

Genotype Analysis of *Malassezia restricta* as the Major Cutaneous Flora in Patients with Atopic Dermatitis and Healthy Subjects

Takashi Sugita^{*,1}, Mami Tajima², Misato Amaya², Ryoji Tsuboi², and Akemi Nishikawa³

¹Department of Microbiology and ³Department of Immunobiology, Meiji Pharmaceutical University, Kiyose, Tokyo 204–8588, Japan, and ²Department of Dermatology, Tokyo Medical University, Shinjuku-ku, Tokyo 160–0023, Japan

Received May 18, 2004; in revised form, June 29, 2004. Accepted July 6, 2004

Abstract: Lipophilic yeasts of the genus *Malassezia* colonize the skin surface of humans and are an exacerbating factor in atopic dermatitis (AD). Two species, *M. restricta* and *M. globosa* are major cutaneous microflora in both AD patients and healthy subjects. We compared the DNA sequences of the intergenic spacer (IGS) region, located between the 26S and 5S rRNA genes of *M. restricta* colonizing the skin surfaces of 13 AD patients and 12 healthy subjects, and of three CBS stock strains as references. The IGS 1 sequences were divided into two major groups, corresponding to AD patients and healthy subjects. These findings suggest that a specific genotype of *M. restricta* plays a significant role in AD, although *M. restricta* commonly colonizes both AD patients and healthy subjects.

Key words: *Malassezia restricta*, Atopic dermatitis, Cutaneous microflora, Genotype, IGS

Lipophilic yeasts in the genus *Malassezia* are part of the normal human cutaneous microflora and are isolated from sebaceous-rich areas, particularly the chest, back, and head. They are a causative factor in *pityriasis versicolor* and seborrheic dermatitis (1, 4). In addition, *Malassezia* species are also thought to exacerbate atopic dermatitis (AD), based on findings that AD patients have specific serum IgE antibodies to *Malassezia* (11, 21, 22). Application of topical antifungal agents by AD patients decreases *Malassezia* colonization and the severity of eczematous lesions (2), suggesting that *Malassezia* species play a role in AD. We have analyzed the cutaneous *Malassezia* microflora of AD patients and healthy subjects using a non-culture method (nested PCR) (16). While *M. restricta* and *M. globosa* were detected in approximately 90% of the AD patients, other *Malassezia* species were found in fewer than 40%. In addition, we analyzed the DNA sequence divergence of the IGS region, which is located between the 26S and 5S rRNA genes of *M. globosa*, and found that the IGS sequence of *M. globosa* from AD patients differed from that from healthy subjects (19). These findings suggest that a specific genotype of this microorganism plays a significant role in AD.

This paper describes the genotype of one of the major *Malassezia* microflora species, *M. restricta*, obtained from the skin of AD patients and healthy subjects.

Materials and Methods

Direct DNA sequencing of the IGS 1 region of *M. restricta* stock strains. Three stock strains obtained from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) were analyzed: CBS 7877 (type strain of *M. restricta*), CBS 7991, and CBS 8747. The first two were isolated from the skin of a healthy subject in the UK, and the third was from the scalp of a healthy subject in Canada (<http://www.cbs.knaw.nl/>). Genomic DNA was extracted using the method of Makimura et al. (12). The IGS region containing 5S rDNA was amplified from each strain using the primer pair 26SBF (5'-AGCTGCTGCCAATGCTAGCTC) and Mala-R (5'-TACTGCTGTGAATGCTCCAGC) (18). The PCR products were sequenced using an ABI 310 DNA sequencer and a BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Perkin-Elmer Applied Biosystems, Calif., U.S.A.) according to the manufacturer's instructions.

*Address correspondence to Dr. Takashi Sugita, Department of Microbiology, Meiji Pharmaceutical University, 2–522–1 Noshio, Kiyose, Tokyo 204–8588, Japan. Fax: +81–424–95–8762. E-mail: sugita@my-pharm.ac.jp

Abbreviations: AD, atopic dermatitis; CBS, Centraalbureau voor Schimmelcultures; IGS, intergenic spacer.

