Genomic Tandem Quadruplication is Associated with Ketoconazole Resistance in *Malassezia pachydermatis*

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**Introduction**

*Malassezia* species are basidiomycetous fungi that are commonly found on the skin of various animals and are considered a residential cutaneous fungal flora. Among this species, *Malassezia pachydermatis* is a normal organism in dogs and is found in the ear canals, on the lips, axillae folds, anal sacs, rectum, and interdigital spaces. However, *M. pachydermatis* can cause diseases such as otitis externa and dermatitis in dogs with compromised immunity or altered skin condition [1, 2]. In particular, comparison of the mycobiota in the ear canals of healthy dogs and dogs with otitis externa based on sequencing data showed an extended population of *M. pachydermatis* in dogs with the disease and confirmed the association of *M. pachydermatis* with this major skin disease in dogs [3]. Although *M. pachydermatis* is primarily zoophilic [4], the yeast frequently colonizes the human skin, of neonates in particular, and causes systemic infections [5, 6].

Ketoconazole, an antifungal azole derivative, is one of the drugs most commonly used to treat canine otitis externa and dermatitis caused by *M. pachydermatis* [4, 7, 8]. In model fungi such as *Saccharomyces cerevisiae* and *Candida albicans*, ketoconazole binds to lanosterol 14α-demethylase encoded by *ERG11* and inhibits the synthesis of ergosterol, which is a major constituent of the fungal membrane [9, 10]. Most *M. pachydermatis* strains are susceptible to ketoconazole; however, the occurrence of resistant strains has been reported [11, 12]. To date, only one study has investigated the mechanisms of azole resistance in *M. pachydermatis* [13]. The study suggested an involvement of drug efflux pumps [13]; however, it used drug efflux pump inhibitors, not a molecular genetic approach.

In the current study, we isolated *M. pachydermatis* strains...
from dogs with various skin diseases, including otitis externa, and the susceptibility of the fungi to ketoconazole was determined. Whereas most of the isolates were susceptible to ketoconazole, three strains showed significantly increased minimal inhibitory concentrations (MICs) implicating that they are resistant to the drug. The strain that displayed the highest MIC was selected for genetic analysis to elucidate its ketoconazole resistance mechanism. Our study revealed the existence of a tandem quadruplication in chromosome 4 of the resistant strain and showed that the quadruplicated region contains genes required for ergosterol synthesis. Moreover, transcriptome analysis suggested that the expression of genes in the quadruplicated region was strongly increased. Our results suggest that the chromosomal region that was quadruplicated in *M. pachydermatis* KCTC 27587 is important in ketoconazole resistance and imply that multiplication of the region contributes to ketoconazole resistance in *M. pachydermatis*.

### Materials and Methods

#### Clinical Sampling

In total, 10 dogs with at least one of the clinical statuses as listed in Table 1, including Cushing’s disease, pancreatitis, mass of cervix or face, heart disease, atopy, mass or cancer in the liver, and hypothyroidism, were presented at our veterinary hospital. The breeds of the dogs were Maltese, Shih-Tzu, Miniature Schnauzer, Poodle, and a mixed breed. Following routine clinical examination, the animals were aseptically swabbed in both ear canals or on a paw or other skin part according to a protocol approved by the Institutional Animal Care and Use Committees at Chung-Ang University (2014-00010). All dogs showed mild ear discharge, and we cleaned the ears rather than administrating antimicrobial or antifungal reagents. Swab samples were transported to the microbiology laboratory at ambient temperature and were processed within 1 h of collection.

