Comparative Ocular Microbial Communities in Humans with and without Blepharitis

Se Hee Lee,[1] *Doo Hwan Oh,*[2] *Ji Young Jung,*[1] *Jae Chan Kim,*[2] *and Che Ok Jeon*[1]

Purpose. The aims of our study were to compare the ocular microbial communities of humans with and without blepharitis in an attempt to elucidate which microorganisms may cause blepharitis.

Methods. Bacterial 16S rRNA genes of eyelash and tear samples from seven blepharitis patients and four healthy controls were sequenced using a pyrosequencing method, and their bacterial community structures were compared bioinformatically.

Results. Phylotypic analysis demonstrated that eyelash and tear samples had highly diverse bacterial communities with many previously undescribed bacteria. Bacterial communities in eyelash samples from subjects with blepharitis were less diverse than those from healthy controls, while the bacterial communities of tear subjects with blepharitis were more diverse than those of healthy subjects. Statistical analyses using UniFrac and a principle coordinate analysis showed that the bacterial communities of tear samples from subjects with blepharitis were well clustered, regardless of individual, while the bacterial communities of all eyelash samples and healthy tear samples were not well clustered due to high interpersonal variability. Bioinformatic analysis revealed that *Propionibacterium*, *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* were the common ocular bacteria. An increase of *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter*, and a decrease of *Propionibacterium* were observed from blepharitis subjects, in terms of the relative abundances.

Conclusions. Higher abundances of *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* in blepharitis subjects suggested that human blepharitis might be induced by the infestations of pollens, dusts, and soil particles. These results will provide valuable information for the prevention and treatment of human blepharitis based on ocular microbial flora. (*Invest Ophthalmol Vis Sci.* 2012;53:5585–5593) DOI:10.1167/iovs.12-9922

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Blepharitis is the general term used to refer to inflammation involving the eyelids and it induces various discomforts, including burning, itching, irritation, and photophobia.[1] It is one of the most commonly encountered conditions in the practice of ophthalmology. However, most of the common causes of blepharitis remain ill-defined, defying detailed pathophysiology with unknown mechanisms. Many researchers have reported that *Demodex* may be an important etiologic factor in chronic blepharitis, conjunctival inflammation, ocular rosacea, and meibomian gland dysfunction, and its occurrence has been shown to be related significantly to age, ocular discomfort, tear film instability, and poor ocular hygiene.[2–7] Until now, very little has been known of the fundamental factors of *Demodex* mites that cause blepharitis symptoms. There have been reports that *Demodex*-related *Bacillus*, rather than *Demodex* itself, might stimulate inflammatory diseases, like rosacea, on the forehead, cheek, chin, or nose due to serum immunoreactivity.[8–11] For example, Lacey et al.[8] and Li et al.[9] reported that 83-kDa and 62-kDa antigen proteins produced by a *Demodex folliculorum*-related bacterium, *Bacillus oleronius*, elicited host immune responses and exacerbated cutaneous inflammation by serum immunoreactivity in blepharitis patients. However, despite the high incidence of *Demodex* in blepharitis patients, it still is controversial as a cause, as blepharitis symptoms are not always present in patients with *Demodex* and often are found in individuals without *Demodex*.[12–14]

On the other hand, many researchers have posited that very diverse microorganisms inhabit the ocular environments, and some of these microorganisms may trigger blepharitis symptoms. Therefore, many studies have been performed using culture-dependent approaches to investigate ocular microbial communities in an attempt to elucidate which microorganisms may cause blepharitis.[15–19] For example, Dougherty and McCulley,[15] and Kulaçoğlu et al.[18] reported that *Staphylococcus* and *Propionibacterium* strains were identified from blepharitis patients as major isolates, and that their elevated levels might contribute to the occurrence of blepharitis. However, culture-based approaches have many limitations, including that many microorganisms are difficult to culture.[20,21] Therefore, culture-independent analysis based on 16S rRNA gene sequences has been developed and applied to investigate human body microbial communities.[22,23] Culture-independent approaches, including PCR, denatured gradient gel electrophoresis (DGGE), and pyrosequencing, have been applied to the study of ocular microbial communities and have revealed that sets of previously cultured isolates from eye environments did not reflect the true microbial compositions of ocular microbial flora.[24–26] In the current study, we applied a massively parallel pyrosequencing strategy to compare more specifically the ocular microbial communities of blepharitis patients and healthy controls. These results will expand our information on the ocular microbial communities, and contribute to the prevention and treatment of blepharitis.

Table 1. Demographics of Blepharitis Patients and Healthy Controls for Eyelash and Tear Sampling

Group	Subject No.	Age (y)	Sex	Demodex*		Allergy†
				Left	Right	
Blepharitis patients (B)	1	63	Female	+	–	–
	2	66	Male	+	+	–
	3	65	Male	+	+	+
	4	66	Male	+	+	+
	5	69	Male	+	+	–
	6	64	Male	+	+	–
	7	76	Female	–	–	–
Healthy controls (H)	1	54	Male	–	–	–
	2	76	Female	–	–	–
	3	25	Male	–	–	–
	4	27	Male	+	–	–

* Positive signs indicate the successful detection of *Demodex* mites from eyelash or tear samples.

† Allergies were determined by symptoms (itching, feeling of irritation, or indisposition) and examination of eyes under a slit-lamp biomicroscope.

