

Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis

Itaru Dekio,^{1,2} Mitsuo Sakamoto,² Hidenori Hayashi,^{2†} Masayuki Amagai,¹ Makoto Suematsu³ and Yoshimi Benno²

Correspondence

Itaru Dekio

dekio@1999.jukuin.keio.ac.jp

¹Department of Dermatology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan

²Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama 351-0198, Japan

³Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan

A previous study using bacterial 16S rRNA gene-based clone libraries revealed that the microbiota in healthy human skin included uncultured micro-organisms, although the micro-organisms in skin exposed to disease conditions remain to be examined. To compare the profiles of skin microbiota in 13 patients with atopic dermatitis (AD) and 10 healthy controls, terminal RFLP analysis of bacterial 16S rRNA genes was applied to 23 swab-scrubbed samples from facial skin. This culture-independent analysis successfully revealed the complex bacterial members of the microbiota as peak patterns following capillary electrophoresis of terminal restriction fragments (T-RFs). Each T-RF peak reflected a micro-organism, and the micro-organism to which each peak was assigned could be identified by computer simulation of T-RF length using the nucleotide sequence data of bacterial species residing in the skin. Among 18 species detected in the study, *Stenotrophomonas maltophilia* was detected significantly more commonly in AD patients (5/13 for AD patients vs 0/10 for controls), whilst *Dietzia maris* was detected significantly more commonly in normal controls (8/10 for controls vs 2/13 for AD patients). Moreover, *Streptococcus* species, which are considered to be uncommon in uninfected skin, were detected in seven patients and eight normal controls. Although further studies should be undertaken to investigate the roles of these micro-organisms in AD, the microbiota were presumed to include hitherto uninvestigated bacterial species in the major population of patients with AD and of healthy controls.

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INTRODUCTION

A classical culture analysis revealed that *Staphylococcus* species are densely colonized on the skin in atopic dermatitis (AD) patients, even at sites that appear normal (Gloor *et al.*, 1982). This phenomenon may result from dry skin due to chronic inflammation and a shortage of ceramide in the epidermis. Recently, it has been shown that *Staphylococcus* colonization occurs due to a decline in the secretion of antibiotic peptides belonging to the dermcidin family in sweat (Rieg *et al.*, 2005). It has been speculated

that the exotoxins and lipase produced by *Staphylococcus* species cause irritation and jeopardize the physiological condition of the skin. However, no other bacteria have been reported to have a direct relationship with AD.

In contrast, molecular techniques allow us to detect a variety of unidentified micro-organisms in human organs, such as the oral cavity, gut, prostate and vagina. These methods enable us to detect bacteria that are difficult to culture and isolate because of technical difficulties. The gene used is a small subunit rRNA-encoding gene known to possess species-specific evolutionary variation of the gene structure (Nelson *et al.*, 2000). This RNA-encoding gene is present in all known bacteria and its conserved region is suitable for amplification; thus, it is convenient for identification purposes (Fredricks, 2001). For the first time, we have reported that clone libraries of the 16S rRNA gene enable us to detect the total profile of bacterial

[†]Present address: Faculty of Engineering, Maebashi Institute of Technology, Kamisatori-Machi, Maebashi, Gunma 371-0816, Japan.

Abbreviations: AD, atopic dermatitis; T-RF, terminal restriction fragment; T-RFLP, terminal RFLP.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences obtained from clone libraries are AB264551–AB264556.

members in skin microbiota, which include uncultured bacteria (Dekio *et al.*, 2005). The data indicated that up to 13 novel 'phylotypes' (yet-to-be-cultured micro-organisms) and 9 novel species exist in the facial skin of healthy humans, in addition to the 10 previously recognized species. A similar molecular analysis from the forearm skin of healthy humans detected 30 phylotypes (Gao *et al.*, 2007), indicating that such molecular methods will inevitably be required to profile microbiota at various sites of the human skin. However, attempts to characterize the profile of microbiota in skin diseases using molecular methods have not yet been reported.

Bacterial 16S rRNA gene-based terminal RFLP (T-RFLP) is one of the molecular methods able to visualize complex microbial communities (Liu *et al.*, 1997; Sakamoto *et al.*, 2003). This molecular approach, which detects a fluorescently labelled fragment of the PCR-amplified 16S rRNA gene, has enabled rapid analysis of the total composition of bacterial samples that include a variety of micro-organisms. We applied T-RFLP analysis to swab-scrubbed skin samples of AD patients and normal subjects to compare the bacterial communities present in the two groups.