#### Isolation and Identification of *M. pachydermatis*

*Malassezia* strains were grown in modified Dixon’s (mDixon) medium (3.6% malt extract, 2% bile salt, 0.6% peptone, 1% Tween-40, 0.2% oleic acid, 0.2% glycerol) at 34°C for 2 days [14]. To identify *Malassezia* isolates, a single colony was taken and suspended in chelating ion exchange resin solution (20% (w/v) Chelex (Bio-Rad, USA) in Tris-ethylenediaminetetraacetic acid buffer) [15]. The mixture was boiled at 100°C for 20 min, cooled, and centrifuged at 16,000 g for 5 min. The supernatant containing genomic DNA was used for amplification of the D1/D2 region of the large subunit of rDNA by PCR using the universal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTCAGACGGG-3') [16]. The PCR product was sequenced for species identification. Sequences from all 10 isolates obtained from each of the dogs showed more than 99% identity to that of the *M. pachydermatis* type strain CBS 1879 and thus, all 10 isolates were identified as *M. pachydermatis*. The identified *M. pachydermatis* strains were deposited to the Korean Collection for Type Culture (KCTC).

#### Determination of Antifungal Susceptibility

To evaluate the susceptibility of *M. pachydermatis* towards ketoconazole, minimal inhibitory concentrations (MICs) of the strains were determined using the modified broth serial dilution method based on the Clinical and Laboratory Standards Institute (CLSI) guideline [17]. A stock solution of ketoconazole was serially diluted two-fold in a total volume of 200 μl of mDixon’s medium in a 96-well plate. *M. pachydermatis* cells (5 × 10^5 to 2.5 × 10^7 CFU/ml) were inoculated into each well of the 96-well plate.
and incubated at 34°C for 2 days. MICs were determined as the lowest concentration at which there was no visible growth of the cells compared to that in medium without ketoconazole. MICs were determined at least three times independently.

Genome Sequencing and Annotation

*M. pachydermatis* KCTC 27587 was grown in mDixon medium at 34°C for 2 days, and genomic DNA was extracted from the cells as described previously [18]. The genomic DNA was fragmented using a g-TUBE device (Covaris, USA) to fragments of 20 kb in length, and a DNA library was constructed for the PacBio platform (PacBio, USA) according to the manufacturer’s instructions. Sequencing was performed on the PacBio Sequel instrument, using one SMRT cell. The raw read sequences were assembled with canu v1.7 [19] with genomeSize set at 7.3 Mb. Contigs were polished using the Arrow consensus caller in PacBio SMRT Link v5.0.1. Telomeric motifs in chromosomal ends and mitochondrial contigs were reviewed by manual curation. Gene prediction was conducted with BRAKER v1.8 [20] and genes were annotated using eggNOG-mapper [21]. When the transcript expression was normalized to TPM (Transcripts Per Kilobase Million), the percentage of genes with TPM value over 10 in *M. pachydermatis* KCTC 27587 strain was 97.1% of total 4,116 genes. Genome sequencing data have been deposited to the Sequence Read Archive database of the National Center for Biotechnology Information (NCBI) under accession no. SRP156512.

Transcriptome Analysis

*M. pachydermatis* KCTC 27587 and CBS 1879 cells were incubated in mDixon’s broth medium at 34°C for 6 h and harvested for RNA extraction. Total RNA was prepared using TransZolUp (Transgen Biotech, China), and the RNA integrity in each sample was evaluated using BioAnalyzer 2100 (Agilent Technologies, USA). Libraries for RNA-sequencing were constructed using the TruSeq Stranded Total RNA Sample Prep Kit (Illumina, USA) following the manufacturer’s instructions. The libraries were sequenced on an Illumina HiSeq 2500 instrument (Illumina, USA) following the manufacturer’s instructions, generating 75-bp paired-end reads for each sample. Raw sequence data were subjected to adapter sequence removal and quality-based trimming with Trimmomatic v. 0.36 using TruSeq adapter sequences [22]. Cleaned reads were mapped to the reference genome using bowtie2 with default parameters settings [23]. FeatureCounts was used to count the reads mapped to each coding sequence [24]. The counts were transformed and normalized for differential expression analysis with DESeq2 [25]. The transcriptome data have been deposited to the Gene Expression Omnibus database of NCBI under accession no. GSE118354.