Methods

Sample Collection

Seven patients with blepharitis who visited a clinic (Chung-Ang University Hospital) for ophthalmic examinations between September 1 and November 30, 2010, and four controls without blepharitis were included in the study. Informed consent was obtained from all participants. This study was approved by the Chung-Ang University Hospital Institutional Review Board, and all methods adhered to the principles of the Declaration of Helsinki. The information from all participants is summarized in Table 1. All participants underwent a complete ophthalmic examination under a slit-lamp biomicroscope. Blepharitis was diagnosed based on clinical evidence of lid margin or tarsal conjunctival erythema, bulbar conjunctival hyperemia, telangiectasia, thickening, or irregularity of the eyelid margins, or meibomian gland orifice inclusions. Eyelash samples were obtained by epilation of four eyelashes (two eyelashes from each lower and upper lids), and the number of *Demodex* mites was counted with an optical microscope. We attempted to epilate as deeply as possible lashes with cylindrical dandruff around the root of the lash. For tear sampling, a small amount of 0.9% (wt/vol) saline solution was dropped on the bulbar conjunctiva of both eyes, and then the participants blinked several times to spread the saline solution to the corner of the bulbar conjunctiva. Tear samples were collected from the corner of the bulbar conjunctiva using hematocrit-capillary tubes (Haematokrit-kapillaren, Eberstadt, Germany), and the presence of *Demodex* was determined using an optical microscope. The obtained eyelash and tear samples were transferred to 0.6 mL tubes and stored in a −80°C freezer until DNA extraction.

DNA Extraction and PCR Amplification for Barcoded Pyrosequencing

To extract total genomic DNA from eyelash and tear samples, 0.3 g of 0.1 mm zirconia/silica beads (Biospec, Bartlesville, OK) and 50 μL of 5% (wt/vol) Chelex-100 (BioRad, Hercules, CA) were added to the 0.6 mL tubes containing eyelash or tear samples, and the tubes then were vortexed vigorously for 2 minutes. After boiling for 10 minutes, the tubes were vortexed vigorously again for 2 minutes and centrifuged for 2 minutes at a maximum speed. The supernatants of the samples were used as templates for PCR amplification of bacterial 16S rRNA genes. For barcoded pyrosequencing, bacterial 16S rRNA genes containing hypervariable regions (V1–V3) were amplified using primer sets, Bac9F (5′-adaptor B-AC-GAG TTT GAT CMT GGC TCA G-3′)/Bac541R (5′-adaptor A-*X*-AC-WTT ACC GCG GCT GCT GG-3′),[27,28] where *X* denotes unique 7–11 barcode sequences inserted between the 454 Life Sciences adaptor A sequence and the common linker, AC (see Supplemental Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9922/-/DCSupplemental). All PCR amplifications were done in a 50 μL C1000 thermal cycler (BioRad) containing 5 μL of template genomic DNA, 20 pmol of each primer, and a Taq polymerase mixture (Solgent, Daejeon, Korea), using a cycling regimen of 94°C for 5 minutes (1 cycle), 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 1 minute (30 cycles), and 72°C for 10 minutes (1 cycle).

Pyrosequencing and Data Analysis

The PCR products were purified using a PCR purification kit (Solgent), and their concentrations were assessed carefully using an ELISA reader equipped with a Take3 multivolume plate (SynergyMx; BioTek, Winooski, VT). A composite DNA sample was prepared by pooling equal amounts of PCR products from each sample. Pyrosequencing of the composite DNA sample was performed on 1/8 plate two times by Macrogen (Seoul, Korea) using a 454 GS-FLX Titanium system (Roche, Branford, CT). Pyrosequencing data were processed and analyzed using the RDP pyrosequencing pipeline (available in the public domain at http://pyro.cme.msu.edu).[29] The sequencing reads were assigned to specific samples based on their unique barcodes sequences, and then the barcodes were removed. The resulting sequencing reads were trimmed by removing beginning and ending bases with a quality score <20 (error rate 0.01), and only sequences >300 base pairs (bp) in length were chosen for further analyses using the Pipeline initial process. Unexpected or nonbacterial reads were removed manually using the RDP classifier.[30] Taxonomic assignments of the processed bacterial reads were performed using the RDP naive Bayesian rRNA Classifier at an 80% confidence threshold. Operational taxonomic units (OTUs) and rarefaction curves were generated using the RDP pyrosequencing pipeline at a 3% dissimilarity level. The Shannon-Weaver[31] and Chao1 biodiversity indices,[32] and evenness were calculated by the RDP pyrosequencing pipeline. The bacterial community structures of eyelash and tear samples were compared using a UniFrac analysis[33] based on the phylogenetic relationships of representative sequences derived from all reads of the individual samples. Briefly, the processed read sequences were clustered into OTUs using CD-HIT[34] with an identity cutoff of 97%. The representative sequences from CD-HIT were aligned using NAST[35] based on the greengenes database,[36] with a minimum alignment length of 300 bp and a minimum identity of 75%. A phylogenetic tree was constructed using the PHYLIP software (ver. 3.6) with the Kimura two-parameter model[37] and was used as an input file for the hierarchical clustering of bacterial communities in the weighted UniFrac analysis. To confirm the multiple community comparison from the UniFrac analysis, a principal coordinate analysis (PCoA) also was performed. The relative bacterial