Subjects. Thirteen AD outpatients (9 males, 4 females; aged 20 to 64 years, mean age 33.9 ± 11.3 years) at Tokyo Medical University Hospital and 12 healthy students (3 males, 9 females; aged 19 to 23 years, mean age 20.7 ± 1.5 years) at Meiji Pharmaceutical University were involved in this study. AD was diagnosed according to the criteria of Hanifin and Rajka (6), and samples were collected from erythematous lesions on the face and neck. Routine skin care, including intermittent applications of mild steroid ointment or petrolatum, was administered before sampling. Written informed consent was obtained from each subject.

Sequencing IGS 1 region from samples. *Malassezia* samples were collected by applying transparent OpSite dressing (Smith and Nephew Medical, Ltd., Hull, U.K.), and fungal DNA was extracted from the OpSite dressing as described previously (16). The species-specific primer pair: Res-IGS-1F (5'-CGACCTAGTCGACTACATCCTAC) and Res-IGS-1R (5'-ATGAGGAGGAAAGGCAGGCAAG) were designed from the IGS 1 sequence analysis. The DNA pellet was resuspended in 30 μ l of TE (10-mM Tris-HCl [pH 8.0], 1-mM EDTA [pH 8.0]). The DNA extracted (10 μ l) from each sample was added to 40 μ l of PCR master mixture, which consisted of 5 μ l of 10 $\times$ PCR buffer (TaKaRa), 4 μ l of 200- μ M deoxynucleoside triphosphates, 10 pmol of each primer, and 0.5 U of TaKaRa Ex Taq DNA polymerase (TaKaRa). PCR was performed with an initial denaturation at 94 C for 1 min, followed by 30 cycles of 30 sec at 94 C, 1 min at 54 C, and 30 sec at 72 C, and a final extension at 72 C for 10 min. The PCR products were cloned using a TA Cloning Kit (Invitrogen Corp., Calif., U.S.A.) and three positive clones were sequenced.

Molecular phylogenetic analysis. The sequences of the IGS 1 region obtained from the samples of AD patients and healthy subjects were aligned using Clustal W (20). For neighbor-joining analysis (13), the distances between sequences were calculated using Kimura's two-parameter model (8). A bootstrap analysis was conducted with 100 replications (5).

Formation of chimeric molecules. To confirm whether chimeric molecules formed under the PCR conditions used in this study, mixed genomic DNA from known *Malassezia* species (*M. dermatis*, *M. furfur*, *M. globosa*, *M. japonica*, *M. obtusa*, *M. slooffiae*, *M. sympodialis*, *M. pachydermatis*, and *M. yamatoensis*) was used for PCR co-amplification of the IGS region. Then, the IGS amplified from the mixed genomes was cloned; 30 clones were selected at random, and their sequences were determined.

Results

IGS 1 Sequence Analysis of *M. restricta*

Stock strains. Complete sequences of the IGS 1 region were determined for three CBS stock strains of *M. restricta*. Their sizes ranged from 620 to 661 bp. *M. restricta* IGS 1 had two short sequence repeats (SSR) of (CA) $_n$ and (CT) $_n$ at positions 125–134 and 178–189 in the IGS sequence of strain CBS7877 (type strain), respectively. The nucleotide sequences determined in this study have been deposited with the DNA Data Bank of Japan (DDBJ) under accession numbers AB178809 (CBS7877), AB178808 (CBS7991), and AB178810 (CBS8747).

