Table 1 Species and strains used in this study.

Species (mating type)	strain	origin
A. benhamiae (+)	VUT-77011 = RV26678	Americano-European race
A. benhamiae (−)	VUT-77012 = RV26680	Americano-European race
A. benhamiae (+)	RV 30000	African race
A. benhamiae (−)	RV 30001	African race
A. benhamiae (+)	VUT-97010	Rabbit (isolated at Hyogo)
A. benhamiae (−)	VUT-00001	Guinea pig (isolated at Hyogo)
A. benhamiae (−)	VUT-00002	Rabbit (isolated at Saitama)
A. benhamiae (−)	VUT-00003	Rabbit (isolated at Saitama)
A. benhamiae (−)	VUT-00003-2	Human (isolated at Saitama)
A. benhamiae (−)	KMU 4137	Rabbit (isolated at Tottori)
A. benhamiae (−)	KMU 4169	Human (isolated at Gifu)
A. benhamiae (−)	KMU 4170	Human (isolated at Gifu)
A. benhamiae (+)	IFM 50998	Hedgehog (isolated at Chiba)
A. benhamiae (−)	IFM 51499	Hedgehog (isolated at Chiba)
A. benhamiae (+)	IFM 51500	Hedgehog (isolated at Chiba)
A. benhamiae (+)	IFM 51502	Hedgehog (isolated at Ibaragi)
A. benhamiae (+)	IFM 51503	Hedgehog (isolated at Okayama)
A. simii (+)	VUT-77009	
A. simii (−)	VUT-77010	
A. vanbreuseghemii (+) VUT-77007		
A. vanbreuseghemii (−) VUT-77008		

KMU, Kanazawa Medical University; VUT, Veterinary Medicine, University of Tokyo, Japan; RV, Institute de Medecine Tropicale Prince Leopold, Antwerpen, Belgium; IFM, Research center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

their identification. In our previous investigation, the nucleotide sequences of *CHS1* from clinical isolates recovered in Japan from humans, rabbits and a guinea pig had proved to be genetically close to the Americano-European race [11]. These results indicated that only the Americano-European race of *A. benhaniae* was found in Japan.

In the present study, nucleotide sequences of the *CHS1* gene from clinical isolates of *A. benhamiae* recovered in Japan from hedgehogs (including four-toed ones), rabbits, a guinea pig and humans were compared with those from the standard strains belonging to both the Americano-European and African race of the fungus in order to better understand the molecular epidemiology of this dermatophyte.

Materials and methods

Strains

Strains studied are listed in Table 1 and were maintained by subculturing on 1/10 Sabouraud dextrose agar at the Department of Pathobiology, Nihon University School of Veterinary Medicine. Five strains of the *A. benhamiae* isolated from the four-toed hedgehogs were obtained from the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University [6]. The mating behavior of the hedgehog isolates was confirmed to be specific and different from that of the two races previously reported [6].

CHS1 and ITS (5.8 rRNA gene and ITS1 and ITS2) region sequence analysis

The dermatophyte isolates (Table 1) were cultured in Sabouraud liquid medium at 27°C for 5 days. The genomic DNA extraction from *A. benhamiae* isolates for *CHS1* sequence analysis was performed by the method previously described [11]. *CHS1* homology relationships and phylogenetic analysis of the isolates and standard strains of *A. benhamiae* were carried out as noted in a previous study [11].

The DNA sequences were compared by Clustal W multiple sequence alignment programs [12] and a phylogenetic tree was constructed by the TREEVIEW displaying phylogenies program [13]. Bootstrap analysis was performed on 1000 random samples and analyzed by the Clustal W programs [14].

To confirm *CHS1* sequence result, we also sequenced the 5.8 rRNA gene and ITS1 and ITS2 region of three clinical isolates (IFM 50998, IFM51499 and IFM 51501) from hedgehogs as described previously [6]. Phylogenetic analysis of the ITS region of the isolates and standard strains of dermatophytes were deposited in EMBL/GenBank as mentioned above.

Results and discussion

Amplification of dermatophyte DNAs with degenerate *CHS1* primers yielded fragments of about 443 bp, consistent with the sizes of fungal *CHS1* gene sequences reported previously [11]. Nucleotide sequence analysis of the *CHS1* gene fragments from the clinical isolates and standard strains of *A. benhamiae* (Americano-European race and African race), indicated that the sequence similarities were more than 90% among them (Fig. 1). Nucleotide sequence similarity

```
VUT-77011 Americano-European race TCGAGTACATGTGCTCGCGCACCAGCAGCAAGACATGGGGCAAAGAAGCCTGGAAGAAGA  60
RV 30000  African race           ************************************************************
IFM 50998 hedgehogs isolate      ************************************************************

VUT-77011 Americano-European race TTGTCGTTTGTATCGTCTCAGACGGTCGTGCAAAGATAAATCCACGTACGAGAGCTGTCC 120
RV 30000  African race           **********C*************************************************
IFM 50998 hedgehogs isolate      ****************T*********************************************

VUT-77011 Americano-European race TTGCCGGTCTTGGTGTTTACCAGGACGGCATTGCCAAACAGCAGGTTAACGGCAAAGACG 180
RV 30000  African race           **********A**********A**************G********T******T******
IFM 50998 hedgehogs isolate      **********C**********A**************G********C*****C******

VUT-77011 Americano-European race TCACTGCTCACATCTACGAATATACCACCCAGATAGGCATGGAGGTCAAGGGCACCCAGG 240
RV 30000  African race           ************************************************C*******
IFM 50998 hedgehogs isolate      ************************************************A*******

VUT-77011 Americano-European race TCATCCTCAAGCCGCGGCCGGGAATGCCGGTCCAGCTCCTCTTCTGTCTCAAAGAGAAGA 300
RV 30000  African race           *T**************A**A**G************************************
IFM 50998 hedgehogs isolate      ****T*********************************************************

IFM 50998 hedgehogs isolate      ACCAGAAGAAGATCAACTCTCACAGATGGTTCTTCCAAGCCTTTGGTCGTGTCCTCGACC 360
RV 30000  African race           ***************************C*****************************T****
IFM 50998 hedgehogs isolate      ***************************A**********************************

VUT-77011 Americano-European race CCAATATCTGTGTTCTCATCGACGCTGGAACAAAACCAGGCGGGCGAAGTATATACCAGC 420
RV 30000  African race           ************************************A************************
IFM 50998 hedgehogs isolate      ************************************G************************

VUT-77011 Americano-European race TCTGGCGTGCTTTTGACCTCGAG 443
RV 30000  African race           ***********************
IFM 50998 hedgehogs isolate      ***********************
```

Fig. 1 Alignment of *CHS1* gene fragments illustrating the sequence divergence among Americano-European race, African race and isolate from hedgehog. Asterisks symbolize identical nucleotides compared to the leader sequence (*A. benhamiae* strain VUT 77011).

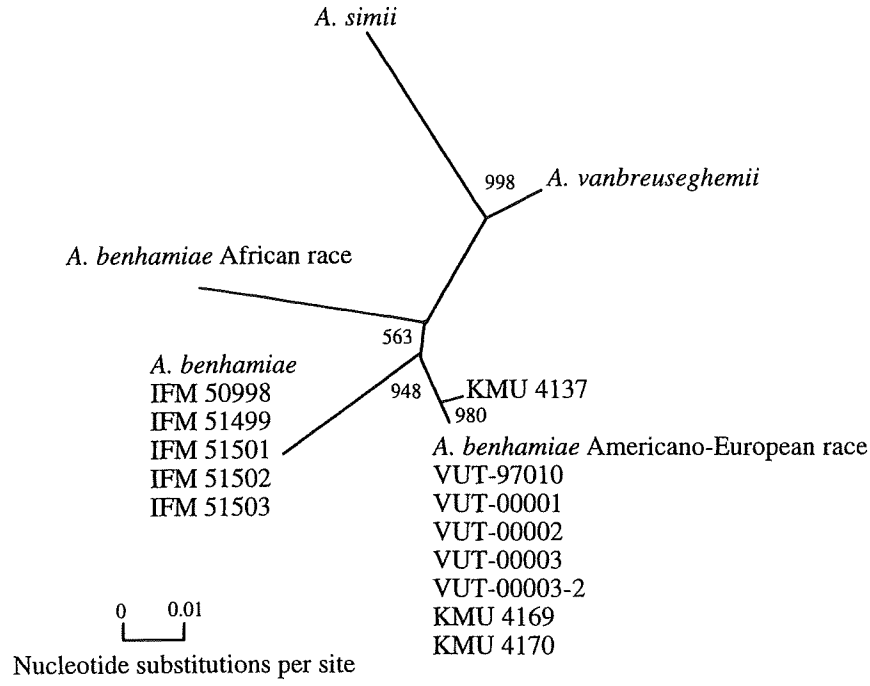


Fig. 2 A tree showing phylogenetic relationships of *CHS1* gene fragments of dermatophyte species. Numbers at branches were determined by the bootstrap analysis indicating the times in 1000 repeat sub samples in monophylogenic grouping.

```
VUT-77011 Americano-European race CCCCACGATAGGAATCAACGTTCCATCAGGGGTGTGCAGATGTGCGCCGGCCTTACGCCC  60
RV 30000  African race           ***********G**************************************************
IFM 50998 hedgehogs isolate      ***********G**************************************************

VUT-77011 Americano-European race CATTCTTGTCTACCTTACTCGGTTGCCTCGGCGGGCCGCGCTCTCCTGGGAGAGTCGTCC 120
RV 30000  African race           ************************************************************
IFM 50998 hedgehogs isolate      ************************************************************

VUT-77011 Americano-European race GGCGAGCCTCTTTGGGGGCTTTAGCTGGATCGCGCCCGCCGG_AGGACAGACATCAAAAA 179
RV 30000  African race           ***********C**************************-***********************
IFM 50998 hedgehogs isolate      ***********C**************************T***********************

VUT-77011 Americano-European race ATCTTGGAAAGCTGTCAGTCTGAGCGTTAGCAAGTAAAATT-AGTTAAAACTTTCAACAA 238
RV 30000  African race           ******A********T******************C****-*C********************
IFM 50998 hedgehogs isolate      ******A********T******************C****-*C********************

VUT-77011 Americano-European race CGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT 298
RV 30000  African race           ************************************************************
IFM 50998 hedgehogs isolate      ************************************************************

VUT-77011 Americano-European race TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGG 358
RV 30000  African race           ************************************************************
IFM 50998 hedgehogs isolate      ************************************************************

VUT-77011 Americano-European race GGGCATGCCTGTTCGAGCGTCATTTCAACCCCTCAAGCCCGGCTTGTGTGATGGACGACC 418
RV 30000  African race           ***********************************C************************
IFM 50998 hedgehogs isolate      ************************************************************

VUT-77011 Americano-European race GTCCGACCTCCTCTTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGGCCAGGCCGCGATTC 478
RV 30000  African race           *****G*****************T*************************************T
IFM 50998 hedgehogs isolate      *****G*****************T*************************************T

VUT-77011 Americano-European race CGGCTT-CCTGGGCGAATGGGCAGTCAAACCAGCGCCCTCAGGACCGGCCGCTCTGGCCT 537
RV 30000  African race           ******-*****************A******C***************************G**
IFM 50998 hedgehogs isolate      ******A*****************A******-***************************C**

VUT-77011 Americano-European race TCCCCCAAATCTCTCTGAGATATTTTTTCAGGTTGACCTCGGATCAGGTAGGGATACCCG 597
RV 30000  African race           **********************T*****************************************
IFM 50998 hedgehogs isolate      **********************T*****************************************

VUT-77011 Americano-European race CT 599
RV 30000  African race           **
IFM 50998 hedgehogs isolate      **
```

Fig. 3 Alignments of complete 5.8 rRNA gene and ITS1 and ITS2 region illustrating the sequence divergence among Americano-European race, African race and isolate from hedgehog. Asterisks symbolize identical nucleotides compared to the leader sequence (*A. benhamiae* strain VUT 77011).

was 100% among *CHS1* gene fragments of the five *A. benhamiae* isolates recovered from the hedgehogs and it was almost as high in Americano-European race (97.9%) and African race (98.4%) strains. The sequences reported in this paper have been deposited in the DDBJ database (accession no. AB353723).

The phylogenetic analysis of *CHS1* sequences of the *T. mentagrophytes* complex revealed that they were divided into five clusters. The first cluster consisted of the Americano-European race and eight Japanese isolates from human, rabbits and guinea pig. The second cluster contained the African race isolates, while the third cluster was composed of the five Japanese isolates of *A. benhamiae* from hedgehogs with skin lesions. Finally, the fourth cluster consisted of the *A. simii* strains and the fifth contained the *A. vanbreuseghemii* strains (Fig. 2).

The finding that the five Japanese clinical isolates from hedgehogs were in the same cluster indicated that these strains were genetically closely related to one another and distinct from the Americano-European and African races of *A. benhamiae.*

The results obtained by 5.8 rRNA gene and ITS1 and ITS region analysis (Fig. 3) were phylogenetically similar to that of the *CHS1* analysis (Fig. 4). The nucleotide sequences of the regions of 3 hedgehog isolates and those of hedgehog isolate deposited in database (DDBJ accession no. AB078898) showed 100% similarity.