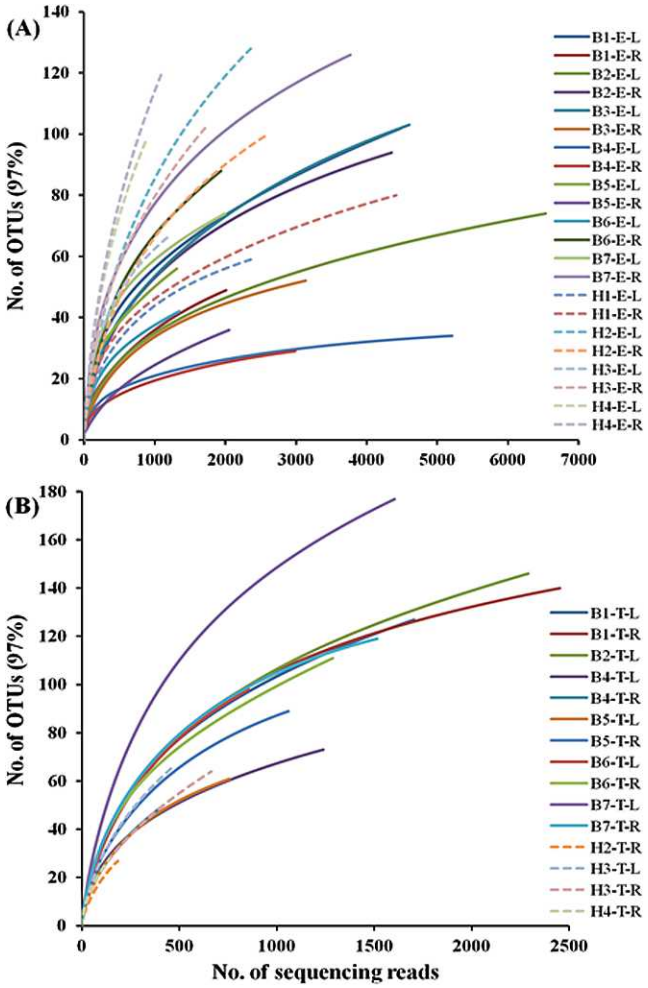

Figure 1. Rarefaction analysis of bacterial 16S rRNA gene sequences from eyelash (**A**) and tear (**B**) samples of blepharitis patients and healthy controls. OTUs were calculated by the RDP pipeline with a 97% sequence similarity cut-off value. B, blepharitis subjects; H, healthy subjects; E, eyelash; T, tear; L, left eye; R, right eye.

mean abundances of eyelash and tear samples from patients with and without blepharitis, shown in Figure 1, were calculated using the mean values of relative phylotypic compositions of respective eyelash and tear samples. The correlations between relative abundance of microbial communities and ocular sample type, shown in Figure 2B, were evaluated statistically by ordination biplot of redundancy analysis (RDA) with the Matlab program (ver. 6.5; MathWorks, Inc., Natick, MA). Genera unclassified by the RDP classifier and H3-T-R were not used in the analysis.

Nucleotide Sequence Accession Numbers

The pyrosequencing data of the 16S rRNA genes are available publicly in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under accession No. SRA050907.

Results

Sampling and Sequencing Analysis of 16S rRNA Genes

To analyze the ocular microbial communities of humans with and without blepharitis, 22 eyelash and 22 tear samples were collected from the left and right eyes of 11 participants (seven blepharitis patients and four healthy controls), respectively (Table 1). Among a total of 44 subjects, seven tear samples did not produce sufficient 16S rRNA gene amplicons for pyrosequencing analysis. From the pyrosequencing of 37 successful PCR amplicons, a total of 96,151 sequencing reads was generated. After the removal of low quality or nonbacterial 16S rRNA sequencing reads, 79,085 high quality reads (82.25% of the total reads) with an average sequence length of approximately 486 bp and an average of >2137 reads for each sample were used for further analysis (Table 2). A rarefaction analysis using the culled 16S rRNA gene sequences was performed to assess the number of microbial communities in eyelash and tear samples were recovered from the pyrosequencing analysis (Fig. 3). Surprisingly, individual rarefaction curves of eyelash and tear samples demonstrated failures to approach asymptotes, which suggested that eyelash and tear samples had highly diverse bacterial communities, and that many unexploited OTUs still remained in the samples. The number of estimated OTUs in each subject by Chao1 richness estimator also was significantly higher than the number of observed OTUs (corresponding to 41.2%–90.7% of the estimated richness), indicating that more sequencing efforts may be required to obtain additional microbial community information. Although the number of OTUs estimated in a subject was a function of the number of pyrosequencing reads obtained, interestingly, Chao1 richness analysis demonstrated that the eyelash microbial communities of blepharitis subjects were relatively less diverse than those of healthy subjects, while the tear microbial communities of blepharitis subjects were relatively more diverse than those of healthy subjects (Table 2), which was supported more clearly by the rarefaction analysis (Fig. 3).