METHODS

Subjects. The experimental protocol was approved by the Ethics Review Committee of Keio University Hospital (approval number 15-70-1) and RIKEN (approval number Wako 3-15-26). The study population comprised 13 patients with AD (aged 19–42 years) and 10 healthy volunteers (aged 22–54 years). They were designated AS1–AS4 (severe AD), AM1–AM9 (moderate AD) and C1–C10 (healthy controls). Their baseline characteristics are shown in Table 1. The AD group included outpatients attending the dermatological clinic of Keio University Hospital and were diagnosed based on the criteria of Hanifin & Rajka (1980). At the time of sampling, they continued to receive the same treatment for more than 2 weeks. The AD severity scores were calculated using the SCORAD scoring system (European Task Force on Atopic Dermatitis, 1993). Patients with scores of ≥ 40 were defined as having severe AD, those with scores of < 40 but ≥ 15 as moderate AD, and those with scores of < 15 as mild AD. The healthy subjects were requested to complete a questionnaire to confirm that they had no history of skin diseases, allergic rhinitis or bronchial asthma. The participants of both groups did not use medicated soap or cosmetics in their daily life. They were advised not to wash, touch or apply cosmetics to the forehead skin (the sampling area) for at least 12 h prior to commencement of the study.

Samples were obtained once from all participants except patients AS3 and AM9. For these two patients, the samples were obtained twice

over 7 days. For AS3, the first sample was obtained when the patient was applying topical steroid ointment (Lidomex ointment) twice a day. On the day on which the first sample was obtained, the treatment was changed to once-a-day application of a topical tacrolimus ointment (Protopic ointment); this had improved the condition of his skin by the day on which the second sample was obtained. AM9 did not apply any ointment during the period when the two samples were obtained.

Collection of samples. The procedure was performed as described previously (Dekio *et al.*, 2005). Briefly, the open end of a 4.9 cm² sterile plastic cylinder was manually placed on the forehead skin. The skin within the enclosed area was scrubbed using a sterile swab moistened with dilution liquid. The tip of the swab was then broken against the wall of a glass tube containing 1 ml dilution liquid, and the tube was immediately capped and shaken to suspend the bacteria. In addition, four control samples were prepared by scrubbing only the inner wall of the cylinder with moistened swabs. A volume of 300 µl suspension was used for culture analysis, and a volume of 250 µl was used for T-RFLP analysis.

Culture analysis. Culture analysis was performed as described previously (Dekio *et al.*, 2005). Briefly, 10⁻¹, 10⁻³ and 10⁻⁵ dilutions of the samples were plated on three non-selective agar plates: trypticase soybean (TS) agar supplemented with 5% horse blood, Eggerth–Gagnon (EG) agar and glucose-blood-liver (BL) agar. Samples plated on TS agar were cultured aerobically at 37 °C for 2–3 days, whilst those plated on EG and BL agar were cultured anaerobically at 37 °C for 7 days. On each agar plate, the cell morphology was examined microscopically by Gram staining, and colonies with comparable macroscopic features were counted to calculate the number of c.f.u. ml⁻¹. The colonies were subcultured, the bacterial DNA extracted and their 16S rRNA genes PCR amplified using the universal primers 27F and 1492R (Lane, 1991). The anterior one-third of the 16S rRNA genes (*Escherichia coli* nt 27–520) was sequenced in both directions using the 27F and 520R primers (Lane, 1991). The sequences were analysed using a BLASTN search (<http://www.ddbj.nig.ac.jp>; Altschul *et al.*, 1997) for identification.

DNA extraction for T-RFLP analysis. The suspension collected for T-RFLP analysis was diluted by adding 800 µl phosphate buffer solution and centrifuged at 14 000 g for 5 min to obtain a pellet. The pellet was suspended in 450 µl extraction buffer [100 mM Tris/HCl (pH 7.6), 40 mM EDTA]; subsequently, 50 µl SDS (final concentration 1%, w/v), 500 µl phenol and 300 mg glass beads (0.1 mm diameter) were added. The cells were degraded under constant shaking using a FastPrep instrument (Bio 101) for 30 s. The mixture was extracted with equal volumes of phenol and phenol:chloroform:isoamyl alcohol (25:24:1). The lysate was subsequently purified and condensed into 20 µl distilled water using a High Pure PCR template preparation kit (Roche) for amplification of the 16S rRNA gene coding region.

