

Original Article

Metalloprotease Gene of *Arthroderma gypseum*

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SUMMARY: The full-length cDNA sequence for metalloprotease (*MEP*) of *Arthroderma gypseum* (one of the teleomorphs of the *Microsporium gypseum* complex) was determined by the 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE methods using cDNA as a template. The full-length cDNA sequence of the *MEP* (2,670 bp) gene was proved to encode 677 amino acids. The amino acid sequence of the *A. gypseum MEP* gene shared about 89 and 66% sequence similarity with the conserved region of the *Microsporium canis MEP* gene and *Aspergillus fumigatus*, respectively. Southern hybridization analysis of genomic DNA with an *MEP* probe gave many distinct bands in *Bam*HI, *Eco*RI and *Hind*III digests of genomic DNA from *A. gypseum*. Reverse transcriptase-PCR analysis suggested that keratin might stimulate the expression of *MEP* mRNA in *A. gypseum*.

INTRODUCTION

Some fungal pathogens, such as dermatophytes and *Candida* spp. are able to penetrate the epithelial skin cells by lysing keratin as a substrate (5,13). The dermatophytes include members of the genera *Trichophyton*, *Microsporium* and *Epidermophyton*, some of which were shown to belong to the genus *Arthroderma*. Many of the dermatophytes frequently cause infections in human and animal keratinized tissues of epidermis, hair and nails (12).

Several keratinases have been purified from dermatophytes, and some of their biochemical characteristics have been analyzed (1-3,15). An extracellular keratinolytic metalloprotease (43.5 kDa) was purified from a feline clinical isolate of *Microsporium canis*, and its characteristics were partially identified (3). Production of this metalloprotease was induced specifically by keratin, suggesting that this enzyme may be one of the virulence-related factors involved in dermatophytosis (3). Recently, Brouta et al. (4) isolated three metalloprotease genes (*MEPs*) from an *M. canis* genomic library, and demonstrated that *MEP3* (DDBJ/EMBL/GenBank accession number: AJ490183) encodes the 43.5 kDa metalloprotease. They also showed that *MEP3* was expressed in vivo during *M. canis* infection of experimental animals.

However, there have been few reports concerning the molecular characteristics of keratinase genes of the other dermatophytes.

Arthroderma gypseum is one of geophilic dermatophytes and is commonly isolated from soil throughout the world. In the process of evolution from soil saprophytes to zoophilic and anthrophilic parasites, changes of keratinase production systems presumably occurred (13). *A. gypseum* and *M. canis* (zoophilic dermatophyte), which are genetically similar (6, 9-12), each show different pathogenicity between humans and

animals (13). To investigate the evolution and pathogenesis of dermatophytes, we cloned and analyzed the *MEP* gene of *A. gypseum*.

MATERIALS AND METHODS

Preparation of cDNA: The standard strain of *A. gypseum*, (+) mating type, VUT-4004 (IAM 12722, Institute of Applied Microbiology, Tokyo, Japan; CMI 86526, C. A. B. International Mycological Institute, Kew, UK) was used in this study. Mycelium was obtained by culturing the strain in hair medium at 24°C for 10 days. The samples collected by centrifugation at 3,000 rpm for 5 min were homogenized in liquid nitrogen. Total RNA was extracted from about 100 µg of samples with an RNeasy total RNA kit (QIAGEN, Valencia, Calif., USA).

A series of 5'- and 3'-rapid amplification of cDNA ends (RACE)-PCR experiments was performed to determine the full-length cDNA sequence of the *MEP* gene of *A. gypseum*.

To clone the *MEP* gene, the sense primer sequences were based on the N-terminal 13 amino acid sequence of the mature protein sequences of *M. canis MEP* (3). The sense primer was 5'-CGCATGGGGCATTAAATGAC-3' and the reverse primer was oligo dT primer (5'-TTTTTTTTTTTTTTTT-3'), which were also used to make the template cDNA of *A. gypseum*.

