Isolation, characterization, and disruption of *dnr1*, the *areA/nit-2*-like nitrogen regulatory gene of the zoophilic dermatophyte, *Microsporum canis*

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A homolog of the major nitrogen regulatory genes *areA* from *Aspergillus nidulans* and *nit-2* from *Neurospora crassa* was isolated from the zoophilic dermatophyte, *Microsporum canis*. This gene, *dnr1*, encodes a polypeptide of 761 amino acid residues containing a single zinc-finger DNA-binding domain, which is almost identical in amino acid sequence to the zinc-finger domains of AREA and NIT-2. The functional equivalence of *dnr1* to *areA* was demonstrated by complementation of an *areA* loss-of-function mutant of *A. nidulans* with *dnr1* cDNA. To further characterize this gene, *dnr1* was disrupted by gene replacement based on homologous recombination. Of 100 transformants analyzed, two showed the results expected for replacement of *dnr1*. The growth properties of the two *dnr1*− mutant strains on various nitrogen sources were examined. Unlike the *A. nidulans areA*− mutant, these *dnr1*− mutants showed significantly reduced growth on ammonia, a preferred nitrogen source for fungi. These mutant strains were also able to utilize various amino acids for growth. In comparison with wild-type *M. canis*, the two *dnr1*− mutants showed reduced growth on medium containing keratin as the sole nitrogen source. This is the first report describing successful production of targeted gene-disrupted mutants by homologous recombination and their phenotypic analysis in dermatophytes.

**Keywords** dermatophyte, major nitrogen regulatory gene, targeted-gene disruption, extracellular protease

**Introduction**

Dermatophytes are a closely related group of keratinophilic fungal pathogens, which are responsible for a superficial cutaneous infection called dermatophytosis (ringworm) in both humans and animals. *Microsporum canis* is a zoophilic fungus, and is the most common causative agent of dermatophytosis in animals. Cats and dogs are regarded as the natural hosts for this fungus, and may even act as reservoirs [1,2], thus contributing to the high prevalence of zoonotic human infections [3–6]. Zoonosis is often caused by direct contact with animals infected with *M. canis* [7].

Dermatophytes commonly gain access to the host via keratinized structures, such as the hair, skin, or nails, which are cornified tissues that form solid structural barriers against invasion. To overcome complex host defense systems and progress the infection cycle, most pathogenic microorganisms produce a variety of extracellular hydrolytic enzymes with different activities both constitutively and inductively. Similarly, dermatophytes appear to use several extracellular hydrolytic enzymes for penetration through cornified tissues and maintenance of fungal growth on host tissues. Among these enzymes, a great deal of attention has been focused on the keratinolytic proteases (the so-called keratinases) that digest keratins, the major constituents
of cornified tissues, as possible virulence-related factors in dermatophytosis.

Although little is currently known about the regulation of expression of extracellular proteases by dermatophytes, extensive studies of protease production in the model filamentous fungi, Aspergillus nidulans and Neurospora crassa, have suggested that extracellular protease expression is regulated by both nitrogen (N) and carbon (C) catabolite repression, i.e., control of nitrogen and carbon availability. In these two fungi, a number of genes have been shown to be expressed in response to restriction of nitrogen and/or carbon source availability. *areA* from *A. nidulans* [8,9] and *nit-2* from *N. crassa* [10,11] are well-characterized genes involved in nitrogen catabolite repression in these fungi. *areA* and *nit-2* encode transcription factors containing a DNA-binding domain consisting of a Cys-2/Cys-2 zinc-finger motif followed by an adjacent basic region. The DNA-binding domains of AREA and NIT-2 are characteristic of the GATA family of transcription factors [12]. These molecules activate expression of a large number of genes encoding permeases and enzymes that mediate uptake and utilization of various secondary nitrogen sources, in the absence of preferred nitrogen sources, such as ammonia and glutamine [13]. These genes include those encoding extracellular proteases. Genes encoding products that are structurally and functionally similar to AREA and NIT-2 have been found in both human and plant pathogenic fungi, such as *Aspergillus fumigatus* [14], *Candida albicans* [15], and *Magnaporthe grisea* [16], and inactivation of the corresponding genes could affect the production of extracellular proteases in these fungi.

