Enhanced gene replacements in Ku80 disruption mutants of the dermatophyte, Trichophyton mentagrophytes

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Abstract

The frequency of targeted gene disruption via homologous recombination is low in the clinically important dermatophyte, Trichophyton mentagrophytes. The Ku genes, Ku70 and Ku80, encode key components of the nonhomologous end-joining pathway involved in DNA double-strand break repair. Their deletion increases the homologous recombination frequency, facilitating targeted gene disruption. To improve the homologous recombination frequency in T. mentagrophytes, the Ku80 ortholog was inactivated. The nucleotide sequence of the Ku80 locus containing a 2788-bp ORF encoding a predicted product of 728 amino acids was identified, and designated as TmKu80. The predicted TmKu80 product showed a high degree of amino acid sequence similarity to known fungal Ku80 proteins. Ku80 disruption mutant strains of T. mentagrophytes were constructed by Agrobacterium tumefaciens-mediated genetic transformation. The average homologous recombination frequency was 73.3 ± 25.2% for the areA/nit-2-like nitrogen regulatory gene (tnr) in Ku80 mutant strains, about 33-fold higher than that in wild-type controls. A high frequency (c. 67%) was also obtained for the Tri m4 gene encoding a putative serine protease. Ku80 mutant strains will be useful for large-scale reverse genetics studies of dermatophytes, including T. mentagrophytes, providing valuable information on the basic mechanisms of host invasion.

Introduction

Molecular genetic studies of pathogenic fungi have made significant contributions to our understanding of the diseases caused by these organisms. Whole genome sequences of several pathogenic fungi, including Aspergillus fumigatus and Cryptococcus neoformans (Loftus et al., 2005; Nierman et al., 2005), have been reported and provide opportunities to extend mechanistic investigations of their pathogenesis through large-scale reverse genetics studies. Gene transfer by transformation (genetic transformation) is an indispensable technique for such studies, which may also be applicable for the development of various gene manipulation techniques. In particular, targeted gene disruption by homologous recombination plays important roles in determining functions and roles of numerous genes isolated from pathogenic fungi.

Dermatophytes are pathogenic fungi of humans and animals, which commonly gain access to the host via keratinized structures, such as the hair, skin, or nails, and cause a superficial cutaneous infection called dermatophytosis (ringworm). To gain insight into the basic mechanisms of host invasion by dermatophytes, several genetic transformation experiments of these fungi have been carried out by protoplast/polyethylene glycol (PEG)-mediated random integration methods (Gonzalez et al., 1989; Kaufman et al., 2005b; Yamada et al., 2005), all of which have low transformation frequencies. Because of the difficulty of genetic manipulation in dermatophytes caused by the low transformation frequencies, there have been few reports regarding the successful production of null mutants by targeted gene disruption via homologous recombination (Fachin et al., 2006; Ferreira-Nozawa et al., 2006; Yamada et al., 2006).

To improve transformation frequency in dermatophytes, we recently developed an Agrobacterium tumefaciens-mediated genetic transformation (ATMT) system for the clinically important dermatophyte, Trichophyton mentagrophytes (Yamada et al., 2009). This ATMT method yielded higher transformation frequencies, as compared with standard protoplast/polyethylene glycol-mediated integration methods.
PEG-mediated transformation methods. Targeted gene disruption by homologous recombination via ATMT was also accomplished in this fungus, the frequency (average about 11%) of which was still lower than in several other fungi, such as A. fumigatus (Bundock et al., 1999; Zhang et al., 2003; Sugui et al., 2005), and varied among disruption experiments. Therefore, the development of an efficient and reproducible targeted gene disruption system is required to promote molecular genetics studies in dermatophytes.

