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Loss of Heterozygosity of *FCY2* Leading to the Development of Flucytosine Resistance in *Candida tropicalis*▽

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As fluconazole resistance becomes an emerging issue for treating infections caused by *Candida tropicalis*, searching for alternative becomes a prominent task. In the present study, 97 clinical isolates of *C. tropicalis* were tested for the susceptibilities to flucytosine (5FC) with the Etest method. Although only one isolate was resistant to 5FC, 30 susceptible isolates could produce resistant progeny after exposure to the drug. Interestingly, 22 of these 30 clinical isolates had a heterozygous G/T at the 145th position on *FCY2*, encoding purine-cytosine permease, whereas their progeny recovered from within the inhibitory ellipses had homozygous T/T, resulting in null alleles for both copies of the gene and produced only truncated proteins, effecting the 5FC resistance. Furthermore, we found that two major fluconazole-resistant clinical clones, diploid sequence type 98 (DST98) and DST140, had a homozygous G/G at the 145th position, and neither was able to produce 5FC-resistant progeny within the inhibitory ellipses. Hence, strains of *C. tropicalis* containing heterozygous alleles may develop 5FC resistance readily, whereas those with homozygous G/G wild-type alleles can be treated with 5FC. Subsequently, a combination of 5FC and another antifungal drug is applicable for treating infections of *C. tropicalis*.

The prevalence of invasive nosocomial *Candida* infections has increased significantly in association with the selective pressure of applying antibiotics, increased number of immunocompromised individuals, and invasive hospital procedures (38, 44). Although *Candida albicans* is the most prominent species causing candidemia in most situations, there has been a shift toward the more treatment-challenged non-*albicans* *Candida* species (8, 28, 29, 36), of which the prevalences were significantly different in various geographic areas (19).

Candida glabrata appears to be the most frequently isolated non-*albicans* *Candida* species in Western countries, whereas, in Asia, it is *Candida tropicalis* (4, 7, 28, 36, 40, 41). In certain regions, it even surpassed *C. albicans* to become the most frequently isolated *Candida* species (4, 40). Furthermore, increasing prevalence of the resistance to fluconazole, the most commonly used antifungal in clinics, is an emerging issue (41, 42) in treating *C. tropicalis* infections.

Flucytosine (5FC) is one of the oldest antifungal drugs for treating human fungal infections such as candidiasis and cryptococcosis (33). Monotherapy with 5FC is limited because of the frequent development of resistance. Therefore, 5FC is mostly used in combination with another antifungal agent. Nevertheless, it may serve as an alternative for treating emerging fluconazole-resistant *C. tropicalis* infections. Thus, it is interesting and important to determine the prevalence of 5FC resistance in clinically isolated *C. tropicalis* and the molecular mechanisms contributing to 5FC resistance.

FC is taken up by fungal cells and converted via 5-fluorouracil to 5-fluorouridine monophosphate (FUMP), which is then catalyzed by either one of two enzymes: cytosine deaminase encoded by *FCY1*and uracil phosphoribosyl transferase (UPRT) encoded by *FUR1*. FUMP is in turn phosphorylated to 5-fluorouridine triphosphate (FUTP), which disturbs the protein synthesis by incorporation into RNA (30, 34). Alternatively, the reduction of FUMP to 5-fluoro-2′-deoxyuridylate monophosphate (FdUMP) leads to the inhibition of the enzyme thymidylate synthetase and thus DNA synthesis (15). *FCY2*, encoding a purine-cytosine permease, is involved in the uptake of 5FC, and *URA3*, encoding an orotidine 5′-phosphate decarboxylase, is involved in the metabolic pathway of uridylmonophosphate (UMP) in nucleic acid synthesis. Mutations on *FCY1*, *FCY2*, *FUR1*, or *URA3* can result in 5FC resistance in certain yeast species (5, 14, 16, 18, 23, 27, 33). Interestingly, 5FC-resistant clinical isolates have been reported to be genetically related (6, 14). Nevertheless, the mechanisms contributing to 5FC resistance in *C. tropicalis* are not clear.

For diploid cells, the phenotypic change to display a recessive trait requires more than one step of genetic alteration. First of all, one of the two original alleles has to mutate to generate a genetic heterozygosity. Then, the other original allele is replaced by the newly mutated allele via mechanisms such as mitotic recombination, which leads to loss of heterozygosity (21). In contrast, if a diploid cell is heterozygous, then only one step is required to complete the loss of heterozygosity and display the mutant phenotype. Recently, Jacques et al. reported that a differential loss of heterozygosity in the diploid *Debaryomyces hansenii*, phylogenetically related to *C. albicans*, may result in the large genetic diversity found among isolates within this species (25). *In vitro*, it occurred in various populations in the presence of antifungal drugs (2, 3, 13) and has

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TABLE 1. Characterization of *C. tropicalis* clinical isolates collected from the TSARY studies