Samples from subjects. Before the IGS sequence analysis of the samples, formation of chimeric molecules was investigated. Thirty clones were chosen at random and sequenced; none were identified as a chimeric molecule. Using the PCR conditions described above, 454- to 515-bp fragments were partially amplified and analyzed. A phylogenetic tree was constructed from 28 IGS 1 sequences obtained from the samples of 13 AD patients, 12 healthy subjects, and three CBS stock strains (Fig. 1). The tree consisted of two major groups separated with 100% bootstrap support. Group 1 included 10 AD patients and three CBS stock strains, and Group 2 contained all 12 healthy subjects and three AD patients. The IGS 1 sequences derived from the AD patients (Group 1) were more diverse than those from the healthy subjects (Group 2). An alignment of the partial sequences of representative strains from Groups 1 and 2 is shown in Fig. 2. The sequence similarity between the two groups was below 70%. Two SSRs, (CA) $_n$ and (CT) $_n$, were also found in the IGS 1 sequence from each sample. For both SSRs, there were more sequence repeats in the IGS 1 region of samples from the healthy subjects than from AD patients: (CA) $_n$, AD patients, 6.8 ± 3.9 repeats; healthy subjects, 14.7 ± 1.2 repeats; (CT) $_n$, AD patients, 6.8 ± 2.0 repeats; healthy subjects, 9.7 ± 1.9 repeats.

Discussion

We have described differences in the IGS 1 genotypes of *M. restricta* colonizing the skin surfaces of AD patients and healthy subjects. rRNA gene sequences have been used for molecular phylogeny, taxonomy, and identification (10, 14). Of the subunits or regions in this gene, the IGS region shows remarkable intraspecies diversity. We have reported the analytical significance of the IGS region using *Cryptococcus neoformans* and *Trichosporon asahii* (15, 17). *M. globosa*, which is a