Here, we report the molecular characterization of the *dnr1* gene from *M. canis*, which is homologous to *areA* and *nit-2*. Complementation of the *A. nidulans areA<sup>−</sup>* mutant with the isolated cDNA and phenotypic analyses of the *dnr1*<sup>−</sup> mutants produced by homologous recombination indicated that *dnr1* is involved in regulation of nitrogen metabolism in *M. canis*. The reduced growth activity of these *dnr1*<sup>−</sup> mutants in liquid medium containing keratin as the sole nitrogen source also suggested a relationship between *dnr1* and expression of extracellular proteases during the infection cycle on host tissues.

**Materials and methods**

**Fungal strains and culture conditions**

The wild-type *M. canis* strain, TIMM4092 [17–19], the wild-type *Aspergillus nidulans* strain, 2373 (*inoB, glrIA1*), and the *A. nidulans areA<sup>−</sup>* loss-of-function mutant strain, 2207 (*wA3, inoB, areA<sup>−</sup>3*), were used in this study. *areA<sup>−</sup>* has the molecular nature of the mutation that the Cys673 of the zinc finger domain was substituted by a tyrosine [20]. *M. canis* strains were maintained at 28°C on solid Sabouraud dextrose (SD) medium with or without an appropriate concentration of hygromycin B, whereas *A. nidulans* strains were maintained at 28°C on solid SD medium, solid *Aspergillus* complete medium (ACM), or minimal medium (AMM) [21] with 10 mM of sodium nitrate (NaN<sub>3</sub>) or ammonium chloride (NH<sub>4</sub>Cl).

**Genomic DNA isolation and Southern blotting analysis**

Genomic DNA of each *M. canis* and *A. nidulans* strain was isolated from the growing mycelia, according to the method of Girardin and Latge [22].

Aliquots of 3 μg of genomic DNA were digested with appropriate restriction enzymes, separated by electrophoresis on 0.7% (w/v) agarose gels, and transferred onto Hybond N<sup>+</sup> membranes (Amersham Biosciences, Piscataway, NJ, USA). Southern blot hybridization was performed using the ECL<sup>TM</sup> Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences).

**Isolation of cDNA and genomic clones encoding the M. canis AREA/NIT-2 ortholog**

Total RNA of the *M. canis* strain, TIMM4092, was isolated from the growing mycelia cultured for 4 days in 50 ml of modified dermatophyte minimal medium (DMM) [23] with 250 mg of dog hair, by using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

A cDNA clone encoding the *M. canis* AREA/NIT-2 ortholog was isolated by RT-PCR and RACE-PCR with the 5’ and 3’ Rapid Amplification of cDNA Ends method with Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). A genomic clone of the *M. canis* AREA/NIT-2 ortholog was also amplified by PCR. Nucleotide sequences of primers (SP1, ASP1, 3’RACE1, ASP2, 5’RACE1, 5’RACE2, SP2 and ASP3) used for amplification of the cDNA and genomic clones were shown in Table 1.

Nucleotide sequencing reactions of all the amplified fragments were performed using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA).

**Construction of transformation vectors**

The transformation vector, pCHSH75-*dnr1*, carrying the ORF of the *dnr1* cDNA under the control of


Cochlibolus heterostrophus promoter 1 (M17304) [24], was constructed from pCHSH75 [25], which is derived from pSH75 [26]. The dnr1-targeting vector, pR-dnr1 (Fig. 3), was also constructed from pCHSH75 [25]. Two regions (fragment 2, nucleotide position: -537-1746; fragment 3, nucleotide position: 1897-2577) of the dnr1 gene were inserted into the upstream site of C. heterostrophus promoter 1 and the downstream site of the termination sequence of the A. nidulans tryptophan C gene (X02390) [27], respectively, to generate pCHSH75-dnr1.