Isolate	MIC (μg/ml)		Source	Code[b]	DST	*FCY2*[c]	Resistant progeny
	FLC[a]	5FC					
YM020112	2	0.25	Blood	M1	ND[d]	G/T	Yes
YM020274	64	0.25	Sputum	S3	153	G/T	Yes
YM020291	0.25	0.5	Sputum	N2	155	G/T	Yes
YM020311	64	0.5	Urine	N2	90	G/T	Yes
YM020347	0.25	<0.125	Blood	N3	ND	G/T	Yes
YM020693	64	<0.125	Blood	M4	90	G/T	Yes
YM020743	2	0.5	Blood	S6	ND	G/T	Yes
YM060088	64	<0.125	Sputum	N9	188	G/T	Yes
YM060146	0.5	<0.125	Pleural effusion	N9	188	G/T	Yes
YM060237	64	0.25	Blood	N2	ND	G/T	Yes
YM060299	64	0.25	Blood	M1	134	G/T	Yes
YM060300	64	0.5	Blood	M1	ND	G/T	Yes
YM060325	0.5	1	Sputum	M4	201	G/T	Yes
YM060330	64	<0.125	Sputum	M4	186	G/T	Yes
YM060371	64	0.125	Blood	M4	187	G/T	Yes
YM060379	1	0.25	Blood	M4	200	G/T	Yes
YM060481	16	<0.125	Urine	S4	27	G/T	Yes
YM060507	0.25	0.5	Sputum	S5	134	G/T	Yes
YM060508	0.5	1	Sputum	S5	134	G/T	Yes
YM060565	1	0.5	Blood	S5	202	G/T	Yes
YM060800	0.5	1	Urine	S1	200	G/T	Yes
YM061047	16	<0.125	Blood	S6	ND	G/T	Yes
YM020438	1	0.5	Blood	S5	ND	G	Yes
YM020671	64	<0.125	Blood	M4	ND	G	Yes
YM020715	16	0.25	Urine	S6	160	G	Yes
YM060075	64	<0.125	Blood	M3	ND	G	Yes
YM060097	64	<0.125	Sputum	N9	149	G	Yes
YM060210	0.125	2	Urine	N2	184	G	Yes
YM060369	8	0.25	Blood	M4	139	G	Yes
YM060616	0.25	0.25	Ascites	M2	ND	G	Yes
YM060512	64	<0.125	Sputum	S5	134	G/T	No
YM020055	4	0.25	Blood	S1	ND	G	No
YM060051	64	<0.125	Sputum	M3	195	G	No
YM060071	0.5	<0.125	Blood	M3	ND	G	No
YM060173	0.25	<0.125	Urine	N3	140	G	No
YM060509	64	ND	Sputum	S5	140	G	No
YM060547	64	ND	Blood	S5	98	G	No
YM060647	64	ND	Sputum	N7	98	G	No
YM060828	64	ND	Blood	S1	140	G	No
YM020136	8	ND	Blood	M1	ND	ND	No
YM020273	4	ND	Sputum	S3	140	ND	No
YM020287	0.25	ND	Urine	N2	154	ND	No
YM020294	16	ND	Urine	N2	144	ND	No
YM020304	4	ND	Blood	N2	ND	ND	No
YM020309	4	ND	Urine	N2	140	ND	No
YM020367	4	ND	Blood	S4	ND	ND	No
YM020434	0.5	ND	Blood	S5	ND	ND	No
YM020449	32	ND	Blood	S5	ND	ND	No
YM020527	0.5	ND	Ascites	N6	ND	ND	No
YM020649	8	ND	Cervix	M4	156	ND	No
YM020659	0.13	ND	Sputum	M4	157	ND	No
YM020709	8	ND	Sputum	S6	159	ND	No
YM020725	8	ND	Urine	S6	161	ND	No
YM020919	1	ND	Sputum	E1	140	ND	No
YM020948	1	ND	Blood	E1	162	ND	No
YM060040	1	ND	Sputum	M3	168	ND	No
YM060064	64	ND	Blood	M3	ND	ND	No
YM060098	64	ND	Sputum	N9	140	ND	No
YM060100	64	ND	Sputum	N9	45	ND	No
YM060102	64	ND	Sputum	N9	140	ND	No
YM060109	64	ND	Sputum	N9	197	ND	No
YM060136	0.5	ND	Blood	N9	168	ND	No
YM060141	0.5	ND	Catheter	N9	192	ND	No
YM060144	64	ND	Urine	N9	180	ND	No

Continued on following page

TABLE 1—*Continued*

Isolate	MIC (μg/ml)		Source	Code[b]	DST	*FCY2*[c]	Resistant progeny
	FLC[a]	5FC					
YM060147	0.25	ND	Catheter	N9	198	ND	No
YM060172	0.5	ND	Urine	N3	171	ND	No
YM060175	64	ND	Urine	N3	179	ND	No
YM060177	64	ND	Urine	N3	149	ND	No
YM060184	4	ND	Blood	N3	ND	ND	No
YM060185	64	ND	Blood	N3	ND	ND	No
YM060302	64	ND	Pleural effusion	M1	185	ND	No
YM060310	64	ND	Blood	M1	ND	ND	No
YM060327	64	ND	Urine	M4	140	ND	No
YM060342	0.25	ND	Urine	M4	196	ND	No
YM060354	1	ND	Sputum	M4	191	ND	No
YM060383	0.5	ND	Blood	M4	189	ND	No
YM060450	64	ND	Sputum	N5	98	ND	No
YM060451	64	ND	Sputum	N5	98	ND	No
YM060500	0.5	ND	Bronchoalveolar lavage	S5	190	ND	No
YM060529	64	ND	Sputum	S5	98	ND	No
YM060533	64	ND	Ascites	S5	ND	ND	No
YM060541	64	ND	Blood	S5	ND	ND	No
YM060559	64	ND	Blood	S5	183	ND	No
YM060590	64	ND	Urine	M2	181	ND	No
YM060607	64	ND	Blood	M2	ND	ND	No
YM060689	64	ND	Blood	M6	ND	ND	No
YM060767	64	ND	Blood	E1	ND	ND	No
YM060776	64	ND	Catheter	E1	179	ND	No
YM060792	0.5	ND	Urine	S1	199	ND	No
YM060804	0.5	ND	Urine	S1	194	ND	No
YM060805	64	ND	Urine	S1	182	ND	No
YM060808	64	ND	Blood	S1	ND	ND	No
YM060812	1	ND	Blood	S1	193	ND	No
YM060925	16	ND	Blood	M5	ND	ND	No
YM060926	16	ND	Blood	M5	ND	ND	No
YM061045	64	ND	Peritoneal fluid	S6	ND	ND	No
YM061051	64	ND	Ascites	N5	ND	ND	No

