps. The cyclodepsipeptides unnarmicin A and unnarmicin C were able to sensitize cells overexpressing azole drug pumps ScPdr5p, CaCdr1p, CgCdr1p, and CgPdh1p to sub-MIC concentrations of fluconazole without affecting the growth of CaCdr2p and CaMdr1p overexpressing cells. Unnarmicin A and unnarmicin C were potent inhibitors of rhodamine 6G efflux of CaCdr1p expressing cells with IC_{50} values of 3.61 and 5.65 μM, respectively. They inhibited the *in vitro* CaCdr1p ATPase activity at IC_{50} values of 0.495 and 0.688 μM, respectively. And most importantly, they were able to sensitize azole-resistant *Candida albicans* clinical isolates to fluconazole. Unnarmicin A and unnarmicin C are candidate efflux pump inhibitors with the potential to be used as adjuvants for antifungal chemotherapy.

Keywords: ATP-binding cassette transporter; *Candida albicans*; Cyclodepsipeptide; Multi-drug resistance; Pump inhibitor; *Saccharomyces cerevisiae*; Antifungal azole; Expression system

The yeast *Candida albicans* is the most common pathogenic fungus that causes oropharyngeal or esophagogastric candidiasis in HIV positive-individuals and AIDS patients [1]. The chemotherapy of patients with fungal infections can pose difficulties because of the limited number of effective antifungal drugs. The widely used triazole drug fluconazole usually provides treatment of these infections and has few side effects. However, prolonged administration of fluconazole in immunocompromised patients can result in the appearance of azole-resistant clinical isolates over-expressing multi-drug efflux transporters including ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporters [1]. Suppressing the activity of these fungal ABC transporters with small molecule multi-drug efflux pump inhibitors could reduce the drug resistance of these pathogenic fungi and therefore help to increase the efficacy of antifungal chemotherapy with triazoles. Compounds including FK506 [2], enniatins [3], milbemycins [4,5], synthetic D-octapeptides [6], and cyclosporine A [7] have been reported to inhibit fungal ABC transporters. These inhibitors are useful tools for the discovery and development of new adjuvants for fungal chemotherapy as well as for studying the molecular mechanisms of ABC transporter activities.

In this study, the cyclodepsipeptides unnarmicin A (UnmA) and unnarmicin C (UnmC) found in extracts from

* Corresponding author. Fax: +81 3 5285 1272.
E-mail address: niimi@nih.go.jp (M. Niimi).
[1] Present address: Department of Microbial Chemical Biology and Drug Discovery, Iwate Medical University School of Pharmacy, 2-1-1 Nishi-tokuda, Yahabacho, Shiwa-gun, Iwate 028-3694, Japan.

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a marine gammaproteobacterium were identified as transporter antagonists in screens using a panel of *Saccharomyces cerevisiae* strains that hyper-express individual transporters responsible for multi-drug resistance in pathogenic fungi [5,8]. These compounds increased the fluconazole susceptibility of the pump-expressing *S. cerevisiae* cells due to the inhibition of the ATPase and drug transport activity of particular ABC transporters. And most importantly, as expected, UnmA and UnmC were also able to strongly sensitize multi-drug resistant *C. albicans* clinical isolates to fluconazole.

Materials and methods

Strains and culture media. The *S. cerevisiae* and *Candida* strains used in this study are listed in Table 1. *S. cerevisiae* strains AD/pABC3, AD/CaCDR1, AD/CaCDR2, and AD/CaMDR1, and vector plasmid pABC3 were created as previously described [5,9]. Complete synthetic medium without uracil (CSM-URA) agars plates contained 0.67% (wt/vol) yeast nitrogen base (Difco, Becton–Dickinson, Sparks, MD), 0.077% (wt/vol) CSM-URA (Bio 101, Vista, CA), 2% (wt/vol) glucose, and 2% (wt/vol) agar. YPD agar plates contained 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose (Difco, Becton–Dickinson, Sparks, MD), and 1.5% agar.

Chemicals and antifungal agents. The compounds tested in this study were provided by Marine Biotechnology Institute Co. Ltd., Iwate, Japan. Other chemicals and antifungal agents were obtained from the following sources: Diflucan (used as fluconazole, Pfizer Ltd., Sandwich, Kent, UK); FK506 (Astellas Pharma Inc., Japan), sodium azide, ATP, dimethyl sulfoxide (DMSO), and phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO); and L-ascorbic acid, ammonium molybdate, (Wako Pure Chemical Industries Ltd., Japan). Chemical library pools and FK506 were prepared as stock solutions dissolved in DMSO.