In eukaryotes, the integration of exogenous genes into chromosomes is thought to be closely related to the DNA double-strand break (DSB) repair systems through two pathways: DSB repair by homologous recombination and sequence homology-independent DSB repair by nonhomologous end joining (NHEJ). In the yeast Saccharomyces cerevisiae, exogenous genes are integrated at homologous sites within the chromosomes through the homologous recombination DSB repair pathway, whereas in humans and plants, the NHEJ pathway seems to be used preferentially for integration of exogenous genes into the chromosomes. The NHEJ process is mediated by the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ku70, Ku80, Xrcc4, and DNA ligase IV (LIG4) (Critchlow & Jackson, 1998). A heterodimer consisting of the Ku70 and Ku80 proteins forms a complex with Xrcc4 and LIG4, and binds tightly to broken DNA ends to stimulate the NHEJ pathway for DSB repair (Walker et al., 2001). Orthologs of Ku70 and Ku80 have also been identified in insects, plants, and fungi (Hefferin & Tomkinson, 2005). Ninomiya et al. (2004) reported remarkable increases in targeted gene disruption frequencies in Ku70 and/or Ku80 deletion mutants of the model filamentous fungus, Neurospora crassa. High frequencies (70–100%) of targeted gene disruption were subsequently achieved in other fungal Ku70 and/or Ku80 deletion mutants (da Silva Ferreira et al., 2006; Krappmann et al., 2006; Takahashi et al., 2006b). These mutants showed normal morphological and phenotypic characteristics, such as mycelial growth and conidial formation, and maintained virulence in animal models.

In this study, we isolated the Ku80 ortholog of T. mentagrophytes, and produced Ku80 disruption mutant strains by targeted gene disruption. The Ku80 disruption mutant strains showed a high frequency of targeted gene disruption at two independent chromosomal loci tested without any phenotypic changes. Thus, these mutant strains have huge potential for large-scale molecular genetics studies of dermatophytes, including T. mentagrophytes.

Materials and methods

Strains and media

The wild-type T. mentagrophytes strain TIMM2789 (teleomorph: Arthroderma vanbreuseghemii) was maintained at 28 °C on solid Sabouraud dextrose medium (SDA) supplemented with 500 μg mL⁻¹ cycloheximide and 50 μg mL⁻¹ chloramphenicol. To maintain transformants, an appropriate concentration of hygromycin B or G418 (Geneticin) was added to the medium. Conidial formation of each T. mentagrophytes strain was induced using modified 1/10 SDA [0.1% (w/v) Bacto peptone, 0.2% (w/v) glucose, 0.1% (w/v) KH₂PO₄, 0.1% (w/v), and MgSO₄·7H₂O] (Uchida et al., 2003). For total DNA and total RNA extraction, the growing mycelia from each T. mentagrophytes strain were collected after incubation on SDA for 4 days at 28 °C.

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) (a gift from Dr K.J. Kwon-Chung, National Institutes of Health, Bethesda, MD) was maintained at 28 °C on solid 2 x YT medium [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl, and 1.5% (w/v) agar] supplemented with 50 μg mL⁻¹ rifampicin and 25 μg mL⁻¹ chloramphenicol.

Extraction and analysis of nucleic acids

Total DNA was extracted from the growing mycelia of each T. mentagrophytes strain according to the method of Girardin & Latge (1994). The total DNA samples were digested with appropriate restriction enzymes, separated by electrophoresis on 0.8% (w/v) agarose gels, and transferred onto Hybond N⁺ membranes (GE Healthcare Limited, Buckinghamshire, UK). Southern hybridization was performed using the ECLTM Direct Nucleic Acid Labelling and Detection System (GE Healthcare Limited).

Total RNA of the wild-type T. mentagrophytes strain TIMM2789 was extracted from the growing mycelia with an RNasey Plant Mini Kit (Qiagen, Hilden, Germany).

Identification of the nucleotide sequence of the T. mentagrophytes Ku80 locus

The nucleotide sequence of a locus containing the T. mentagrophytes Ku80 homolog-coding region was identified by PCR. A pair of degenerate primers, MP-F1 and MP-R1, were designed based on the amino terminal and internal short amino acid sequences highly conserved among known fungal KU80 proteins, and a partial DNA fragment was amplified from total DNA of T. mentagrophytes by PCR with these primers. The amplified product (fragment 1) was cloned and sequenced. The 5’ end of fragment 1 was amplified by inverse PCR with a pair of specific primers, 5’IPC-R1 and 5’IPC-R1. The amplified product, which contained the translational initiation codon (ATG), was cloned and sequenced. The 3’ end of fragment 1 was amplified by rapid amplification of cDNA ends (RACE)-PCR using a specific primer, SP1, designed based on the nucleotide sequence of fragment 1. Aliquots of 5 μg of total RNA were reverse transcribed according to the 3’RACE
method with Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA). The amplified product (fragment 2), which contained the translational termination codon (TAG), was cloned and sequenced. A genomic DNA fragment containing the ORF of the T. mentagrophytes Ku80 homolog was amplified by PCR with a pair of specific primers, SP2 and ASP1, and designated as TmKu80.