Amplification was carried out for 30 cycles consisting of template denaturation (1 min at 94°C), primer annealing (1 min at 60°C) and polymerization (2 min at 72°C). The PCR products were gel-purified and cloned into pCRII vector (Invitrogen, Carlsbad, Calif., USA). The plasmid DNAs from more than three clones of *MEP* gene products were extracted with a QIAGEN plasmid kit (QIAGEN) and sequenced by the dideoxy chain termination method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA).

The sequences of gene-specific primers for the 5' and 3' methods were designed from the sequences of progressively amplified products beginning with the sequences of the *MEP* gene fragment, respectively. RACE procedures were carried

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out according to the 5'/3' RACE kit 2nd instruction manual (Roche Diagnostics GmbH, Penzberg, Germany). The PCR products were gel-purified and cloned into pCRII vector (Invitrogen). The plasmid DNAs from more than three clones of 5' and 3' fragments were extracted with a QIAGEN plasmid kit (QIAGEN) and sequenced by the dideoxy chain termination method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Southern hybridization analysis: For Southern hybridization analysis, an MEP probe was constructed from the PCR products of the partial *MEP* gene, which were labeled with digoxigenin by a DIG-High Prime DNA Labeling and Detection Kit I (Roche Diagnostics).

The primer sequences used for amplification of *MEP* were

5'-CCGTCTAAAGCCCCGTGACG-3' (primer AGMEP1S; nucleotides [nt] 1412-1431 in the *A. gypseum MEP* sequence DDBJ-AB126116) and 5'-CATCACGGCCTGGACGAAG-3' (primer AGMEP1R; nt 1742-1761 in the same sequence). It was expected that a 450-bp fragment from *A. gypseum MEP* would be amplified with these primers.

The cDNA samples (100 ng) were amplified by PCR in a volume of 30 μ l, using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM each deoxynucleoside triphosphate, 1.0 unit of *Taq* polymerase (Takara, Kyoto, Japan) and 0.5 μ g of each of a pair of primers. Amplification was carried out for 24, 27 and 30 cycles consisting of template denaturation (1 min at 94°C), primer annealing (2 min at 63°C) and polymerization

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A.gypseum  MHGLLLAGLLALPMNVLAHPAEHHASNVLSRRGVDIDSFRLPLKAKYMDN-EAAAEKIQA  59
M. canis    *****QQT*S*****S-D*T*Q*****
A.fumigatus MR*****A***AS*F***--*Q*YG*N**T**NA**KSL***VNAT*TVI*APSS

A.gypseum  LSF*TKDDDYVSTATKLVKSTFPKSTFRVVDHDIYIGTNGIGHVHFQKTAHGPDIIDNSDFNV  119
M. canis    ***S*E*****L*****
A.fumigatus FAPF*PQS**EV**QH**MIA*DA****D***V*D**VA***R**N*L***A****

A.gypseum  NIDRDGKVFVSGNSFFTGEIPKENPMVKRAFSDPVKALKGAVKALSLPVKSDNAKAKNVA  179
M. canis    *****S*****E*****N*****E*****T*E
A.fumigatus *VGK*****Y***Y*TGQ***SAALT**D*****T*NT*****TV*S*SSESTE

A.gypseum  GKETVEFMGTSGALSAPKAKLVYLQKDDGTLLTWRVETDVENWLLSYVDANNSETVHN  239
M. canis    ***S*F**Q*****E**S*****K*****D*****H*****
A.fumigatus E**SYV*K*V**T*V*****F*V*****A*****I*D*****T**K*SG*E*I*G

A.gypseum  VVDYVASAEYKVFVGLNDPTEGNPTSIKDPWT-DASPYTWNSDGNKTYPTTRGNNAIAQ  298
M. canis    *****F*****A*S**F**H***T*****
A.fumigatus *****E*D*Q*Y***I*****E*R*V*K***D*V**E*F**I***S*TN*T*S***G**

A.gypseum  DNPTGGSQYLNNYRQSPNLFYSYPSATATPPSSYKDFSI*TLFYTTNRFHDL*LYSFGF  358
M. canis    *****T*****A*****N**T*AM*****Y*****
A.fumigatus S**S**PS*****S*SS*S*K**Y*VSSS*****I*A*****A*I*Y*****T*L**