[a] FLC, fluconazole.
[b] That is, the location of the collection source.
[c] The nucleotide at position 145 of *FCY2*.
[d] ND, not determined.

been suggested to contribute to drug resistance in clinical *C. albicans* isolates. For example, loss of heterozygosity was found at and around *ERG11*, the target of azole drugs, to decrease susceptibility to fluconazole (22, 37). In addition, homozygosity for gain-of-function mutations in *TAC1*, an activator of ABC transporters, resulted in elevated levels of azole resistance (11). Similar phenomena have also been reported for mutations on multidrug resistance regulator (*MRR1*), a regulator for multidrug resistance, *MDR1*, an efflux pump contributing to azole resistance (17), and on *GSC1* (*FKS1*), a glucan synthase catalytic subunit, involved in micafungin resistance (26). Loss of heterozygosity in *C. tropicalis* has also been reported (24). Nevertheless, whether loss of heterozygosity contributes to drug resistance in this species has not been reported.

In the present study, we screened and selected several 5FC-resistant *C. tropicalis* to show that isolates with a null mutation in one of the *FCY2* allele, when exposed to 5FC, were readily to undergo loss of heterozygosity to effect the homozygous state with the mutant allele and lead to resistance. Hence, strains containing homozygous G/G wild-type alleles can be treated with 5FC, whereas those containing heterozygous G/T will require different medication. In light of the emerging fluconazole-resistant *C. tropicalis* infection, a combination of 5FC and another antifungal drug other than fluconazole is a reasonable choice for treatments.

MATERIALS AND METHODS

Strains and media. The *C. tropicalis* clinical isolates collected during the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) studies in 2002 and 2006 (43, 45) were used for screening the 5FC resistance strains listed in Table 1. Yeast-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, and 2% dextrose) and synthetic dextrose (SD; 0.67% yeast nitrogen base without amino acid and 2% dextrose) were prepared as described previously (32). Cells were grown in either YPD or SD unless otherwise noted. The compounds for addition to media were from Difco unless otherwise noted.

Constructions of different *FCY2* alleles. FCY2ORF, a 2,258-bp KpnI-XhoI fragment comprising of the entire *FCY2* coding region and its flanking sequences, was amplified from the genomic DNA of YM020192 by using the primers HJL1420 and HJL1424 (Table 2) and cloned into pSF2A containing t*he SAT1* flipper cassette (31). FCY2d, a 528-bp SacII-SacI fragment complementary to the 46 bp at the 3′ end sequence of *FCY2* open reading frame (ORF), as well as its downstream region, was amplified by primers HJL1422 and HJL1423 and cloned into pSF2A containing the FCY2ORF fragment. LOB319 contained the G allele of *FCY2*, whereas LOB320 contained the T allele. The KpnI-SacI digested fragments of LOB319 containing homozygous G alleles were transformed into YM020291 and YM060800 to obtain YLO415 and YLO447, respectively. The KpnI-SacI digested fragments of LOB320 containing homozygous T alleles were transformed into YM020291 and YM060800 to obtained YLO417 and YLO440, respectively. The point mutation was generated with fusion PCR,