On the other hand, the frequency of homologous recombination is often closely related to the size of the homologous DNA fragments flanking the selectable marker within the disruption construct. Thus, to construct the TmKu80-targeting vector containing larger homologous DNA fragments, additional nucleotide sequence data for the TmKu80 locus were required. Therefore, the ORF of TmKu80 was extended in the downstream direction by inverse PCR using a pair of specific primers, 3\\'IPCR-F1 and 3\\'IPCR-R1, and the amplified product was cloned and sequenced.

All the amplified fragments were sequenced with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA).

The nucleotide sequences of primers used for identification of the genomic DNA fragment containing the ORF of TmKu80 are shown in Table 1.

### Table 1. Primers used in this study

<table>
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<th>Name</th>
<th>Sequence (5’→3’)</th>
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<tbody>
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<td>MP-F1</td>
<td>ATGGCACA(G,C,T)GAA(G,C,T)AAAGAGGAAAGCACG (TmKu80)</td>
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<tr>
<td>MP-R1</td>
<td>CG(A)(T)(A,C,G,T)CCG(T)TTTCTGAAAGGAAAGCACG</td>
</tr>
<tr>
<td>SP1</td>
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</tr>
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<td>5\'IPCR-F1</td>
<td>GGATCCCTCTATTGCGGAGG</td>
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<td>5\'IPCR-R1</td>
<td>GGATCCCTCTATTGCGGAGG</td>
</tr>
<tr>
<td>SP2/ClaI</td>
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</tr>
<tr>
<td>ASP1/BamHI</td>
<td>ATGGAGTTTGGGATCTGTTG</td>
</tr>
<tr>
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<td>Spel</td>
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</tr>
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<tr>
<td>Apal</td>
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<td>TrmA-R1</td>
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<tr>
<td>TrmA-F2/Spel</td>
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</tr>
<tr>
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<tr>
<td>TrmA-R3/EcoRI</td>
<td>CTGCGGCTGAGGTGAGGAAAGAGGG</td>
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</table>

Restriction enzyme recognition sites are indicated in italics.

### Sequence alignment

The amino acid sequences of fungal Ku80 proteins were aligned using CLUSTAL W. The neighbor-joining method (Saitou & Nei, 1987) was used to construct the phylogenetic tree with MEGA4 (Kumar et al., 2008). Data consistency was tested by bootstrapping the alignments 1000 times with corrections for multiple substitutions.

### Construction of transformation vectors

The T. mentagrophytes Ku80 homolog-targeting vector pAg1-TmKu80/T (Fig. 1a) was constructed from pAg1-hph (Yamada et al., 2009). Two regions (fragment 3, nucleotide position: 550 to 1444; fragment 4, nucleotide position: 1533–3533) of the TmKu80 locus were amplified by PCR with the primers TmKu80-F1/Spel and TmKu80-F2/BamHI and TmKu80-R2/KpnI, respectively. The amplified fragments were double digested with Spel/Apal or BamHI/KpnI, and inserted into the upstream Spel/Apal and downstream BamHI/KpnI sites of the hygromycin B phosphotransferase gene (hph) cassette (2.3 kb) within pAg1-hph, respectively, to generate pAg1-TmKu80/T.

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**Fig. 1.** Phylogenetic tree of fungal Ku80 orthologs. Alignment was performed using CLUSTAL W. The neighbor-joining method (Saitou & Nei, 1987) was used to construct the phylogenetic tree with MEGA4 (Kumar et al., 2008). Data consistency was tested by bootstrapping the alignments 1000 times with corrections for multiple substitutions. Numbers at each node indicate Aspergillus fumigatus (XP_749397.1), Aspergillus oryzae (BAE78503.1), Aspergillus terreus (XP_001209605.1), Coccidioides immitis (EAS36450.1), Magnaporthe grisea (XP_365937.2), Neurospora crassa (BAD16623.1), Penicillium marneffei (XP_002151653.1), and Saccharomyces cerevisiae (CAL35976.1).
The *T. mentagrophytes* are nit-2-like gene *tnr* (accession no. AB364680)-targeting vector pAg1-*TmKu80*T, and genomic organization of the *TmKu80* gene. pAg1-*TmKu80*T was constructed from pAg1-*hph* (Yamada et al., 2009). Pch, *Cochliobolus heterostrophus* promoter 1 (Turgeon et al., 1987); *hph*, *Escherichia coli* hygromycin B phosphotransferase gene (Gritz & Davies, 1983); TrpC, terminator sequence of *Aspergillus nidulans* tryptophan C gene (Mullaney et al., 1985); A, ApaI; Ba, BamHI; Bg, BglII; C, ClaI; E, EcoRI; K, KpnI; S, SpeI; and Xh, XhoI. (b) Southern-blotting analysis of total DNA samples from *T. mentagrophytes* Ku80 mutant strains produced by ATMT. Total DNA from each strain was digested with Xhol and separated by electrophoresis on a 0.8% (w/v) agarose gel. Lane 1, total DNA sample from the parental wild-type control of *T. mentagrophytes*; Lanes 2–7, total DNA samples from *T. mentagrophytes* Ku80 mutant strains. A partial fragment (645 bp, nucleotide position: 1533–2177) of the *TmKu80* locus was used as a hybridization probe. DNA standard fragment sizes are shown on the left.