Ocular Microbial Communities of Blepharitis and Healthy Subjects

To compare the ocular bacterial taxa compositions of blepharitis and healthy subjects, the bacterial 16S rRNA sequencing reads of individual subjects were classified using the RDP naive Bayesian rRNA Classifier at both phylum and genus levels (Fig. 4). At a 80% confidence threshold in the RDP Classifier, the 16S rRNA gene sequencing reads of eyelash and tear samples were classified into 12 bacterial phyla, and most sequences were affiliated predominantly with five phyla: *Actinobacteria* (0.06%–95.53%), *Proteobacteria* (0.45%–99.67%), *Firmicutes* (0.17%–84.23%), *Cyanobacteria* (0–44.38%), or *Bacteroidetes* (0–32.48%), which together accounted for 88.89% to 100% of all sequencing reads (Fig. 4A). The relative abundances of the five prevalent phyla in each subject were significantly variable, depending on individual and sample type. Interestingly, some subjects were predominated by a single phylum. For example, subject B4-E-L was predominated by *Proteobacteria*, with 99.67% abundance of total sequencing reads, while subject B3-E-L was predominated by *Actinobacteria*, with 95.53% abundance, and *Proteobacteria* accounting for only 3.03% of the total sequencing reads in the same sample. Eyelash samples had higher variability than tear samples in terms of the relative abundances of the prevalent phyla in each sample, possibly because eyelids have less consistent conditions due to environmental exposures, as compared to the bulbar conjunctiva. The bacterial reads belonging to *Fusobacteria* (0–2.61%), *Planctomycetes* (0–0.61%), *Acidobacteria* (0–1.02%), *OP10* (0–0.69%), *TM7* (0–0.25%), *Deinococcus-Thermus* (0–0.45%), and *Spirochaetes* (0–0.44%) also were found as minor groups.

At the genus level, most 16S rRNA gene sequencing reads from eyelash and tear samples were categorized into 24 bacterial genera (Fig. 4A). Among these, five genera, *Propionibacterium*, *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter*, were identified as common ocular bacteria in most subjects; however, B4-E-L (0.29%) and B4-E-R (13.42%)

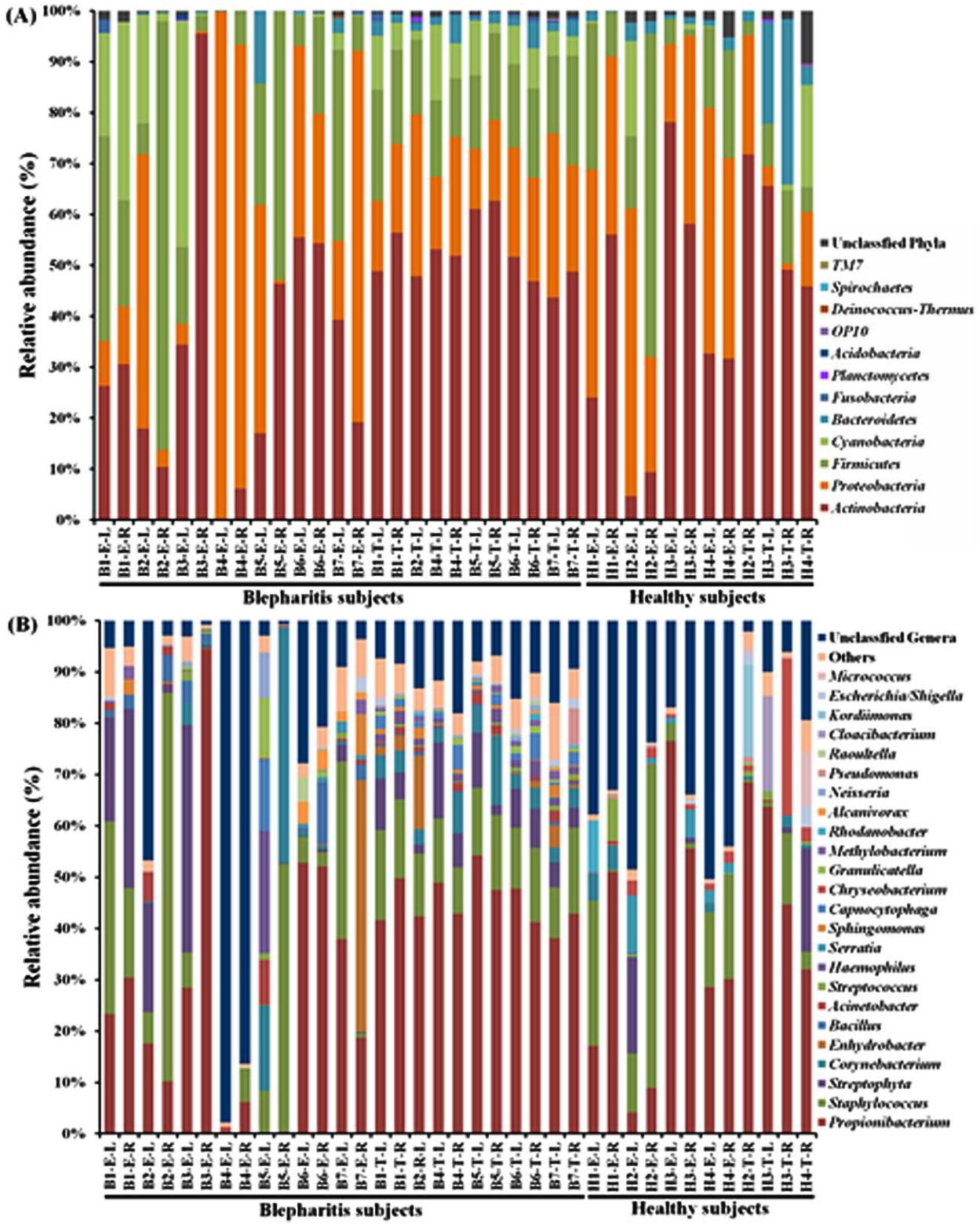

FIGURE 2. Relative bacterial compositions of eyelash and tear samples from blepharitis patients and healthy controls. Partial 16S rRNA gene sequences were classified into phylum (**A**) and genus (**B**) levels using the RDP naive Bayesian rRNA Classifier based on the RDP 16S rRNA gene database at an 80% confidence threshold. Others in panel (**B**) are composed of the genera, each showing a percentage of reads <3.0% of the total reads in all of the subjects.

