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（Summary）

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（要　旨）

Penicillium marneffei is a thermally dimorphic fungus that exist in the mycelial form at 25°C and switch to the yeast-like form at 37°C and formerly regarded as rare fungal pathogen for human. However, *P. marneffei* infection, penicilliosis, has become significant incident since the AIDS pandemic and emerged as the third common opportunistic disease, after tuberculosis and cryptococcosis, among patients in many Asian countries. To date, penicilliosis is successfully treated with amphotericin B combined with itraconazole or using voriconazole alone. Although no drug resistance *P. marneffei* strains have been reported, it is possible that the clinical resistant strains might develop anytime due to the prolonged azole therapy as previously found in *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. Therefore it is important to undertake research into the possible *P. marneffei* drug resistance mechanisms as resistance is a serious threat considering the high mortality rate of untreated penicilliosis. The azole resistant *P. marneffei* might develop through the overexpression of ABC transporters similar to the other pathogenic fungi. To prove this hypothesis, the ABC transporters that could possibly cause azole resistance in *P. marneffei* were identified, cloned, expressed, and examined their azole drug resistance activity in *Saccharomyces cerevisiae* ADΔ.

P. marneffei ABC proteins were identified by DELTA-BLAST search for homologs of NBD1 of *S.*

cerevisiae S228C Mdl1p within the protein sequence database of *P. marneffei* ATCC 18224 resulting in fifty-eight candidate proteins. Then all ABC proteins were further identified their conserved domains and classified into ABCB to ABCG subfamilies using the NCBI Conserved Domain Search. *P. marneffei* contains 46 ABC transporter homologs (two ABC transporters were considered as two isoforms of the same half-size ABC transporter, another two ABC transporters were assumed to form one full-size ABC transporter) and eight soluble ABC proteins. Forty-six *P. marneffei* ABC transporters were divided into 9 ABCB, 23 ABCC, 2 ABCD, and 12 ABCG type ABC transporters. Eight ABC proteins were grouped into 1 ABCE and 5 ABCF subfamilies and two non-classified ABC proteins.

Five ABC transporter genes in *P. marneffei* were chosen for further investigation of their drug resistance activity by BLAST search against *C. albicans* Cdr1p (ABCG subfamily) or *A. fumigatus* Mdr1p and Mdr2p (ABCB subfamily). Three ABC transporter genes belonging to ABCG subfamily were designated as *PmABC1*, *PmABC2*, and *PmABC3*. The other two ABC transporters genes were from ABCB subfamily and named as *PmMDR1* and *PmMDR2*. *PmABC1* was amplified from *P. marneffei* F4 cDNA because its genomic gene contains four introns whereas the other proteins were amplified from *P. marneffei* F4 genomic DNA by recombinant PCR amplification because they contain only one or two introns. These five genes were cloned and expressed in *S. cerevisiae* ADΔ. The drug resistance of these five yeast transformants were analyzed and compared with ADΔ/pABC3 and ADΔ/pABC3-CaCDR1, the parent and multidrug resistant strains, respective. *Pm*Abc1p could recognize almost all *Ca*Cdr1p efflux pump substrates except cerulenin (fluconazole, itraconazole, ketoconazole, miconazole, voriconazole, terbinafine, rhodamine 6G, cycloheximide, latrunculin A,

verapamil, cytochalasin D, bafilomycin A1, and nigericin) but showed a lower resistance level. *Pm*Abc3p could confer multidrug resistance phenotype to yeast cells only against fluconazole, terbinafine, latrunculin A, cycloheximide, and cerulenin. *Pm*Abc2p expressing yeast cells showed no change in drug susceptibilities compared to ADΔ/pABC3 strain. The azole susceptibilities of ADΔ/pABC3, ADΔ/pABC3-PmABC1, ADΔ/pABC3-PmABC2, and ADΔ/pABC3-PmABC3 were also confirmed by MIC test. The results showed that ADΔ/pABC3-PmABC1 had an MIC four- to eight-fold higher than ADΔ/pABC3 while, interestingly, the MIC of fluconazole for ADΔ/pABC3-PmABC3 were 128-fold higher than ADΔ/pABC3. Expression of *Pm*Mdr1p in *S. cerevisiae* ADΔ cells showed the resistance only to cytochalasin D. *Pm*Mdr2p expression gave the same result as *Pm*Mdr1p but it also increased resistance to cerulenin. From these results, it indicated that *Pm*Abc1p and *Pm*Abc3p could function as multidrug efflux pumps and might play an important role in drug resistance in *P. marneffei*. *Pm*Mdr1p and *Pm*Mdr2p were not considered as potential multidrug efflux pumps because they could not recognize azoles which were the widely used antifungal drugs. These two proteins might involve in transport of other substances or other cellular mechanisms as predicted from the function of their yeast homologs.

The localization of *Pm*Abc1p, *Pm*Abc2p, *Pm*Abc3p, *Pm*Mdr1p, and *Pm*Mdr2p in *S. cerevisiae* ADΔ cells were observed by using their C-terminal GFP-fusion derivatives. All proteins, except *Pm*Abc2p, were localized at the plasma membrane whereas *Pm*Abc2p accumulated inside the cell. Addition of GFP-tag to transporters had no affected in their drug resistance activities except for *Pm*Mdr1p-GFP-expressing strain that was increased resistance to cytochalasin D. Moreover, the effect of temperature on localization of all five

proteins were also examined and revealed that at 37°C *Pm*Abc1p and *Pm*Mdr1p were aggregated in the cytosol rather than trafficked to plasma membrane as previously observed at 30°C. The azole resistance of *Pm*Abc1p was also abolished at 37°C.