TABLE 2. Primers used in this study

HJL designation	Primer	Sequence (5′–3′)[a]	Application
HJL1205	CtFCY1-f	ATCATTAGTTCAGATGGTAAAGTCTTG	PCR and sequencing
HJL1206	CtFCY1-r	CCTTTTTAGTAACATGTCTATTCTCCA	PCR and sequencing
HJL1211	CtFUR1-f	TCATCAAAACCATGTCTGCTG	PCR and sequencing
HJL1212	CtFUR1-r	AAGTGTATGTAGTGATAATTGCTATGC	PCR and sequencing
HJL1413	CtURA3-f	ATTGGATAGTCCCTCTAAACTCACTACTA	PCR and sequencing
HJL1414	CtURA3	AGCATTAGTTATATCACTCCACGATGAA	Sequencing
HJL1415	CtURA3	TGCCGATATTGGAAATACAGTTA	Sequencing
HJL1416	CtURA3-r	AATCAACTATTCAAGTTGACCG	PCR and sequencing
HJL814	CaSAT1-f	CTCAACATGGAACGATCTAGC	PCR
HJL1207	CtFCY2-f	TGCCCATAAATTAAATGCAGAA	Sequencing
HJL1208	CtFCY2-r	GGAAGCAACAAACCCAAAAA	Sequencing
HJL1209	CtFCY2-f	TGCTGCCGATTATGTTGTTT	Sequencing
HJL1210	CtFCY2-r	GTGAAAACGAGCCAATCCAT	Sequencing
HJL1420	CtFCY2-f (KpnI)	ggtaccTCAACTCAACCCCAAAGT	Fusion PCR and sequencing
HJL1421	CtFCY2-r (XhoI)	ctcgagCCCAAGGAGAAAGTAGCA	PCR
HJL1422	CtFCY2-f	CGGATTCAATGTAGCCAG	PCR
HJL1423	CtFCY2-r	GTCATTCCATGTCGTGGT	PCR
HJL1424	CtFCY2-r (XhoI)	ctcgagGTCATTCCATGTCGTGGT	Fusion PCR and sequencing
HJL1477	CtFCY2-r out of B (3′)	CTGTTGCTCCAGGTGAATCA	PCR
HJL1753	CtFCY2f	TCGTTGCTTGTGTTGGTTGG	Sequencing
HJL2100	CtFCY2-145Gf	CATAAATTAAATGCAGAAACTAAAGGTATTG	Fusion PCR
HJL2101	CtFCY2-145Gr	CAATACCTTTAGTTTCTGCATTTAATTTATG	Fusion PCR
HJL2102	CtFCY2-145Tf	CATAAATTAAATGCATAAACTAAAGGTATTG	Fusion PCR
HJL2103	CtFCY2-145Tr	CAATACCTTTAGTTTATGCATTTAATTTATG	Fusion PCR
HJL2104	CtFCY2-f	CTTCTCCTTAACTACCTTTTCCTCC	Sequencing

[a] Restriction enzyme sites are indicated by lowercase letters; mutation sites are indicated by underlining.

in which three separate PCRs were conducted as following. Primers HJL1420 and HJL2103 were used to amplified 5′ end of fragment from LOB319 and HJL2102 and HJL1424 were used to amplify 3′ end of the fragment from LOB319. The FCY2LOB319T fragment amplified by primers HJL1420 and HJL1424 using 5′ and 3′ end fragments as templates was used to replace the KpnI-XhoI fragment of LOB319 to generate LOB383. *FCY2* 5′ and 3′ end fragments were amplified from LOB320 by primer pairs HJL1420/HJL2101 and HJL2100/HJL1424, respectively. The FCY2LOB320G fragment, generated by amplification by the primers HJL1420 and HJL1424 and with the 5′ and 3′ end fragments as templates, then replaced the KpnI-XhoI fragment of LOB320 to generate LOB384. The KpnI-SacI-digested fragments of LOB383 and LOB384 were transformed into YM020291 competent cells by electroporation to generate YLO468 and YLO466, respectively. Finally, the mutant isolates were confirmed by colony PCR and sequencing.

Antifungal susceptibility tests. Susceptibilities to 5FC of all *C. tropicalis* isolates collected in TSARY 2002 and 2006 (43, 45) were tested. The Etest assay was used to determine the susceptibilities to antifungal agents for *C. tropicalis* isolates. Homogenized colonies from an overnight YPD agar medium were transferred in 0.85% NaCl to achieve a density of 5×10^6 cell/ml. A sterile cotton swab was dipped into the inoculum suspension and used to swab the entire agar surface of the RPMI medium (Gibco-BRL) evenly. The 5FC (from 0.002 to 32 μg/ml) drug strips (AB Biodisk, Solna, Sweden) were then applied onto the RPMI agar medium when the excess moisture was absorbed completely. Two colonies (when applicable) were selected within the inhibition ellipses of each of

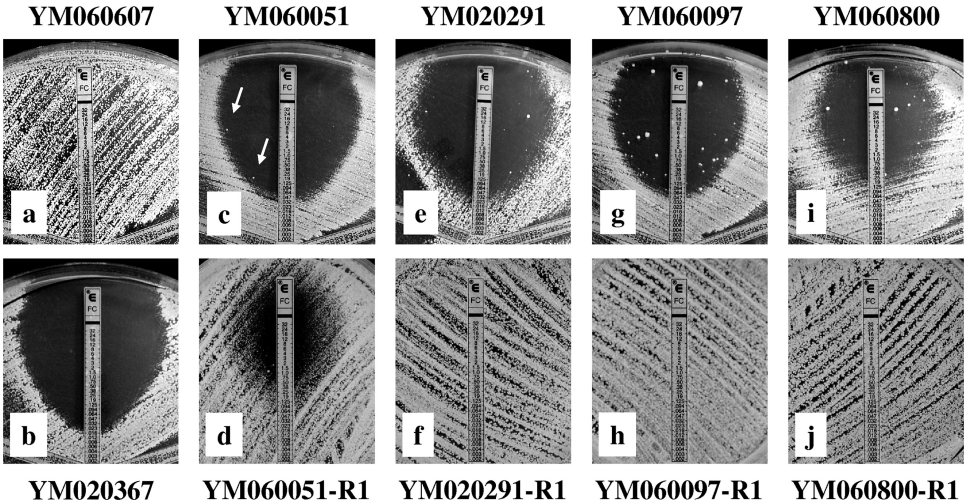

FIG. 1. Isolation of flucytosine-resistant progeny from the clinical isolates using Etest. (a) Resistant isolate YM060607; (b) susceptible isolate with clear inhibitory ellipses YM020367; (c to j) susceptible isolates producing progeny within inhibitory ellipses YM060051 (c), YM020291 (e), YM060097 (g), and YM060800 (i) and their progeny YM060051-R1 (d), YM020291-R1 (f), YM060097-R1 (h), and YM060800-R1 (j). The results were photographed after 72 h (a, b, c, e, g, and i) or 48 h (d, f, h, and j) of incubation at 35°C. Arrows indicate small colonies.