Table 2. Summary of the Pyrosequencing and Statistical Data of Bacterial Communities of Eyelash and Tear Samples from Blepharitis Patients and Healthy Controls

Subject*	No. of Reads	No. of High Quality Reads	Average Read Length (bp)	OTUs†	Shannon-Weaver Index (H')†	Chao1†	Evenness (E)†
B1-E-L	4784	4488	482	102	2.47	168.23	0.53
B1-E-R	2109	2010	477	49	1.86	70.11	0.48
B1-T-L	2161	1706	484	127	3.11	183.89	0.64
B1-T-R	2785	2454	487	140	3.07	179.06	0.62
B2-E-L	7147	6532	485	74	1.78	101.00	0.41
B2-E-R	4684	4357	495	94	1.76	123.06	0.39
B2-T-L	2519	2292	487	146	2.98	235.44	0.60
B3-E-L	4899	4608	471	103	2.06	140.00	0.45
B3-E-R	3300	3134	485	52	1.05	69.00	0.27
B4-E-L	5905	5213	507	34	0.78	37.50	0.22
B4-E-R	3360	2988	506	29	0.94	34.14	0.28
B4-T-L	1470	1240	483	73	2.57	100.00	0.60
B4-T-R	737	420	487	50	2.42	96.43	0.62
B5-E-L	1388	1312	490	56	2.82	87.63	0.70
B5-E-R	2283	2056	490	36	0.99	64.50	0.28
B5-T-L	1120	758	483	61	2.30	82.00	0.56
B5-T-R	1270	1061	487	89	2.75	109.71	0.61
B6-E-L	1453	1352	486	42	1.90	62.00	0.51
B6-E-R	2102	1942	490	88	2.19	145.27	0.49
B6-T-L	1000	857	485	98	2.89	137.00	0.63
B6-T-R	1702	1288	484	111	3.13	209.00	0.66
B7-E-L	2149	2001	485	74	2.40	111.50	0.56
B7-E-R	4051	3772	482	126	2.50	171.56	0.52
B7-T-L	1725	1606	482	177	3.60	242.21	0.70
B7-T-R	1995	1516	486	119	3.11	136.22	0.65
H1-E-L	2579	2360	496	59	2.13	78.13	0.52
H1-E-R	4726	4424	493	80	2.06	107.00	0.47
H2-E-L	2649	2358	477	128	2.29	195.50	0.47
H2-E-R	2835	2613	488	100	2.06	139.06	0.45
H2-T-R	3284	184	476	27	1.78	40.00	0.54
H3-E-L	1494	1217	480	67	2.56	92.30	0.61
H3-E-R	2006	1710	480	102	2.50	159.50	0.54
H3-T-L	1183	468	487	66	2.59	106.62	0.62
H3-T-R	1991	665	487	64	2.53	126.33	0.61
H4-E-L	1058	879	490	98	2.98	176.40	0.65
H4-E-R	1298	1100	490	120	3.08	246.00	0.64
H4-T-R	2950	144	481	28	2.44	68.00	0.73

OTUs were calculated by the RDP pipeline with a 97% OTU cutoff of the 16S rRNA gene sequences.

* B, blepharitis subjects; H, healthy subjects; E, eyelash; T, tear; L, left eye; R, right eye.

† Diversity indices of the microbial communities were calculated using the RDP pyrosequencing pipeline based on the 16S rRNA gene sequences.

had very low overall abundances of the five major genera because these subjects contained high proportions of the previously unclassified genera. The relative abundances of the prevalent genera and unclassified bacterial phylotypes also varied significantly depending on individual and sample type. For example, subject B4-E-L was predominated by only a single genus, *Propionibacterium*, with 94.48% abundance of total reads, while trace sequencing reads belonging to *Staphylococcus* and *Corynebacterium* were detected from the same subject (<0.017%). Also, the genus *Propionibacterium* accounted for only 0.29% in subject B5-E-R, but *Staphylococcus* and *Corynebacterium* represented 52.24% and 45.87% of the total reads in the same subject, respectively.

Statistical Comparisons of Ocular Microbial Communities of Blepharitis and Healthy Subjects

The bacterial compositions of eyelash and tear subjects from humans with and without blepharitis were assessed statistically using a phylogeny-based metric, UniFrac based on representative sequences derived from all culled 16S rRNA gene sequences of the individual subjects. As shown in Figure 5, although there were some exceptions, intrapersonal bacterial communities of eyelash subjects were relatively well clustered compared to the interpersonal bacterial communities, which was consistent with previous results that interpersonal variability was high, whereas individuals exhibited less internal variability.[22] Bacterial communities of tear samples from blepharitis subjects were well clustered from those of eyelash samples or those of healthy tear subjects. PCoA also demonstrated that tear samples from blepharitis subjects were distinguished clearly from those of other subjects, which suggested that some genera or microbiota representing human blepharitis may be present in the conjunctiva in these patients. However, eyelash samples from subjects with blepharitis were not clearly statistically different (Fig. 6), which might be explained by the fact that microbial communities of eyelashes can be influenced easily by external factors.