Nucleotide sequence analysis of the *CHS1* gene fragments from the clinical isolates and standard

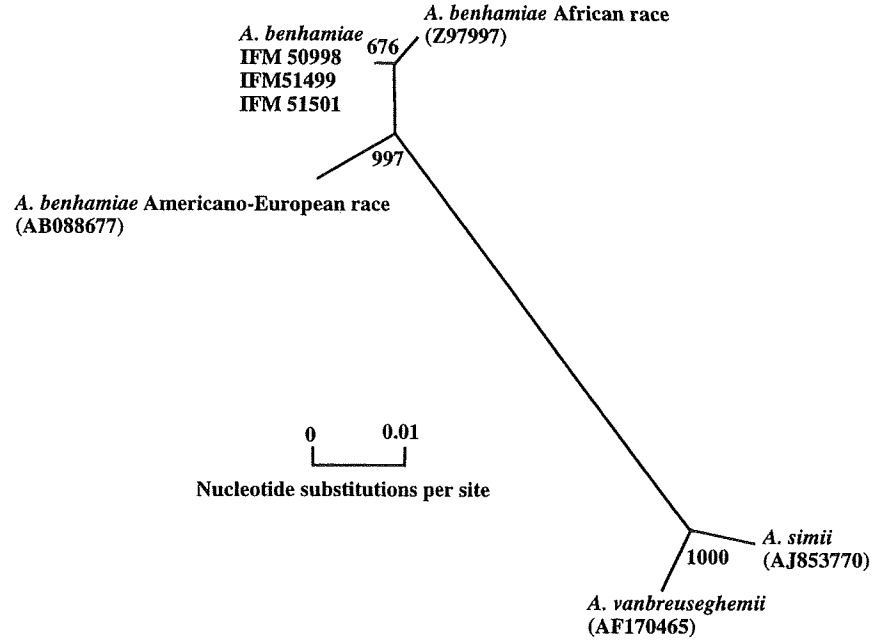


Fig. 4 A tree showing phylogenetic relationships of obtained 5.8 rRNA gene and ITS1 and ITS2 region analysis of dermatophyte species. Numbers at branches were determined by the bootstrap analysis indicating the times in 1000 repeat sub samples in monophylogenic grouping. () indicates DDBJ session number of 5.8 rRNA gene and ITS1 and ITS2 region analysis of dermatophyte species.

strains of *A. benhamiae* (Americano-European race and African race), indicated that the sequence similarities were more than 90% among them (Fig. 1). Therefore, all of these isolates were included in the same species, since the *CHS1* sequence similarities of different species of dermatophytes were at least 10% distant from one another. Moreover, nucleotide sequence analysis of the 5.8 rRNA gene and ITS1 and ITS2 from the clinical isolates and standard strains of *A. benhamiae* (Americano-European race and African race), indicated that the sequence similarities were 97.5–99.3% among them. It has been reported that ITS sequence similarities of different species in dermatophytes were at least 3% distant from one another. The ITS sequence homology among synonymy of geophylic dermatophytes was more than 98% [15]. Therefore, ITS region analysis as *CHS1* sequence analysis in this study indicated that the clinical isolates and standard strains of *A. benhamiae* (both races) might be treated as the same species.

Takahashi et al. reported that the seven clinical isolates from hedgehogs in Japan, five of which were included in this study, showed a distinct mating behavior when compared with the isolates of Americano-European and African races [6]. They implied that these isolates presented a new race [6]. This was confirmed by phylogenetic analysis which revealed that the five Japanese hedgehog isolates were genetically clustered differently from the Americano-European and African races (Figs. 2 and 4). From these results, we propose a new grouping based on the genotypes of *A. benhamiae* on the view points of mating behavior as well as molecular differences; Genotype I consisting of Americano-European race isolates, genotype II containing the African races and finally genotype III composed of our (Japanese) isolates from hedgehogs. We speculated that genotype III may have been transported to Japan from Africa, which is where these hedgehogs (*Atelerix albiventris*) originated. Further molecular investigations on the relatedness of the *A. benhamiae* complex are required to identify the isolates in detail and to know the dissemination route of the genotype III isolates of *A. benhamiae*.

Acknowledgements

This study was supported by grants from the Academic Frontier Project of The ministry of Education, Culture, Sports, Science and Technology (MEXT) and Cooperative Research Program of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

References

1 Ajello L, Cheng S-L. The perfect state of *Trichophyton mentagrophytes*. *Sabouraudia* 1967; **4**: 230–234.
2 Stockdale MP, Mackenzie WRD, Austwick KCP. *Arthroderma simii* sp. nov., the perfect state of *Trichophyton simii* (Pinoy) comb. nov. *Sabouraudia* 1967; **4**: 112–123.
3 Takashio M. The *Trichophyton mentagrophytes* complex. In: Iwata K (ed.). *Recent Advances in Medical and Veterinary Mycology*. Tokyo: University of Tokyo Press, 1977: 271–276.

4 Hironaga M, Watanabe S. Mating behavior of 334 Japanese isolates of *Trichophyton mentagrophytes* in relation to their ecological status. *Mycologia* 1980; **72**: 1159–1170.
5 Kano R, Nakamura Y, Yasuda K, *et al.* The first isolation of *Arthroderma benhamiae* in Japan. *Microbiol Immunol* 1998; **42**: 575–578.
6 Takahashi Y, Sano A, Takizawa K, *et al.* The epidemiology and mating behavior of *Arthroderma benhamiae* var. *erinacei* in household four-toed hedgehogs (*Atelerix albiventris*) in Japan. *Jpn J Med Mycol* 2003; **44**: 31–38.
7 Takashio M. Observation of African and European strains of *Arthroderma benhamiae. Intern J Dermatol* 1974; **13**: 94–101.
8 Kano R, Aihara S, Nakamura Y, Watanabe S, Hasegawa A. Chitin synthase 1 (*Chs1*) gene sequences of *Microsporum equinum* and *Trichophyton equinum. Vet Microbiol* 2001; **78**: 85–90.
9 Hirai A, Kano R, Nakamura Y, Watanabe S, Hasegawa A. Molecular taxonomy of dermatophytes and their related fungi by chitin synthase 1 (*CHS1*) gene sequences. *Antonie van Leeuwenhoek* 2003; **83**: 11–20.
10 Kano R, Nakamura Y, Watanabe S, Hasegawa A. Chitin synthase 1 and 2 genes of dermatophytes. *Stud Mycol* 2002; **47**: 49–56.
11 Kano R, Hirai A, Hasegawa A. Chitin synthase 1 gene of *Arthroderma benhamiae* isolates in Japan. *Mycoses* 2002; **45**: 277–281.
12 Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acid Res* 1994; **22**: 4673–4680.
13 Page RDM. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. *Appl Biosci* 1996; **12**: 357–358.
14 Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985; **39**: 783–791.
15 Sharma R, Rajak RC, Pandey AK, Graser Y. Internal Transcribed Spacer (ITS) of rDNA of appendaged and non-appendaged strains of *Microsporum gypseum* reveals *Microsporum appendiculatum* as its synonym. *Antonie Van Leeuwenhoek* 2006; **89**: 197–202.

Journal of Medical Microbiology (2008), 57, 50–57 DOI 10.1099/jmm.0.47216-0

Development of a loop-mediated isothermal amplification method for diagnosing *Pneumocystis* pneumonia

Natsu Uemura,[1,2] Koichi Makimura,[1] Masanobu Onozaki,[3] Yoshihito Otsuka,[4] Yasuhiro Shibuya,[5] Hirohisa Yazaki,[6] Yoshimi Kikuchi,[6] Shigeru Abe[1] and Shoji Kudoh[2]

Correspondence
Natsu Uemura
s3016@nms.ac.jp

[1]Teikyo University Institute of Medical Mycology, 539 Otsuka, Hachioji, Tokyo 192-0395, Japan
[2]Department of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, Tokyo, Japan
[3]Kanto Chemical Co. Inc., Tokyo, Japan
[4]Social Insurance Central General Hospital, Tokyo, Japan
[5]Tokyo Metropolitan Hiroo General Hospital, Tokyo, Japan
[6]AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

Loop-mediated isothermal amplification (LAMP) is a novel, rapid nucleic acid amplification method with high specificity and sensitivity under isothermal conditions. In this study a LAMP assay for diagnosing *Pneumocystis* pneumonia (PCP) was developed. Oligonucleotide primers specific for *Pneumocystis* species were designed corresponding to 18S rRNA gene sequences. The assay, performed for 30 min at 61 °C, was capable of detecting 50 copies per tube (2×10^3 copies ml^{-1}) in 30 min and did not show cross-reactivity to other species of fungi, including the genera *Candida*, *Aspergillus* and *Cryptococcus*. A total of 21 of 24 clinical specimens (sputum and bronchoalveolar lavage fluid) from patients with suspected PCP tested positive using the LAMP assay by real-time fluorescence detection. The results of the LAMP reaction were also observed by real-time turbidity detection and end-point visual turbidity or fluorescence detection. With real-time fluorescence detection, melting curves of the products were effective at distinguishing specific amplification from non-specific amplification or self-amplification. Visual detection was also possible as a rapid and easy assay using only a heat block and a black light.

Received 7 February 2007
Accepted 13 September 2007

INTRODUCTION

Pneumocystis is a fungus belonging to the class Archiascomycetes, phylum Ascomycota. The human pathogen was known as *Pneumocystis carinii* f. sp. *hominis*, but has been renamed *Pneumocystis jirovecii* based on the results of molecular biological studies (Frenkel, 1999; Stringer *et al.*, 2002). This opportunistic fungus causes *Pneumocystis* pneumonia (PCP) in patients infected with human immunodeficiency virus (HIV), as well as other immunocompromised patients. Recently, human-to-human transmission (Helweg-Larsen *et al.*, 1998; Hocker *et al.*, 2005) and colonization by *P. jirovecii* have been reported in the general population (Medrano *et al.*, 2005). Although the standard method for diagnosis of PCP is based on microscopic examination of clinical specimens [bronchoalveolar lavage fluid (BALF), sputum or lung biopsy], the sensitivity of this method is poor except in the hands of highly skilled examiners (Flori *et al.*, 2004). Therefore, PCR-based methods are widely used in Japan for the diagnosis of *Pneumocystis* infection. However, conventional PCR methods require a thermal cycler and electrophoresis for detection, whilst real-time PCR allows simultaneous amplification and detection, but requires expensive equipment and therefore is not available in general clinical laboratories.

Recently, a new specific DNA amplification technique called loop-mediated isothermal amplification (LAMP) was developed (Nagamine *et al.*, 2002; Notomi *et al.*, 2000), and has already been applied to the detection of pathogenic viruses (Poon *et al.*, 2005), bacteria (Iwamoto *et al.*, 2003), parasites (Poon *et al.*, 2006) and fungi (Endo *et al.*, 2004). LAMP, which does not involve the use of PCR, allows the rapid amplification of DNA with high specificity under isothermal conditions using DNA polymerase with

Abbreviations: BALF, bronchoalveolar lavage fluid; BLF, bronchial lavage fluid; HIV, human immunodeficiency virus; LAMP, loop-mediated isothermal amplification; PCP, *Pneumocystis* pneumonia.

The GenBank/EMBL/DDBJ accession number for the 18S rRNA gene sequence of *Pneumocystis jirovecii* reported in this paper is AB266392.

strand-displacement activity. Specific nucleotide extension is achieved with four primers recognizing six distinct regions on the target. The amplification time can be further shortened by using an additional two primers, termed the loop primers (Nagamine *et al.*, 2002). Moreover, LAMP products can be detected not only using specialized equipment but also by visual observation of turbidity or fluorescence (Mori *et al.*, 2001). Here, we report the development of a *Pneumocystis*-specific LAMP assay, which has been applied successfully to clinical specimens for diagnosis of PCP.

METHODS

Fungal strains. A total of 69 strains from 51 species of fungi were used as controls in the present study (Table 1).

Preparation of DNA from fungal cells. With the exception of *P. jirovecii*, all fungal strains were grown on Sabouraud dextrose agar [1 % (w/v) peptone; 1 % (w/v) glucose; 1.5 % (w/v) agar] at 27 or 37 °C for 1–5 days. Rapid preparation of DNA from the fungi was performed by a modification (Makimura *et al.*, 1994) of a method described by Cenis (1992). Briefly, small samples of mycelia grown on Sabouraud dextrose agar were placed in lysis buffer [200 mM Tris/HCl (pH 8.0), 0.5 % (w/v) SDS, 250 mM NaCl, 25 mM EDTA] and crushed with a conical grinder. These samples were incubated at 100 °C for 15 min, mixed with 150 µl 3.0 M sodium acetate, kept at −20 °C for 10 min and then centrifuged at 10 000 *g* for 5 min. The supernatants were extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and once with chloroform. DNA was precipitated with an equal volume of 2-propanol at −20 °C for 10 min, washed with 0.5 ml 99 % ethanol, dried and resuspended in 50 µl ultrapure water (MilliQ Synthesis A10; Millipore). Aliquots of 1 µl of the resulting solutions were used as templates for PCR.

DNA from yeasts was prepared by a modification of the method described by Makimura *et al.* (1994). Briefly, small samples of individual yeast colonies were suspended in 100 µl lysis buffer. The samples were mixed using a vortex for 5 s and incubated at 100 °C for 15 min. Next, 100 µl 3.0 M sodium acetate was added and the preparations were mixed. After incubation at −20 °C for 10 min, the samples were centrifuged at 10 000 *g* for 5 min, after which the supernatants were transferred to fresh tubes. DNA was precipitated with an equal volume of 2-propanol, washed with 0.5 ml 99 % ethanol, dried and resuspended in 100 µl ultrapure water.