A.gypseum  NEAAGNFQVNNNGKGRGNDFAIVNAQDGSNTNANFATPPDGS*PGRMRMYNWTARPNR  418
M. canis    *****N*****K*****
A.fumigatus T*K***E*Y*TN*GQ**L***Y*V*L*****Q*****V**E*ST*Y*Y

A.gypseum  DGCLEAGIVLHEHYAHGLSNRLCGGPANSGCLNALES*GGMGEGW*GDFYATAIRL*PKPRDAKN  478
M. canis    *****T*****A*****T**
A.fumigatus **S*F*****T*****T*****N*****S**M*****P*G*KRS

A.gypseum  TNYSMGAWAANNPKGIRAYLYSTNLQTNPYMYT*SVNSLRE*VHQYGT*VWAT*MLYDL*MLWALI  538
M. canis    *****D*****N*****G*I*****E***G***
A.fumigatus *D*T**E**S*RAG**Q*P**S**L*T**S**N*A**A*I*T**S**E*V*W*N**

A.gypseum  EAHGNTYSANPVFRNGV*PQDGRHLAMKLVMDGMALQPCNP*FVQARDAI*IDADR*AL*NTSA  598
M. canis    *****D*****N*****G*I*****E***G***
A.fumigatus **G***D*****I*****G**

A.gypseum  NKCTIWKAFKRLGLY*GAKYDSRNRTGSN*KLPPGC-  633
M. canis    *****P*T*****Q*****
A.fumigatus *Q*E**T*****A***S***V***T*E*V*S*V*C

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Fig. 1. Comparison between homologous amino acid sequences of *A. gypseum MEP* and other fungal *MEPs*, *M. canis MEP* (GenBank D21269) and *A. fumigatus MEP* (GenBank L29566-1). An asterisk indicates identity with the amino acid found in the *A. gypseum MEP* sequence. The box indicates the zinc-binding domain that contains the highly conserved *MEP* signature sequence "HEXXH".

(2 min at 72°C). The PCR product was analyzed on a 2% agarose gel.

Genomic DNA samples of *A. gypseum* were isolated as previously reported by Kano et al. (8). Thirty micrograms of genomic DNA was digested, in separate procedures, with the restriction endonucleases *Bam*HI, *Eco*RI and *Hind*III. The digests were electrophoresed through 1% agarose gel and transferred to a hybridization membrane, GeneScreen Plus (NEN Research Products, Boston, Mass., USA). The membrane was hybridized with a DIG-labeled MEP probe as per the DIG-High Prime DNA labeling and detection kit user manual (Roche Diagnostics).

Reverse transcription (RT)-PCR assay of MEP mRNA of *A. gypseum* cultured with and without keratin: *A. gypseum* was cultured on Sabouraud's dextrose agar (12), corn meal agar (12), diluted Sabouraud's dextrose agar (12), hair medium and keratin azure (Sigma, St. Louis, Miss., USA) for 10 days at 24°C. The mycelial samples were homogenized in liquid nitrogen. Total RNA was extracted from samples with an RNeasy total RNA kit (QIAGEN). RT of the poly(A)⁺ RNA was performed with an Omniscript™ Reverse Transcriptase kit (QIAGEN).

The primer pairs used for amplification were AGMEP1S/AGMEP1R, as described above.

The cDNA samples (100 ng) were amplified by PCR in a volume of 30 μl in the same reaction mixture and using the same amplification conditions as described above for the Southern hybridization analysis.

RESULTS AND DISCUSSION

The epithelial surface of humans and animals represents the principal passive barrier against microbial invasion. Keratin plays a preventive role in the infection. However, some fungi such as dermatophytes dissolve the keratin with keratinases including MEPs. In the present study, the sequence of the MEP gene of *A. gypseum* was fully determined and the expression of MEP mRNA was confirmed in cells growing on medium with keratin.

The full-length cDNA sequence of the MEP gene (2,009 bp) contained a single long open reading frame of 1,899 bp coding a protein of 633 amino acids, beginning with a putative initiating methionine (ATG).