Generation of T-RFLP patterns. The procedure was performed as described by Sakamoto *et al.* (2004) with some modifications. Two universal primers, 6-carboxyfluorescein (6-FAM)-labelled-27F (5'-6-FAM-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTAC-CTTGTTACGACTT-3') (Lane, 1991), were used to amplify the bacterial 16S rRNA gene coding region. Amplification reactions were performed in a total volume of 100 µl using the following program: 1 cycle of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min, with a final extension period of 72 °C for 10 min. The size of the amplified DNA was verified on a gel and purified using PEG solution.

The purified PCR product (2 µl) was digested with 20 U *HhaI* or *MspI* (TaKaRa) in a total volume of 10 µl at 37 °C for 3 h. The length

Table 1. Baseline characteristics of participants

Population	AD patient group (n=13)	Normal control group (n=10)
Race	Japanese	Japanese
Gender (male/female)	11/2	8/2
Age (mean $\pm$ SD) (years)	29.0 $\pm$ 7.3	34.2 $\pm$ 11.8
SCORAD scores (mean $\pm$ SD)	36.0 $\pm$ 18.5	Not calculated

of the terminal restriction fragments (T-RFs) was determined on an ABI PRISM 3100 genetic analyzer (Applied Biosystems) in the GeneScan mode (15 kV, 100 μ A and 60 °C for 40 min for each sample) (Fig. 1). The fragment sizes were estimated using the Local Southern method of the GeneScan 3.7 software (Applied Biosystems). T-RFs with a peak height of less than 60 fluorescent units were excluded from the analysis, and the percentage of each peak area compared with the total area was calculated as described by Matsumoto & Benno (2007). The predicted T-RFLP patterns of the 16S rRNA genes of known bacterial species were obtained using the GENETYX-MAC program (Software Development).

Identification of unassigned peaks by clone library analysis.

Using sequence data from the DDBJ/EMBL/GenBank databases and the results of our previous clone library analysis (Dekio *et al.*, 2005), we attempted to assign the T-RFs to micro-organisms. However, we noticed three common T-RFs that could not be assigned to any bacterium. To attempt to assign these peaks, we performed gel extraction and clone library analysis. In brief, we performed gel electrophoresis of the *Hha*I-restricted PCR products that were used for the T-RFLP analysis, and the bands that were suspected to form the fluorescent peaks in the T-RFLP analysis were excised manually. DNA was extracted from this gel slice using an UltraClean GelSpin kit (Mo Bio), and the double-stranded DNA adaptor for the *Hha*I site developed by Mengoni *et al.* (2002) was ligated to the *Hha*I-restricted site using a DNA ligation kit (TaKaRa). PCR amplification was performed using primer 27F and an adaptor primer (p*Hha*I: 5'-CCATGCG-TCAGATGCTCCGC-3'). Clone libraries were constructed as described by Hayashi *et al.* (2002), and sequence analysis was performed using BLASTN to identify the species that formed the *Hha*I/*Msp*I peak sets.

The term phylotype is used for clusters of 16S rRNA gene clone sequences that differ from known species by more than 2 % and are at least 98 % similar to the members of their cluster (Suau *et al.*, 1999; Paster *et al.*, 2001; Dekio *et al.*, 2005). 'Phylotype A' and 'phylotype B' refer to species presented in a previous report (Dekio *et al.*, 2005). These were characterized by bacterial 16S rRNA gene sequences obtained from swab-scrubbed skin samples of healthy humans (GenBank accession nos AB161079 and AB161080).

RESULTS

Culture analysis

Propionibacterium acnes and *Staphylococcus* species were isolated from the samples from all ten healthy controls. In contrast, of the 13 samples from AD patients, *P. acnes* was not isolated from 4 samples (31 %) and *Staphylococcus* species was not isolated from 1 sample (8 %) (Table 2). All isolates of *Staphylococcus* species were coagulase-negative, and *Staphylococcus aureus* was not isolated from any sample examined in this study. All of the cultured bacteria were the known classical components of skin microbiota, and the total number of c.f.u. cm^{-2} of the samples was in the same order as that reported by Marples & McGinley (1974) and Evans (1975).