Each binary vector was introduced into *A. tumefaciens* strain EHA105 by electroporation (MicroPulser; Bio-Rad, Hercules, CA), in accordance with the manufacturer’s instructions (2.2 kV, 600 μF).

All the amplified fragments cloned in the vectors were sequenced to confirm that no nucleotide substitutions had occurred during amplification.

The nucleotide sequences of primers used for construction of transformation vectors are shown in Table 1.

**Fungal genetic transformation**

Transformation of each *T. mentagrophytes* strain was carried out by the ATMT method (Yamada et al., 2009).

**Phenotypic analysis**

Sensitivity to chemical mutagens was tested as follows: small conidia (1 × 10⁴ conidia) from each *T. mentagrophytes* strain were inoculated onto solid RPMI1640 medium containing several concentrations of methyl methane sulfonate (MMS)
(Wako Chemical, Osaka, Japan), ethyl methane sulfonate (EMS) (Wako Chemical), hydroxyurea (Wako Chemical), or phleomycin (Sigma, St. Louis, MO), and incubated at 28°C for 4 days.

To examine the sensitivity of each *T. mentagrophytes* strain to temperature, small conidia (1 × 10⁴ conidia) were inoculated onto solid RPMI1640 medium, and incubated at 28, 37, and 40°C for 4 days.

Small conidia were collected from 7-day-old mycelium cultures grown on modified 1/10 SDA (Uchida et al., 2003). Conidial germination frequency was assayed by spreading 2 × 10⁵ conidia onto SDA plates. The numbers of colonies regenerating on the plates were calculated after 4 days of incubation at 28°C.

**Assay of growth properties of *T. mentagrophytes* on various nitrogen sources**

Aliquots of 10μL of conidial suspensions containing about 1 × 10⁶ cells of each *T. mentagrophytes* strain were spotted onto solid Aspergillus minimal medium (Barratt et al., 1965) supplemented with various nitrogen sources as follows: 10 mM NaNO₃, NaNO₂, and NH₄Cl, 1 mM L-tyrosine, 5 mM other amino acids, and urea. *Trichophyton mentagrophytes* conidia were incubated at 28°C for 5–6 days and growth of fungal colonies was compared.

**Nucleotide accession number**

The nucleotide sequence data of *TmKu80* from *T. mentagrophytes* have been deposited in DDBJ/EMBL/GenBank under accession no. AB427108.

**Results**

**Identification of the nucleotide sequence of the *T. mentagrophytes* Ku80 locus**

A PCR-based strategy was chosen to determine the nucleotide sequence of the Ku80 locus in *T. mentagrophytes*. A pair of degenerate primers, MP-F1 and MP-R1, were designed based on the amino terminal and internal regions highly conserved among known fungal Ku80 proteins, and used to amplify a partial genomic DNA fragment corresponding to the *T. mentagrophytes* Ku80 homolog. Nucleotide sequencing of the obtained DNA fragment of about 1.4 kb (fragment 1) revealed that the predicted amino acid sequence contained several regions conserved among known fungal Ku80 proteins. Subsequently, 3′ RACE-PCR and inverse PCR were carried out to extend fragment 1 in both directions, and a nucleotide sequence of a genomic DNA fragment of about 4.1 kb, containing an ORF of 2788 bp, was finally identified and designated as *TmKu80*. The ORF of *TmKu80* was interrupted by 10 introns (< 100 bp). The positions of the ORF, exon, and intron were estimated on the basis of similarity to known fungal Ku80 proteins and the GT-AG splicing rule. Figure 1 shows the alignment of the predicted amino acid sequence of *TmKu80* with those of other known fungal Ku80 proteins. The ORF of *TmKu80* consisted of 728 amino acid residues, and shared about 70% amino acid sequence identity with *Coccidioides immitis* Ku80 protein, about 60% identity with Ku80 proteins from *Penicillium marneffei*, and *Aspergillus* species, and 40–50% identity with *Magnaporthe grisea* and *N. crassa* Ku80 proteins. Two Ku80 functional domains, the N-terminal α/β domain and the internal β-barrel domain, were found within the *TmKu80* polypeptide.