Although the bacterial communities of tear samples from blepharitis patients were relatively well distinguished from

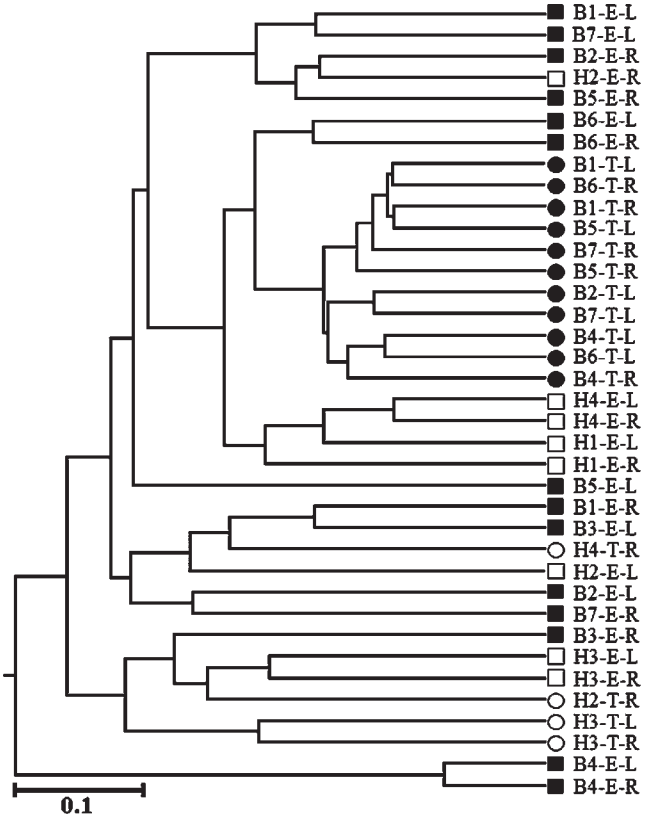

Figure 3. Hierarchical clustering of bacterial communities using the weighted UniFrac UPGMA clustering method in eyelash and tear samples of blepharitis patients and healthy controls. Scale bar represents the weighted UniFrac distance. Respective symbols represent bacterial communities of eyelash (■) and tear (●) samples from blepharitis patients, and eyelash (□) and tear (○) samples from healthy controls.

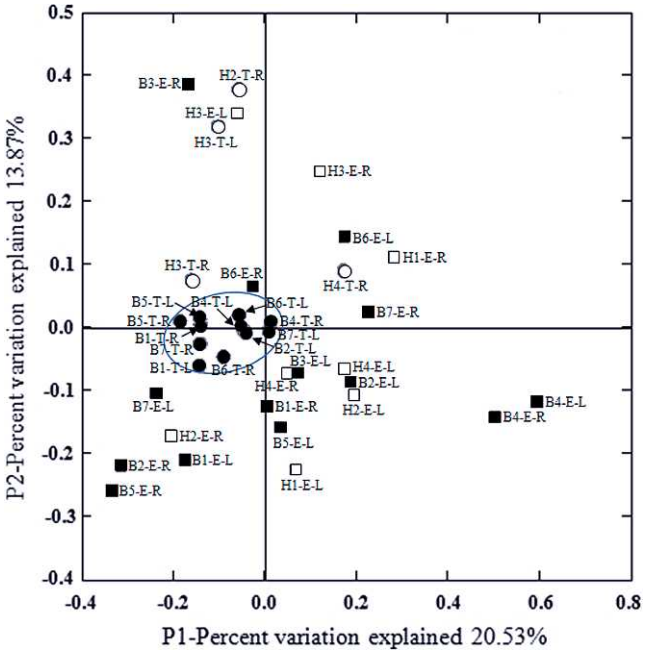

Figure 4. PCoA results showing the relationships of bacterial communities in eyelash and tear samples of blepharitis patients and healthy controls. The PCoA plot was constructed using the weighted UniFrac method. Respective symbols represent bacterial communities of eyelash (■) and tear (●) samples from subjects with blepharitis, and eyelash (□) and tear (○) samples from healthy subjects.

those of other tear and eyelash samples, their bacterial communities had too much interpersonal variability to compare their bacterial differences. Therefore, relative bacterial mean abundances of eyelash and tear samples of blepharitis patients and healthy controls were calculated (Fig. 1). At the phylum level, the results clearly showed that five phyla, *Actinobacteria*, *Proteobacteria*, *Fimicutes*, *Cyanobacteria*, and *Bacteroidetes*, were predominant in all eyelash and tear samples (Figs. 1A, 4A). The relative proportions of *Actinobacteria* in eyelash and tear samples with blepharitis were slightly lower than those in healthy subjects. The relative proportions of *Proteobacteria* and *Fimicutes* in eyelash samples from blepharitis patients and healthy controls were similar, while the relative proportions of *Proteobacteria* and *Fimicutes* in tear samples from patients with blepharitis were clearly higher than those in tear samples from healthy controls. Surprisingly, the relative proportions of *Cyanobacteria*, whose source may be plant material, such as pollen, in eyelash and tear samples from blepharitis patients were clearly higher than in those from healthy controls. Interestingly, tear samples of healthy controls had a significantly higher proportion of *Bacteroidetes* than did other tear and eyelash samples, which suggests that *Bacteroidetes* might be important as a resident commensal microbiota and may contribute to the prevention of blepharitis.[38] The genus level analysis demonstrated that *Propionibacterium*, *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* were common ocular bacteria in all eyelash and tear samples, regardless of the presence of blepharitis (Fig. 1B); these results differed slightly from prior results that *Pseudomonas*, *Bradyrhizobium*, *Propionibacterium*, *Acinetobacter*, *Corynebacterium*, and *Staphylococcus* were dominant in healthy human conjunctiva.[24] This discrepancy may be caused by differences of individuals, sampling methods, and sample types.