Quantitative Real-Time PCR

For the validation of gene copy numbers, quantitative real-time PCR (Q-RT-PCR) was conducted using genomic DNA as a template. Gene-specific primers for Q-RT-PCR were designed using Primer Express software v. 3.0 (Applied Biosystems, USA) and are listed in Table S1. Q-RT-PCRs were run on a 7500 Real-Time PCR System (Applied Biosystems, Foster, USA). The relative copy number of each gene of interest was calculated by the 2^{-ΔΔCT} method [26]. The translation elongation factor 2 (*TEF2*) gene was used as an endogenous control for normalization.

Generation of a Ketoconazole-Resistant *M. pachydermatis* Strain by In Vitro Evolution

The ketoconazole-susceptible strain *M. pachydermatis* KCTC 27575 was used for in vitro evolution according to a previously reported method with modification [27]. The susceptible strain was periodically propagated in mDixon agar containing ketoconazole for seven transfers. For each transfer, a single colony was transferred to medium containing 2-fold serially increasing concentrations of ketoconazole (from 0.06 μg/ml to 8 μg/ml) and incubated for 5 days at 34°C between transfers. Four independently generated resistant strains showing MICs equivalent to that of *M. pachydermatis* KCTC 27587 were obtained.

Results and Discussion

Isolation of Ketoconazole-Resistant *M. pachydermatis*

The MICs of 10 clinically isolated *M. pachydermatis* strains to ketoconazole were determined according to a modified CLSI broth microdilution method. The MICs of seven *M. pachydermatis* isolates, KCTC 27575, KCTC 27824, KCTC 27825, KCTC 27826, KCTC 27827, KCTC 27828, and KCTC 27829, were between 0.125 μg/ml and 0.5 μg/ml, which was the range for the ketoconazole-susceptible *M. pachydermatis* type strain, CBS 1879. However, three out of the 10 isolates, KCTC 27587, KCTC 27591, and KCTC 27593, showed significantly higher MICs of 4–8 μg/ml, suggesting that they are resistant to ketoconazole (Table 1). Among the resistant strains, *M. pachydermatis* KCTC 27587, which was isolated from a dog suffering from otitis externa, atopic dermatitis, and food allergy, showed the highest MIC and was therefore selected for further investigation of the mechanism underlying ketoconazole resistance of *M. pachydermatis*.

Whole Genome Sequencing of *M. pachydermatis* KCTC 27587

To understand the mechanism of ketoconazole resistance of *M. pachydermatis* KCTC 27587, the whole genome of the yeast isolate was sequenced using the PacBio platform and compared with that of type strain *M. pachydermatis* CBS 1879. The genome and mitochondrial genome sizes of *M. pachydermatis* KCTC 27587 are 8,283,742 and 35,575 bp, respectively. In total, six chromosomes were assembled, and 4,116 protein-coding regions were predicted. The
mitochondrial genome contains 18 coding regions (Table 2). Wu et al. recently analyzed the genome of the *M. pachydermatis* type strain CBS 1879 using Illumina HiSeq and found that it has a 8.2-Mbp genome harboring 4,328 genes. However, they did not completely assemble the chromosomes, probably because of a limitation of the sequencing technology they used. In the current study, we were able to assemble all six individual chromosomes of *M. pachydermatis* with corresponding telomeres (Fig. 1). Most of the genome features of KCTC 27587 were similar to those of the type strain CBS 1879.

### Table 2. Summary of the genome analysis.

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<td>Coverage (fold)</td>
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<table>
<thead>
<tr>
<th></th>
<th>Nuclear</th>
<th>Mitochondrial</th>
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<tbody>
<tr>
<td>Total assembly size (bp)</td>
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<td>35,575</td>
</tr>
<tr>
<td>GC (%)</td>
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<td>31.2</td>
</tr>
<tr>
<td>Scaffold</td>
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<td>1</td>
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<tr>
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<td>2</td>
</tr>
<tr>
<td>tRNA</td>
<td>77</td>
<td>19</td>
</tr>
</tbody>
</table>