Protoplast preparation and genetic transformation

Protoplast preparation and polyethylene glycol (PEG)-mediated transformation of the M. canis strain, TIMM4092, and the A. nidulans areA/C28 mutant strain, 2207, were performed, according to the method of Yamada et al. [25], except that the selection of A. nidulans transformants.

Assay of growth properties of A. nidulans and M. canis on various nitrogen sources

The conidial suspensions of each A. nidulans strain were spotted onto solid AMM supplemented with various nitrogen sources added to the medium as follows: 10 mM of NaNO₃, sodium nitrite (NaNO₂), and NH₄Cl, 1 mM L-tyrosine, and 5 mM of other amino acids, adenine, urea, and formamide, 1 mM hypoxanthine, 1.0% (w/v) skimmed milk. After incubation at 37°C for 2 to 3 days, growth of fungal colonies was compared.

Protoplast suspensions of each M. canis strain were spotted onto solid DMM supplemented with 1.2 M D-sorbitol and the above nitrogen sources plus 0.5% (w/v) BSA. After incubation at 28°C for 7 to 8 days, growth of reproduced fungal colonies was compared.

Nucleotide sequence accession number

The nucleotide sequence data of dnr1 from M. canis reported here have been deposited in the DDBJ/EMBL/GenBank under Accession No. AB201458.

Results

Isolation of cDNA and genomic clone encoding M. canis AREA/NIT-2 ortholog

An isolated cDNA, designated as dnr1, contained an open reading frame (ORF) of 2283 bp encoding a predicted product of 761 amino acid residues (Fig. 1). Alignment between the dnr1 cDNA and genomic sequences revealed that the ORF of dnr1 was interrupted by 2 introns of 80 bp and 74 bp. The existence of these two introns was similar to nit-2 from N. crassa [10,11], and nut1 and chr1 from the plant fungal pathogens Magnaporthe grisea [16] and Colletotrichum lindemuthianum [28]. In contrast, the areA genes from A. nidulans [9] and other Aspergillus species [29,30] have only a single intron in their coding regions. Southern blotting analysis using a Cys-2/Cys-2 zinc-finger domain (Fig. 1, shaded box) as a probe suggested the existence of the dnr1 gene as a single copy in the

| Table 1 Primers used in this study |
|----------------------------------|
| **Name** | **Sequence** |
| SP0 | 5’-TGTACA(C, G, T)AAT(T)GGC(T)GCT(T)AACA(C, G, T)CA-3’ |
| ASP0 | 5’-CGAGG(TA(T)GAAT(T)ATGAAT(T)GTTG-3’ |
| SP1 | 5’-ATGGAAGA(T)CG(T)TATGTA(AC, G, T)CGA(C, G, T)CA-3’ |
| ASP1 | 5’-AGACCAAGGAGCCGCTACA-3’ |
| 3’ RACE1 | 5’-TATGTAACGCCTGTGGCCTG-3’ |
| ASP2 | 5’-AGGGATATGGTCTCCTG-3’ |
| 5’ RACE1 | 5’-CTGCTACTTCTGTTGGCCTG-3’ |
| 5’ RACE2 | 5’-TCAAATCTTGCGCCTG-3’ |
| SP2 | 5’-AGGGCTGCGGCTTCTGCGCCTG-3’ |
| ASP3 | 5’-TGGCAGGCAAAATGCAAGCTCA |
| SP6 | 5’-C AGAGCTGTCTCATATGCGA-3’ |
| SP7 | 5’-ACGC(AA)ACTACTCCCT-3’ |
Fig. 1 Comparison of the amino acid sequence of the predicted *M. canis* *dnr1* protein with fungal AREA/NIT-2 orthologs. AREA (X52491) [8,9], NIT-2 (AAB03891) [11], and NUT1 (AAB03415) [16] are from *A. nidulans*, *N. crassa*, and the plant pathogenic fungus, *Magnaporthe grisea*, respectively. The zinc-finger region is boxed. Horizontal arrows indicate annealing sites of the oligonucleotide primers used for isolation of the *dnr1* cDNA.