TABLE 3. Sequence of different genes of 33 *C. tropicalis* isolates

Strain	5FC MIC (μg/ml)	Gene sequence[a]									
		FCY2 (1,317 nt)									
		18	27	51	**145**	**201**	315	438	**486**	963	969
YM020112	0.25	G/A	C/T	T	G/T	G	A/C	G/C	G/T	A/G	C/T
YM020715	0.25	G/A	C/T	C/T	G	G/A	A/C	G	G	A	C
YM060369	0.25	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060616	0.25	G/A	C/T	T	G	G	A/C	G	G	A	C
YM020291	0.5	A	C	T	G/T	G	C	G/C	G/T	A/G	C/T
YM020743	0.5	A	C	T	G/T	G	C	G/C	G/T	A/G	C/T
YM060800	0.5	A	C	T	G/T	G	C	G/C	G/T	A/G	C/T
YM020438	0.5	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060210	2	G/A	C/T	T	G	G	A/C	G	G	A	C
YM020438-1	8	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060616-1	8	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060088-2	16	A	C	T	T	G	C	C	T	G	T
YM020347-1	32	A	C	T	T	G	C	C	T	G	T
YM060607	64	A	C	T	G/T	G	C	G/C	G/T	A/G	C/T
YM020112-1	64	A	C	T	T	G	C	C	T	G	T
YM020112-2	64	A	C	T	T	G	C	C	T	G	T
YM020347-2	64	A	C	T	T	G	C	C	T	G	T
YM060088-1	64	A	C	T	T	G	C	C	T	G	T
YM060800-2	64	A	C	T	T	G	C	C	T	G	T
YM020715-1	64	G	T	T	G	A	A	G	G	A	C
YM020715-2	64	G	T	T	G	A	A	G	G	A	C
YM020438-2	64	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060369-2	64	G/A	C/T	T	G	G	A/C	G	G	A	C
YM020347	<0.125	G/A	C/T	T	G/T	G	A/C	G/C	G/T	A/G	C/T
YM060088	<0.125	G/A	C/T	T	G/T	G	A/C	G/C	G/T	A/G	C/T
YM020291-1	>64	A	C	T	T	G	C	C	T	G	T
YM020291-2	>64	A	C	T	T	G	C	C	T	G	T
YM020743-1	>64	A	C	T	T	G	C	C	T	G	T
YM020743-2	>64	A	C	T	T	G	C	C	T	G	T
YM060210-1	>64	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060800-1	>64	A	C	T	T	G	C	C	T	G	T
YM060369-1	>64	G/A	C/T	T	G	G	A/C	G	G	A	C
YM060616-2	>64	G/A	C/T	T	G	G	A/C	G	G	A	C

[a] Numbers in boldface indicate a change in amino acid due to different nucleotides. Genetic details: (i) *FCY2*, 145th G, Glu; T, stop; 201th G, Trp; A, stop; 486th G, Me; T, Ile; and (ii) *URA3*, 775th G, Ala; A, Thr; 791th C, Thr; A, Ile. nt, nucleotides.

the 35 isolates and grown on YPD agar medium in the absence of drug for 2 days before they were kept in 50% glycerol at −80°C for further analysis.

The susceptibilities to 5FC of the 67 progeny within the inhibition ellipses of the 35 isolates along with their parental isolates were determined by the broth microdilution method according to the procedures in previous study (45), which is modified from the guidelines of Clinical and Laboratory Standards Institute (10). First, all isolates were grown on the YPD agar medium overnight. The RPMI medium 1640 (Gibco-BRL catalog no. 31800-022), which contains 0.2% glucose, was used for the testing. Strains from the American Type Culture Collection, including *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019), were used as the standard controls. The concentration of 5FC and fluconazole ranged from 0.125 to 64 μg/ml. Cell growth was determined by using spectrophotometric measurement with a Biotrak II plate reader (Amersham Biosciences, Biochrom, Ltd., Cambridge, England) after a 48-h incubation at 35°C. For fluconazole, isolates with MICs of ≥64 μg/ml were considered to be resistant, whereas those with an MIC ≤8 μg/ml were susceptible. Isolates with MICs falling in between (16 to 32 μg/ml) were susceptible-dose dependent. For 5FC, isolates with MICs ≥32 μg/ml were considered resistant, whereas those with MICs of ≤4 μg/ml were susceptible. Isolates with MICs falling in between (8 to 16 μg/ml) were intermediate. In addition, we used Etest to verify the susceptibilities of 5FC of at least one resistant progeny from each clinical parental isolate.

