Diversity of Bacteria at Healthy Human Conjunctiva

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Purpose. Ocular surface (OS) microbiota contributes to infectious and autoimmune diseases of the eye. Comprehensive analysis of microbial diversity at the OS has been impossible because of the limitations of conventional cultivation techniques. This pilot study aimed to explore true diversity of human OS microbiota using DNA sequencing-based detection and identification of bacteria.

Methods. Composition of the bacterial community was characterized using deep sequencing of the 16S rRNA gene amplicon libraries generated from total conjunctival swab DNA. The DNA sequences were classified and the diversity parameters measured using bioinformatics software ESPRIT and MOTHUR and tools available through the Ribosomal Database Project-II (RDP-II).

Results. Deep sequencing of conjunctival rDNA from four subjects yielded a total of 115,003 quality DNA reads, corresponding to 221 species-level phylotypes per subject. The combined bacterial community classified into 5 phyla and 59 distinct genera. However, 31% of all DNA reads belonged to unclassified or novel bacteria. The intersubject variability of individual OS microbiomes was very significant. Regardless, 12 genera—*Pseudomonas, Propionibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas, Staphylococci, Aquabacterium, Sphingomonas, Streptococcus, Streptophyta,* and *Methylobacterium*—were ubiquitous among the analyzed cohort and represented the putative "core" of conjunctival microbiota. The other 47 genera accounted for <4% of the classified portion of this microbiome. Unexpectedly, healthy conjunctiva contained many genera that are commonly identified as ocular surface pathogens.

Conclusions. The first DNA sequencing-based survey of bacterial population at the conjunctiva have revealed an unexpectedly diverse microbial community. All analyzed samples contained ubiquitous (core) genera that included commensal, environmental, and opportunistic pathogenic bacteria. (*Invest Ophthalmol Vis Sci.* 2011;52:5408–5413) DOI:10.1167/iovs.10-6939

Together with corneal epithelium and tear film, the conjunctival epithelium forms a barrier to ocular infection. This epithelium also represents an initial interface between the diverse and abundant skin microbiota and the ocular surface (OS) of the host. Numerous studies have examined the microbiota of the OS using traditional microbiology techniques.[1–5] However, traditional culture-based methods detect only a fraction of the microbiota, whereas even a limited survey of approximately 1000 individual 16S rRNA sequences indicated the presence of a significantly more abundant bacterial community on healthy conjunctiva.[6,7]

The commensal microbiota is thought to have coevolved with humans. It has been shown to "educate" the immune system during maturation[8] and to induce "tolerogenic" dendritic cells and "attenuated" macrophages during gut colonization.[9,10] Although the molecular details of the crosstalk between the microbiota and the host remain to be investigated, it was shown to involve the participation of pattern-recognition receptors.[11–13] Pathologic shifts in the indigenous microbial community can cause dramatic overexpression of these receptors in allergic tissues, such as in the conjunctiva of patients with vernal keratoconjunctivitis.[12] Mutually beneficial coexistence of the host with microbiota in the gut, oral and nasal cavities, lungs, and urogenital epithelia raised the following questions about ocular microbiota: is ocular microbiome a stable community of commensal species or is it composed of random transient species introduced from the environment? Does healthy OS normal microbiome contain opportunistic pathogens? Do OS infections result from the invasion of exogenous pathogens or from an increased virulence of indigenous species? In search of answers to these questions, we used the 16S rRNA gene-based sequencing approach to characterize bacteria at the ocular surface.

Materials and Methods

Conjunctival Swab Collection

The authors of this study adhered to the tenets of the Declaration of Helsinki. All experiments were performed in compliance with the protocol (no. 20070960) approved by the University of Miami Miller School of Medicine Institutional Review Board. Written informed consent was obtained from all study subjects (see the form in Appendix 4). To minimize variability introduced by sex, age, race, and ethnicity, we sampled a very narrow population of four healthy, non-Latino, Caucasian, 26- to 48-year-old male volunteers. Volunteer information included sex, age, general health status, and ocular health status. The volunteers did not wear contact lenses and had medical histories free of systemic and ocular diseases, ocular traumas/transplantations, and

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recent (6-month) history of antibiotic treatment. We chose to select this homogeny of the subject group to assess basal individual variability of conjunctival microbiome and to determine bacteria representing the core of the conjunctiva microbiome.