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*M. canis* genome (data not shown). The predicted protein of *dnr1* shares extensive regions of high similarity with the known AREA/NIT-2 orthologs (Fig. 1). Of these conserved regions, the carboxy-terminal conserved region, together with the zinc-finger domain, was shown to be involved in interaction with another nitrogen regulatory protein in *N. crassa* [31,32]. As the results of estimation by the unweighted pair group method with arithmetic mean (UPGMA), the predicted *dnr1* protein product showed amino acid sequence similarities of 22.0%, 15.0%, and 14.0% to AREA, NIT-2, and NUT1, respectively.

**Functional analysis of the M. canis dnr1 gene**

To determine whether *M. canis* *dnr1* is capable of complementing the functions of *areA*, the *A. nidulans areA* mutant strain was transformed with pCHSH75-*dnr1* carrying the *dnr1* cDNA. Of 19 transformants regenerated on solid AMM containing different nitrogen sources. As shown in Table 2, the *A. nidulans areA* mutant strain (2207) was able to grow only on medium containing ammonium, glutamine, and urea. In contrast, the wild-type *A. nidulans* strain (2373) and the two *A. nidulans* transformants showed similar growth properties and were able to grow on all of the media tested (Table 2), demonstrating that the *dnr1* gene product has functions equivalent to those of AREA. The both *A. nidulans* transformants were found to also produce extracellular proteases on the skimmed milk-containing medium (Table 2).

**Table 2** Growth properties of *A. nidulans* wild-type, mutant, and complemented strains on *Aspergillus* minimal medium supplemented with various nitrogen sources.

| Nitrogen source | *A. nidulans* strains |
|-----------------|-----------------------|
|                 | 2373 (wild-type)      | 2207 (areA<sup>-</sup>) | NT10<sup>‡</sup> | NT11<sup>‡</sup> |
| Nitrate (NO<sub>3</sub>) | + + +                | −                    | + + +               | + + +               |
| Nitrite (NO<sub>2</sub>) | + + +                | −                    | + + +               | + + +               |
| Ammonia (NH<sub>4</sub>) | + + +                | + + +               | + + +               | + + +               |
| Glutamine       | + + +                | +                    | + + +               | + + +               |
| Hypoxanthine    | + + +                | −                    | + + +               | + + +               |
| Adenine         | + + +                | −                    | + + +               | + + +               |
| Urea            | + + +                | +                    | + + +               | + + +               |
| Formamide       | +                    | −                    | + + +               | + + +               |
| Alanine         | + + +                | −                    | + + +               | + + +               |
| Aspartate       | + + +                | −                    | + + +               | + + +               |
| Glutamate       | + + +                | −                    | + + +               | + + +               |
| Phenylalanine   | + + +                | −                    | + + +               | + + +               |
| Glycine         | + + +                | −                    | + + +               | + + +               |
| Isoleucine      | + + +                | −                    | + + +               | + + +               |
| Leucine         | + + +                | −                    | + + +               | + + +               |
| Asparagine      | + + +                | −                    | + + +               | + + +               |
| Proline         | + + +                | −                    | + + +               | + + +               |
| Arginine        | + + +                | −                    | + + +               | + + +               |
| Serine          | + + +                | −                    | + + +               | + + +               |
| Threonine       | + + +                | −                    | + + +               | + + +               |
| Valine          | + + +                | −                    | + + +               | + + +               |
| Tryptophan      | + + +                | −                    | + + +               | + + +               |
| Tyrosine        | + + +                | −                    | + + +               | + + +               |
| Ornithine       | + + +                | −                    | + + +               | + + +               |
| Skimmed milk    | + + + +h             | −                    | + + + +h            | + + + +h            |