RESULTS AND DISCUSSION

Screening flucytosine-resistant isolates of *C. tropicalis*. A total of 27 and 70 *C. tropicalis* clinical isolates collected for the TSARY studies in 2002 and 2006, respectively (43, 45), were tested for susceptibilities to 5FC by the Etest method (Table 2). Only one isolate, YM060607 (Fig. 1a), was resistant to 5FC. It was also resistant to fluconazole (Table 2). This low prevalence of 5FC resistance may be due to the rare use of this drug in Taiwan. The inhibition ellipses of 61 isolates were clear, such as that of YM020367 (Fig. 1b). In contrast, colonies appeared within the inhibition ellipses of the remaining 35 isolates. Few had small colonies on the edges of the inhibition ellipses such as that of YM060051 (Fig. 1c), whereas others had colonies evenly distributed within the inhibition ellipses, such as those of YM020291 (Fig. 1e), YM060097 (Fig. 1g), and YM060800 (Fig. 1i). The 5FC susceptibilities were determined for the 67 isolates recovered from within the inhibitory ellipses, as well as their parental isolates by the broth microdilution method (Table 2). Of the 67 isolates 55 (82.1%), derived from 30 different clinical isolates, still displayed resistance to 5FC, whereas their parental isolates were susceptible. For the remaining five clinical isolates, YM060512 produced a progeny, YM060512-1, with intermediate susceptibility to 5FC, and the progeny from the other four clinical isolates, YM020055, YM060051, YM060071, and YM060173, were still 5FC susceptible. The 5FC resistance phenotype of at least one progeny of

TABLE 3—*Continued*

Gene sequence[a]								
FUR1 (657 nt)						*URA3* (807 nt)		
21	93	127	431	501	507	345	775	**791**
A	T	A/G	C	T	T	A	G	C/T
A	T	A/G	C	T	T	A	G	C/T
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A	T	A/G	C	T	T	A	G	C/T
A	C/T	A	C/T	C/T	C/T	A/G	A/G	C
A	T	A/G	C	T	T	A	G	T
A	T	A/G	C	T	T	A	G	T
A	T	A/G	C	T	T	A	G	C/T
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A	T	A/G	C	T	T	A	G	C/T
A	T	A/G	C	T	T	A	G	C/T
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A	T	A/G	C	T	T	A	G	C/T
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A/T	T	A	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C
A	C/T	G	C	C/T	C/T	A	G	C

each clinical parental isolate was confirmed by Etest, such as those of YM020291-R1 (Fig. 1f), YM060097-R1 (Fig. 1h), and YM060800-R1 (Fig. 1j). In contrast, YM060051-R1 was still susceptible to 5FC, which is also consistent with the results of the broth microdilution method (Fig. 1d).

Sequencing the four known genes involved in flucytosine resistance. To determine the mechanisms contributing to 5FC resistance, we sequenced the *FCY1*, *FCY2*, *FUR1*, and *URA3* ORFs of 33 isolates (Table 3), a group comprising 1 resistant isolate (YM060607), 11 susceptible parental isolates (randomly selected from the 30 isolates producing 5FC-resistant progeny), and 21 progeny from these isolates. Unlike *FCY1* in either *C. albicans* or *C. lusitaniae* (20, 23), the sequence of *FCY1* in the present study is highly conserved. Neither single-nucleotide polymorphism (SNP) nor any other variation was detected among all of the tested isolates. For *FUR1*, six SNPs were detected. Nevertheless, all were synonymous alterations since they did not change the amino acid residues in the encoded proteins. There were three SNPs detected in *URA3*. The SNP at position 345 was a synonymous alteration. The one at 775th position allowed the 259th amino acid residue to be either threonine (ACC) or alanine (GCC), and that at 971st position made the 264th residue to be either threonine (ACC) or isoleucine (ATC).

When we compared the *FCY2* sequences of the isolates from the present study to that of CTRG_02059 from the *C. tropicalis* database of the Broad Institute (http://www.broadinstitute.org/annotation/genome/candida_group/GeneDetails.html?sp=S7000000625961821), we found that CTRG_02059 contained a nonsense mutation at the 201st position, which caused the ATG at positions 214 to 216 to be denoted as the translation initiation site. Therefore, *FCY2* in fact encodes a 509-amino-acid purine-cytosine permease (HQ166001), and its translational initiation site corresponds to position 2136158 at the supercontig 2 (Fig. 2). Of 10 SNPs detected in *FCY2*, 7 were synonymous alterations. In contrast, both the 145th nucleotide alteration, G to T, and the 201st nucleotide alteration, G to A, resulted in truncated purine-cytosine permeases. The remaining one was at the 486th position, a G-to-T alteration changing methionine to isoleucine at the 162nd amino acid residue.

Of the 11 susceptible parental isolates, 6, including YM020112, YM020291, YM020347, YM020743, YM060088, and YM060800, had a heterozygous G/T at position 145, and their progeny had a homozygous T/T in *FCY2*. Three isolates (YM020112, YM020347, and YM060088) had eight SNPs, and the other three (YM020291, YM020743, and YM060800) had five SNPs within the *FCY2* ORF. We assessed the results by cloning PCR products of *FCY2* from YM020291 into a vector and sequencing several independent clones. We found that there were two distinct *FCY2* alleles in the YM020291 isolate.