Detection of T-RFLP peak patterns

A set of two patterns (corresponding to the *Hha*I and *Msp*I digests) was derived from each sample (Fig. 1). The presence of each major bacterial species was reflected as one peak in the *Hha*I pattern and one peak in the *Msp*I

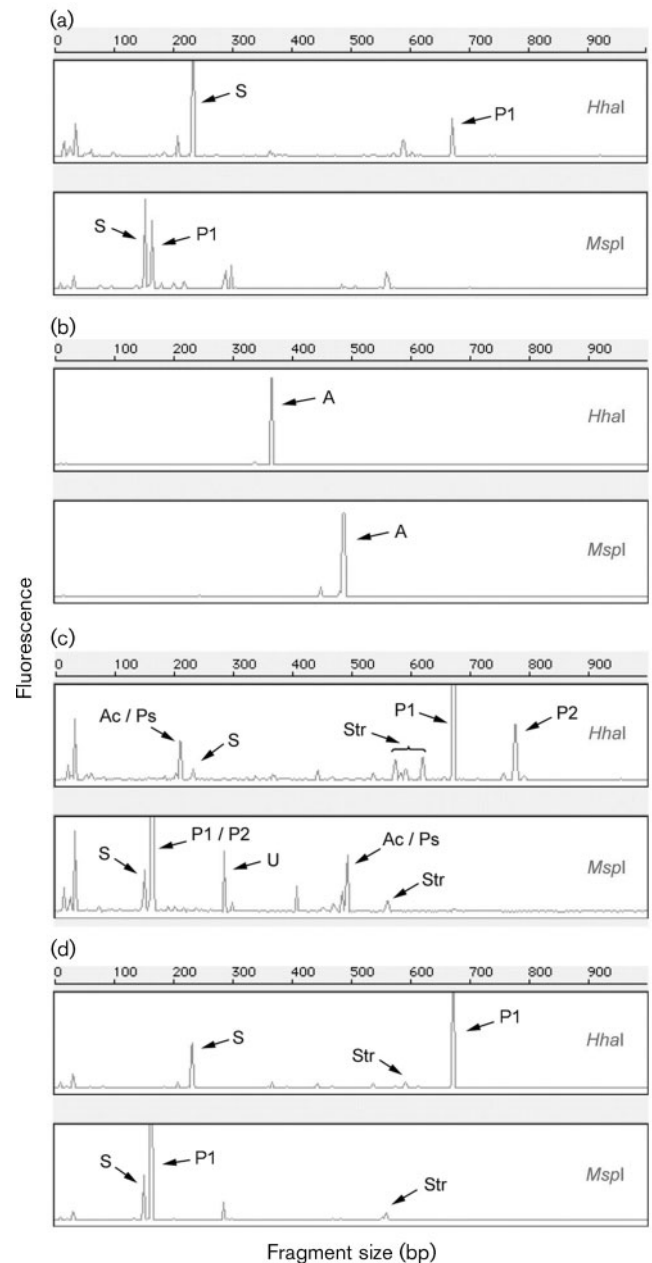


Fig. 1. Examples of peak patterns obtained by T-RFLP analysis. The patterns shown are (a) AS4, (b) AM4, (c) C9 and (d) C8. The peak heights indicate the relative strength of fluorescence. A, Peak set IX (phylotype A); Ac/Ps, peak set IV (*Acinetobacter* sp. or *Pseudomonas* sp.); P1, peak set XVI (*P. acnes*); P2, peak set XVII (*P. acnes*); S, peak set VII (*Staphylococcus* spp.); Str, peak set XIV (*Streptococcus* spp.); U, peak set XI (unidentified).

pattern; thus, we defined the two peaks of a single species as a 'peak set'. Eighteen major corresponding peak sets were detected from the samples (Table 3). Large differences were observed among the participants with regard to the peak sets, which reflected differences in the diversity of the bacterial communities.

Table 2. Results of culture analysis

The log count of each species was calculated from the colony count; undetected samples were not included. ND, Not detected.