Aliquots of 10 μl of each conidial suspension containing about 5 × 10<sup>4</sup> conidia were spotted on media and growth of fungal colonies was compared after incubation of 2 to 3 days at 37°C. The ability to grow on different media, and protein degradation by extracellular proteases are indicated as ‘−’ (no growth) to ‘+++’ (vigorous growth) and ‘h’ (halo), respectively.

<sup>‡</sup>The two *A. nidulans* strains complemented with the *M. canis dnr1* cDNA; NT10 and NT11, exhibited the same morphological characteristics.

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and screened for replacement of dnrl by the hph cassette. Southern blotting analysis (Fig. 3) using the fragment 3 as a probe and PCR with three pairs of primers (SP6 & ASP1, SP7 & ASP3, and SP7 & ASP1 shown in Table 1) (data not shown) suggested the replacement of the dnrl by the hph cassette in the genomes of M47 and M58. No morphological differences were detected between TIMM4092 and M47 and M58 (data not shown).

**Growth properties of the M. canis dnrl⁻ mutant strains on various nitrogen sources**

The growth properties of the *M. canis* dnrl⁻ mutant strains, M47 and M58, on solid DMM containing different nitrogen sources were compared with those of the wild-type strain, TIMM4092, and the ectopic transformant, M55. As TIMM4092 showed absence of conidia productivity within a few passages in subculture, none of the derivatives of TIMM4092 examined here, i.e., M47, M58, and M55, was capable of producing conidia. Therefore, we used protoplasts prepared from growing mycelia. As shown in Table 3, TIMM4092 and M55 were able to grow on all of the nitrogen sources tested, while both M47 and M58 showed reduced or complete loss of ability to utilize nitrate, nitrite, and several other nitrogen compounds. However, unlike the *A. nidulans* areA⁻ mutant, M47 and M58 maintained their growth activity on glutamine and many other amino acids. Furthermore, both strains showed difficulties in growth on ammonia, one of the preferred nitrogen sources for fungi (Table 3), and addition of much higher levels of ammonia did not improve their reduced growth activity.

TIMM4092 and M55, but not M47 and M58, showed production of extracellular proteases on skimmed milk-containing medium (Table 3). M47 and M58 also showed different growth properties on the medium containing keratin, an important nutrient source for dermatophytes, as compared with TIMM4092 and M55. Mycelia from TIMM4092 and
M55 had already begun to grow after 2 days of incubation, whereas those from M47 and M58 showed visible growth only after 7 days of incubation (Fig. 4). Subsequently, their mycelia were incubated for a further 4 days, the extent of which was reduced as compared with TIMM4092 and M55 (data not shown).

**Discussion**

In the present study, we report the isolation of the *areA*-like nitrogen regulatory gene (*dnr1*) from *M. canis*. Comparison of the growth properties between *M. canis* wild-type and *dnr1* mutant strains on different nitrogen sources (Table 3) indicated that the *dnr1* is involved in the control of nitrogen metabolism in this species. The *dnr1* mutant strains showed significantly reduced growth on ammonia (Table 3). Similar results were reported previously in an *areA* mutant strain of *A. oryzae* [29], except that growth activity could be restored in this mutant by increasing the amount of ammonia present in the medium. Based on the observation that high levels of ammonia could overcome the poor growth activity of the *A. oryzae areA* mutant strain, Christensen et al. [29] concluded that the uptake of ammonia, rather than its subsequent assimilation cycle, should be the main factor responsible for the reduced growth of the *A. oryzae areA* mutant strain on this nitrogen source. In contrast, growth activity of the *dnr1* mutant strains was not improved even by the presence of a very high level of ammonia (100 mM) (Table 3), suggesting that the intracellular ammonia assimilation cycle, together with its uptake, may be related to ammonia availability in *dnr1* mutant strains.