The bulbar conjunctiva on the inferior lids of both eyes (including fornices) were sampled concurrently with small cotton swabs (Dacron; Medical Packaging Corp., Camarillo, CA) two or three times to obtain a pooled sample containing an average of 241 ng total DNA from each volunteer. Samples were collected using repetitive dry cotton swabs applied with slight pressure. We collected four tarsal conjunctival samples using small dry cotton swabs (Puritan; Medical Packaging Corp.). To test whether the mucosal layer of the conjunctiva contained a distinct microbial community, one volunteer was resampled with a wet cotton swab applied on the tarsal conjunctiva with minimal pressure 1 week after the initial sampling.

DNA Extraction and PCR Amplification

The conjunctival swabs were placed into a 1.7-mL tube containing 300 μL aqueous suspending solution (Beads Solution; Bangs Laboratories, Fishers, IN) and was vortexed for 5 minutes before 60 μL solution (MD1; PowerSoil DNA Isolation Kit, MoBio, Carlsbad, CA) was added. The bead tubes were capped and processed according to the manufacturer's instructions to ensure complete homogenization and microbial cell lysis. The extracted DNA quality was assessed by platform analysis (Bioanalyzer; Agilent Technologies, Santa Clara, CA) and quantitative PCR amplification of the 16S rRNA gene, where the absence of nonspecific bands was closely monitored. Universal primers (PSL forward, 5′-AGG ATT AGA TAC CCT GGT AGT-3′; PSR reverse, 5′-ACT TAA CCC AAC ATC TCA CGA CAC-3′) were used for the 16S rRNA-encoding DNA, as reported earlier.[7] The PCR conditions were 96°C for 5 minutes, followed by 40 cycles of 96°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Serial dilutions of the known quantities of *Escherichia coli* 16S rRNA amplicon were used to generate standard curves. Sample blanks consisted of unused swabs processed through DNA extraction and tested to contain no 16S amplicons.

MDA Amplification and 16S rRNA Gene Sequencing

After the assessment of genomic DNA quality and concentration, two replicates of multiple displacement amplification (MDA) were performed with each biological sample (GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ). After MDA, PCR-generated amplicon libraries were constructed using sequencing primers specific to the V3-V4 region of the 16S gene (*E. coli* positions 338–802[14]) reported by Fierer et. al.[15] The 27F-5 forward primer (5′-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3′) contained the 454 Life Sciences primer B, the broadly conserved bacterial primer 27F, and a two-base linker sequence (TC″). The reverse primer (5′-GCCTCCCTCGCGCCATCAGNNNNNNNNCATGCTGCCTCCCGTAGGAGT-3′) contained the 454 Life Sciences primer A, the bacterial primer 338R, a CA″ inserted as a linker between the rRNA primer and a unique 8-bp bar code used to tag each PCR product (designated by NNNNNNNN). Primers contained 454-specific adapter sequences as well as a bioinformatic "bar code" key sequence, as described earlier.[16] This approach allows sequencing multiple samples without physical partitioning.[13,17]

Each 30 μL PCR reaction was performed in triplicate using *Taq* DNA polymerase (Platinum High Fidelity; Invitrogen, Carlsbad, CA) and contained 1 μM of each forward and reverse primers, 3 μL template DNA, and 22.5 μL PCR super mix (Platinum; Invitrogen). Samples were denatured at 94°C for 3 minutes and amplified for 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. Final extension at 72°C for 10 minutes ensured complete amplification of the target region. All tagged samples were sequenced in a single 454 run of the reagent (GS-FLX 454; Roche Life Sciences, Branford, CT) instrument run to avoid interexperimental variation. Negative controls, including no template and template from unused swabs, were included in all steps to control potential primer or sample DNA contamination.