Subjects with blepharitis had lower proportions of *Propionibacterium* than those of healthy subject, whereas the relative proportions of *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* in eyelash and tear samples from blepharitis patients were higher than those of healthy controls. The relative proportions of *Staphylococcus* in eyelash samples from blepharitis patients and healthy controls were similar, while the relative proportions of *Staphylococcus* in tear samples from blepharitis patients clearly were higher than those in tear samples from healthy controls. Figure 1B shows that *Chryseobacterium* was identified as one of the major populations from tear samples of healthy controls (6.35%). However, because its high proportion in healthy tear samples was found in only one tear sample (H3-T-R; Fig. 4B), the high proportion of *Chryseobacterium* was not considered to be normal flora of the ocular microbiome, and *Chryseobacterium* was excluded from the following discussion. The correlation between sample type and microbial community was confirmed by redundancy analysis (Fig. 2). The distributions of subject type and microbial community in the ordination space, as determined by RDA, clearly highlighted that subjects with blepharitis had more abundant *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* than healthy subjects (Fig. 1B). The RDA results also highlighted the uniqueness of the tear samples, mainly due to the abundance of the genus *Propionibacterium* (Fig. 2).

Discussion

An understanding of the ocular microbial community is essential for the prevention and treatment of blepharitis, as the ocular microbiota contributes to infection and prevention of

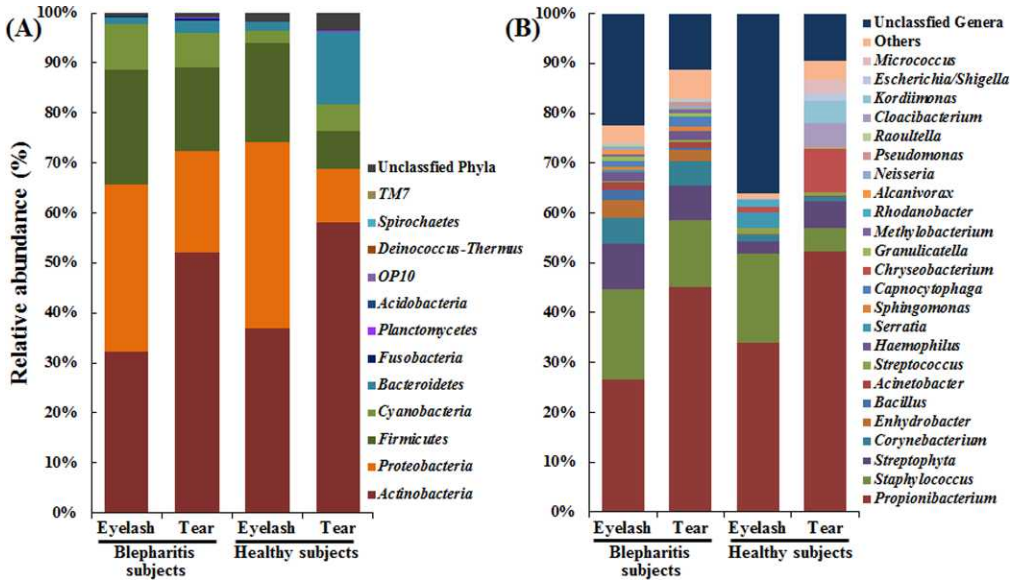

Figure 5. Relative bacterial mean abundances in eyelash and tear samples from blepharitis patients and healthy controls at phylum (**A**) and genus (**B**) levels. The relative bacterial mean abundances were calculated by the mean values of relative phylotypic compositions of respective eyelash and tear samples. Others in panel (**B**) are composed of the genera each showing a percentage of reads <3.0% of the total reads in all subjects.

eye diseases. Many researchers have analyzed ocular microbial communities, and members of the genera *Propionibacterium*, *Staphylococcus*, *Acinetobacter*, and *Corynebacterium* have been identified as major microbiota from eye conjunctivas or lids with blepharitis.[16,19,39,40] Comparative community analysis using culture-based approaches found that the ocular microbial communities of patients with and without blepharitis could differ in terms of their relative abundance proportions,[15,16,18] which suggested that the differences in ocular microbial communities, especially in the relative abundance of *Propionibacterium* and *Staphylococcus*, might contribute to the occurrence of blepharitis. However, culture-based approaches have many limitations in terms of the culturability of microorganisms. Recently, a culture-independent approach based on pyrosequencing demonstrated that *Pseudomonas*, *Propionibacterium*, *Bradyrhizobium*, *Corynebacterium*, *Acinetobacter*, *Brevundimonas*, and *Staphylococcus*, as well as many other previously undescribed bacteria, were identified from healthy human conjunctiva.[24] Therefore, in our current study, we applied a massively parallel pyrosequencing strategy to compare the ocular microbial communities of humans with and without blepharitis, which potentially will be very helpful in understanding the occurrence and treatment of human blepharitis on the basis of ocular microbial flora.