Agar diffusion chemosensitization assay. YPD (50 ml) containing fluconazole at 1/2–1/8 of the MIC for each test strain was solidified with 1.2% agar in a Petri dish (230 by 82 by 18 mm; Radia Industry Co. Ltd., Japan) [5]. Yeast cells ($1–2 \times 10^5$) were added to melted YPD agar containing fluconazole at 55 °C and poured immediately. An appropriate volume of a compound (<5 μl) was applied to a sterile 3 MM blotting paper disk (5-mm diameter; Whatman International Ltd., Kent, UK), dried at room temperature for 30 min, and placed onto the surface of these plates. The cells were incubated at 30 °C for 24–48 h until clear growth inhibitory zones appeared around the disks. YPD agar plates without fluconazole were used to assess the effects of test compounds alone on the growth of the yeast strains.

Fluconazole chemosensitization assay. Checkerboard drug chemosensitization assays were performed as described elsewhere [6]. Synergisms between fluconazole and individual test compounds were evaluated based on their fractional inhibitory concentration (FIC) index [10].

In vitro assays. (i) Isolation of plasma membranes. Cells (1×10^7) were grown in 150 ml YPD liquid medium at 30 °C to $OD_{600} \sim 6$. Plasma membrane fractions of *S. cerevisiae* cells were prepared as described previously [6] with slight modifications of how the cells were broken. Instead of using a beads-beater, cells were resuspended in breaking buffer containing 8 g glass beads (0.5 mm diameter) and disrupted by beating them vigorously with a MULTI-BEADS SHOCKER (Yasui Kikai corporation, Japan) using ice-cold ethanol as coolant. Cells were broken by beating them for five 1-min periods at 2700 rpm with alternating 1-min cooling periods in between. After isolation of the plasma membranes, the protein concentrations were determined with the BCA assay (Pierce Biotechnology, Inc., Rockford, IL) using bovine serum albumin as the protein standard.

(ii) Inhibition of heterologously expressed ABC transporter ATPase activity. Plasma membrane fractions were isolated as described above from *S. cerevisiae* strains AD/CaCDR1 or AD/CaCDR2. ATPase assays were carried out at 30 °C and pH 7.5 for 30 min in the presence of different concentrations of candidate compounds as described previously [11] and using 1.5 μg of total plasma membrane protein per assay.

(iii) Efflux of rhodamine 6G (R6G). R6G efflux from cells was measured as described previously [6]. Cell samples (50 μl) were preincubated at 30 °C for 5 min with or without test compounds and 50 μl of 0.8% glucose added to start the reaction. After 8 min, the cells were filtered, and the amount of R6G in the supernatant was quantitated fluorometrically (DTX-880; Beckman Coulter, Inc. Fullerton, CA) by using a standard curve for R6G in HEPES buffer.

Results

Screening of specific inhibitors of fungal ABC transporters

Novel inhibitors of fungal ABC transporters were identified by screening a library of 8640 crude extracts and 80 pure compounds extracted from marine derived fungi and bacteria and using a panel of *S. cerevisiae* strains overexpressing fungal ABC transporters as tester strains. Novel inhibitors of fungal ABC transporters were screened for their ability to chemosensitize fluconazole resistant *S. cerevisiae* strains overexpressing the two major *C. albicans* multi-drug efflux pumps *CDR1* and *CDR2* (AD/CaCDR1 and AD/CaCDR2, respectively) to the presence of sub-MIC concentrations of fluconazole. The strain expressing the *C. albicans* multi-drug efflux pump *MDR1* (AD/CaMDR1), encoding a MFS type multi-drug transporter that is

Table 1
Fungal strains used in this study

Species and strain	Description	Reference
Candida albicans		
CaAD	Cdr1p-overexpressing clinical isolate, Janssen Research Foundation	[14]
B59630	Cdr1p-overexpressing clinical isolate, Janssen Research Foundation	[15]
Saccharomyces cerevisiae		
AD/pABC3	AD1-8u$^-$ containing the empty transformation cassette integrated at the *PDR5* locus	[5]
AD/CaCDR1	AD1-8u$^-$ overexpressing *C. albicans CDR1* ORF from the *PDR5* locus	[5]
AD/CaCDR2	AD1-8u$^-$ overexpressing *C. albicans CDR2* ORF from the *PDR5* locus	[5]
AD/CaMDR1	AD1-8u$^-$ overexpressing *C. albicans MDR1* ORF from the *PDR5* locus	[5]
AD/CgCDR1	AD1-8u$^-$ overexpressing *C. glabrata CDR1* ORF from the *PDR5* locus	[5]
AD/CgPDH1	AD1-8u$^-$ overexpressing *C. glabrata PDH1* ORF from the *PDR5* locus	[5]
AD/ScPDR5	AD1-8u$^-$ overexpressing *S. cerevisiae PDR5* ORF from the *PDR5* locus	[5]