Bioinformatics Analysis

Sequences were trimmed of primers and classified using bioinformatics tools available through the RDP-II Classifier and assigned to the corresponding sample based on the 8-bp sample identifier tag. Only sequences that were longer than 200 bp and had average quality scores ≥25 according to 454 Roche quality control, were included in further analyses. They were grouped into operational taxonomic units (OTUs) using the furthest-neighbor algorithm and 3% genetic difference as a cutoff to define phylotypes using a high-performance software package (ESPRIT).[18] Therefore, all the sequence reads sharing at least 97% identity were clustered as distinct "species-level" phylotypes for each sample. The microbial diversity in individual OS samples was estimated using rarefaction analysis and Shannon diversity index (SDI), computed with the MOTHUR package (www.mothur.org). 16S rRNA–based sequences were classified from phylum down to the genus level using the RDP Classifier (version 2.2) and a 90% confidence cutoff. We calculated the Chao1 estimator to assess the species richness of the samples, as previously described.[19] Depersonalized bacterial sequence data and the results of initial analysis are available at the project's Web site (http://www.microbiota.org/ocular/).

Results and Discussion

16S rRNA Sequences from OS Samples

Sequencing of four biological samples from four subjects in two replicates generated a total of 39.7 million base pairs (bp) corresponding to an average of 16.8 thousand 16S rRNA gene reads per sample. After the removal of sequences of insufficient quality, a total of 115,003 high-quality reads (average length, 236 bp) were used in the further analysis of the composition of the OS microbiome. These sequences were classified according to bacterial taxonomy using the RDP Classifier (version 2.2)[20] with a 90% confidence cutoff threshold and were further analyzed for ecological diversity measurements. Overall, the quality sequencing data averaged 14,375 reads per replicate, ranging between 3,360 and 25,601 reads. Rarefaction analysis of sequencing "depth" (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental) indicated that species representation in each conjunctival sample had entered the plateau phase (i.e., novel bacteria would unlikely be recovered with additional sequencing efforts). In this study, to increase the absolute amount of total bacterial DNA in human OS samples, we applied MDA before 16S amplicon library construction using the GenomiPhi V2 protocol, which is known to produce near linear DNA amplification with the least amount of bias.[21,22] However, a recent study[23] indicated that MDA may introduce a bias affecting subsequent quantitative analysis. Therefore, though MDA can effectively compensate for low bacterial biomass in human OS samples, this additional amplification step may be omitted if quantitative analysis is the primary focus.

On average, we were able to classify 87.9% ± 16.4% of the obtained 16S sequences to the phylum level (Fig. 1A; Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental) and 69.3% ± 23.5% to the genus level (Fig. 1B; Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). Using the 3% cutoff level as the chosen parameter to distinguish "species-level" nonredundant bacterial phylotypes (OTUs) in the RDP-II Classifier, we identified an average of 2,137 unique phylotypes per sample. This corresponded to an average of 221 phylotypes per individual microbiome (Table 1), which is in line with the 266 phylotypes