Groden et al.[16] demonstrated that members of *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, and *Acinetobacter* were identified as the most common isolates from all lids, but that normal skin bacteria, such as *Staphylococcus* and *Propionibacterium*, were isolated in greater quantities from lids with blepharitis. Kulaçoğlu et al. reported that mixed skin microbial flora, including *P. acnes*, were found in blepharitis patients and healthy controls, but *Staphylococcus* and some other skin microbial flora, such as *Prevotella* and *Bacteriodes*, were not found in healthy controls.[18] These previous reports suggest that skin microbial flora can be a cause of human blepharitis on the basis of ocular microbial flora. Our analysis demonstrated that *Propionibacterium* and *Staphylococcus*, as well as *Streptophyta*, *Corynebacterium*, and *Enhydrobacter*, were identified as the most common ocular microbial flora, regardless of the occurrence of blepharitis, but that their compositions were different depending on sample types and the occurrence of blepharitis (Fig. 1B). The relative proportions of *Staphylococcus*, *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* were higher in subjects with blepharitis than in healthy subjects, especially in tear samples; however, surprisingly, the proportions of *Propionibacterium* clearly were lower in subjects with blepharitis than in healthy subjects (Fig. 1B), which suggests that *Propionibacterium* might be important as a resident commensal microbiota for the prevention of blepharitis. The relative proportion of *Staphylococcus* was clearly higher especially in tear samples from subjects with blepharitis than in healthy tear samples, which supports previous results that found that elevated levels of skin microbial flora, such as *Staphylococcus*, in the eye can be a cause of human blepharitis.[16,25,41] The

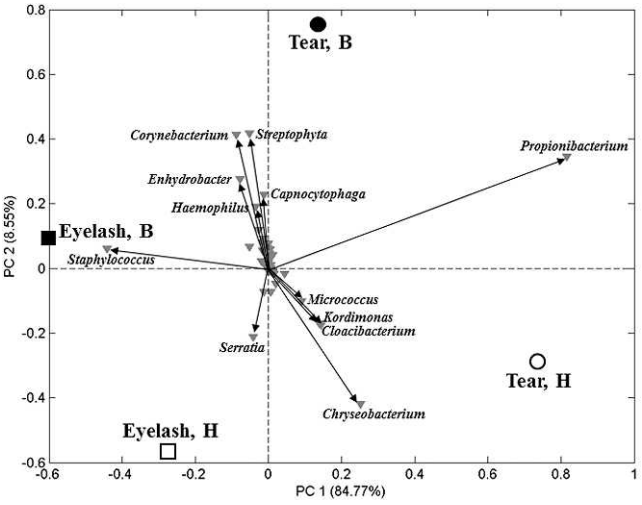

Figure 6. Ordination biplot of RDA showing correlations between subject types and microbial communities of Figure 5B. Subject types are represented by *circles* and *rectangles* (*closed* or *open*), and genera are represented by *inverted triangles*. Genera unclassified by the RDP classifier were not used in the analysis. *Arrow*: directions point toward maximal abundance, and their lengths are proportional to the maximal rate of change between subject types.

phylotypic and statistical redundancy analyses demonstrated clearly that the relative abundances of *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* were higher in tear samples from subjects with blepharitis than in healthy tear subjects (Figs. 1, 2), which is supported by a previous report that *Corynebacterium* elicited human blepharitis by immunoreactivity.[17] These results suggested that human blepharitis might be induced by infestations of mixed skin microbial flora, as well as plant pollens, dusts, and soil particles, because pollens, dusts, and soil are the main sources of the genera *Streptophyta*, *Corynebacterium*, and *Enhydrobacter*. In previous studies, *Pseudomonas aeruginosa* was cultured from blepharitis subjects[18,42] and *Pseudomonas* represented one of the major genera in healthy conjunctiva; however, our analysis showed that *Pseudomonas* was detected in minor abundance in all subjects, regardless of the occurrence of blepharitis (Figs. 4B, 1B).

Demodex mites are the most common permanent ectoparasites in human skin.[43] They are easily found, especially from infundibular portions of pilous follicles of the eyelash, small hair sebaceous glands, meibomian glands, face, and external otic tract, where active sebum excretion provides a favorable habitat for breeding.[44] Some prior studies have reported that *Demodex* or *Demodex*-related *Bacillus* might contribute to the occurrence of blepharitis.[8–11] However, in our analysis, the incidence of *Demodex* mites did not demonstrate a clear correlation with ocular microbial community, although sample sizes were too small to allow for a statistical comparison. Despite the finding that *Demodex* mites are found more frequently in blepharitis patients, there is controversy as to whether *Demodex* is a cause of blepharitis, since blepharitis symptoms often are found in humans not associated with blepharitis.[12,13] Therefore, the frequent discovery of *Demodex* mites from blepharitis patients may not reflect the cause of blepharitis occurrence, but rather a result of blepharitis because active sebum excretion caused by blepharitis can provide favorable conditions for *Demodex* mites. Although *Demodex* mites may worsen blepharitis symptoms, additional investigations are required to clarify these hypotheses. In our current study, we compared ocular microbial communities of humans with and without blepharitis using pyrosequencing and suggested that some ocular microbiota can contribute to infection and prevention of human blepharitis. Our analysis demonstrated that many bacteria known as ocular surface pathogens were identified with high abundance in ocular samples, regardless of the occurrence of blepharitis, as reported previously,[24,26] but that their compositions were different depending on the occurrence of blepharitis. These results suggested that the balance or the commensal growth between ocular microbiota might be important for the prevention of blepharitis, because ocular health and blepharitis may depend on the interplay between the eye and the ocular microbial community. However, further studies at species or strain levels will be required to test the validity of this hypothesis. Continued investigations of ocular microbial communities are required to add valuable information for the prevention and treatment of human blepharitis, because the roles of the ocular microbial community in humans with and without blepharitis are unknown.

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