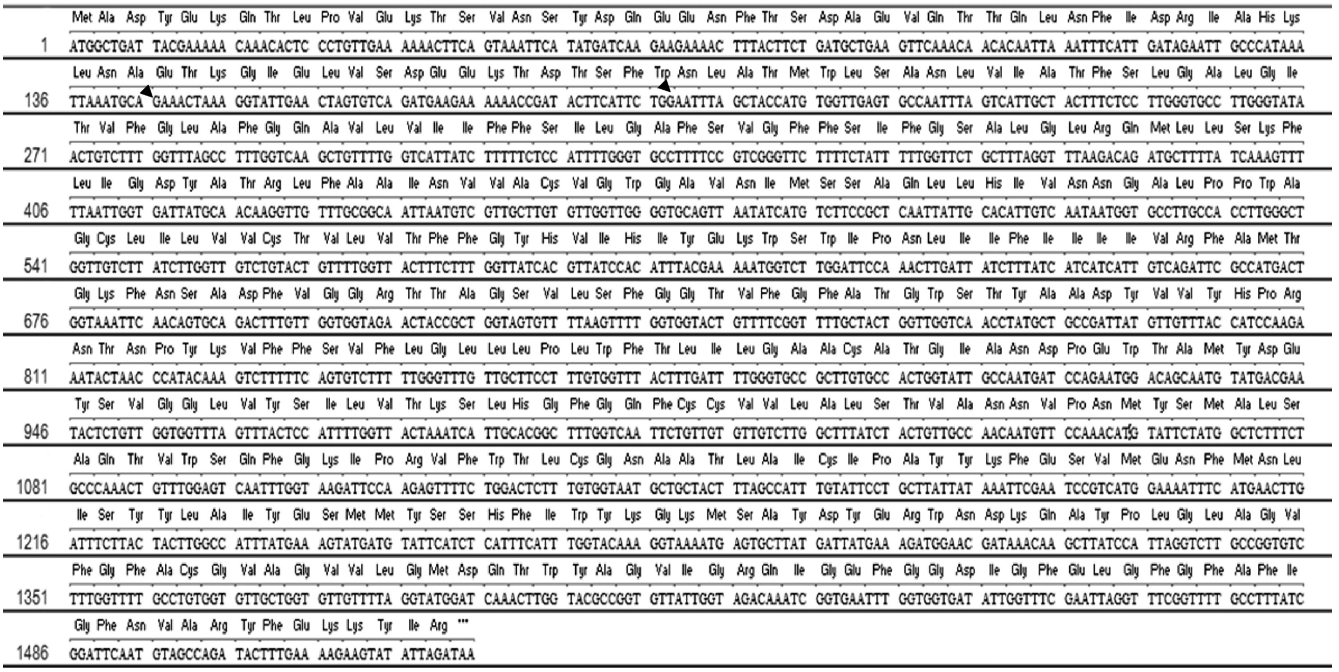

FIG. 2. Sequence of *FCY2*. The *FCY2* encodes a purine-cytosine permease consisting of 509 amino acids. The A of the translational initiation site of *FCY2* corresponds to position 2136158 at the supercontig 2. Positions 145 and 201 are marked by arrowheads.

One allele was noted as a G allele, containing G, G, G, A, and C at positions 145, 438, 486, 963, and 969, respectively, and the other as the T allele, containing T, C, T, G, and T at the same positions. Hence, the G/T to T/T alteration at position 145 did not occur via single site mutations. Most likely, it was achieved via replacing the G allele with the T allele.

Loss of heterozygosity in *FCY2* heterozygous nonsense mutants leading to 5FC resistance. Strains with homozygous G alleles and T alleles of *FCY2* were constructed in YM020291 and YM060800 strains. The YLO415 and YLO 447 (GGGAC/GGGAC) isolates, harboring homozygous G alleles, were susceptible to 5FC (Fig. 3c and d), whereas YLO417 and YLO440 (TCTGT/TCTGT), harboring homozygous T alleles, were resistant to 5FC (Fig. 3e and f). Furthermore, we used YM020291 as the parental stain to determine the effect of the nonsense mutation at position 145 of *FCY2*. We performed site-directed mutagenesis to construct the YLO466 (TGGAC/TCTGT) strain, in which the G at position 145 in the G allele of *FCY2* of YM020291 (GGGAC/TCTGT) was replaced by a T, and the YLO468 (GGGAC/GCTGT) strain, in which the T at position 145 in the T allele was replaced by a G. We found that the YLO468 isolate, with a homozygous G, was susceptible to 5FC (Fig. 3g), whereas the YLO466 isolate, containing a homozygous T at position 145, was resistant to 5FC (Fig. 3h). These results demonstrated that a nonsense mutation in *FCY2* at position 145, when in a homozygous state, sufficiently contributed to the 5FC-resistant phenotype.

Of the 30 clinical isolates producing 5FC-resistant progeny, 22 had the G/T SNP at position 145 of *FCY2* (the first 30 isolates in Table 1). Furthermore, all but one (YM060416-2) progeny derived from these 22 clinical isolates had a homozygous T/T at that position. In fact, except for YM060512, all parental clinical isolates containing the G/T SNP at position 145 produced 5FC-resistant progeny. In contrast, all eight tested parental clinical isolates with clear inhibitory ellipses had a homozygous G/G at position 145. Hence, a nonsense mutation in *FCY2*, followed by loss of heterozygosity can be a major mechanism contributing to 5FC resistance. This is the first study to demonstrate that loss of heterozygosity and alteration in 5FC susceptibility can be readily detected in *C. tropicalis* after exposure to the drug.

Loss-of-heterozygosity can be due to one of the following: chromosome loss and duplication, break-induced replication, allelic recombination, and gene conversion (1, 11, 17, 26). Various mechanisms may reflect different expenses for fitness under diverse environmental conditions. Previous studies in *C. albicans* suggest that loss of heterozygosity under *in vitro* laboratory culture conditions mainly resulted from chromosome loss and duplication, whereas in clinical isolates it occurred via mitotic recombination (12, 17, 39). Our preliminary result suggests that the loss of heterozygosity of *FCY2* predominantly resulted from break-induced replication/allelic recombination (unpublished data).