*M. pachydermatis* KCTC 27587 Harbors a Tandem Quadruplication in Chromosome 4

During the sequence assembly, we found that coverage of the region between the chromosomal positions 907,643 and 992,110 on chromosome 4 was 350–400×, which was significantly higher than the average coverage (97.76×) of the remainder of chromosome 4 and other chromosomes, suggesting that the region is tandemly quadruplicated (Fig. 2). In the quadruplicated region, 52 protein-coding genes were identified, including MP87_02948 and MP87_02952, homologs of *S. cerevisiae* ERG11 and ERG4, which encode lanosterol 14-α demethylase and sterol C-24 reductase, respectively, involved in the ergosterol biosynthesis pathway (Fig. 3). To confirm the existence of the tandemly quadruplicated region in chromosome 4, we determined the relative copy numbers of three genes (MP87_02934, MP87_02948, and MP87_02974, which are homologs of *S. cerevisiae* CCA1, ERG11, and PET8 respectively) in the quadruplicated region by Q-RT-PCR. The genes MP87_00784, MP87_01226, MP87_02511, and MP87_03488, which encode translation elongation factor 2, GAPDH, eIF-5α, and...
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α-tubulin, respectively, located in a non-quadruplicated region, were used as references. The Q-RT-PCR results showed that the copy numbers of MP87_02934, MP87_02948, and MP87_02974 were approximately four times higher than those of the reference genes (Fig. 4). These data confirmed the existence of the tandemly quadruplicated region in chromosome 4 suggested by the whole genome sequencing data, and led us to hypothesize that the chromosomal rearrangement might be contributed to the significantly reduced ketoconazole susceptibility of M. pachydermatis KCTC 27587.

Transcriptome Analysis

Earlier studies in C. neoformans have shown that multiple duplication of a chromosome in azole-resistant strains results in increased expression of genes within the chromosome, although the level of increase may or may not correspond with the amount of duplication [28, 29]. Therefore, we investigated whether the quadruplication in chromosome 4 influenced on the transcription of genes in that region. The transcriptome of M. pachydermatis KCTC 27587 was analyzed by RNA-sequencing and was compared with that of the type strain M. pachydermatis CBS 1879. Overall, the

Fig. 3. Organization of the quadruplicated region.
In total, 52 protein-coding genes were found in the tandemly quadruplicated region, including homologs of S. cerevisiae ERG4 and ERG11, which encode sterol C-24 reductase and lanosterol 14-α demethylase, respectively.

Fig. 4. Confirmation of gene copy numbers in the quadruplicated region.
(A) Results of Q-RT-PCR using genomic DNA extracted from M. pachydermatis KCTC 27587 as a template confirmed the existence of quadruplication in chromosome 4. Data were obtained from three independent experiments and an asterisk indicates statistical significance (p < 0.05). (B) Primers specific to MP87_02934 (CCA1), MP87_02948 (ERG11) and MP87_02974 (PET8) genes, which are located in the quadruplicated region, and MP87_00784 (TEF2), MP87_01226 (GAPDH), MP87_02511 (eIF-5α) and MP87_03488 (α-tubulin) genes, which are located in the non-quadruplicated region as references, were used for Q-RT-PCR.
transcript levels of genes within the quadruplicated region were higher than those in the type strain: 45 out of 52 genes showed a more than two-fold increase (Fig. 5). Particular attention was paid to two genes, MP87_02948 and MP87_02952, the homologs of \textit{S. cerevisiae} \textit{ERG11} and \textit{ERG4}, because increased expression of genes in the ergosterol pathway contribute to azole resistance in various pathogenic fungi. For example, elevated mRNA levels of \textit{ERG4} and \textit{ERG11} have been observed in azole-resistant \textit{C. albicans} clinical isolates [30]. As we expected, the transcript levels of the \textit{ERG11} and \textit{ERG4} homologs in the quadruplicated region in \textit{M. pachydermatis} KCTC 27587 were significantly higher (3.68- and 2.81-fold, respectively) than those in the type strain. Considering that increased expression of \textit{ERG11} and \textit{ERG4} is one of the common mechanisms of azole resistance in other pathogenic fungi, we concluded that the overexpression of the \textit{ERG4} and \textit{ERG11} homologs, which may be caused by quadruplication of the genomic region, might be the main cause of ketoconazole resistance in \textit{M. pachydermatis} KCTC 27587. We note that the genes encoding homologs of ferric reductases, MP87_02943 and MP87_02944, in the quadruplicated region were also more strongly expressed in \textit{M. pachydermatis} KCTC 27587, which may also influence susceptibility of the yeast to ketoconazole as the enzyme has been shown to contribute to azole resistance [31, 32]. Transcript levels of two other genes in the quadruplicated region, encoding a hypothetical protein (MP87_02949) and the homolog of \textit{S. cerevisiae} \textit{Sip1} domain containing protein (MP87_02972), respectively, were also significantly increased by >10-fold. However, the function of these genes in relation with azole resistance in fungi is unknown, and therefore, further investigation is required to define the contribution of these genes to ketoconazole resistance, although such contribution is unlikely.