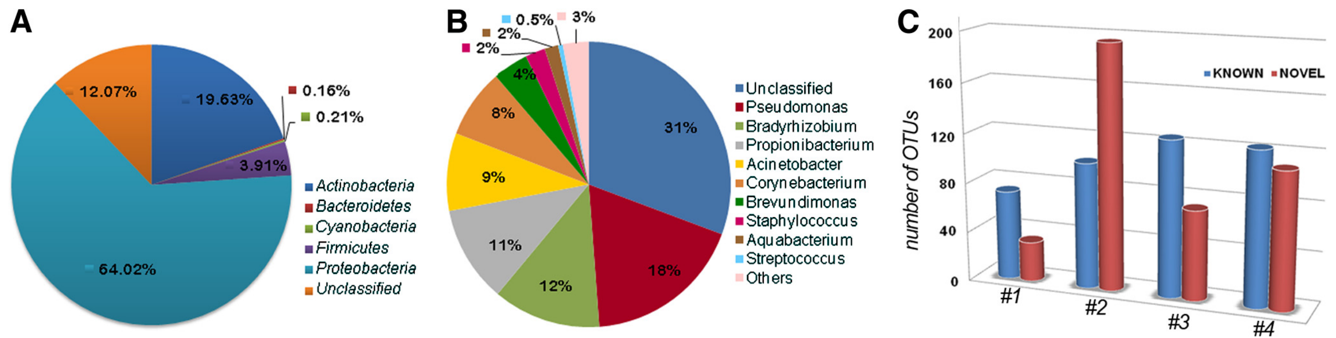

FIGURE 1. Relative abundance of bacterial taxa in the conjunctiva. (**A**) Phylum-level representation of the bacteria at the OS of the four subjects calculated according to relative abundance of classified 16S rRNA gene reads. The percentage of reads that failed to classify to known bacterial phyla is indicated as Unclassified, shown in orange. The circular diagram presents average values calculated for all analyzed subjects. Color-coding legend on the right shows taxonomic identities of the classified bacteria. (**B**) Genus-level representation of the bacteria at the OS. Unclassified reads (31% of the total 115,003 sequences) are shown in *dark blue*. (**C**) Relative abundance of known (16S-classified) and novel (unclassified) bacterial phylotypes at the conjunctiva of the individual subjects. All percentages were calculated relative to the total number of qualified DNA reads for each individual.

identified in the microbiome of the oral mucosa.[24] The estimated numbers of OTUs are meant to provide an upper bound of the phylotypes in the observed ocular microbiome because potential sequencing errors and chimeric sequences may inflate the true number of species. Genera representation in pairs of technical replicates was nearly identical (see Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental), indicating that the sequence data indeed represent a snapshot of the homeostatic microbiomes rather than random events such as contamination. Interestingly, the RDP-II Classifier was unable to classify an average of 31% of sequences to the genus level (Fig. 1B). The phylotypes in this category are missing in the Classifier RDP-II database, suggesting that most of them likely represent novel bacteria. The representation of unclassified bacterial phylotypes (designated as novel) varied significantly among the subjects (Fig. 1C).

Bacterial Community Composition at Tarsal Conjunctiva

To identify bacterial taxa composition of the human ocular microbiome, the 16S rRNA sequences were classified at both the phylum and the genus levels (Supplementary Table S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). At the 90% confidence cutoff level set in the RDP Classifier software, all DNA reads represented five bacterial phyla, three of which—*Proteobacteria* (64%), *Actinobacteria* (19.6%), and *Firmicutes* (3.9%)—accounted for >87.9% of all sequences (Fig. 1A). The other two phyla, *Cyanobacteria* and *Bacteroidetes*, were found in contamination-level quantities (0.21% and 0.16%, respectively). Therefore, these bacteria were not considered to be normal components of ocular microbiome and were excluded from further analysis.

At the genus level, 69.3% of all the sequence reads were categorized into 59 distinct bacterial genera. Twelve of those genera were ubiquitous among the subjects examined. Five of those—*Pseudomonas*, *Bradyrhizobium, Propionibacterium, Acinetobacter*, and *Corynebacterium*—were the most abundant and accounted for 58% of all detected sequence reads and for >92% of RDP-classified sequence reads (known bacteria). Together with four other genera (*Brevundimonas, Sphyngomonas, Staphylococcus*, and *Steptococcus*), these ubiquitous bacteria accounted for >96% of classified sequence reads (Fig. 1B).

As has often been reported in microbiologic studies, commensal bacterial population of the ocular surface is dominated by Gram-positive species of *Staphylococci, Corynebacterium, Propionibacterium,* and *Streptococci*.[5,25–27] Therefore, our discovery of the diverse microbiome of a different and more complex structure at the OS was surprising. According to our PubMed search, 42 of 59 the classified bacterial genera have not been previously reported in healthy eyes (Supplementary Table S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). The OS microbiome differs from that in the oral cavity, which is dominated by *Firmicutes* (*Neisseria* and *Streptococcus*),[28] or in airways, which are largely populated by *Actinobacterium* (*Corynebacterium, Aureobacterium,* and *Rhodococcus*).[29] At the same time, the OS microbial community bears similarity with that on the skin, which is also enriched with the *Proteobacteria* phylum.[30]