Clinical specimens. A total of 38 clinical specimens (24 from PCP and 14 from non-PCP patients) were examined. Twenty-four specimens, comprising eight sputum samples and sixteen BALF samples, were collected from twenty-one patients diagnosed with PCP and admitted to three hospitals in Tokyo (Social Insurance Central General Hospital, Tokyo Metropolitan Hiroo Hospital or the International Medical Center of Japan) between January 1998 and July 2006. In the PCP group (Table 2), PCP was diagnosed by microscopic examination, or symptoms were improved by PCP therapy (co-trimoxazole, i.e. sulfamethoxazole/trimethoprim and/or pentamidine isethionate). In addition, 1 BALF sample and 13 bronchial lavage fluid (BLF) samples were collected from the 14 non-PCP patients (Table 3). The subjects in the non-PCP group, which comprised 1 patient with idiopathic pulmonary fibrosis and 13 with lung cancer, did not have fever or inflammatory signs. The PCP group included six patients with collagen disease (rheumatoid arthritis) treated with steroids or immunosuppressive agents, and one lung cancer patient treated with chemotherapy. The patients in the non-PCP group received no treatment with anti-*Pneumocystis* agents.

DNA preparation from clinical specimens. Clinical specimens were stored at −80 °C before DNA extraction using a NucliSENS kit manually with MiniMAG (bioMérieux) in accordance with the manufacturer's instructions. Samples of 200–1000 µl sputum, BALF or BLF were used for extraction and sample DNA was stocked at −80 °C before use.

Primer design. Oligonucleotide primers specific for the genus *Pneumocystis* were designed corresponding to regions of the 18S rRNA genes of *P. jirovecii* reported by Shah *et al.* (1996) and to genomic sequences determined in our laboratory. The following Archiascomycetes, Hemiascomycetes and human sequences were also used as references: *P. carinii* (host, ferret; GenBank accession no. S83267), *P. carinii* (host, rat; L27658), *Pneumocystis murina* (AY532651), *P. carinii* (X12708), *Taphrina carnea* (AB000948), *Taphrina virginica* (AB000960), *Taphrina robinsoniana* (AB000958), *Taphrina nana* (AB000955), *Taphrina wiesneri* (D12531), *Taphrina communis* (AB000949), *Taphrina ulmi* (AB000959), *Schizosaccharomyces pombe* (X58056), *Candida albicans* (E15168), *Saccharomyces cerevisiae* (Z75578) and *Homo sapiens* (M10098). Sequences were analysed using GENETYX software (Genetyx).

The sequences of our newly designed oligonucleotide PCR primers were as follows: 5′-TTCGGGGCTTACTTTGGTC-3′ (Pjf9), 5′-GTAGTTAGTCTTCAATAAATCT-3′ (Pjr4), 5′-AGGCCTACCATG-GTTTCG-3′ (Pjf8) and 5′-CTTCGGAGGACCGGGCCGT-3′ (Pjr8). In addition to these primers, the 18S rRNA gene-based universal fungal primers NS1, NS3, NS5, NS4, NS6 and NS8 (White *et al.*, 1990) were also used for nested PCR and sequencing as shown in Fig. 1.

As LAMP primers, a set of six oligonucleotides comprising two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FL and BL) recognizing eight distinct regions on the target sequence were designed using LAMP primer design support software (Primer Explorer version 3.0; Eiken Genome; http://primerexplorer.jp/). The FIP primer consisted of F2 and the complementary strand of F1 (F1c). The BIP primer consisted of B2 and the complementary strand of B1 (B1c). The sequences and positions of these primers are shown in Table 4 and Fig. 1. All oligonucleotides were synthesized by Sigma-Aldrich.

PCR and plasmid preparation. Nested PCR was performed to amplify part of the 18S rRNA gene using primers NS1, NS8, Pjf9 and Pjr4. Each PCR mixture contained 10 µl 10× reaction buffer (GE Healthcare Bio-Sciences), 200 µM each dATP, dCTP, dGTP and dTTP (Toyobo), 2.5 U *Taq* polymerase (GE Healthcare Bio-Sciences), 30 pmol each primer and 1 µl DNA template solution. Ultrapure water was added to a final volume of 100 µl. First-round PCR was performed with primers NS1 and NS8 under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min; and 72 °C for 10 min. Second-round PCR was performed with primers Pjf9 and Pjr4 under the following conditions: 94 °C for 4 min; 25 cycles of 94 °C for 1 min, 60 °C for 15 s and 72 °C for 15 s; and 72 °C for 10 min. PCR products were separated by electrophoresis on 1.2 % agarose gels, stained with ethidium bromide and visualized by UV irradiation.

The PCR product amplified with the Pjf9 and Pjr4 primers was ligated into the TA cloning vector pCR 2.1 (Original TA cloning kit; Invitrogen). The ligated product was introduced into *Escherichia coli* DH5α (Nippon Gene) by transformation. After propagation and purification of the plasmid, the concentration (copies μl^{-1}) was calculated from the measurement of A_{260} and the molecular mass of the plasmid.

Table 1. Fungal species and strains used in the LAMP assay to determine specificity

Species	Strain
Candida albicans	ATCC 10231, TIMM 1768, TIMM 1308
Candida glabrata	ATCC 90030, CBS 138
Candida krusei	TIMM 3404, ATCC 6258
Candida dubliniensis	CBS 7987, CBS8500
Candida tropicalis	ATCC 750, Lucy colony S 9/1
Candida parapsilosis	ATCC 90018, ATCC 22019
Candida guilliermondii	TIMM 0257, TIMM 3400
Clavispora lusitaniae	JCM 1610, TIMM 4124
Saccharomyces cerevisiae	ATCC 9763
Schizosaccharomyces pombe	L972h-
Geotrichum candidum	TIMM 0693
Cryptococcus gattii	CN 03010907, CN 03010906
Cryptococcus neoformans	ATCC 90113, CN 03010904
Pichia anomala	TIMM 3420, TIMM 3826
Trichosporon asahii	CBS 2497, 1/26 No.43
Aspergillus fumigatus	TIMM 0108, TIMM 1776
Aspergillus fumigatus var. *fumigatus*	JCM 10253
Aspergillus niger	JCM 10254, TIMM 0114, TIMM 0115
Aspergillus flavus	JCM 2061, TIMM 0057, TIMM 0059
Aspergillus conicus	JCM 1725
Aspergillus restrictus	JCM 1727
Aspergillus ustus	JCM 1928
Aspergillus sclerotiorum	JCM 1962
Aspergillus unguis	JCM 2256
Aspergillus tamarii	JCM 2259
Aspergillus avenaceus	JCM 2719
Aspergillus sydowii	JCM 2722
Aspergillus nidulans var. *nidulans*	JCM 2728
Aspergillus varians	JCM 2760
Aspergillus clavatus	JCM 10080
Aspergillus oryzae	JCM 10114
Aspergillus clavatonanicus	JCM 10183
Aspergillus candidus	JCM 10250
Aspergillus ochraceus	JCM 10255
Aspergillus penicillioides	JCM 10256
Aspergillus terreus var. *terreus*	JCM 10257
Aspergillus versicolor	JCM 10258
Eurotium amstelodami	JCM 1565
Eurotium chevalieri	JCM 1568
Eurotium herbariorum	JCM 1575
Neosartorya fischeri var. *fischeri*	JCM 1740
Neosartorya quadricincta	JCM 1855
Petromyces alliaceus	JCM 1948
Emericella rugulosa	JCM 2729
Fennellia nivea	JCM 2731
Penicillium marneffei	TIMM 4090
Penicillium expansum	TIMM 1293
Pseudallescheria boydii	TIMM 0886
Rhizopus oryzae	TIMM 0921
Mucor circinelloides	TIMM 3177
Fusarium moniliforme	TIMM 1294

For nested PCR to detect *P. jirovecii* from clinical specimens, we used the *Pneumocystis*-specific primers Pjf9 and Pjr4 for first-round PCR and Pjf8 and Pjr8 for second-round PCR. The other PCR components were as described above. Both first- and second-round PCRs were performed under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min; and 72 °C for 10 min.

18S rRNA gene sequencing. The 18S rRNA gene DNA extracted from clinical sample number 1 (PCP group) was sequenced. Fig. 1 shows the location of the primers on the 18S rRNA gene. Both strands of the primary PCR product (generated with primers NS1 and NS8) were sequenced directly using an ABI PRISM BigDye terminator v1.1 cycle sequencing kit; Applied Biosystems) using primers NS1, NS3, NS4, NS5, NS6 and NS8 as reported by White *et al.* (1990) and an automated sequencer (ABI PRISM 310 genetic analyser; Applied Biosystems), in accordance with the manufacturer's instructions.

LAMP assay. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical) in reaction mixtures composed of 40 pmol each of primers FIP and BIP, 5 pmol each of primers F3 and B3, 20 pmol each of primers FL and BL, 12.5 μl 2 × reaction mixture, 1 μl *Bst* DNA polymerase, 2 μl DNA sample and distilled water up to a final volume of 25 μl. The mixtures were incubated at 61 °C for 30 min (Realoop-30; Eiken Chemical) or 60 min (LightCycler V3; Roche Diagnostics), and then heated at 80 °C for 2 min to terminate the reaction. When using the LightCycler, 20 mU *Tth* pyrophosphatase ml^{-1} (thermostable; Roche Diagnostics) and 0.25 μg YO-PRO1 ml^{-1} (a propidium dye; Molecular Probes) were added to the reaction mixtures. The temperature transition rate was 0.2 °C s^{-1}, increasing from 60 to 98 °C, and the cooling temperature was 30 °C for 1 min for melting curve analysis. For visual fluorescence detection, 1 μl fluorescent detection reagent (Eiken Chemical) was added to the reaction mixture. Control samples of plasmid solution (10^2, 10^4 and 10^6 copies per tube, i.e. 4×10^3, 4×10^5 and 4×10^7 copies ml^{-1}, respectively) were checked each time.

Detection of LAMP products. LAMP products were detected by real-time fluorescence detection with a LightCycler, real-time turbidity detection with a Realoop-30, and end-point turbidity or visual fluorescence detection, the latter being judged under UV irradiation. When using the LightCycler, melting curves of the products were analysed and specific reactions were identified.

Sensitivity and specificity of the LAMP assay. The sensitivity of the LAMP assay was examined using serial dilutions of plasmid solution (1, 10, 10^2, 10^3, 10^4 and 2×10^6 copies per tube, i.e. 40, 4×10^2, 4×10^3, 4×10^4, 4×10^5 and 8×10^7 copies ml^{-1}, respectively) with triplicate samples. Specificity was examined for 51 fungal species (69 strains), as shown in Table 1.

RESULTS AND DISCUSSION

Specificity

A plasmid construct containing the nucleotide sequence of the 18S rRNA gene of *P. jirovecii* (GenBank accession no. AB266392) was amplified by the LAMP method. The experiment using LAMP primers for *P. jirovecii* did not show cross-reactivity with pathogenic or environmental fungi. DNA samples extracted from 69 strains of 51 species of fungi shown in Table 1, including the genera *Candida*,

Table 2. Clinical information on specimens collected from PCP patients

Sample no.	Case no.	Clinical specimen	Underlying disease	Result				
				Microscopic examination	LAMP			PCR
					Real-time fluorescence detection	Copy no.*	Visual detection†	
1	C1	BALF	HIV carrier	+	+	$<10^6$‡	ND	+
2	C2	BALF	RA	+	+	$<10^6$‡	ND	+
3	C3	Sputum	RA	−	+	10^2–10^4	ND	−
4	C4	BALF	WG	+	+	10^6	ND	+
5	C5	Sputum	HIV carrier	+	+	10^6	ND	+
6	C6	BALF	HIV carrier	+	+	10^4	ND	+
7	C6	Sputum	HIV carrier	+	+	10^2–10^4	ND	+
8	C7	BALF	RA	+	+	10^2	ND	−
9	C8	Sputum	RA	+	+	$<10^2$	ND	−
10	C9	BALF	RA	−	−	−	ND	−
11	C9 (after Tx)§	Sputum	RA	−	−	−	ND	−
12	C10	Sputum	LC (chemo)‖	+	+	$<10^2$	ND	+
13	C10 (after Tx)§	Sputum	LC (chemo)‖	−	−	−	ND	−
14	C11	BALF	IPF	−	+	$<10^2$	ND	−
15	C12	BALF	HIV carrier	+	+	10^4	ND	+
16	C13	Sputum	HIV carrier	+	+	10^2	ND	+
17	C14	BALF	HIV carrier	+	+	10^4	+	+
18	C15	BALF	HIV carrier	+	+	10^2–10^4	+	+
19	C16	BALF	HIV carrier	+	+	10^2–10^4	+	+
20	C17	BALF	HIV carrier	+	+	10^2–10^4	+	+
21	C18	BALF	HIV carrier	+	+	10^2–10^4	+	+
22	C19	BALF	HIV carrier	+	+	10^2–10^4	+	+
23	C20	BALF	HIV carrier	+	+	10^2–10^4	+	+
24	C21	BALF	HIV carrier	+	+	10^4–10^6	+	+

IPF, Idiopathic pulmonary fibrosis; LC, lung cancer; ND, not determined; RA, rheumatic arthritis; WG, Wegener's granulomatosis.
*When clinical specimens were examined by LAMP assay with the LightCycler, 10^6, 10^4 and 10^2 DNA copies of *P. jirovecii* plasmid were included together. The estimate of copy number for each sample is based on these amplification curves.
†Visual detection was by end-point visual turbidity and fluorescence detection. Both methods gave identical results. ND indicates that experiments to check visual detection could not be done as the amount of clinical specimen was too small.
‡As the amount of clinical specimens was small, experiments to check quantitative capability could not be performed.
§After PCP therapy.
‖Undergoing chemotherapy.

Aspergillus and *Cryptococcus*, were not detected by the LAMP method.