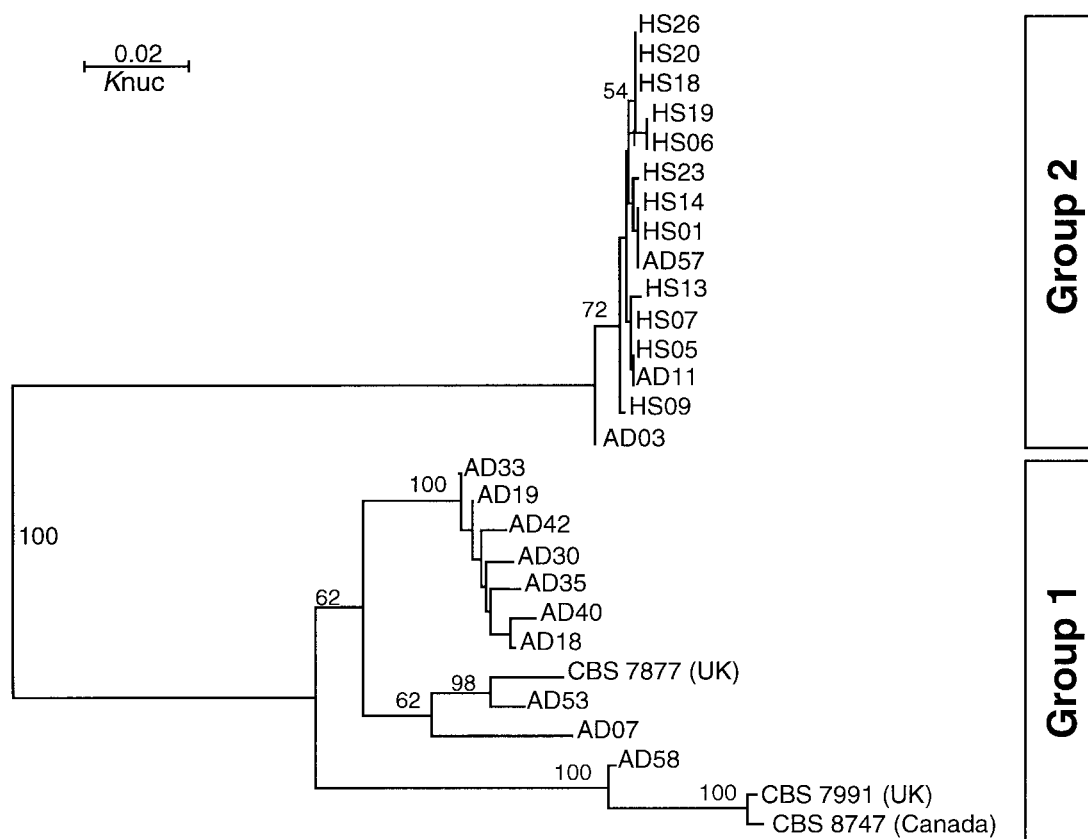


Fig. 1. Phylogenetic tree of *M. restricta* colonizing the skin surfaces of AD patients and healthy subjects based on DNA sequences of the IGS 1 region. AD, patient with atopic dermatitis; HS, healthy subject. The numbers are the confidence levels from 100 replicate bootstrap samplings (frequencies less than 50% are not shown). K_{mc} , Kimura's parameter (8).

```
AD33      1 CGACCTAGTCGACTACATCCTACTGTATCCTACGCAATGGAGGTGTGTATGGTAGGTAC
HS01      1 CGACCTAGTCGACTACATCCTACTGTATTCTACGCAATGGAGGTGTGTATTGTAGGACAC
          *****

AD33     61 ATCCTACCCCACTTTCCCATTCTTCTATACCTTACCCCCCTACCCCCTCTCTACGTCACC
HS01     61 AT-----ACACCCACCCACAT-A--
          **                               ** * * * * *

AD33    121 CGTGCACACACACA-----TTCACGCCTGTCTATGCGTCCCCCTC
HS01     79 ---CACACACACACACACACACACACACACAGTCGTCACCC-----
          *****                      **       *

AD33    161 CTCCAAGAGTCGAATTTCTCTCTCTCTCT-----CGTCAGTCTACTTGCCCATGG
HS01    119 -----CTCTCTCTCTCTCTCTCTCTCTCTCTCTCGTCAGTCTACTTGGCCATGT
          *****                      *****
```

Fig. 2. Alignment of the DNA sequences of the IGS 1 regions of representative sequences. AD, patient with atopic dermatitis; HS, healthy subject. The SSRs are underlined: (CA)_n and (CT)_n.

major cutaneous microflora, also has four genotypes (19). Of the four genotypes, two groups were found in AD patients; one group was from healthy subjects, and the other included samples from both AD patients and

healthy subjects. This suggests that a specific genotype of *M. globosa* plays a significant role in AD. The present study also indicates that the IGS genotype of *M. restricta* obtained from AD patients is different from

that of healthy subjects. Three CBS stock strains were included in this study as reference strains; although they were isolated from healthy subjects, they are located in the AD cluster in the tree (Fig. 1). The CBS strains were from British or Canadian subjects. It is still not known whether our finding is specific for the Japanese population, or the CBS strains are an exception. This requires further analysis of *M. restricta* strains from other countries. The *M. restricta* IGS, like that of *M. globosa*, also has SSRs that could be used to distinguish microorganisms present in AD patients and healthy individuals.

The reason for the difference in the genotypes found in each population is unclear, but might reflect selection against *M. restricta* strains. There are three possible causes: (1) the effect of skin surface lipids, (2) the effect of ointments or cream bases, or (3) antifungal drug susceptibility. The main skin surface lipids are triglycerides, squalene, wax esters, cholesterol, ceramides, and free fatty acids (3). Although the lipid composition in AD patients is generally not different from that of healthy subjects, a significant decrease in ceramide 1 and differences in the concentrations of the related linoleate and oleate molecules have been reported (7, 24). Such differences in composition might affect colonization by *Malassezia* strains with different lipid requirements. The base ingredient of an ointment or cream can affect the growth of microorganisms, as with the growth of *M. furfur*, *M. globosa*, and *M. sympodialis* (9), but effects on *M. restricta* are unknown. Antifungal drug susceptibility might be responsible for selection of strains. A correlation between antifungal susceptibility and genotype has been reported in *Candida albicans* (23). However, because no patient in this study received antifungal therapy, this possibility can be excluded.

In conclusion, our IGS sequence analysis showed that the genotypes of *M. restricta* colonizing the skin surface of AD patients and healthy subjects are significantly different. Therefore, genotype should be considered when studying the relationship between *M. restricta* and AD.

This study was supported in part by Research Grant C (16590127) from the Japan Society for the Promotion of Science (TS).