The amino acid sequence of the conserved region in the *A. gypseum* MEP gene shared about 89 and 66% sequence similarity with those in the *M. canis* and *Aspergillus fumigatus* MEP genes, respectively (Fig. 1). The amino acid sequence similarity among MEP genes of the fungi analyzed was relatively high, particularly in the zinc-binding domain that contains the highly conserved MEP signature sequence "HEXXH" (4,14). The signature sequence was also found in the *A. gypseum* MEP gene as in the *M. canis* MEP gene and *A. fumigatus* MEP gene (Fig. 1).

The sequence reported was deposited in the DNA data bank of Japan (DDBJ) database (DDBJ accession no.- AB126166, *Arthroderma gypseum* MEP mRNA for metalloprotease, complete cds).

Upon Southern hybridization analysis with *A. gypseum* genomic DNA, the MEP probe gave distinct bands in *Bam*HI, *Eco*RI and *Hind*III digests (Fig. 2). On the other hand, Southern hybridization analysis of genomic DNA with the MEP probe gave one band in three enzyme digests of genomic DNA from *M. canis* (16). Phylogenetic analysis indicated that the *M. canis* zoophilic dermatophyte and *A. gypseum* geophilic

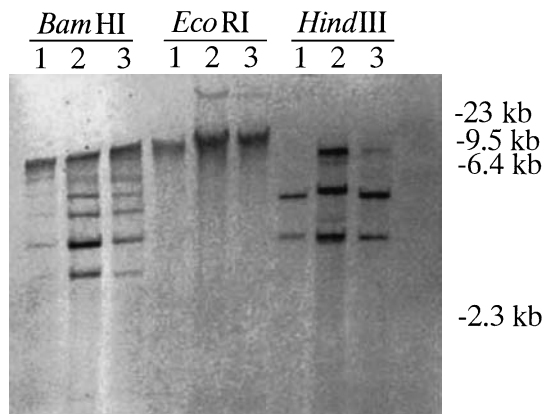


Fig. 2. Southern hybridization analysis of genomic DNA of *A. gypseum* with an MEP probe. The lanes for the various digests are: *Bam*HI, *Eco*RI and *Hind*III. Lane 1, *A. gypseum* VUT-4004; lane 2, *A. gypseum* VUT-4005; lane 3, *A. gypseum* VUT-99011.

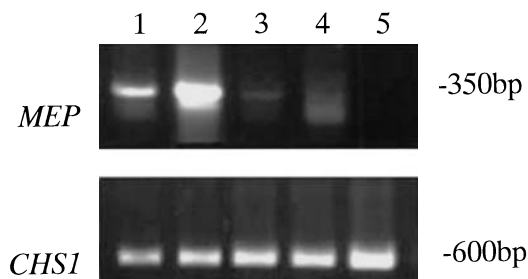


Fig. 3. Reverse transcription-PCR (RT-PCR) assay of *A. gypseum* VUT-4004 MEP induced by keratin. Lane 1, *A. gypseum* cultured on hair medium; lane 2, *A. gypseum* cultured on keratin azure; lane 3, *A. gypseum* cultured on Sabouraud's dextrose agar; lane 4, *A. gypseum* cultured on corn meal agar; lane 5, *A. gypseum* cultured on diluted Sabouraud's dextrose agar. The *CHS1* (chitin synthase 1) gene was used as a control for gene expression to compare the levels of mRNA (10).

dermatophyte were genetically close (6,9-12). The results of phylogenetic analyses on nucleotide and amino acid sequences of MEP genes from these dermatophytes and *A. fumigatus* were consistent with the fact that dermatophytes and *Aspergillus* belong to the same taxonomic group of Ascomycetes producing prototunicate asci in ascocarps (7). The results of restriction fragment length polymorphism (RFLP) analysis might be useful to understand the evolution and pathogenesis of dermatophytes.

After 30 amplification cycles in RT-PCR, MEP mRNA was detected in *A. gypseum* cultured on hair medium and keratin azure, and was undetectable on Sabouraud's dextrose agar, corn meal agar and diluted Sabouraud's dextrose agar (Fig. 3). Since MEP mRNA was detectable in *A. gypseum* cultured with keratin but not without keratin, keratin could induce the expression of MEP in this species.

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