It is important to emphasize that our data showed dramatically different prevalence and greater diversity at the genus level than typically revealed by culture-based methods. Microbiologic surveys of OS performed earlier by us[25,31] and others[32,33] revealed the highest incidence of *Staphylococci, Propionibacterium,* and *Corynebacterium* species. In contrast, the 16S rRNA gene sequencing showed the highest prevalence of *Pseudomonas*, *Propionibacterium*, and *Bradyrhizobium*, with only 4% of *Staphylococcus* spp. Our current analysis reveals more than three times higher diversity than culture methods.[31] The disparity between the microbiologic and molecular approaches most likely reflects the fact that culture-

TABLE 1. OTU-Based Analysis of Conjunctival DNA Sequencing Data

Sample Type	Sample ID*	Total OTUs (3% difference)	Chao 1 (diversity)	SDI†
Surface swab	2ss/R1	249	322	2.72
	2ss/R2	236	311	2.47
Deep swab	1/R1	104	150	3.10
	1/R2	102	278	3.07
	2/R1	240	355	3.13
	2/R2	351	437	3.37
	3/R1	232	291	3.21
	3/R2	160	186	2.92
	4/R1	203	258	2.72
	4/R2	332	449	3.18
	Average	**220.9**	**303.7**	**3.09**
	SDEV	83.4	95.6	0.23

Bold numbers are the averages of all sequenced samples.
* R1/R2, technical replicates.
† Shannon Diversity Index.

based detection is biased toward fast-growing bacteria that can be easily cultivated on standard media.[34–37]

Does the OS Microbiome Have a Core of Ubiquitous Species?

Studies of human microbiome were divisive on determining the "core" bacterial taxa of gut and skin.[24,38,39] The latest analysis suggests that instead of the core taxa, homeostatic communities are defined by the presence of a core microbial gene set that encodes essential metabolic pathways.[40,41] However, our analysis of genus prevalence revealed that 12 of 59 genera were ubiquitous among all examined subjects. This qualifies these genera as a putative core of the OS microbiome. The 10 most prevalent (defined as >1% of all detected genera) included *Pseudomonas* (20%), *Propionibacterium* (20%), *Bradyrhizobium* (16%), *Corynebacteria* (15%), *Acinetobacter* (12%), *Brevundimonas* (5%), *Staphylococcus* (4%), *Aquabacterium* (2%), *Sphingomonas* (1%), and *Streptococcus* (1%) (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). Given the small sample size in this pilot study, it is premature to conclude that this genus subset is the true core of this microbiome. However, the fact that the nine genera accounted for 96% of known bacterial sequences (Fig. 1B) strongly suggests that the healthy conjunctiva is colonized by ubiquitous homeostatic taxa.

Individual Variability and Potential Contribution of the Environment

There was significant variability among the analyzed subjects in terms of the relative abundance of prevalent genera (Fig. 2). The SDI measure of individual variability among all samples was 3.09 + 0.18 (Table 1). Interestingly, some subjects showed high domination by a single genus. For example, *Corynebacteria* accounted for 47% of total identifiable reads (OTUs) in subject 2 and species of *Pseudomonaceae* represented 69% in subject 4, while the second most abundant genus, *Propionibacterium,* accounted for only 1.2% in the same sample (Fig. 2; Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). Examples of a single genus overgrowing other genera are not unique to the eye and were reported for other human microbiomes, such as at the skin.[42]

Despite intersubject variability, 12 bacterial genera with the most abundant DNA reads were shared among subjects (Fig. 3). This suggests that the distinct environment of the OS, which has