Sensitivity

With a LAMP assay specific for *Pneumocystis*, it was possible specifically to detect *Pneumocystis* DNA in 30 min, with lower limits of detection of 50 or 100 copies (2×10^3 or 4×10^3 copies ml^{-1}) of template DNA using a LightCycler or by visual detection, respectively.

Fig. 2 shows the results of real-time fluorescence detection using a LightCycler. DNA amplification is indicated by the rising curve of fluorescence in Fig. 2(a). With the LAMP assay, it was possible to detect 100 copies per tube (4×10^3 copies ml^{-1}), with the curve beginning to rise at 12–13 min and reaching a plateau at 22–23 min. Detection of 50 copies per tube (2×10^3 copies ml^{-1}) could be achieved in 30 min. In reactions of 10 copies per tube (4×10^2 copies ml^{-1}), products were detected in two out of three tubes, with no product detected in the remaining tube.

On visual detection, the results were judged at a reaction time of 30 min. By both visually determined turbidity and fluorescence detection, the assays could detect 100 copies of the target sequence per tube (4×10^3 copies ml^{-1}). However, the results were unstable at concentrations below 50 copies per tube (2×10^3 copies ml^{-1}). Fig. 3 shows the results of visual fluorescence detection (Fig. 3a) and turbidity detection (Fig. 3b).

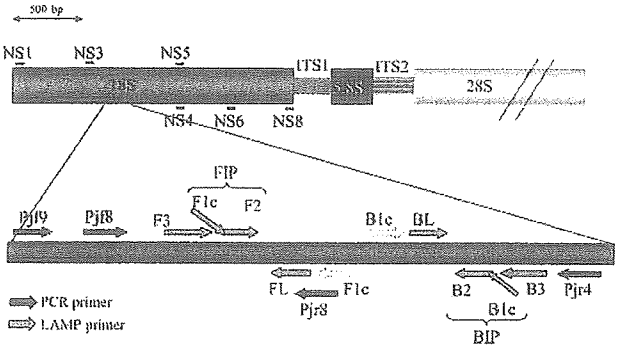


Fig. 1. Map showing the location of primers on the 18S rRNA gene of *P. jirovecii*. ITS, Internal transcribed spacer.

both base sequence and GC content. Amplification of primer-dimers or non-specific products will be observed as peaks with different T_m values. Bu *et al.* (2005) reported identification of PCR products amplified from *Candida* spp. and *Aspergillus* spp. using melting-curve analysis, and specific probes have been applied to melting-curve analysis typing of hepatitis virus (Haverstick *et al.*, 2004) and differentiation of *Mycobacterium* species (Shrestha *et al.*, 2003). This is believed to be the first report of melting-curve analysis of LAMP products. Identification of LAMP products may theoretically be determined by probes. However, as a relatively large number of oligonucleotides are already used, this would add to the complexity of this assay.

Clinical samples

A total of 22 samples (21 from PCP patients and 1 from a non-PCP subject) tested positive in the LAMP assay using the LightCycler (Tables 2 and 3). The diagnostic sensitivity of this assay was 87.5 % (21/24 PCP cases). In comparison, *P. jirovecii* DNA was detected in only 17 samples from PCP patients by nested PCR. The nested PCR used in the present study could detect 100 copies of plasmid per tube (10^3 copies ml^{-1}) (data not shown) and the sensitivity of this method was 20 times lower than a quantitative real-time PCR reported by Brancart *et al.* (2005). However, this nested PCR did not detect target DNA in any specimens from non-PCP cases or LAMP reaction-negative specimens. With regard to quantitative capability, we speculated that the target DNA copy number in sample 12 in Fig. 4(a) was equivalent to less than 100 copies per tube (4×10^3 copies ml^{-1}). Similarly, clinical samples were examined with control plasmid (10^2, 10^4 and 10^6 copies per tube, i.e. 4×10^3, 4×10^5 and 4×10^7 copies ml^{-1}, respectively) each time, and we estimated the equivalent DNA copy numbers of clinical specimens, as shown in Table 2.

Only one patient (case 22) in the present study showed colonization with *P. jirovecii*. There have been a number of recent reports of *P. jirovecii* colonization in both the general population and in patients with respiratory disease (Maskell *et al.*, 2003; Medrano *et al.*, 2005; Vidal *et al.*, 2006), with colonization rates of about 20 % in healthy subjects (Medrano *et al.*, 2005) and about 30 % in patients with interstitial lung disease (Vidal *et al.*, 2006). Further evaluation of this LAMP method with larger numbers of clinical specimens is currently under way in our laboratory.

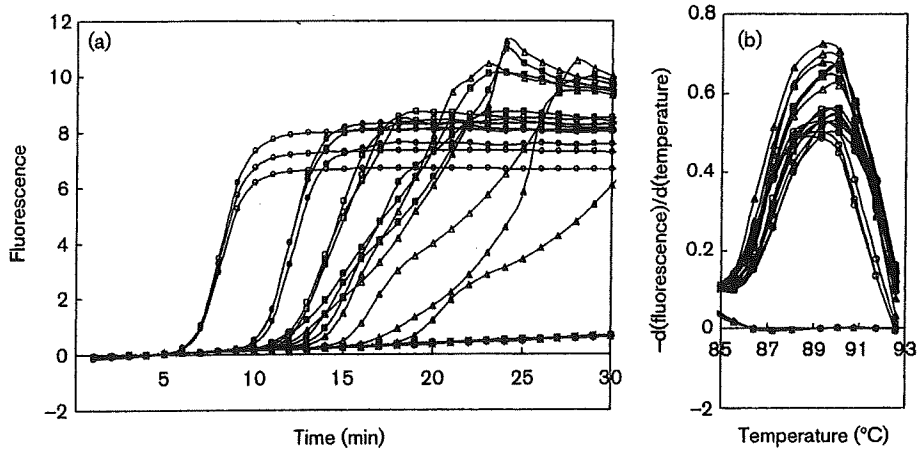


Fig. 2. Sensitivity of the LAMP assay. Triplicate samples of serial dilutions of *P. jirovecii* plasmid solution were used as DNA template. LAMP products were amplified and analysed using a LightCycler. (a) Amplification analysis is shown, where the rising curves of fluorescence indicate DNA amplification. (b) In the melting-curve analysis, the melting temperature of each sample is shown as a peak. Number of *P. jirovecii* plasmid copies: ○, 2×10^6 copies (8×10^7 copies ml^{-1}); ●, 10^4 copies (4×10^5 copies ml^{-1}); □, 10^3 copies (4×10^4 copies ml^{-1}); ■, 10^2 copies (4×10^3 copies ml^{-1}); △, 50 copies (2×10^3 copies ml^{-1}); ▲, 10 copies (4×10^2 copies ml^{-1}); □, 1 copy (40 copies ml^{-1}); ◆, distilled water.

method, although its sensitivity is slightly lower than that of the real-time PCR-based method. In patients with suspected PCP, this LAMP method is competent to diagnose PCP. Therefore, visual detection of the LAMP reaction is faster and easier than the PCR-based method and may be applied in general clinical laboratories.

ACKNOWLEDGEMENTS

This study was partly supported by Health Science Research Grants for Research on Emerging and Re-emerging Infectious Disease from the Ministry of Health, Labour and Welfare of Japan (to K. M.).

REFERENCES

Brancart, F., Rodriguez-Villalobos, H., Fonteyne, P. A., Peres-Bota, D. & Liesnard, C. (2005). Quantitative TaqMan PCR for detection of *Pneumocystis jiroveci*. *J Microbiol Methods* **61**, 381–387.

Bu, R., Sathiapalan, R. K., Ibrahim, M. M., Al-Mohsen, I., Almodavar, E., Gutierrez, M. I. & Bhatia, K. (2005). Monochrome LightCycler PCR assay for detection and quantification of five common species of *Candida* and *Aspergillus*. *J Med Microbiol* **54**, 243–248.

Cenis, J. L. (1992). Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res* **20**, 2380.

Endo, S., Komori, T., Ricci, G., Sano, A., Yokoyama, K., Ohori, A., Kamei, K., Franco, M., Miyaji, M. & Nishimura, K. (2004). Detection of *gp43* of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. *FEMS Microbiol Lett* **234**, 93–97.

Fischer, S., Gill, V. J., Kovacs, J., Miele, P., Keary, J., Silcott, V., Huang, S., Borio, L., Stock, F. & other authors (2001). The use of oral washes to diagnose *Pneumocystis carinii* pneumonia: a blinded prospective study using a polymerase chain reaction-based detection system. *J Infect Dis* **184**, 1485–1488.

Flori, P., Bellete, B., Durand, F., Raberin, H., Cazorla, C., Hafid, J., Lucht, F. & Sung, R. T. (2004). Comparison between real-time PCR, conventional PCR and different staining techniques for diagnosing *Pneumocystis jiroveci* pneumonia from bronchoalveolar lavage specimens. *J Med Microbiol* **53**, 603–607.

Frenkel, J. K. (1999). *Pneumocystis* pneumonia, an immunodeficiency-dependent disease (IDD): a critical historical overview. *J Eukaryot Microbiol* **46**, 89S–92S.

Haverstick, D. M., Bullock, G. C. & Bruns, D. E. (2004). Genotyping of hepatitis C virus by melting curve analysis: analytical characteristics and performance. *Clin Chem* **50**, 2405–2407.

Helweg-Larsen, J., Tsolaki, A. G., Miller, R. F., Lundgren, B. & Wakefield, A. E. (1998). Clusters of *Pneumocystis carinii* pneumonia: analysis of person-to-person transmission by genotyping. *QJM* **91**, 813–820.

Hocker, B., Wendt, C., Nahimana, A., Tonshoff, B. & Hauser, P. M. (2005). Molecular evidence of *Pneumocystis* transmission in pediatric transplant unit. *Emerg Infect Dis* **11**, 330–332.

Iwamoto, T., Sonobe, T. & Hayashi, K. (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *J Clin Microbiol* **41**, 2616–2622.

Jaijakul, S., Saksirisampant, W., Prownebon, J., Yenthakam, S., Mungthin, M., Leelayoova, S. & Nuchprayoon, S. (2005). *Pneumocystis jiroveci* in HIV/AIDS patients: detection by FTA filter paper together with PCR in noninvasive induced sputum specimens. *J Med Assoc Thai* **88**, S294–S299.

Larsen, H. H., Masur, H., Kovacs, J. A., Gill, V. J., Silcott, V. A., Kogulan, P., Maenza, J., Smith, M., Lucey, D. R. & Fischer, S. H. (2002). Development and evaluation of a quantitative, touch-down, real-time PCR assay for diagnosing *Pneumocystis carinii* pneumonia. *J Clin Microbiol* **40**, 490–494.

Makimura, K., Murayama, S. Y. & Yamaguchi, H. (1994). Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol* **40**, 358–364.

Maskell, N. A., Waine, D. J., Lindley, A., Pepperell, J. C., Wakefield, A. E., Miller, R. F. & Davies, R. J. (2003). Asymptomatic carriage of *Pneumocystis jiroveci* in subjects undergoing bronchoscopy: a prospective study. *Thorax* **58**, 594–597.

Medrano, F. J., Montes-Cano, M., Conde, M., de la Horra, C., Respaldiza, N., Gasch, A., Perez-Lozano, M. J., Varela, J. M. & Calderon, E. J. (2005). *Pneumocystis jirovecii* in general population. *Emerg Infect Dis* **11**, 245–250.

Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001). Detection of loop mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* **289**, 150–154.

Nagamine, K., Hase, T. & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* **16**, 223–229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**, E63.

Poon, L. L., Leung, C. S., Chan, K. H., Lee, J. H., Yuen, K. Y., Guan, Y. & Peiris, J. S. (2005). Detection of human influenza A viruses by loop-mediated isothermal amplification. *J Clin Microbiol* **43**, 427–430.

Poon, L. L., Wong, B. W., Ma, E. H., Chan, K. H., Chow, L. M., Abeyewickreme, W., Tangpukdee, N., Yuen, K. Y., Guan, Y. & other authors (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem* **52**, 303–306.

Shah, J. S., Pieciak, W., Liu, J., Buharin, A. & Lane, D. J. (1996). Diversity of host species and strains of *Pneumocystis carinii* is based on rRNA sequences. *Clin Diagn Lab Immunol* **3**, 119–127.

Shrestha, N. K., Tuohy, M. J., Hall, G. S., Reischl, U., Gordon, S. M. & Procop, G. W. (2003). Detection and differentiation of *Mycobacterium tuberculosis* and nontuberculous mycobacterial isolates by real-time PCR. *J Clin Microbiol* **41**, 5121–5126.

Stringer, J. R., Beard, C. B., Miller, R. F. & Wakefield, A. E. (2002). A new name (*Pneumocystis jirovecii*) for pneumocystis from humans. *Emerg Infect Dis* **8**, 891–896.

Vidal, S., de la Horra, C., Martin, J., Montes-Cano, M. A., Rodriguez, E., Respaldiza, N., Rodriguez, F., Varela, J. M., Medrano, F. J. & other authors (2006). *Pneumocystis jirovecii* colonisation in patients with interstitial lung disease. *Clin Microbiol Infect* **12**, 231–235.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego, CA: Academic Press.