Species	AD patient group		Normal control group	
	Detection rate	log count (mean $\pm$ SD)	Detection rate	log count (mean $\pm$ SD)
<i>Staphylococcus epidermidis</i>	12/13	4.7 $\pm$ 0.8	10/10	3.0 $\pm$ 1.4
<i>Staphylococcus capitis/Staphylococcus caprae</i>	3/13	4.4 $\pm$ 0.9	1/10	3.6
<i>Propionibacterium acnes</i>	9/13	4.8 $\pm$ 1.2	10/10	5.5 $\pm$ 1.1
<i>Propionibacterium granulosum</i>	0/13	ND	1/10	4.2
<i>Streptococcus</i> spp.	0/13	ND	2/10	3.5 $\pm$ 1.2

Assignment of T-RFLP peaks using computer simulation

In order to assign the 18 major peak sets to bacterial species, we carried out computer simulation of T-RFLP for each bacterial member existing in the skin based on sequences in the DDBJ/EMBL/GenBank databases (Table 4). Peak sets IV, VII, XIV and XVI were assigned to 16S rRNA sequences of well-known skin bacteria in the databases, and peak sets V, VI, IX and X were assigned to those obtained from our previous clone library analysis (Dekio *et al.*, 2005). The computer simulation successfully identified 8 of the 18 major peak sets; among these, *Stenotrophomonas maltophilia*, *Dietzia maris*, and phylotype A and phylotype B (yet-to-be-cultured bacteria) are members that have not been reported previously to be isolated from human skin.

To verify this assignment, we compared the computer simulation data with the actual T-RFLP data for single live strains obtained from human skin (Table 4). Differences of up to 6 bp were observed between the predicted and observed peaks. A similar phenomenon was observed in an analysis of microbiota in saliva by Sakamoto *et al.* (2003); the reason for this is unknown.

Assignment of T-RFLP peaks using clone library analysis

Despite the extensive computer simulation, 10 of the 18 major peak sets remained unassigned. To identify the organisms producing these peaks, we performed gel electrophoresis using a mixture of the T-RFs used for T-RFLP analysis, constructed clone libraries from the excised gel bands and sequenced them. Based on the obtained nucleotide sequences (GenBank accession nos AB264551–AB264556), we assigned the peak sets to *Alcaligenes xylosoxidans* (peak set XII), *Staphylococcus* species (peak set XIII) and *P. acnes* (peak set XVII). So the clone library analysis successfully identified three peak sets; among these, *A. xylosoxidans* has not been reported previously to be isolated from human skin.

At this stage, 7 of the 18 peak sets remained unidentified. These peaks were difficult to assign on the basis of gel extraction and data from clone libraries; some peaks were too short for the analysis, others were difficult to excise

from the gel because the bands on the gel were in extremely close proximity to other bands.

Heterogeneity in the distribution of bacterial species among individual samples

We detected species that were observed more frequently in AD patients than in the controls and vice versa. In AD patients, *Stenotrophomonas maltophilia* (peak set VI) was significantly more common (5/13 AD patients and 0/10 controls, $P = 0.045$; Table 5). On the other hand, *D. maris* (peak set X) was significantly more common in the controls (2/13 AD patients and 8/10 controls, $P = 0.003$; Table 5).

Although the difference between the AD and control groups was significant with regard to the microbiota found in the skin, no significant difference was observed between the moderate AD and severe AD groups with respect to the specific bacterial species in each group.

High detection rate of *Streptococcus* species and phylotypes

Streptococcus species (peak set XIV), which has been considered previously to reside only in infected lesions in human skin, was detected in both AD patients and healthy controls. We detected this species in 7/13 (54 %) patients and in 8/10 (80 %) controls.

Other observations in non-typical participants

Patients AS1, AS4, AM2 and AM3 continuously applied topical tacrolimus (Protopic ointment). Although this drug is known to reduce the number of *Staphylococcus* species on the skin (Pournaras *et al.*, 2001; Remitz *et al.*, 2001), the number of *Staphylococcus* species in the skin of these patients appeared to be the same as that observed in other patients in the analysis of both the molecular T-RF peaks and cultured colonies. This ointment thus appears to be less effective in reducing the number of *Staphylococcus* species in AD patients *in vivo*.

For two patients (AS3 and AM9), samples were taken twice over 7 days. Although culture analysis revealed a slight

Table 3. Profiles of *HhaI*-digested T-RFs determined by 16S rRNA gene T-RFLP analysis of skin samples

The largest peak in each T-RFLP pattern is indicated in bold.