References

- 1) Ashbee, H.R., and Evans, E.G. 2002. Immunology of diseases associated with *Malassezia* species. Clin. Microbiol. Rev. **15**: 21–57.
- 2) Back, O., Scheynius, A., and Johansson, S.G. 1995. Ketoconazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. Arch. Dermatol. Res. **287**: 448–451.
- 3) Downing, D.T., Stewart, M.E., and Strauss, J.S. 1999. Lipids of the epidermis and the sebaceous glands, p. 144–155. In Freedberg, I.M., Eisen, A.Z., Wolff, K., Austen, K.F., Goldsmith, L.A., Katz, S.I., and Fitzpatrick, T.B. (eds), Fitzpatrick's dermatology in general medicine, 5th ed, McGraw-Hill, N.Y.
- 4) Faergemann, J. 2002. Atopic dermatitis and fungi. Clin. Microbiol. Rev. **15**: 545–563.
- 5) Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783–791.
- 6) Hanifin, J.M., and Rajka, G. 1980. Diagnostic features of atopic dermatitis. Acta Derm. Venereol. **92**: 4–47.
- 7) Hara, J., Higuchi, K., Okamoto, R., Kawashima, M., and Imokawa, G. 2000. High-expression of sphingomyelin deacylase is an important determinant of ceramide deficiency leading to barrier disruption in atopic dermatitis. J. Invest. Dermatol. **115**: 406–413.
- 8) Kimura, M. 1980. A simple method for estimation evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**: 111–120.
- 9) Koyama, T., Kanbe, T., Kikuchi, A., and Tomita, Y. 2002. Effects of topical vehicles on growth of the lipophilic *Malassezia* species. J. Dermatol. Sci. **29**: 166–170.
- 10) Kurtzman, C.P., and Robnett, C.J. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35**: 1216–1223.
- 11) Leung, D.Y. 1995. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. J. Allergy Clin. Immunol. **96**: 302–318.
- 12) Makimura, K., Murayama, Y.S., and Yamaguchi, H. 1994. Detection of a wide range of medically important fungal species by polymerase chain reaction (PCR). J. Med. Microbiol. **40**: 358–364.
- 13) Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.
- 14) Sugita, T., Nishikawa, A., Ikeda, R., and Shinoda, T. 1999. Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J. Clin. Microbiol. **37**: 1985–1993.
- 15) Sugita, T., Ikeda, R., and Shinoda, T. 2001. Diversity among strains of *Cryptococcus neoformans* var. *gattii* as revealed by a sequence analysis of multiple genes and a chemotype analysis of capsular polysaccharide. Microbiol. Immunol. **45**: 757–768.
- 16) Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T., and Nishikawa, A. 2001. Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. J. Clin. Microbiol. **39**: 3486–3490.
- 17) Sugita, T., Nakajima, M., Ikeda, R., Matsushima, T., and Shinoda, T. 2002. Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. J. Clin.

- Microbiol. **40**: 1826–1830.
- 18) Sugita, T., Takashima, M., Kodama, M., Tsuboi, R., and Nishikawa, A. 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.
- 19) Sugita, T., Kodama, M., Saito, M., Ito, T., Kato, Y., Tsuboi, R., and Nishikawa, A. 2003. Sequence diversity of the intergenic spacer region of the rRNA gene of *Malassezia globosa* colonizing the skin of patients with atopic dermatitis and healthy individuals. J. Clin. Microbiol. **41**: 3022–3027.
- 20) Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673–4680.
- 21) Werfel, T., and Kapp, A. 1998. Environmental and other major provocation factors in atopic dermatitis. Allergy **53**: 731–739.
- 22) Wessels, M.W., Doekes, G., Van Ieperen-Van Kijk, A.G., Koers, W.J., and Young, E. 1991. IgE antibodies to *Pityrosporum ovale* in atopic dermatitis. Br. J. Dermatol. **125**: 227–232.
- 23) Xu, J., Ramos, A.R., Vilgalys, R., and Mitchell, T.G. 2000. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. J. Clin. Microbiol. **38**: 1214–1220.
- 24) Yamamoto, A., Serizawa, S., Ito, M., and Sato, Y. 1991. Stratum corneum lipid abnormalities in atopic dermatitis. Arch. Dermatol. Res. **283**: 219–223.