Inactivation of the *areA*-like genes in many fungi results in loss or a significant reduction in growth activity on most of the amino acids, with the exception of glutamine, but the *M. canis dnr1* mutants were still

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**Table 3** Growth properties of the wild-type and *dnr1* mutant strains of *M. canis* on dermatophyte minimal medium supplemented with various nitrogen sources.

| Nitrogen source                  | TIMM4092 (wild-type) | M47 (*dnr1*) | M58 (*dnr1*) | M55<sup>a</sup> |
|---------------------------------|----------------------|-------------|-------------|-----------------|
| Nitrate (NO<sub>3</sub>⁻)       | +                    | –           | –           | +               |
| Nitrite (NO<sub>2</sub>⁻)       | +                    | –           | –           | +               |
| Ammonia (NH₄⁺) (10 mM)          | + + +               | +           | +           | + + +           |
| Ammonia (NH₄⁺) (100 mM)         | + + +               | +           | +           | + + +           |
| Glutamine (5 mM)                | + + +               | +           | +           | + + +           |
| Glutamine (50 mM)               | + + +               | +           | +           | + + +           |
| Hypoxanthine                    | +                    | –           | –           | +               |
| Adenine                         | +                    | –           | –           | +               |
| Urea                            | + +                  | –           | –           | +               |
| Formamide                       | +                    | –           | –           | +               |
| Alanine                         | + + +               | + +         | + +         | + + +           |
| Aspartate                       | + + +               | +           | +           | + + +           |
| Glutamate                       | + + +               | +           | +           | + + +           |
| Phenylalanine                   | + + +               | + +         | + +         | + + +           |
| Glycine                         | + + +               | –           | –           | + + +           |
| Isoleucine                      | + + +               | +           | +           | + + +           |
| Leucine                         | + + +               | + +         | + +         | + + +           |
| Asparagine                      | + + +               | + +         | + +         | + + +           |
| Proline                         | + + +               | +           | +           | + + +           |
| Arginine                        | + + +               | +           | +           | + + +           |
| Serine                          | + + +               | –           | –           | + + +           |
| Threonine                       | + +                  | –           | –           | + + +           |
| Valine                          | + +                  | –           | –           | + + +           |
| Tryptophan                      | + + +               | +           | + +         | + + +           |
| Tyrosine                        | + + +               | +           | + +         | + + +           |
| Ornithine                       | + + +               | +           | + +         | + + +           |
| Skimmed milk                    | + + h               | –           | –           | + + h           |
| Bovine serum albumin (BSA)      | + +                 | –           | –           | + +             |

Aliquots of 10 µl of protoplast suspensions containing about 1 × 10⁵ cells were spotted onto media and growth of fungal colonies was compared after incubation of 7 to 8 days at 28°C. The ability to grow on different media, and protein degradation by extracellular proteases are indicated as ‘_’ (no growth) to ‘+ + +’ (vigorously growing) and ‘h’ (halo), respectively.