T-RFLP peak set	<i>HhaI</i> / <i>MspI</i> fragment size (bp)	Severe AD				Moderate AD									Normal control									
		AS1	AS2	AS3	AS4	AM1	AM2	AM3	AM4	AM5	AM6	AM7	AM8	AM9	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Peak area (%)																								
I	53/53	18.9				6.9	18.0	1.2			0.8			53.7										
II	166/NS	4.5				1.9	4.3	0.9				0.5		8.9										1.3
III	190/NS					1.2		0.2			1.8										0.7			1.4
IV	207/491				4.2		3.0							8.2					3.9	0.5			2.3	
V	208/141										0.9	2.5										1.5		
VI	216/465			5.5		0.4		2.5			2.2		0.2											
VII	235/152	76.5	100	92.9	66.7	0.8	66.1	86.4			63.4	93.5	11.3	22.6	70.4	3.8		13.0	12.0	4.8	1.6	23.3	1.0	1.3
VIII	338/450								4.2															
IX	367/490			0.6	2.7	0.7		0.1	94.6		2.0		1.7			0.7	1.2	1.0		1.8	1.0	2.1	1.7	8.8
X	444/159					0.6							1.0			0.7	0.9	0.8		0.9	0.7	1.7	0.5	0.6
XI	537/299					0.6							1.8			1.3	1.8	0.9		1.6	1.0	2.3	0.4	0.7
XII	568/490								1.1															
XIII	575/152										1.7	0.2	0.9			0.6	0.5			1.0	0.8		2.1	0.3
XIV	591/560			0.8	10.5		8.3	5.1			8.4	1.9		3.6	11.2	1.8		1.3	12.0	0.7	0.9	3.5	1.3	
XV	621/NS												4.5			1.2	0.9	4.6		0.7			2.7	4.8
XVI	673/165				15.6	85.8		3.1		100	18.2	1.0	78.3	2.7	18.3	88.6	93.7	78.2	57.9	87.5	91.2	65.2	82.1	67.9
XVII	781/165					0.6										1.0	0.7				1.7		5.3	12.4
XVIII	NS/286				*	*				*				*			*				*		*	*
Cultured colonies (log c.f.u. cm ⁻²)																								
<i>Staphylococcus</i> spp.		4.6	4.8	5.8	3.3	3.6	4.3	5.9	4.9	ND	4.5	5.6	3.9	5.8	4.6	4.7	3.6	4.9	2.8	3.9	1.6	2.1	1.6	2.0
<i>Propionibacterium</i> spp.		4.0	3.6	ND	3.3	6.1	ND	ND	5.8	4.1	4.0	ND	6.2	6.3	4.8	6.9	6.6	6.3	4.5	5.6	5.5	3.3	3.9	6.6

ND, Not detected; NS, not specified.

*Peak set specified only in *MspI* digestion.

change, the T-RFLP patterns and the appearance of the skin showed no apparent change. This suggests that the skin microbiota do not change in a short time period in patients, irrespective of a change in the ointment used, unless a change in the skin condition is observed.

Participant C1, a healthy control, had a unique set of micro-organisms; only *Staphylococcus* species, *Streptococcus* species and *P. acnes* were detected, and the largest peak set corresponded to *Staphylococcus* species. This pattern was specific to AD patients. The culture result supported the finding that the count of *Staphylococcus* species was atypically as high as that of *Propionibacterium* species. However, this individual did not have any allergic diseases and was experiencing a very good skin condition. This seemingly unexplainable phenomenon may have occurred due to the unique host–parasite relationship in this individual; a molecular analysis of her faeces revealed that she had a unique set of micro-organisms in the intestine when compared with that observed in other healthy individuals (data not shown).

DISCUSSION

In the present study, molecular analyses successfully revealed the comprehensive profile of skin microbiota including uncultured bacteria. The microbiota differed diversely between patients with AD and healthy controls.