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2007, p. 3737–3742 Vol. 45, No. 11
0095-1137/07/$08.00+0 doi:10.1128/JCM.01243-07

Revised Culture-Based System for Identification of *Malassezia* Species[▽]

Takamasa Kaneko,[1,2] Koichi Makimura,[2]* Michiko Abe,[3] Ryoko Shiota,[4] Yuka Nakamura,[5] Rui Kano,[6] Atsuhiko Hasegawa,[6] Takashi Sugita,[7] Shuichi Shibuya,[8] Shinichi Watanabe,[8] Hideyo Yamaguchi,[2] Shigeru Abe,[2] and Noboru Okamura[9]

Kanto Chemical Co., Inc., Marusan Bldg. 11-5, Nihonbashi Honcho, 3-Chome, Chuo-ku, Tokyo 103-0023, Japan[1]; Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan[2]; Department of Medical Technology, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan[3]; Department of Microbiology, Takinomiya General Hospital, 486 Takinomiya, Ryonan, Ayaka, Kagawa 761-2393, Japan[4]; Kyoritsu Seiyaku Co. 1-12-4 Kudankita, Chiyoda-ku, Tokyo 102-0073, Japan[5]; Department of Pathobiology, School of Veterinary Medicine, Nihon University, 1866, Kameino, Fujisawa Kanagawa 252-8510, Japan[6]; Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan[7]; Department of Dermatology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan[8]; and Laboratory of Microbiology and Immunology, Graduate School of Health Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan[9]

Received 19 June 2007/Returned for modification 27 August 2007/Accepted 9 September 2007

Forty-six strains of *Malassezia* spp. with atypical biochemical features were isolated from 366 fresh clinical isolates from human subjects and dogs. Isolates obtained in this study included 2 (4.7%) lipid-dependent *M. pachydermatis* isolates; 1 (2.4%) precipitate-producing and 6 (14.6%) non-polyethoxylated castor oil (Cremophor EL)-assimilating *M. furfur* isolates; and 37 (34.3%) *M. slooffiae* isolates that were esculin hydrolyzing, 17 (15.7%) that were non-tolerant of growth at 40°C, and 2 (1.9%) that assimilated polyethoxylated castor oil. Although their colony morphologies and sizes were characteristic on CHROMagar *Malassezia* medium (CHROM), all strains of *M. furfur* developed large pale pink and wrinkled colonies, and all strains of *M. slooffiae* developed small (<1 mm) pale pink colonies on CHROM. These atypical strains were distinguishable by the appearance of their colonies grown on CHROM. Three clinically important *Malassezia* species, *M. globosa*, *M. restricta*, and *M. furfur*, were correctly identified by their biochemical characteristics and colony morphologies. The results presented here indicate that our proposed identification system will be useful as a routine tool for the identification of clinically important *Malassezia* species in clinical laboratories.

Members of the genus *Malassezia* are among the microbiological flora of the skin of homoiothermic animals. Most species of this genus are lipid-dependent yeasts which colonize the seborrheic part of the skin, and they have been reported to be associated with pityriasis versicolor, seborrheic dermatitis, *Malassezia* folliculitis, and atopic dermatitis (1, 6, 19, 20, 24, 29). Although *M. furfur* was previously thought to be the causative agent or trigger factor in all of these skin disorders, Guého et al. (9) reclassified this genus into five species in 1996. *Malassezia* has since been reclassified into seven species based on molecular biological analysis of nuclear ribosomal DNA/RNA (9, 10), and the results agreed with those of mitochondrial ribosomal DNA analyses (30). As members of the genus *Malassezia* share similar morphological and biochemical characteristics, it was thought that differentiating between them based on phenotypic features would be difficult. While molecular biological techniques are the most reliable for the identification of *Malassezia*, they are not available in most clinical laboratories. Therefore, culture methods for the identification of *Malassezia* species are required. Some of these identification or differentiation methods have been reported previously. Guillot et al. reported a method of identification based on lipid usage pattern, catalase reaction, growth temperature, and cell shape (11). Hammer and Riley reported the production of a precipitate by some *Malassezia* strains on Dixon's agar (12); for example, *M. furfur*, *M. obtusa*, and *M. slooffiae* were precipitate-negative strains, while *M. sympodialis* and *M. globosa* were precipitate-positive strains. Mayser et al. reported that some *Malassezia* species hydrolyzed esculin and assimilated polyethoxylated castor oil (Cremophor EL; Sigma, St. Louis, MO) (18), and these properties could be used to differentiate among species (8). However, it is necessary to develop an updated phenotype-based identification method applicable to new *Malassezia* species (25, 26). We reported previously that CHROMagar *Candida* medium with vital growth factors for *Malassezia*, CHROMagar *Malassezia* medium (CHROM), could be used for isolating and differentiating between *Malassezia* and *Candida* spp. simultaneously (14) and that a biological feature-based identification method was developed for nine species of *Malassezia* (15). However, we found that some strains showed atypical features in fresh clinical isolates. Here, we (i) report the incidence of atypical biochemical features in *Malassezia* species and (ii) propose a culture-based identification system for three clinically important *Malassezia* species, *M. furfur*, *M. globosa*, and *M. restricta*.

* Corresponding author. Mailing address: Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan. Phone and fax: 81-426-78-3256. E-mail: makimura@main.teikyo-u.ac.jp.

▽ Published ahead of print on 19 September 2007.

TABLE 1. Origin and distribution of *Malassezia* strains

Species	No. of total strains	No. of stock strains	No. of fresh isolates	Origin	Comments
M. pachydermatis	43	1		CBS 1879	
			30	Dog, external ear	External otitis
			12	Dog, external ear	Healthy dogs
M. sympodialis	84	1		CBS 7222	
			80	Human	Healthy adults
			1	Human	Psoriasis vulgaris
			1	Human	Atopic dermatitis
			1	Human, external ear	External otitis
M. globosa	14	1		CBS 7966	
			10	Human	Pityriasis versicolor
			3	Human	Seborrheic dermatitis
M. dermatis	5	2		JCM11348 and JCM11470	
			2	Human	Healthy adults
			1	Human	Seborrheic dermatitis
M. furfur	41	1		CBS 1878	
			38	Human	Healthy adults
			1	Human	Psoriasis vulgaris
			1	Human, external ear	External otitis
M. slooffiae	108	1		CBS 7956	
			95	Human, external ear	Healthy adults
			8	Human, external ear	External otitis
			3	Human	Seborrheic dermatitis
			1	Human	Psoriasis vulgaris
M. obtusa	4	1		CBS 7876	
			3	Human	Healthy adults
M. restricta	71	1		CBS 7877	
			31	Human, external ear	Healthy adults
			35	Human	Seborrheic dermatitis
			2	Human	Psoriasis vulgaris
			2	Human	Pityriasis versicolor
M. japonica	2	2	0	M9966, M9967	
M. yamatoensis	5	0	2	Human	Healthy adults
			2	Human	Psoriasis vulgaris
			1	Human	Seborrheic dermatitis
Total	377	11	366		

MATERIALS AND METHODS

Organisms. Three hundred sixty-six fresh clinical isolates of *Malassezia* (42 *M. pachydermatis*, 83 *M. sympodialis*, 13 *M. globosa*, 40 *M. furfur*, 107 *M. slooffiae*, 3 *M. obtusa*, 70 *M. restricta*, 3 *M. dermatis*, and 5 *M. yamatoensis* isolates) obtained from human subjects or dogs, as described below, as well as type and standard strains of *Malassezia* (Table 1), were used in this study. All strains were identified by molecular biological analysis (16, 17).

Culture media. Strains of *Malassezia* were maintained on modified Leeming and Notman agar composed of (per liter) 10 g of peptone (Oxoid, Basingstoke, United Kingdom), 10 g of glucose, 2 g of yeast extract (Oxoid), 8 g of ox bile (Oxoid), 10 ml of glycerol, 0.5 g of glycerol monostearate, 5 ml of Tween 60, 20 ml of olive oil, and 15 g of agar (Oxoid) and sterilized by autoclaving.

The following specific media were used in this study. CHROMagar Malassezia medium (CHROM) was composed (per liter) of 56.3 g of CHROMagar Malassezia basal medium (CHROMagar, Paris, France) and 10 ml of Tween 40 (15). Sabouraud's dextrose agar (SDA) was composed (per liter) of 10 g of mycological peptone, 40 g of glucose, and 15 g of agar. Cremophor EL (Sigma, St. Louis, MO) agar (EL slant) was composed (per liter) of 65 g of SDA and 10 ml of Cremophor EL (15). Tween 60-esculin agar (TE slant) was composed (per liter) of 10 g of peptone, 10 g of glucose, 2 g of yeast extract, 5 ml of Tween 60, 0.5 g of ferric ammonium citrate, 1 g of esculin, and 15 g agar (15).

Clinical specimens. Two hundred eighteen clinical specimens from the body surface of patients with atopic dermatitis, seborrheic dermatitis, psoriasis vulgaris, and external otitis and from healthy adults were obtained from Teikyo University Hospital (Tokyo, Japan), Kitasato University Hospital (Kanagawa, Japan), and Takinomiya General Hospital (Kagawa, Japan) and from 65 dogs with and without external otitis from Nihon University Veterinary Hospital (Kanagawa, Japan) and veterinary clinics in the Kanto area. Samples from the body surface were taken using adhesive tape (10 mm by 10 mm) as reported by Padilha-Goncalves (22), which was then placed on CHROM. External ear samples were taken with swabs and then streaked on CHROM. All isolates observed for CHROM were checked by colony morphology and size after incubation in air at 32°C for 4 to 7 days.

Phenotypic feature testing. The "typical phenotypic features" of *Malassezia* species were defined as shown in Table 2. All isolates of *Malassezia* were inoculated onto CHROM and specific media (SDA, EL slant, and TE slant) and incubated at 32°C for 4 days before observation. SDA was used to determine the isolates' lipid dependence, EL slants for the isolates' abilities to utilize polyethoxylated castor oil, and TE slants for the isolates' abilities to hydrolyze esculin and utilize Tween 60. Fresh cultures grown on CHROM were subjected to catalase testing with 3% hydrogen peroxide. Colony size on CHROM was determined by measuring well-isolated single colonies and assessed as small (<1 mm), medium (1 to 2 mm), or large (2 to 5 mm). Additional phenotypic characterization of the ability to grow at 40°C was performed if the isolate was thought to be a strain of *M. slooffiae*. All test strains of *Malassezia* species were identified by molecular analysis.

Molecular analysis. DNA was extracted by the procedure of Makimura et al. (16). The internal transcribed spacer 1 (ITS1) region was sequenced directly from PCR products by using the primer pair 18SF1 and 58SR1 (17). The PCR products were sequenced on an ABI PRISM 310 genetic analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

RESULTS

Phenotypic features of *Malassezia* and incidence of atypical phenotypic features found in *Malassezia* species. The phenotypic features of nine *Malassezia* species are shown in Table 3.

TABLE 2. Typical phenotypic features in nine species of *Malassezia*[a]

Species	Growth on SDA	Growth on mDixon at			Precipitate production on CHROM	Utilization of				Esculin	Polyethoxylated castor oil	Catalase reaction
		32°C	37°C	40°C		10% Tw 20	0.5% Tw 40	0.5% Tw 60	0.1% Tw 80			
M. pachydermatis[b]	+	+	+	+	+[f]	+	+	+	+	±[e]	±[e]	+
M. sympodialis[b]	−	+	+	+	+[f]	−	+	+	+	+[e]	−[e]	+
M. globosa[b]	−	+	± or −	−	+[f]	−	−	−	−	−[e]	−[e]	+
M. dermatis[c]	−	+	+	+	+[f]	+	+	+	+	−[f]	−[f]	+
M. furfur[b]	−	+	+	+	−[f]	+	+	+	+	−[e]	+[e]	+
M. slooffiae[b]	−	+	+	+	−[f]	± or +	+	+	−	−[e]	−[e]	+
M. obtusa[b]	−	+	± or +	−	−[f]	−	−	−	−	+[e]	−[e]	+
M. restricta[b]	−	+	+	−	−[f]	−	−	−	−	−[e]	−[e]	−
M. japonica[d]	−	+	+	−	−[f]	−	±	+	−	+[f]	−[f]	+

[a] mDixon, modified Dixon agar; Tw, Tween; +, positive; −, negative; ±, weakly positive.
[b] Guého et al. (9).
[c] Sugita et al. (25).
[d] Sugita et al. (26).
[e] Mayser et al. (18).
[f] Kaneko et al. (15).

(i) Precipitate production and colony morphology on CHROM. All of the type and reference strains and fresh clinical isolates of *M. pachydermatis* (43/43), *M. sympodialis* (84/84), *M. globosa* (14/14), and *M. dermatis* (5/5) produced precipitates after incubation at 32°C for 4 days on CHROM. Other strains of the species *M. furfur* (40/41), *M. slooffiae* (108/108), *M. obtusa* (4/4), *M. restricta* (71/71), and *M. japonica* (2/2) did not produce such precipitates, with the exception of only

TABLE 3. Biological features and incidence of a typical phenotype of nine species of *Malassezia*

Species identified by molecular biological analysis	No. of strains	Colony characteristic on CHROM (% of incidence)[a]			Growth characteristic (% of incidence)[b]				Catalase reaction
		Size	Color/morphology	Precipitate	SDA	TE slant	EL slant	40°C	
M. pachydermatis	43	Large (100)	Pale pink/smooth (100)	+ (100)	Growth (95.3)[d]	Growth and produced a black zone (95.3)[d]	Growth (95.3)[d]	NT	+ (100)
M. sympodialis	84	Large (100)	Pale pink/smooth (100)	+ (100)	No growth (100)	Growth and produced a black zone (100)	No growth (100)	NT	+ (100)
M. globosa	14	Small (100)	Purple/smooth (100)	+ (100)	No growth (100)	No growth and no change (100)	No growth (100)	NT	+ (100)
M. dermatis	5	Large (100)	Pale pink to purple/smooth (100)	+ (100)	No growth (100)	Growth and no change (100)	No growth (100)	NT	+ (100)
M. furfur	41	Large (100)	Pale pink/wrinkled (100)	− (97.6)[c]	No growth (100)	Growth and produced a black zone (100)	Growth (85.4)	NT	+ (100)
M. slooffiae	108	Small (100)	Pale pink/smooth (100)	− (100)	No growth (100)	Growth and no change (65.7)[c]	No growth (98.1)	Growth (84.3)[e]	+ (100)
M. obtusa	4	Medium (100)	Pink/rough (100)	− (100)	No growth (100)	No growth but produced a black zone (100)	No growth (100)	NT	+ (100)
M. restricta	71	Small (100)	Pink/smooth (100)	− (100)	No growth (100)	No growth and no change (100)	No growth (100)	NT	− (100)
M. japonica	2	Large (100)	Pink/smooth (100)	− (100)	No growth (100)	Growth and produced a black zone (100)	No growth (100)	No growth (100)	+ (100)

[a] Incubated at 32°C for 4 to 7 days.
[b] Incubated at 32°C for 4 days. NT, not tested; +, positive; −, negative.
[c] Only one strain of fresh clinical isolate produced a precipitate.
[d] Only two strains of fresh clinical isolates did not grow on SDA and EL and did not produce a black zone on TE.
[e] Thirty-seven strains of fresh clinical isolates produced a black zone on TE, and 17 strains did not grow on modified Leeming and Notman agar at 40°C for 4 days.