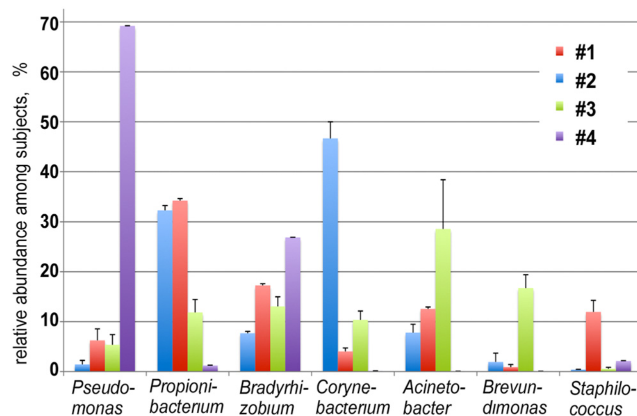

FIGURE 2. Individual variability of most abundant bacteria at the conjunctiva. Prevalence of the seven most abundant genera in the OS of the four analyzed subjects. Values represent percentages of DNA reads corresponding to the indicated genera relative to all classified reads generated from an individual sample (coded by colors).

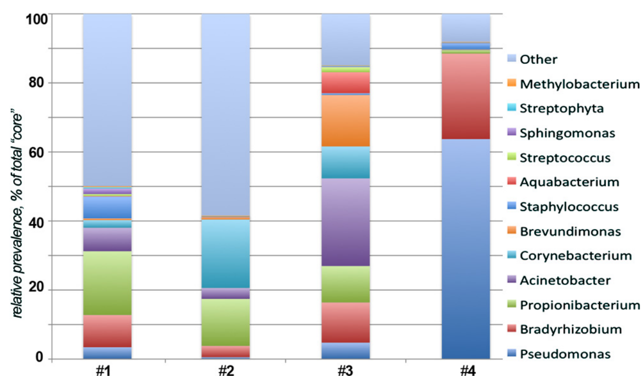

FIGURE 3. Putative core microbiome of the ocular surface. Genus-level representation of the 12 most prevalent bacteria at the OS of the four subjects calculated according to relative abundance of classified DNA reads. Percentage of DNA reads that fail to classify beyond bacteria is indicated as "other." Color-coding legend on the right side of the panel designates each genus.

less energy flow and exposure to external microbiota than other human niches, could be a significant factor that shapes the composition of the OS microbiome. Individual (between-subject) variation seemed to have impacted only relative abundances of DNA reads representing this microbial community, but not its composition. The importance of a specific environment was reported for the microbiomes of other human niches.[24,38,39] However, one cannot exclude the substantial contribution from physical interaction with the proximal human microbial communities, including skin at the eyelid margins or hands. In addition, the contact with airborne "dust" particles and contaminated water may contribute a plethora of exogenous microbial species. The latter may explain the origin of diverse environmental bacteria in the examined subjects, including autotrophic *Rhizobium* and *Bradyrhizobium*, typical soil bacteria. Overall, the species of *Bradyrhizobium* were the second most abundant genus at the conjunctiva and part of the "core" constituents of the OS microbiome, but their source remains unknown. Significantly, several studies reported abundant *Rhizobium* and *Bradyrhizobium* species in human samples from different organs.[43–45] It is noteworthy that species of *Bradyrhizobium* are common endosymbionts of the protozoans *Amoeba* and *Acanthomoeba,* which often pollute tap water and air-conditioning systems.[44,46–48] These protozoans are known to infect lungs and eyes.[45,49] Considering that the protozoan *Wolbachia* is an example of pathogen dissemination in river blindness,[50] it is reasonable to hypothesize that *Amoeba* and *Acanthamoeba* have a similar role in disseminating *Bradyrhizobia*. In line with this hypothesis, other typical endosymbionts of clinically isolated *Acanthamoeba*[51] were detected at the OS, including *Corynebacterium* spp, *Mycobacterium spp*, and *Methylobacterium* spp. Whether protozoan endosymbionts, indeed serve as "Trojan horses" in shuttling commensal and pathogenic microbiota to the OS is a subject of our future investigations.