We used forehead skin as the sampling site. According to published studies using culture techniques, skin microbiota vary largely among different sites (Marples, 1982). To examine differences between the data obtained in this study and those in a previous report on the 16S rRNA gene-based bacterial profiling of forehead skin samples in healthy individuals (Dekio *et al.*, 2005), we used the same sampling site. The selected site has one of the greatest bacterial counts on the human skin and is considered to be stable despite environmental changes (Evans, 1975; Dekio *et al.*, 2005). Such a high population appears to be beneficial to reduce the contamination effect from the environment. As a result, the number of c.f.u. cm⁻² determined in our study was comparable to that in published reports (Marples & McGinley, 1974; Evans, 1975) (Table 2), suggesting that the sampling procedure was reliable for the analyses of physiological steady-state conditions with minimal contamination.

Staphylococcus species were detected as two different peak sets (peak sets VII and XIII) in our T-RFLP analysis. This was probably attributable to strain variation of nt 238, which is located in the *HhaI* restriction site. Analysis of the sequence data for several strains in the nucleotide databases revealed that *Staphylococcus aureus* should be detected only as peak set VII, but other *Staphylococcus* species strains should be detected as either peak set VII or XIII. The sequence data from our clone library analysis (GenBank

Table 4. Computer simulation and real observation of the T-RF lengths of peak sets

Species or phylotype	T-RF length of peak set (bp)				Sequence data used for prediction (GenBank accession no.)
	<i>HhaI</i>		<i>MspI</i>		
	Predicted	Observed	Predicted	Observed	
<i>Acinetobacter johnsonii</i>	207	ND	491	ND	Z93440
<i>Bacillus cereus</i>	579	586	147	144	X55060
<i>Bacillus subtilis</i>	240	235	145	141	AB042061
<i>Dietzia maris</i> *	441	440	161	159	X79290
<i>Propionibacterium acnes</i>	671	673	165	163	AB042288
<i>Propionibacterium granulosum</i>	367	365	202	201	AJ003057
<i>Pseudomonas aeruginosa</i>	207	ND	490	ND	AF094713
<i>Staphylococcus aureus</i>	238	ND	155	ND	X68417
<i>Staphylococcus capitis</i> / <i>Staphylococcus caprae</i>	238	234	155	152	AB009935
<i>Staphylococcus epidermidis</i>	238	235	155	152	D83363
<i>Stenotrophomonas maltophilia</i> *	215	ND	465	ND	X95923
<i>Streptococcus agalactiae</i>	581	ND	555	ND	AB023574
<i>Streptococcus mitis</i>	579	587	83	77	AF003929
<i>Streptococcus oralis</i>	581	588	555	556	AF003932
<i>Streptococcus pyogenes</i>	543	ND	517	ND	AB002521
Phylotype A (<i>Methylophilus methylotrophus</i> 97 %)*	367	ND†	490	ND†	AB161079
Phylotype B (<i>Ideonella dechloratans</i> 94 %)*	208	ND†	141	ND†	AB161080

ND, Not done.

*Uncultured micro-organisms from the skin.

†Not performed as the live strain was not available.

Table 5. Table 5. Detection rate of peak sets

Peak set	Fragment (bp)		Identification	AD patient group	Normal control group	Fisher's <i>P</i> value
	<i>HhaI</i>	<i>MspI</i>				
I	53	53	Unidentified	6/13	0/10	0.019*
II	166	NS	Unidentified	6/13	1/10	0.088
III	190	NS	Unidentified	3/13	2/10	1.0
IV	207	491	<i>Acinetobacter</i> spp. or <i>Pseudomonas</i> spp.	3/13	3/10	1.0
V	208	141	Phylotype B	2/13	1/10	1.0
VI	216	465	<i>Stenotrophomonas maltophilia</i>	5/13	0/10	0.045*
VII	235	152	<i>Staphylococcus</i> spp. 1	11/13	9/10	1.0
VIII	338	450	Unidentified	1/13	0/10	1.0
IX	367	490	Phylotype A	7/13	8/10	0.381
X	444	159	<i>Dietzia maris</i>	2/13	8/10	0.003†
XI	537	299	Unidentified	2/13	8/10	0.003†
XII	568	490	<i>Alcaligenes xylosoxidans</i>	1/13	0/10	1.0
XIII	575	152	<i>Staphylococcus</i> spp. 2	3/13	6/10	0.102
XIV	591	560	<i>Streptococcus</i> spp.	7/13	8/10	0.378
XV	621	NS	Unidentified	1/13	6/10	0.018*
XVI	673	165	<i>Propionibacterium acnes</i> 1	8/13	10/10	0.045*
XVII	781	165	<i>Propionibacterium acnes</i> 2	1/13	5/10	0.051
XVIII	NS	286	Unidentified	4/13	4/10	0.685

NS, Not specified.