Chromosomal Rearrangement Occurs in Ketoconazole-Resistant \textit{M. pachydermatis} Strains Generated by In Vitro Evolution

We next questioned whether chromosomal rearrangement, such as the tandem quadruplication observed in the current study, commonly occurs in ketoconazole-resistant \textit{M. pachydermatis} strains. To answer this question, we artificially generated ketoconazole-resistant strains by in vitro evolution using the ketoconazole-susceptible strain KCTC 27575, which was isolated from a healthy dog. In total, four independent resistant strains showing MICs equivalent to that of KCTC 27587 were obtained and were named “75IVE-1,” “75IVE-2,” “75IVE-3,” and “75IVE-4.” In parallel, the susceptible strain KCTC 27575 was periodically transferred to drug-free media. The reference strain generated in this way to observe any effect of spontaneous mutation was named “75IVE-C.”

The copy numbers of the chromosomal region that is quadruplicated in \textit{M. pachydermatis} KCTC 27587 in strains 75IVE-1, 75IVE-2, 75IVE-3, and 75IVE-4 were determined by Q-RT-PCR. We first determined the relative gene copy number of MP87_02948, the \textit{ERG11} homolog, within the quadruplicated region and found that two out of the four artificially-generated resistant strains, i.e., 75IVE-1 and
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75IVE-4, showed more than three times higher copy numbers than the reference strain 75IVE-C (Fig. 6A). These results implied the existence of quadruplication in the chromosomal region containing MP87_02948 (ERG11) in strains 75IVE-1 and 75IVE-4. To confirm this, we designed primers targeting MP87_01226 (GAPDH) and MP87_02511 (eIF-5α) located in the non-quadruplicated region and used as reference genes, as well as primers targeting MP87_02934 (CCA1), MP87_02948 (ERG11), and MP87_02974 (PET8) located within the quadruplicated regions. As shown in Fig. 6B, the relative copy numbers of MP87_02934 (CCA1), MP87_02948 (ERG11), and MP87_02974 (PET8) were greater than those of the reference genes MP87_01226 (GAPDH) and MP87_02511 (eIF-5α), in strains 75IVE-1 and 75IVE-4 confirming the existence of multiplication of the chromosomal region. These results suggested that gene multiplication occurred in the artificially-generated ketoconazole-resistant M. pachydermatis strains.

Chromosomal rearrangements, such as duplication and translocation, often cause alteration of phenotypic characteristics in various organisms [33–35]. In fungi, numerous cases of chromosomal duplication and translocation as well as gene amplification have been observed and are considered mechanisms of resistance to growth inhibitors and abiotic stresses [33, 34, 36, 37]. It has been suggested that highly repeated sequences in chromosomes are involved in chromosomal duplication and translocation; however, the underlying mechanism remains to be elucidated.

In the current study, we found that M. pachydermatis underwent extensive chromosomal rearrangement, i.e., tandem quadruplication, to adapt to the pressure of ketoconazole, which has resulted in increased expression levels of the genes involved in the pathway targeted by the drug. Moreover, our in vitro evolution assay revealed that chromosomal rearrangement might be one of the strategies of M. pachydermatis to survive in the presence of high concentrations of ketoconazole.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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