The Effect of Sampling Depth

Collecting samples from the same individual with different swab pressure revealed significant changes in relative abundances of many microbial genera. Thus, using "deep" (dry cotton swab applied with pressure) rather than "soft" (moist cotton applied with minimal pressure) swabbing of the same conjunctiva, we observed a significantly higher abundance of reads that classify as *Proteobacteria* (*Bradyrhizobium, Delftia,* and *Sphingomonas*) in the former sample (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). In contrast, the bacterial

population retrieved by the soft swab showed overrepresentation of *Firmicutes (Staphylococci)* and *Actinobacteria (Corynebacterium* spp.) and a major reduction in *Proteobacteria* (Supplementary Table S6, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). In addition, soft swabbing recovered multiple sequences of opportunistic pathogens *Rothia* and *Herbaspirillum,* as well as environmental *Leptothrichia* and *Rhizobium* that were either minor or not detected in deep swab samples. These differences in community structure may reflect substantial vertical stratification of the conjunctival microbiome: *Staphylococci* and *Corynebacteriae* localize mostly to the mucosal layer, and *Proteobacteria* show a strong association with the conjunctival epithelium. The soft swab-captured bacteria most likely represent transient species being commonly removed from the OS by mucus flow. This is consistent with previous findings that genus composition at the surface of human skin differs from that in deeper epidermal layers.[30] Deep rather than soft swabbing is, therefore, required to recover the full diversity of the conjunctival microbiota.

Known Ocular Pathogens at Healthy OS

Many known ocular pathogens belong to the 12 core genera at the OS, including *Pseudomonas*, *Acinetobacter*, *Propionibacterium, Corynebacterium, Staphylococcus*, *Streptococcus*, and *Sphingomonas*. Six other genera— *Gordoni, Kocuria, Pantoea, Oligella, Ralstonia,* and *Delftia* (Supplementary Table S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental)—which were occasionally recovered from infected eyes (D. Miller, personal communication, 2010), are also known to contain pathogenic strains causing infection in other tissues. Overall, the OS of healthy subjects contained 24 genera that included many species of common ocular pathogens. In fact, only four genera, *Chrysobacterium, Enterobacter*, *Flavimonas*, and *Nocardia* (Supplementary Table S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental) that harbor well-known ocular pathogen strains remained undetected in this survey. The remarkable abundance and diversity of potentially pathogenic bacteria suggest that healthy OS has powerful mechanisms suppressing microbial pathogenicity. These could involve an interaction with the immune system and with commensal microbiota, as recently demonstrated.[25,52–55] It stands to reason that some ocular infections can be caused by resident opportunistic pathogens, after their virulence is enhanced by external factors, rather than by external invaders. An increase in virulence in response to trauma, stress, aging, and the depletion of resident commensal microbiota has been previously detected in other human niches.[56,57] However, further studies at species and strain levels are required to test the validity of this hypothesis.

Putative Novel Bacteria at the OS

To estimate the occurrence of potentially novel bacterial taxa in the ocular microbiome, we assessed the percentage of reads and of OTUs in each sample that fail to be assigned to any known genus in the RDP-II database. At the 90% confidence level, 30.7% of all sequence reads from the deep swab samples were assigned to this category. Even with a much more relaxed 60% confidence level, 26.5% of all reads remained unclassified (Supplementary Table S5, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6939/-/DCSupplemental). In each deep swab sample, we detected an average of 97 such reads per 216 total OTUs (Fig. 1C). This finding implies that nearly one-third of the entire bacterial population detected by 16S rRNA phylogeny is represented by novel species-level phylotypes (Fig. 3, "other"). Although a limited number of unclassified sequences can result from PCR errors or sequencing artifacts, such an abundance of unclassified reads argues for a significant presence of novel species. Our discovery of numerous novel bacteria in the OS is consistent with massive identification of novel species in other human niches.[58,59] A detailed sequence-based analysis of uncharacterized reads and their phylogeny is an interesting direction of future research; however, it was outside the scope of this pilot study.

Conclusions

This study establishes the presence of a diverse bacterial community at the healthy human OS. This community is overrepresented by a relatively small number of core genera, but it also contains a significant proportion of known pathogens. Large numbers of 16S sequence reads do not correspond to known bacteria and can be grouped into hundreds of potentially novel phylotypes at putative species level. The role of indigenous OS bacterial community in health and disease is unknown and must be investigated across population groups.

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