*Significant ($P < 0.05$).†Significant ($P < 0.01$).

accession nos AB264552–AB264555) verified this possibility. In contrast, *P. acnes* was also detected as two different peak sets (XVI and XVII), but this was unlikely to have resulted from a difference at the restriction point as in the case of *Staphylococcus* species. The sequence of our clone library from peak set XVII (fragment sizes 781 bp/165 bp) (GenBank accession no. AB264556) exhibited the *HhaI*-restrictive sequence at nt 671 and not nt 781; therefore, there was no apparent reason for the generation of the 781 bp fragment. One hypothesis is that the conformational structure of the single strands allowed cleavage by the restriction enzyme at this point ('pseudo-T-RF', discussed below).

Using the computer simulation and clone library technique, we successfully detected five species/phylotypes that have not been reported previously to be isolated from human skin; *Stenotrophomonas maltophilia*, *D. maris*, *A. xylosoxidans*, and phylotype A and phylotype B. *Stenotrophomonas maltophilia* is an environmental bacterium isolated from soil and crops (Hauben *et al.*, 1999). As this bacterium is related to dermatitis in sheep (London & Griffith, 1984), its presence may worsen the condition of atopic skin. Furthermore, it is an emerging pathogen in the respiratory and urinary tracts of immunocompromised hosts (Vartivarian *et al.*, 1996; Senol, 2004); our findings suggest that the skin microbiota of AD patients serve as a reservoir for such opportunistic pathogens. *D. maris* is a common bacterium found in ponds and on the surface of

fish. It is possible that it exists commonly in humans and maintains a healthy environment on the skin. *D. maris* was observed to coexist with unspecified bacteria (peak set XI), suggesting a symbiotic relationship between them.

Compared with previous culture studies, our molecular analysis detected a higher rate of *Streptococcus* species. This species has been reported previously to occur only in infected lesions in human skin and not on normal atopic or healthy skin. In our study, peaks corresponding to *Streptococcus* species were much smaller relative to those corresponding to *Staphylococcus* species in every sample. This suggests the coexistence of a small number of *Streptococcus* species and a large number of *Staphylococcus* species. The reason for the low detection rate of *Streptococcus* species by culture analysis is unknown, but we hypothesize that the ratio of the population of *Streptococcus* species to that of *Staphylococcus* species is very small in culture plates; therefore, their colonies remained unrecognized. Our molecular method enabled the detection of even these minor components of microbiota.

Despite the use of computer simulation and the clone library technique to assign peak sets to bacterial species, 7 of the 18 major peak sets remained unidentified. Whilst these could be real micro-organisms, such peaks could also be 'pseudo-T-RFs', which have been described by Egert & Friedrich (2003). Single-stranded DNA molecules, which result from a large number of PCR cycles, form partial double strands within themselves. New restriction sites are

generated after the formation of a complex structure consisting of covalent bonds within a strand and hydrogen bonds between the two strands of the partial double strand; this results in the generation of 'pseudo-T-RFs'. To avoid such peaks, pre-treatment using single-strand-specific mung bean nuclease should be performed before capillary electrophoresis.

In previous analyses of skin microbiota in AD patients, interest was limited to the number and ratio of *Staphylococcus* species to *P. acnes* (Leyden *et al.*, 1974; Ogawa *et al.*, 1994). However, such culture analyses were unable to categorize participants clearly based on the condition of their skin, and only tendencies were observed. In contrast, our method enabled a clearer clustering of skin microbiota, although a small number of exceptions existed. This suggests that bacteria other than *Staphylococcus* species and *P. acnes* should also be analysed in AD patients. Furthermore, a total analysis of skin microbiota should also include analysis of *Stenotrophomonas maltophilia*, *D. maris*, *Streptococcus* species and phylotype A.

Our aim is to search for novel micro-organisms that are beneficial or harmful to AD patients. Our high-throughput molecular analysis successfully captured the micro-organisms that were relatively specific to AD patients and the controls. However, it is impossible to determine whether the composition of microbiota is the cause, a worsening factor or only the result of AD, as the data are solely observational.

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