<sup>a</sup> The ectopic transformant, M55, showed the same morphological characteristics as the wild-type strain.
able to use glutamine and many other amino acids (Table 3). The availability of a variety of amino acids in the \(dnr1^-\) mutant strains may be related to nutritional environments encountered by the fungi on the host tissues. In general, dermatophytes grow exclusively in the superficial keratinized tissues, such as the hair, skin, nails, or stratum corneum, which appear to be poor in available nutrient sources. Thus, available exogenous small compounds, such as amino acids and short peptides, which are produced by digestion of keratin-based nutrients, appear to become indispensable nutrients for the fungi for the maintenance of growth during infection. The uptake of exogenous amino acids is usually carried out by specific membrane proteins (the so-called permeases). Of the amino acid permeases identified in yeasts and filamentous fungi, \(GAP1\), the general amino acid permease from \(S. cerevisiae\) [34], and \(prnB\), the proline transporter from \(A. nidulans\) [35], are known to be under control of the \(areA/nit-2\)-like genes (\(S. cerevisiae\), \(GAT1/NIL1\) and \(GLN3\) [36]; \(A. nidulans\), \(areA\)). The loss or significant reduction of the availability of several amino acids (glycine, proline, etc.) in the \(dnr1^-\) mutants suggests that \(M. canis\) should have a common exogenous amino acid uptake system under control of the \(areA/nit-2\)-like gene. In addition to this specific system, the fungus may also develop different exogenous amino acid uptake systems that contribute cooperatively to achieve efficient utilization of exogenous nutrients in poor nutritional environments.

The production of extracellular proteases by \(M. canis\) was affected by \(dnr1\) (Table 3). The \(dnr1^-\) mutant strains showed significantly retarded growth on medium containing keratin as the sole nitrogen source, as compared with the wild-type strain (Fig. 4). Several extracellular keratinolytic proteases from \(M. canis\) were produced during culture in medium containing keratin as the sole nitrogen source [23,37]. This growth property of these mutants may be related to the production of extracellular keratinolytic proteases. Furthermore, it has been reported that \(areA/nit-2\)-like genes in some human and plant pathogenic fungi play roles in host infection [14,15,28]. Experiments are currently underway in our laboratory to identify the extracellular proteases of \(M. canis\) under control of the \(areA/nit-2\)-like genes in some human and plant pathogenic fungi play roles in host infection [14,15,28]. Experiments are currently underway in our laboratory to identify the extracellular proteases of \(M. canis\) under control of the \(areA/nit-2\)-like gene in some human and plant pathogenic fungi play roles in host infection. These studies will provide more detailed information regarding the roles of \(dnr1\) and extracellular proteases in dermatophytosis.

\[\text{Fig. 4 Observation of growth properties of the } M. canis \text{ } dnr1^- \text{ mutant strains on keratin. Mycelia of the four } M. canis \text{ strains grown in liquid modified DMM containing keratin as the sole nitrogen source, after 2, 4, and 7 days of incubation at } 28^\circ \text{C, were observed by stereomicroscopy. Bar } = 2.5 \text{ mm.}\]
Targeted gene disruption mediated by genetic transformation is indispensable for determination of the functions and roles of genes isolated from pathogenic fungi. Disruption of the *dnr1* was confirmed in only 2 of the 100 transformants analyzed, a lower frequency of homologous recombination than reported previously in other filamentous fungi, such as *A. nidulans*. The low efficiency of homologous recombination in dermatophytes is attributed mainly to their low transformation frequency [24,38]. To increase the transformation frequency of dermatophytes, several aspects of the transformation procedure must be altered. One candidate is alteration of the transformation vector conformation (i.e., circular vs. linear). Linearization of transformation vectors by restriction enzymes, the so-called restriction enzyme-mediated integration (REMI) procedure [39], has been shown to improve transformation frequency in several filamentous fungi [40,41]. Kaufman et al. [42] recently reported enhanced transformation of *T. mentagrophytes* called restriction enzyme-mediated integration (REMI) procedure. In addition, Bird and Bradshaw [43] reported that nucleotide length of homologous fragments within the transformation vectors, together with their conformation, was correlated with the efficiency of homologous recombination in *A. nidulans*; an increase in the size of the homologous fragments resulted in an increase in the efficiency of homologous recombination (1.0% for 0.6 kb, 14.0% for 0.9 kb, 27.0% for 1.2 kb). One of the homologous fragments inserted in the pR- *dnr1* was only about 0.7 kb in length (Fig. 2). Thus, use of larger homologous fragments may make a contribution to the increase in the efficiency of homologous recombination in dermatophytes.

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