YM020715 had G/A at position 201. The YM020715-1 and YM020715-2 recovered from within the inhibitory ellipse had homozygous A/A at that position (Table 3). This G-to-A substitution, causing a nonsense mutation, followed by converting to A/A, contributed to the 5FC-resistant phenotype of YM020715-1 and YM020715-2. Of the isolates collected in Taiwan, we found that the 145th SNP occurred at a much higher frequency than the 201st one (23 versus 1, thus far). Furthermore, 42 of the 43 resistant progeny from the 22 clinical parental isolates with the G/T alleles contained homozygous T/T alleles. Hence, genomic alterations, resulting in homozygosity occur more frequently than the acquisition of an independent mutation in the second allele or a mutation on a

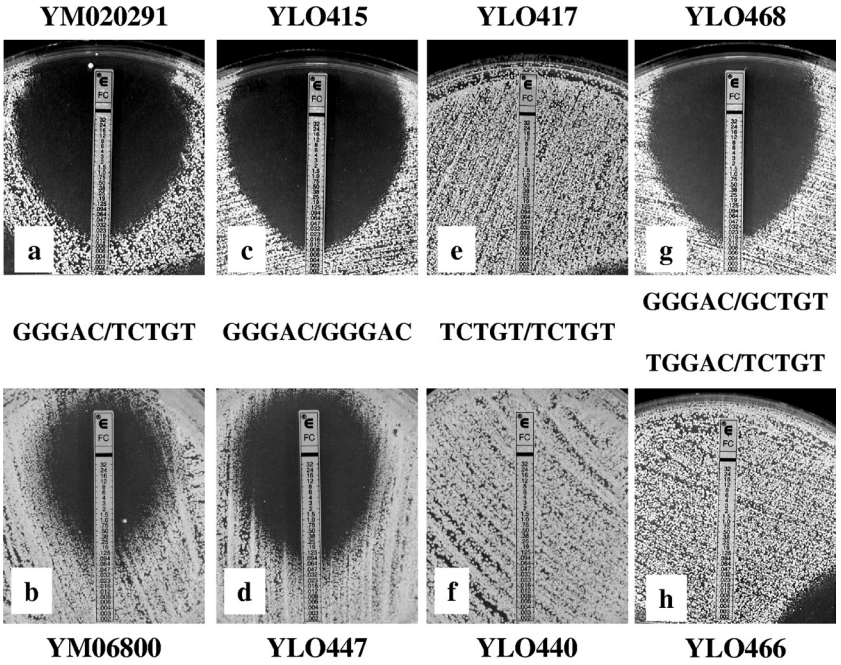

FIG. 3. Effects on flucytosine susceptibility of different *FCY2* mutants. The susceptibilities of different strains were determined using Etest. Parental isolates YM020291 (a) and YM060800 (b) with GGGAC/TCTGT at positions 145, 438, 486, 963, and 969 of *FCY2* are shown. Additional isolates: YLO415 (c) and YLO447 (d) with GGGAC/GGGAC, YLO417 (e) and YLO440 (f) with TCTGT/TCTGT, YLO468 with GGGAC/GCTGT (g), and YLO466 with TGGAC/TCTGT (h). The results were photographed after 48 h of incubation at 35°C.

different gene. Similar phenomena have been reported in *C. albicans* in the mechanistic studies of fluconazole resistance. One involved two hyperactive *TAC1* alleles from isolates overexpressing *CDR1* and *CDR2* (11) and another two different *MRR1* mutants overexpressing *MDR1* (17).

Existence of resistance with unknown mechanisms. The YM060607 isolate was the only 5FC-resistant clinical isolate among our collection in the TSARY studies. It had a G/T at position 145 of *FCY2* and an A/G at position 775 of *URA3*. Hence, the development of resistance is not based on the mechanism and genes mentioned above. In addition to mutations within ORFs, alterations on the level of gene expression due to mutations in the untranslated regions or their trans-regulators may also result in resistance. The mechanisms contributing to the increase in 5FC MICs of other progeny are under investigation. These progeny included 10 resistant isolates (YM020438-2, YM060210-1, YM060075-1, YM020671-1, YM020671-2, YM060097-1, YM060097-2, YM060369-1, YM060369-2, and YM060616-2) and 3 intermediate isolates (YM020438-1, YM060616-1, and YM060512-1).

Conclusion. In the present study, we found that *FCY2*'s loss of heterozygosity is the major molecular mechanism contributing to the 5FC-resistant phenotype of *C. tropicalis*. The increasing rate of reduced susceptibility to fluconazole in *C. tropicalis* has considerable clinical importance. In addition, approximately half of the fluconazole-resistant *C. tropicalis* isolates collected in Taiwan belonged to diploid sequence type 98 (DST98) and DST140 (9, 35). In the present study, we found that DST98 and DST140 isolates had homozygous G/G at position 145, and none produced 5FC-resistant progeny within the inhibitory ellipses. Among all of the tested isolates, only one, YM060607, was resistant to both 5FC and fluconazole. Hence, 5FC in combination with another antifungal drug can be considered for treating fluconazole-resistant *C. tropicalis*.

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