**Disruption of *TmKu80* by homologous recombination**

To disrupt the *T. mentagrophytes* Ku80 homolog *TmKu80*, we constructed a gene-targeting vector, pAg1-*TmKu80*/T (Fig. 2a), carrying the *hph* cassette flanked by the *TmKu80* sequences. Transformation of the wild-type *T. mentagrophytes* strain TIMM2789 mediated by *A. tumefaciens* containing pAg1-*TmKu80*/T produced a large number of hygromycin B-resistant colonies on selective medium, 30 of which were chosen at random and analyzed by molecular biological methods. PCR analysis suggested that homologous recombination between the disruption construct and *Tmk80* locus occurred in six colonies (data not shown). On Southern-blotting analysis with a *TmKu80*-specific probe, all six colonies exhibited the banding pattern expected for homologous recombination via double crossover without additional ectopic integration of the disruption construct (Fig. 2b). Mycelia of the six *Ku80*’ mutant strains growing on SDA with or without hygromycin B were examined by light microscopy, and no morphological differences were detected between the parental wild-type strain TIMM2789 and *Ku80*’ mutant strains (data not shown). The six *Ku80*’ mutant strains also exhibited normal conidial formation on modified 1/10 SDA. Subsequently, the sensitivities of the two *Ku80*’ mutant strains (*TmKu80Δ49* and *TmKu80Δ52*) to the chemical mutagens MMS, EMS, hydroxyurea, and phleomycin were compared with the wild-type strain (Fig. 3a). The sensitivities to these agents were not significantly different between the parental wild-type and *Ku80*’ mutant strains. They were able to grow on solid RPMI1640 medium containing 0.1% (w/v) MMS, but not on that containing this agent at 0.5% (w/v) (data not shown). They were also able to grow on solid RPMI1640 medium containing 0.2% (w/v) EMS, 1.0 mg mL⁻¹ hydroxyurea, and 100 μg mL⁻¹ (w/v) phleomycin, but their growth activity was reduced. In addition to chemical mutagens, the temperature sensitivities of the parental wild-type strain and one *Ku80*’ mutant strain (*TmKu80Δ49*) were compared. Neither strain showed...
growth at 40 °C, but no differences in growth were observed at 28 or 37 °C (Fig. 3b). Furthermore, the conidial germination frequencies in the parental wild-type strain and TmKu80Δ49 were compared. Based on the results of triplicate experiments, the average conidial germination frequencies were estimated to be 80 ± 7.1% (parental wild-type strain) and 77 ± 6.2% (TmKu80Δ49), respectively. Thus, there were no obvious differences in conidial germination activity between these strains. In addition, small conidia of the TmKu80Δ49 inoculated onto the modified 1/10 SDA medium at 28 °C for more than a week exhibited vigorous conidial germination activity and normal conidial formation (data not shown).

**Evaluation of the influence of deletion of the Ku80 ortholog on the frequency of targeted gene disruption in T. mentagrophytes**