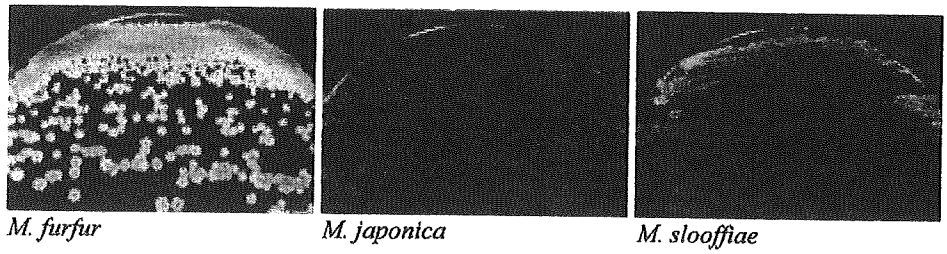


FIG. 1. Colony characteristics of *M. furfur*, *M. japonica*, and *M. slooffiae* on CHROM were observed after incubation at 32°C for 4 days. Colonies of *M. furfur* were large, pale pink, and wrinkled and did not produce precipitates. Colonies of *M. japonica* were larger (2 to 5 mm) than those of *M. slooffiae* (<1 mm). Their sizes were measured in well-isolated single colonies.

one (2.4%) strain of a fresh clinical isolate of *M. furfur*. *Malassezia furfur* colonies, including this one atypical strain, were easily distinguishable from those of other *Malassezia* species on CHROM due to their characteristically large pale pink and wrinkled colonies (Fig. 1). Colony size on CHROM was measured from well-isolated single colonies, and isolates were divided into three groups: small (*M. globosa*, *M. slooffiae*, and *M. restricta*), medium (*M. obtusa*), and large (*M. pachydermatis*, *M. sympodialis*, *M. dermatis*, *M. furfur*, and *M. japonica*).

(ii) Lipid dependence. Only *M. pachydermatis* (41/43) grew on the lipid-free culture medium (SDA), and other *Malassezia* species did not grow. This biological feature of *M. pachydermatis* was specific, but two (4.7%) atypical strains of lipid-dependent *M. pachydermatis* were obtained.

(iii) Utilization of polyethoxylated castor oil. EL slants were used to determine the ability to utilize polyethoxylated castor oil, and we obtained six (14.6%) atypical strains of *M. furfur* and two (4.7%) of *M. pachydermatis* that did not grow on EL slants. Two (1.9%) *M. slooffiae* strains grew on EL slants. This biological feature was not acceptable as a key identifying feature of *M. furfur*.

(iv) Catalase reaction. *Malassezia restricta* was the only catalase-negative species. This biological feature was acceptable as a key identifying feature of *M. restricta*.

(v) Hydrolysis of esculin and utilization of Tween 60. Forty-one (95.3%) isolates of *M. pachydermatis*, 84 (100%) of *M. sympodialis*, 41 (100%) of *M. furfur*, and 2 (100%) of *M. japonica* showed production of a black zone around the colonies due to esculin hydrolysis products and ferrous iron in TE slants. On the other hand, 5 (100%) *M. dermatis* and 71 (65.7%) *M. slooffiae* isolates did not produce such a zone. None of the strains of *M. globosa* or *M. restricta* utilized Tween 60 or grew on TE slants. None of the strains of *M. obtusa* utilized Tween 60, but they hydrolyzed esculin and produced a black zone on TE slants.

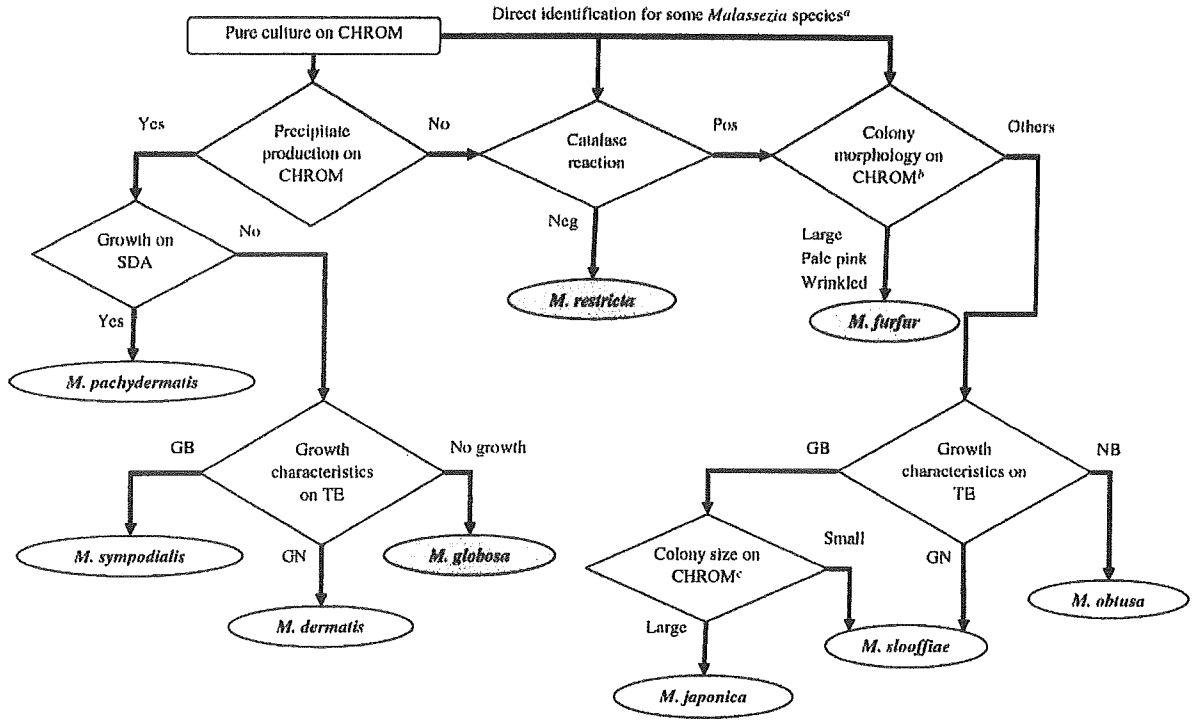


FIG. 2. Proposed identification workflow for nine species of *Malassezia*. Pos, positive; Neg, negative; GB, growth and black zone; GN, growth and no change; NB, no growth and black zone. *a*, Direct identification by catalase reaction and features of colonies on CHROM for *M. restricta* and *M. furfur*. *b*, Colony morphology on CHROM as shown in Fig. 1. *c*, Colony size of *M. japonica* and *M. slooffiae* on CHROM as shown in Fig. 1.

TABLE 4. Identification results and sensitivity and specificity of the proposed identification system

Species identified by molecular biological analysis	No. of test strains	No. of correct results	No. of incorrect results	Sensitivity (%)	Specificity (%)
M. pachydermatis	43	41	0	95.4	100
M. sympodialis	84	84	2[a]	100	97.7
M. globosa	14	14	0	100	100
M. dermatis	5	5	2[b]	100	71.4
M. furfur	41	41	0	100	100
M. slooffiae	108	108	3[c]	100	97.3
M. obtusa	4	4	0	100	100
M. restricta	71	71	0	100	100
M. japonica	2	2	0	100	100
M. yamatoensis	5	0	0	NA[d]	NA
Total	377	370	7		

[a] Two strains of fresh clinical isolates of *M. yamatoensis* were incorrectly identified as *M. sympodialis* by this modified identification method.
[b] Two strains of fresh clinical isolates of *M. pachydermatis* were incorrectly identified as *M. dermatis* by this modified identification method.
[c] Three strains of fresh clinical isolates of *M. yamatoensis* were incorrectly identified as *M. dermatis* by this modified identification method.
[d] NA, this method was not applicable.

(vi) Tolerance of 40°C. None of the strains of *M. japonica* and 17 (15.7%) *M. slooffiae* strains did not grow on modified Leeming and Notman agar at 40°C. This biological feature was not acceptable for distinguishing between *M. slooffiae* and *M. japonica* species. Other *Malassezia* species were not tested.

Proposal for an identification system for *Malassezia* species. Our proposed identification system for nine species of *Malassezia* using 366 fresh isolates and 11 type and reference strains identified by molecular analysis is shown in Fig. 2. First, some *Malassezia* species could be identified directly. *M. furfur* developed characteristically large pale pink and wrinkled colonies on CHROM and could be differentiated from other *Malassezia* species. *M. restricta* was the only catalase-negative species. Second, only *M. pachydermatis* grew on SDA, but 2 (4.7%) atypical strains were lipid dependent and were incorrectly identified as *M. dermatis*. Third, with the exception of *M. yamatoensis*, other *Malassezia* species were correctly identified by this system. This system was not applicable for the identification of *M. yamatoensis*. Table 4 shows the numbers of correct and incorrect results and the sensitivity and specificity of our identification system. Three clinically important *Malassezia* species, *M. furfur* (100% [41 of 41]), *M. globosa* (100% [14 of 14]), and *M. restricta* (100% [71 of 71]), were correctly identified by this system, and the rate of concordance of this system with molecular analysis was 98.1% (370/377).

Cost effectiveness of the proposed identification system. Cost comparison results were based on the commercial price of each system (Table 5), but the labor costs associated with testing were not included. The cost of identifying *Malassezia* was reduced by about $9,100 in this study.

DISCUSSION

The biological tests described above were designed to identify *Malassezia* species in clinical laboratories. We investigated the variation of phenotypic features of 377 *Malassezia* species in this study.

TABLE 5. Cost comparison in U.S. dollars of the molecular biological analysis system and that of the proposed system

Item	Molecular biological analysis		Proposed biological identification system	
	Cost per test	Total cost[a]	Cost per test	Total cost[a]
CHROM	2.00	566	2.00	566
Molecular biological analysis	25.00	9,425	0	0
3% hydrogen peroxide[b]	0	0	0.417	0.0264
SDA slant	0	0	0.417	157.2
TE slant	0	0	0.417	157.2
Total	27.0	9,991	3.251	880

[a] During the present study.
[b] Catalase test.

First, CHROMagar was used as the primary culture medium, and we obtained 1 (2.4%) atypical strain of *M. furfur* that produced precipitates on CHROM. On the other hand, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, and *M. dermatis* were recognized from their precipitates in the agar, as reported by Kaneko et al. (15). The atypical strain of *M. furfur* developed characteristically large pale pink and wrinkled colonies on CHROM, and all test strains of *M. furfur* were characteristically similar without precipitate production. Therefore, *M. furfur* was identified correctly by colony morphology on CHROM. Although *M. globosa* and *M. obtusa* were similar to each other in terms of their phenotypic features (9), lipid usage patterns, catalase reactions, and growth temperatures, their characteristics with regard to precipitation on CHROM were different. Furthermore, *M. dermatis* and *M. japonica* are new species reported by Sugita et al. (25, 26), and their biological properties resembled those of *M. slooffiae* and *M. sympodialis*, except for their lipid usage patterns, respectively, but their precipitations on CHROM were different (Tables 2 and 3).

Second, SDA was used to determine the lipid dependence; none of the lipid-dependent species grew on SDA. We obtained two (4.7%) atypical *M. pachydermatis* strains that could not be cultured on lipid-free culture medium (SDA), and the sensitivity and specificity of this culture medium for *M. pachydermatis* were 95.4% and 100%, respectively. The atypical strains were incorrectly identified as *M. dermatis* based on their biological features. Duarte et al. (5) reported that one lipid-dependent variant strain of *M. pachydermatis* was isolated in an investigation of 964 cattle and 6 dogs, and unambiguous identification required sequencing.

Third, EL slants were used to determine the ability to utilize polyethoxylated castor oil for *M. furfur*. Mayser et al. (18) showed that polyethoxylated castor oil was metabolized only by five strains of *M. furfur* in the agar diffusion test, but we obtained six atypical strains (14.6%) of *M. furfur*, two (4.7%) of *M. pachydermatis*, and two (1.9%) of *M. slooffiae*. Their biological features were not acceptable as key features of *M. furfur*, and this culture medium's sensitivity and specificity for *M. furfur* were 85.4% and 97.3%, respectively. However, all test strains of *M. furfur* were directly identified by colony morphology on CHROM as described above.