In our previous study (Yamada et al., 2009), we developed an ATMT system for the wild-type T. mentagrophytes strain TIMM2789, and succeeded in disruption of the specific gene by homologous recombination via ATMT. To evaluate the effects of deletion of the TmKu80 locus on the targeted gene disruption frequency in T. mentagrophytes, the specific genes were disrupted in the TmKu80 wild-type strain TmKu80ΔA49 by ATMT. We chose trn as the target gene, which is an ortholog of areA/nit-2 from Aspergillus nidulans and N. crassa, and constructed the trn-targeting vector pAg1N-trn/T. areA/nit-2 encoding GATA-type transcription factors regulate expression of a large number of genes involved in nitrogen metabolism in these fungi (Stewart & Vollmer, 1986; Fu & Marzluf, 1990; Kudla et al., 1990; Langdon et al., 1995; Scazzocchio, 2000). The trn-targeting vector pAg1N-trn/T (Fig. 4a) containing the nptII cassette was constructed and introduced into the T. mentagrophytes Ku80\(^{-}\) mutant strain TmKu80ΔA49 by ATMT. Transformation of the T. mentagrophytes Ku80\(^{-}\) mutant strain TmKu80ΔA49 produced a large number of G418-resistant colonies on the selective medium, 10 of which were chosen at random, and analyzed by molecular biological methods. Southern-blotting analysis with a trn-specific probe revealed that all 10 colonies exhibited the banding pattern expected for homologous recombination via double crossover without additional ectopic integration of the disruption construct (Fig. 4b). Disruption of trn in such transformants was finally confirmed by phenotypic analyses. Two transformants (T23 and T24) were chosen at random and their growth properties on solid Aspergillus minimal medium supplemented with different nitrogen sources were examined (Table 2). The recipient strain TmKu80ΔA49 and the parental wild-type strain TIMM2789 showed similar growth properties on all nitrogen sources examined. Both transformants showed reduced or complete loss of the ability to utilize nitrate and several other nitrogen compounds, which were similar to the trn\(^{-}\) mutant strain produced in the previous study (Yamada et al., 2009). Based on the results of three independent disruption experiments, the average frequency of homologous recombination at the trn locus without additional ectopic integration of the disruption construct was estimated to be 73.3 ± 25.2% (Table 3). In addition to the
TmKu80Δ49, the tnr-targeting vector pAg1N-tnr/T was introduced into the parental wild-type strain TIMM2789 by ATMT. Thirty of the G418-resistant colonies produced on the selective medium were chosen at random and analyzed by Southern-blotting analysis. Three independent disruption experiments were carried out, in all of which tnr-disrupted mutant strains were obtained (Table 4). However, the average frequency of homologous recombination at the tnr locus without additional ectopic integration of the disruption construct was estimated to be 2.2 ± 1.9%. Subsequently, the putative serine protease gene (Tri m4) (accession no. AY929335) was chosen to examine the targeting frequency at the other T. mentagrophytes locus. The corresponding disruption construct (pAg1N-Trim4/T) (data not shown) was introduced into TmKu80Δ49 by ATMT. Twelve of the G418-resistant colonies produced on the selective medium were chosen at random and analyzed by Southern-blotting analysis with a Tri m4-specific probe. Of these, eight colonies (about 67%) exhibited the banding pattern expected for homologous recombination via double crossover without additional ectopic integration of the disruption construct (Table 3).

The above results indicate that deletion of TmKu80 led to improvement of targeted gene disruption frequency in T. mentagrophytes.

Discussion
In the present study, we carried out characterization of the Ku80 ortholog in T. mentagrophytes, and also succeeded in producing Ku80Δ mutant strains by homologous recombination. Ku genes have been reported to be involved in the NHEJ pathway of DSB repair (Critchlow & Jackson, 1998), and to play a role in the maintenance of telomere length in species as diverse as yeasts, plants, and mammals (Boulton & Jackson, 1996; Bailey et al., 1999; Riha et al., 2002). To estimate the influence of deletion of the Ku80 ortholog on the developmental processes of T. mentagrophytes, we carried out several morphological and phenotypic comparisons of the corresponding Ku80Δ mutant strains with the parental wild-type strain. Mycelial growth and conidial formation were intact in the Ku80Δ mutant strains, and no obvious differences in temperature sensitivity and conidial germination activity were seen between these strains (Fig. 3b).
Table 2. Growth properties of the parental and tnr− mutant strains of Trichophyton mentagrophytes on Aspergillus minimal medium supplemented with various nitrogen sources

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<th>Nitrogen source</th>
<th>T. mentagrophytes strains</th>
<th>No. of transformants</th>
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<th>Multiple copies of the disruption construct</th>
<th>Total</th>
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<td>Nitrate (NO₃⁻)</td>
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<td>1</td>
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<td>2</td>
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<tr>
<td>Ammonia (NH₄⁺)</td>
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</tr>
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<td>Tyrosine</td>
<td>+ + + + + + + + + + + + + +</td>
<td>30</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Urea</td>
<td>+ + + + + + + + + + + + + +</td>
<td>30</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. Frequency of homologous recombination between the disruption construct and the target locus

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of transformants analyzed</th>
<th>Single copy of the disruption construct</th>
<th>Multiple copies of the disruption construct</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 (tnr)</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>#2 (tnr)</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>#3 (tnr)</td>
<td>20</td>
<td>14</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>#4 (Tri m4)</td>
<td>12</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

*The number of transformants exhibiting homologous recombination at the tnr locus without additional ectopic integration of the disruption construct.

**The number of transformants exhibiting homologous recombination at the target locus with additional ectopic integration of the disruption construct.

Furthermore, the Ku80− mutant strains did not show sensitivity to the chemical agents MMS, EMS, hydroxyurea, and phleomycin (Fig. 3a). Similar results for these chemical mutagens have been reported in Ku80− mutant strains of the koji molds Aspergillus oryzae and Aspergillus sojae (Takahashi et al., 2006a). In contrast, Ku80− mutant strains of A. fumigatus and N. crassa showed mild sensitivity to MMS (Ninomiya et al., 2004; da Silva Ferreira et al., 2006). Taken together, these observations suggested that dermatophytes and the koji molds may have Ku-independent DSB repair systems. On the other hand, the change in virulence caused by deletion of the Ku80 ortholog will retard the progress of molecular genetics studies of the host invasion mechanisms by pathogenic fungi. To ensure that deletion of the Ku80 ortholog does not impair virulence of T. mentagrophytes, the Ku80− mutant strain TmkKu80ΔA49 is currently being tested in an experimental animal model. However, from previous reports in Ku80− mutant strains of several other pathogenic fungi (da Silva Ferreira et al., 2006; Goins et al., 2006; Villalba et al., 2008), we expect that deletion of Ku80 will not result in any loss or decrease of virulence in this fungus.

As shown in Table 3, the average frequency of homologous recombination at the tnr locus without additional ectopic integration of the disruption construct was approximately 11.0% and varied among disruption experiments (0–30%). These results indicate that deletion of the Ku80 ortholog led to the improvement of the frequency and reproducibility of targeted gene disruption in T. mentagrophytes. Ku80− mutant strain TmkKu80ΔA49 was estimated to be about 73%. A similar targeting frequency (average 67%) was also obtained at the Tri m4 locus. In contrast, the average targeting frequency of tnr in the parental wild-type strain TIMM2789 was estimated to be only about 2.2% (Table 4). In a previous study (Yamada et al., 2009), specific disruption of tnr by homologous recombination via ATMT was also accomplished in TIMM2789, the average frequency of which was about 11.0% and varied among disruption experiments (0–30%). These results indicate that deletion of the Ku80 ortholog led to the improvement of the frequency and reproducibility of targeted gene disruption in T. mentagrophytes. On the other hand, the M. grisea Ku80− mutant strains have recently been reported to have high frequencies (> 75%) of targeted gene disruption at nine loci located on different chromosomes (Villalba et al., 2008). However, the targeted gene disruption frequency has been shown to vary among chromosomal loci. These variations may be closely related to locus-specific properties, such as differences in chromatin structure. Thus, further studies of T. mentagrophytes may identify loci for which the frequency of targeted gene disruption is not increased regardless of the presence or absence of Ku80 and the NHEJ pathway.
As described above, the frequency of targeted gene disruption was improved markedly in
T. mentagrophytes using *Ku80* mutant strains. In addition, the *T. mentagrophytes* *Ku80* mutant strains produced here did not show any differences in morphological or phenotypic characteristics from the parental wild-type strain. Once the desired null mutant strains are produced, the functions of *Ku80* may be easily restored, if necessary, by complementation with a functional *Ku80* gene, allowing the analysis of such mutant strains in the otherwise wild-type genetic background. High throughput gene analysis methodologies, such as EST sequencing, differential cDNA screening, and cDNA-based microarray analysis, provide large amounts of valuable information regarding the basic mechanisms of host invasion by pathogenic organisms. Such methodologies have recently begun to be applied for molecular genetic studies of dermatophytes (Kaufman et al., 2005a; Wang et al., 2006; Liu et al., 2007; Maranha et al., 2007; Zaugg et al., 2009), providing useful information on many types of dermatophyte genes and their expression profiles under different growth conditions. *Ku80* mutant strains will be powerful tools for investigating the functions and roles of such genes and their relationships to virulence, leading to a better understanding of the molecular mechanisms of host invasion by these fungi.

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References