Fourth, TE slants were used to determine the ability to hydrolyze esculin and utilize Tween 60 as key features for

differentiation among the precipitate-producing group (*M. sympodialis*, *M. dermatis*, and *M. globosa*) and the nonprecipitate-producing group (*M. japonica*, *M. slooffiae*, and *M. obtusa*). Mayser et al. (18) reported that two tested strains of *M. sympodialis* were able to split esculin with the black zone, while two tested strains of *M. slooffiae* remained negative. We confirmed these results previously with three strains each of *M. sympodialis* and *M. slooffiae* (15). However, 37 atypical strains (34.3%) of *M. slooffiae* hydrolyzed esculin and showed a black zone around the colonies on TE slants. In addition, 17 (15.7%) strains of *M. slooffiae* were not tolerant of growth at 40°C. Therefore, the previously reported biological features (15, 18) were not useful for differentiation between *M. slooffiae* and *M. japonica*. On the other hand, all test strains of *M. slooffiae* developed pale pink colonies that were smaller than those of *M. japonica* on CHROM and could be differentiated based on colony size.

Fifth, catalase reaction was used as a key feature of *M. restricta*, and this reaction allowed correct recognition of all test strains except *M. restricta*.

The results presented here indicated that the clinically important species *M. globosa*, *M. restricta*, and *M. furfur* were identified correctly using our proposed method. Recently, data from several institutions have suggested that *M. globosa* is associated with pityriasis versicolor (19, 20), that *M. restricta* is associated with seborrheic dermatitis (24, 29), and that *M. furfur* infections have been observed in hospitalized neonates with very low birth weight receiving intravenous lipid emulsions (2, 3, 4, 7, 23, 28). In agreement with these reports, we isolated mainly *M. globosa* from pityriasis versicolor and *M. restricta* from seborrheic dermatitis in the present study.

A simple, reliable, and cost-effective identification method is required in most clinical laboratories. CHROM with two specific media (SDA and TE slant) and catalase reactions allowed identification of *Malassezia* species easily, quickly, and at reasonable cost without requiring any expensive or specialized equipment. For example, automated sequencers are very expensive in Japan (about $84,000). In addition, we estimated that the use of this system will result in cost savings equivalent to about $9,100 per 377 samples in our laboratory. This biological identification system was used to correctly identify three clinically important *Malassezia* species, and we believe that this system can be adopted easily by most clinical laboratories and thus reduce labor costs and enable rapid reporting of clinically relevant laboratory results. Recently, *M. nana*, *M. yamatoensis*, and *M. equi* were reported as new species of *Malassezia* (13, 21, 27). Although these species are very rare, we hope that a technique for the easy differentiation of these species will be developed in future. The results presented here indicate that this system will be a useful tool for the routine identification of clinically important *Malassezia* species.

ACKNOWLEDGMENT

This study was supported in part by Health Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan (K.M.).

REFERENCES

1. **Ashbee, H. R., and E. G. Evans.** 2002. Immunology of diseases associated with *Malassezia* species. Clin. Microbiol. Rev. **15**:21–57.
2. **Boekhout, T., and R. W. Bosboom.** 1994. Karyotyping of *Malassezia* yeasts: taxonomic and epidemiological implications. Syst. Appl. Microbiol. **17**:146–153.
3. **Boekhout, T., M. Kamp, and E. Guého.** 1998. Molecular typing of *Malassezia* species with PFGE and RAPD. Med. Mycol. **36**:365–372.
4. **Danker, W. M., S. A. Spector, J. Fierer, and C. E. Davis.** 1987. *Malassezia* fungemia in neonates and adults complication of hyperalimentation. Rev. Infect. Dis. **9**:743–753.
5. **Duarte, E. R., M. A. Lachance, and J. S. Hamdan.** 2002. Identification of atypical strains of *Malassezia* spp. from cattle and dog. Can. J. Microbiol. **48**:749–752.
6. **Faergemann, J.** 2002. Atopic dermatitis and fungi. Clin. Microbiol. Rev. **15**:545–563.
7. **Gueho, E., R. Simmons, W. Pruitt, S. Meyer, and D. Ahearn.** 1987. Association of *Malassezia pachydermatis* with systemic infections of humans. J. Clin. Microbiol. **25**:1789–1790.
8. **Guého, E., T. Boekhout, H. R. Ashbee, J. Guillot, A. Van Belkum, and J. Faergemann.** 1998. The role of *Malassezia* species in the ecology of human skin and as pathogens. Med. Mycol. **36**:220–229.
9. **Guého, E., G. Midgley, and J. Guillot.** 1996. The genus *Malassezia* with description of four new species. Antonie Leewenhoek **69**:337–355.
10. **Guillot, J., and E. Guého.** 1995. The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNA comparisons. Antonie Leeuwenhoek **67**:297–314.
11. **Guillot, J., E. Guého, M. Lesourd, G. Midgley, G. Chevrier, and B. Dupont.** 1996. Identification of *Malassezia* species. J. Mycol. Med. **6**:103–110.
12. **Hammer, K. A., and T. V. Riley.** 2000. Precipitate production by some *Malassezia* species on Dixon's agar. Med. Mycol. **38**:105–107.
13. **Hirai, A., R. Kano, K. Makimura, E. R. Duarte, J. S. Hamdan, M. A. Lachance, H. Yamaguchi, and A. Hasegawa.** 2004. *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. Int. J. Evol. Microbiol. **54**:623–627.
14. **Kaneko, T., K. Makimura, M. Onozaki, K. Ueda, Y. Yamada, Y. Nishiyama, and H. Yamaguchi.** 2005. Vital growth factors of *Malassezia* species: presumptive identification of *Malassezia* and *Candida* species on modified CHROMagar Candida agar. Med. Mycol. **43**:699–704.
15. **Kaneko, T., K. Makimura, T. Sugita, and H. Yamaguchi.** 2006. Tween 40-based precipitate production observed on modified chromogenic agar and development of biological identification kit for *Malassezia* species. Med. Mycol. **44**:227–231.
16. **Makimura, K., Y. S. Murayama, and H. Yamaguchi.** 1994. Detection of a wide range of medically important fungi by the polymerase chain reaction. J. Med. Microbiol. **40**:358–364.
17. **Makimura, K., Y. Tamura, M. Kudo, K. Uchida, H. Saito, and H. Yamaguchi.** 2000. Species identification and strain typing of *Malassezia* species stock strains and clinical isolates based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J. Med. Microbiol. **49**:29–35.
18. **Mayser, P., P. Haze, C. Papavassilis, M. Pickel, K. Gruender, and E. Guého.** 1997. Differentiation of *Malassezia* species: selectivity of cremophor EL, castor oil and ricinoleic acid for *M. furfur*. Br. J. Dermatol. **137**:208–213.
19. **Morishita, N., Y. Sei., I. Takiuchi, and T. Sugita.** 2005. Examination of the causative agent of pityriasis versicolor. Jpn. J. Med. Mycol. **46**:169–170.
20. **Nakabayashi, A.** 2002. Identification of causative species in *Malassezia*-associated dermatoses. Jpn. J. Med. Mycol. **43**:65–68.
21. **Nell, A., S. A. James, C. J. Bond, and M. E. Herrtage.** 2002. Identification and distribution of a novel *Malassezia* yeast species on normal equine skin. Vet. Rec. **150**:395–398.
22. **Padilha-Goncalves, A.** 1996. A single method to stain *Malassezia furfur* and *Corynebacterium minutissimum* in scales. Rev. Inst. Med. Trop. Sao Paulo **38**:299–302.
23. **Richet, H. M., M. M. McNeil, M. C. Edwards, and W. R. Jarvis.** 1989. Cluster of *Malassezia furfur* pulmonary infections in infants in a neonatal intensive-care unit. J. Clin. Microbiol. **27**:1197–1200.
24. **Sei, Y.** 2006. *Malassezia* related disease. Jpn. J. Med. Mycol. **47**:75–80.
25. **Sugita, T., M. Takashima, T. Shinoda, H. Suto, T. Unno, R. Tsuboi, H. Ogawa, and A. Nishikawa.** 2002. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. J. Clin. Microbiol. **40**:1363–1367.
26. **Sugita, T., M. Takashima, M. Kodama, R. Tsuboi, and A. Nishikawa.** 2003. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. J. Clin. Microbiol. **41**: 4695–4699.
27. **Sugita, T., M. Tajima, M. Takashima, M. Amaya, M. Saito, R. Tsuboi, and A. Nishikawa.** 2004. A new yeast, Malassezia yamatoensis, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. Microbiol. Immunol. **48**:579–583.
28. **Surmont, I., A. Gavilanes, J. Vandepitte, H. Devlieger, and E. Eggermont.** 1989. *Malassezia furfur* fungemia in infants receiving intravenous lipid emulsions: a rarity or just underestimated? Eur. J. Pediatr. **148**:435–438.
29. **Tajima, M.** 2005. *Malassezia* species in patients with seborrheic dermatitis and atopic dermatitis. Jpn. J. Med. Mycol. **45**:137–142.
30. **Yamada, Y., K. Makimura, K. Ueda, Y. Nishiyama, K. Uchida, H. Yamaguchi, and M. Osumi.** 2003. DNA base alignment and taxonomic study of genus *Malassezia* based upon partial sequences of mitochondrial large subunit ribosomal RNA gene. Microbiol. Immunol. **47**:475–478.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 356–362
BBRC
www.elsevier.com/locate/ybbrc

Activation of prothrombin by two subtilisin-like serine proteases from *Acremonium* sp.

Chunli Liu [a], Yasuhiko Matsushita [b], Kosuke Shimizu [a], Koichi Makimura [c], Keiji Hasumi [a,*]

[a] *Department of Applied Biological Science, Tokyo Noko University, 3-5-8 Saiwaicho, Fuchu-shi, Tokyo 183-8509, Japan*
[b] *Gene Research Center, Tokyo Noko University, 3-5-8 Saiwaicho, Fuchu-shi, Tokyo 183-8509, Japan*
[c] *Teikyo University Institute of Medical Mycology, 359 Ohtsuka, Hachioji, Tokyo 192-0395, Japan*

Received 13 April 2007
Available online 30 April 2007

Abstract

Two novel subtilisin-like serine proteases (AS-E1 and -E2) that activate prothrombin have been identified in a culture of the fungus *Acremonium* sp. The enzymes were purified through repeated hydrophobic interaction chromatography. The N-terminal sequences of AS-E1 (34.4 kDa) and AS-E2 (32 kDa) showed high similarity to the internal sequences of two distinct subtilisin-like hypothetical proteins from *Chaetomium globosum*. Both enzymes proteolytically activated prothrombin to meizothrombin(desF1)-like molecules, while the activation cleavage seemed to occur at a site (Tyr^{316}-Ile^{317}) that is four residues proximal to the canonical Xa cleavage site (Arg^{320}-Ile^{321}). Both enzymes inhibited plasma clotting, possibly due to extensive degradation of fibrinogen and production of meizothrombin(desF1)-like molecule.

Keywords: Subtilase; Subtilisin-like serine protease; Prothrombin; Meizothrombin(desF1); Blood coagulation; Hemostasis

The blood coagulation is an important defense system, protecting the body against blood loss from injured vessels. Prothrombin is a vitamin K-dependent zymogen that is converted to thrombin during the penultimate step of the blood coagulation cascade. Under physiological conditions, prothrombin is activated to thrombin on cell surfaces by the prothrombinase complex consisting of Xa, Va, and phospholipid membranes [1]. Although Xa alone is capable of the activation, the rate is $<10^{-5}$ times as compared to the activation by the prothrombinase [2]. Thrombin promotes plugging of damaged vessels by activating platelets and converting fibrinogen to a fibrin clot [3,4]. In addition to the hemostatic role, thrombin is also involved in the inflammation processes [5]. Proteases from foreign sources are thought to be virulence factors in inflammatory events occurring at infected sites. An example is several snake venom enzymes that activate prothrombin [6]. With regard to microbial proteases that activate prothrombin, only a few enzymes have been studied in detail. These include metalloproteinases from *Staphylococcus aureus* [7] and *Bacillus megaterium* (bacillolysin MA) [8], and cysteine proteinases from *Porphyromonas gingivalis* (gingipains) [9].

In our attempts of a screen of microorganisms that modulate coagulation and fibrinolytic systems, we have found that a fungus produced potent enzymes that activate prothrombin. In the present study, we describe the identification, purification, and properties of two novel enzymes belonging to a family of the serine protease subtilase.

Experimental procedures

Microorganism and purification of AS-E1 and -E2. Strain F11177 was originally isolated from a soil sample and identified as *Aremonium* sp. based on morphological studies and 28S rDNA D1–D2 domain sequence (Supplementary Methods). The enzymes were produced as described in Supplementary Methods. Culture supernatant (451 ml) was brought to

* Corresponding author. Fax: +81 42 367 5708.
E-mail address: hasumi@cc.tuat.ac.jp (K. Hasumi).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.04.133

75% saturation of $(NH_4)_2SO_4$ for 30 min and centrifuged to obtain pellet, which was then suspended with 9 ml of TBS (20 mM Tris–HCl and 150 mM NaCl, pH 7.4). The resulting supernatant was diluted with four volumes of TBS, brought to 40% saturation with $(NH_4)_2SO_4$ and subjected to chromatography on a Butyl-Toyopearl column (20 ml) equilibrated with TBS/$(NH_4)_2SO_4$ (40% saturation). The column was washed sequentially with 63 ml each of TBS containing 40%, 30%, and 20% saturations of $(NH_4)_2SO_4$. Activity was eluted with 63 ml of TBS/$(NH_4)_2SO_4$ (10% saturation). Active fraction was brought to 40% saturation of $(NH_4)_2SO_4$ and subjected to HPLC on a Protein-pack G-butyl column (Waters; 10 × 100 mm) equilibrated with TBS/$(NH_4)_2SO_4$ (40% saturation). The column was developed for 30 min with TBS/$(NH_4)_2SO_4$ (40% saturation), 60 min with a gradient of $(NH_4)_2SO_4$ (40–0%) in TBS, and 70 min with TBS at a flow rate of 0.5 ml/min, affording two purified proteins with an activity to promote prothrombin activation.

Enzyme assay. For determination of amidolytic activity, appropriate amount of enzyme was incubated with 0.1 mM Spectrozyme TH (H-D-hexahydrotyrosol-Ala-Arg-*p*-nitroanilide; American Diagnostica, Greenwich, CT, USA) in 50 μl of TBS/T/Ca (TBS containing 0.1% Tween 80 and 2 mM $CaCl_2$) at 37 °C. The hydrolysis of the chromogenic substrate was monitored at 405 nm. For determination of prothrombin activation, enzyme was incubated with 0.1 mM Spectrozyme TH in the presence or absence of 20 nM human prothrombin (Haematologic Technologies, Essex Junction, VT, USA) in 50 μl of TBS/T/Ca at 37 °C, and the change in absorbance at 405 nm was measured. The differences between values obtained in the presence and absence of prothrombin were plotted against t^2 to obtain initial rate of generation of thrombin activity, which was normalized using human α-thrombin (Sigma, St. Louis, MO, USA) as standard and expressed as α-thrombin equivalent.

Zymography. For casein zymography, AS-E1 (3 ng) or AS-E2 (15 ng) was resolved on nonreduced SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel containing 1 mg/ml casein. After electrophoresis, gels were washed for 30 min with 2.5% (wt/vol) Triton X-100 twice and then incubated in TBS/T/Ca for 16 h at 37 °C. After staining with Coomassie brilliant blue R-250, proteolytic activity was observed as a clear area on a blue background. For fibrinogen zymography, prothrombin (2 μM) was treated with 3 nM of either AS-E1 or -E2 at 37 °C for 30 min, and aliquot of the mixture was subjected to nonreduced SDS–PAGE on a 12.5% gel. The gel was washed as described above and finally washed thrice with water, followed by overlaying the gel to a fibrinogen indicator gel [2 mg/ml human fibrinogen and 0.8% agarose in 75 mM Tris–HCl and 22 mM NaCl, pH 7.8] [10]. After incubation at 37 °C for 18 h, the indicator gel was processed for image scanning.

Characterization of prothrombin cleavage. Prothrombin (2 μM) was incubated with either AS-E1 or -E2 at 37 °C for 30 min in TBS/T/Ca and then subjected to reduced or nonreduced SDS–PAGE on 12.5% gels. When using Xa (25 pM), the reaction mixture was further supplemented with Va (100 pM) and 50 μM phosphatidylcholine–phosophatidylserine (3:1, mol/mol). Where indicated, 3 μM dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA; Haematologic Technologies) was included in the reaction mixture to inhibit thrombin-mediated cleavages.

Measurement of clot formation. Citrated human plasma (200 μl) was mixed with 60 μl of AS-E1 or -E2 (0.07–4 μM) in PBS. Plasma clot formation was initiated by adding 40 μl of 133 mM $CaCl_2$, and thrombelastogram was recorded at 37 °C on a ROTEM Gamma (Pentapharm, Munich, Germany). For measurement of fibrinogen clotting, 120 μl of 10 mg/ml human fibrinogen and 120 μl of varying concentrations of AS-E1 or -E2 were mixed in TBS/T/Ca at 37 °C for 30 min. Clot formation was initiated by adding 60 μl of 10 nM α-thrombin. Turbidometric assay for fibrinogen clotting was assayed in microplates using similarly prepared reaction mixtures. After addition of α-thrombin, the change in absorbance at 630 nm was monitored.

Results and discussion

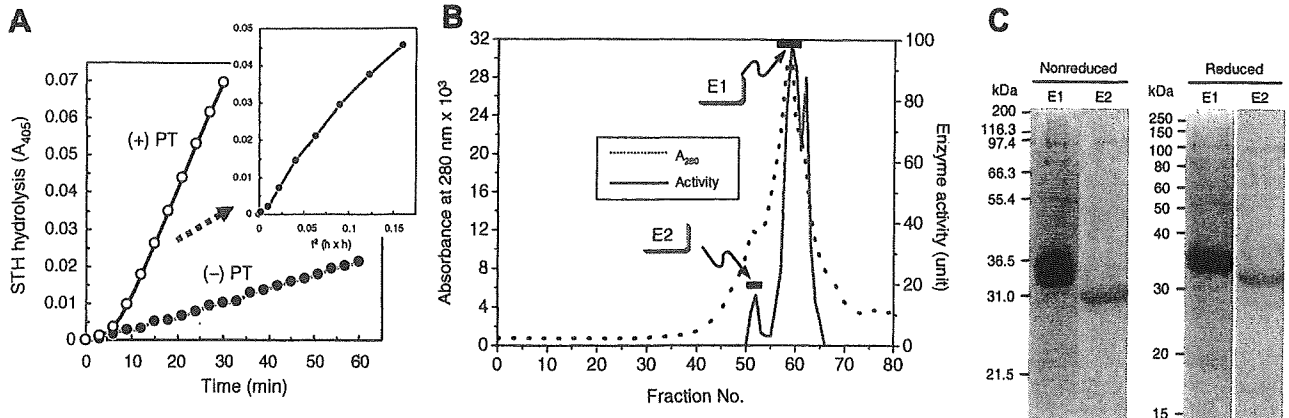
From a screen of microorganisms that modulate coagulation and fibrinolytic systems, we identified a soil isolate F11177 as a producer of a potent activity that promoted prothrombin activation. The strain was taxonomically classified based on morphological studies and nucleotide sequence of the D1–D2 domain of 28S rDNA (see Supplementary Methods). The D1–D2 domain sequence was highly similar (98% identity) to the sequence of the ascomycetous fungi *Chaetomium* species, including *C. globosum*. The morphological studies, however, suggested that strain F11177 did not have characteristic ascospores but had features of the genus *Acremonium* sp. Therefore, the strain was identified as *Acremonium* sp. F11177, an anamorph of *Chaetomium* sp. When incubated with prothrombin, the culture supernatant had significant activity to hydrolyze Spectrozyme TH (a chromogenic substrate for thrombin), while slight activity was seen in the absence of prothrombin (Fig. 1A). The activity to promote prothrombin activation could be obtained by subtracting values obtained with the incubation in the absence of prothrombin from values obtained in its presence, followed by plotting the differences versus t^2 (Fig. 1A, *inset*).

Purification of AS-E1 and -E2

From 451 ml of culture supernatant, 120 and 570 μg of AS-E1 and E2 were purified through salting out and repeated hydrophobic interaction chromatographies (Fig. 2B), where AS is named after "*Acremonium* subtilase". Both proteins were nearly homogeneous on both reduced and nonreduced SDS–PAGE (Fig. 1C), with apparent molecular masses of 34.4 kDa (AS-E1) and 32 kDa (AS-E2), as judged from reduced gels. The N-terminal sequences of both proteins partly resemble to each other (Fig. 1D and E), and following database search suggested that the two proteins belonged to a family of subtilisin-like serine proteases, called subtilases (Supplementary Fig. 1). Each enzyme appeared to form a dimer (as well as oligomers) as judged from size-exclusion chromatography (Fig. 1F). The zymography on casein gel suggested that the major active enzyme species was a dimer for AS-E1, while additional active smear bands corresponding to multimers were seen in AS-E2 (Fig. 1G).

Identification of AS-E3 from cDNA cloning

An initial database search with AS-E1 N-terminal sequence identified several related sequences. From the consensus of these sequences, we designed several combinations of degenerate oligonucleotide primers to clone cDNAs by reverse transcription-polymerase chain reaction on RNA from strain F11177. One combination resulted in the production of a cDNA segment, from which a full-length clone was obtained (see Supplementary Methods). Amino acid sequence deduced from the cDNA matched the N-terminal sequence of neither AS-E1 nor -E2. Therefore, the cDNA was designated AS-E3. From recent database search, we found that AS-E3 was highly similar to a hypothetical protein from *C. globosum*, Q2H5N4 (TrEM-



D

Cycle	1	2	3	4	5	6	7	8	9	10
Amino acid	D	N	V	P	W	G	L	A	R	I
Recovery (pmol)	12.4	9.5	12.3	8.2	7.9	6.1	8.0	11.5	5.3	11.1
	11	12	13	14	15	16	17	18	19	20
	S	H	R	T	T	G	A	T	S	Y
	3.7	3.8	4.3	3.3	4.3	5.2	10.0	3.0	2.9	4.3
	21	22	23	24	25	26	27	28	29	30
	V	Y	D	D	S	A	G	E	G	T
	6.8	5.1	5.2	6.5	2.9	8.3	3.5	1.6	3.2	2.4
	31	32	33	34	35	36	37	38	39	40
	C	S	Y	I	I	D	T	G	I	Y
	-	2.0	2.5	2.9	2.9	3.1	1.6	3.1	2.2	1.9

E

Cycle	1	2	3	4	5	6	7	8	9	10
Amino acid	A	Y	V	S	Q	S	G	A	P	W
Recovery (pmol)	13.2	22.6	23.4	10.2	19.3	8.9	9.9	13.4	10.7	8.0
	11	12	13	14	15	16	17	18		
	G	L	G	R	I	S	H	K		
	9.6	11.8	10.4	6.9	14.2	3.5	3.3	5.3		

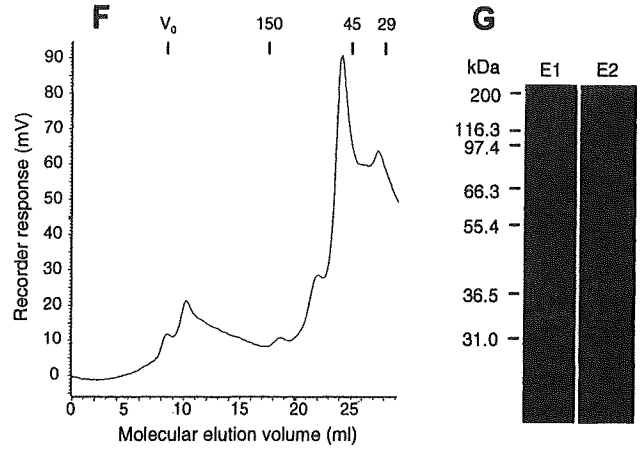


Fig. 1. Purification of proteases that activate prothrombin. (A) A representative result of a screen for prothrombin-activating activity. Culture supernatant (0.01 μl) from *Acremonium* sp. F11177 was incubated with Spectrozyme TH (STH) in the presence or absence of prothrombin (PT). The difference between the two values ["(+) PT" subtracted by "(−) PT"] was also plotted against t^2 (*inset*) to obtain initial rate of thrombin activity generation. (B) Final enzyme purification was achieved by chromatography on a Protein-pack G-butyl column. Bars represent fractions pooled for AS-E1 and -E2. Bar represents fractions pooled. One unit is defined as the activity that hydrolyzes 1 nmol of STH per hour under the standard assay conditions. (C) Purified AS-E1 (7.5 μg) and -E2 (3 μg) were treated with 2 mM PMSF (to prevent enzyme autoproteolysis), boiled for 5 min in SDS-sample buffer and subjected to SDS–PAGE under nonreducing and reducing conditions. (D,E) N-terminal amino acid sequences of AS-E1 and -E2. (F) Size-exclusion chromatography of AS-E2. (G) Casein-gel zymography after resolving on nonreduced SDS–PAGE. The samples were treated neither with PMSF nor boiling before electrophoresis.

BL) (Supplementary Fig. 1). Similarly, 38/40 and 15/18 of amino acids in N-terminal sequences of AS-E1 and -E2, respectively, were found to be identical to internal sequences of two other distinct hypothetical proteins from *C. globosum* [Q2GUF9 (TrEMBL) and Q2GYU7 (TrEMBL), respectively]. Thus, we concluded that AS-E1, -E2, and -E3 were variants of corresponding hypothetical proteins from *C. globosum*, while functional properties of these have remained unknown in spite of their sequence similarity to subtilisin.

Characterization of enzyme activity

Substrate specificity of AS-E1 and -E2 is shown in Supplementary Table 1. Among the chromogenic substrates tested, Spectrozyme TH was the preferred substrate for both enzymes (2.92 and 0.86 nmol/h/pmol enzyme under standard conditions). Succinyl-Ala-Ala-Ala-*p*-nitroaniline was hydrolyzed by AS-E2 (but not by E1) at a significantly slower late (0.06 nmol/h/pmol). As shown in Fig. 2A, the activities of AS-E1 and -E2 to hydrolyze Spectrozyme TH were roughly 10 and 3 times higher than the activity of subtilisin A (0.26 nmol/h/pmol). With regard to prothrombin activation, AS-E1 was 7 times more potent than AS-E2 (Fig. 2B). To date no subtilase had been recognized to activate prothrombin, while the present data suggest that some types of subtilases may activate prothrombin. Indeed, subtilisin A was positive in activating prothrombin, whereas its activity was 1/20 and 1/3 of AS-E1 and -E2, respectively (Fig. 2B).

As shown in Fig. 2C, both enzymes were strongly inhibited by antipain and phenylmethanesulfonyl fluoride (PMSF). Benzamidine (50 mM) inhibited AS-E1 by 80%, while its inhibition of AS-E2 was 49%. On the other hand, AS-E2 was completely inhibited by aprotinin at 1000 KIU/ml, whereas its inhibition of AS-E1 was only partial (17%).