Hand bacterial communities vary across two different human populations

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This study utilized pyrosequencing-based phylogenetic library results to assess bacterial communities on the hands of women in Tanzania and compared these communities with bacteria assemblages on the hands of US women. Bacterial population profiles and phylogenetically based ordinate analysis demonstrated that the bacterial communities on hands were more similar for selected populations within a country than between the two countries considered. Organisms that have commonly been identified in prior human skin microbiome studies, including members of the Propionibacteriaceae, Staphylococcaceae and Streptococceae families, were highly abundant on US hands and drove the clustering of US hand microbial communities into a distinct group. The most abundant bacterial taxa on Tanzanian hands were the soil-associated Rhodobacteraceae and Nocardioidaceae. These results help to expand human microbiome results beyond US and European populations, and the identification and abundance of soil-associated bacteria on Tanzanian hands demonstrated the important role of the environment in shaping the microbial communities on human hands.

INTRODUCTION

Touch is a common way in which humans physically interact with each other, with indoor living spaces and with the outdoor environment. Clear links between bacterial communities on human skin and bacterial communities located on surfaces, flooring and in the air of the indoor environment have recently been established (Flores et al., 2011; Hospodsky et al., 2012; Kembel et al., 2012). Hands are unique in that they are frequently implicated in human exposure to fomite and water-borne pathogens, and ultimately in the transmission of infectious disease (Aiello & Larson, 2002; Luby et al., 2005; Pickering et al., 2011). An enhanced understanding of factors that shape and drive bacterial communities on human hands would be beneficial for the continuing efforts to describe the content and relevance of the human skin microbiome in health and disease.

DNA-based cutaneous bacterial diversity studies have been largely limited to US populations, and hand microbial diversity has been addressed mostly in the scope of full-body cutaneous microbial diversity assessments. In these prior observations, highly abundant bacterial taxa included members of the Propionibacteria and Corynebacteria genera, and the Streptococcaeae, Staphylococcaeae, Lactobacilaceae and Moraxellaceae families (Costello et al., 2009; Fierer et al., 2008). Additionally, populations isolated from the hypothenar palm regions showed enrichments of Gamma- and Betaproteobacteria (Grice et al., 2009). Significant differences in relative phylotype abundance within participants of one study were also observed based on gender and handedness (Fierer et al., 2008). Previous culture- and DNA-based bacterial ecology results supported the distinction of hand microbial populations between micro-organisms adapted to the skin habitat of the human host (such as pH, temperature, presence of eccrine, apocrine or sebaceous glands and thickness of epidermal cell layer) and micro-organisms that make ephemeral or spatially disconnected appearances depending on contact with the environment (Grice et al., 2009; Marples, 1965; Price, 1938). Variations in human behaviour that give

Abbreviations: OTU, operational taxonomic unit; PCoA, principal coordinate analysis; q, quantitative.

Tag information and the unprocessed DNA sequences obtained in this study have been deposited in the MG-RAST database archive under accession numbers 4503472.3, 4503473.3, 4503474.3 and 4503475.3.

One supplementary table and three supplementary figures are available with the online version of this paper.
rise to variable skin microbe populations may include hand-washing frequency, contact with different types of surfaces and use of cosmetics (Fierer et al., 2008; Giacomoni et al., 2009). Thus, both the presence of microbiota adapted to the skin habitat and contact-driven environmental populations in human hands may be influenced by human behaviour and cultural practices.

Whilst the recent application of next-generation DNA sequencing has brought new insights to the human skin microbiome (Fierer et al., 2008; Grice et al., 2008, 2009; Kong, 2011), studies have not been conducted to explore whether human host population diversity impacts human hand bacterial populations. Given the potential influences that interactions with the environment have on hand microbial habitat structure, we hypothesized that the human hand microbiome differs significantly across human populations that are involved in disparate daily activities. To provide support for this hypothesis, hand-wash samples were collected from two populations separated by geography and lifestyle, and included adult females in Tanzania who were caregivers to young children and adult females in the US who were graduate students. Analysis included multiplexed next-generation DNA sequencing of hand-wash samples to perform bacterial community assessment. These results indicate how interaction with local environments may influence the character and abundance of bacterial populations on hands.

**METHODS**

**Sampling.** Micro-organisms from the hands of US adult women (n = 15) as well as Tanzanian adult women (n = 29) were collected. Female participants were chosen to eliminate previously observed gender biases associated with human hand bacterial populations (Fierer et al., 2008). In the US sample population, out of 15 participants, 13 were white and of European ancestry, and two were Chinese-American. Additional information for each individual, including frequency of hand washing, is provided in Table S1 (available in the online Supplementary Material). For the US population, samples were collected in March 2009 from a graduate student population using the ‘glove juice’ method (Sickbert-Bennett et al., 2005). Both left and right hands were sampled separately using 0.2 μm pore size, filter-sterilized PBS (10 mM phosphate, 137 mM sodium chloride, pH 7.4) solution. The liquid samples from both hands of one individual were pooled subsequently. Each subject provided information on the time since last hand washing, frequency of hand washing and handedness.

In the Tanzanian population, left and right hand-wash samples were taken in July 2009 by the ‘glove juice’ method modified to use one sterile Whirl-Pak bag (NASCO) for both hands (Pickering et al., 2010). The 29 samples came from adult women who were caregivers to children <5 years and lived in a low-income urban community. Respondents were asked how many times they had washed their hands with and without soap during the previous day. Both left and right hands were sampled consecutively in the same bag of rinse solution. In the Tanzania samples, the washing solution was commercial bottled drinking water produced by Uhai that was treated by reverse osmosis. Yale University and Stanford University human subjects committees approved the study protocols and written informed consent was obtained from each of the participants.

**DNA extraction.** To extract DNA from all 44 samples, each hand-wash solution was filtered onto a sterile 0.45 μm pore size, 47 mm diameter MF-Millipore membrane filter (EMD Millipore). A previously described DNA extraction method was then utilized (Yamamoto et al., 2012), during which cells on one-quarter of each filter were lysed by physical disruption through bead beating, and DNA was purified and concentrated using spin columns and reagents of the Mobio PowerMax Soil DNA Isolation Kit (Mobio). Exceptions to the referenced DNA extraction method include eluting DNA from the kit’s spin column twice with 50 μl 10 μM Tris buffer (pH 8). To test for PCR inhibition in the hand-wash samples, standard curves for spiked Bacillus atrophaeus DNA were produced in hand-wash sample DNA extracts in accordance with previously described quantitative (q) PCR methods and universal bacterial primers (Qian et al., 2012). No inhibitory effects, as defined by a difference of ± 2 cycle threshold values at a concentrations of 10^5 genomes in the template were observed between standards run with and without sample extracts. DNA extracts from water and buffers used for hand washes from gloves and Whirl-Paks prior to their use were run as PCR negative controls. These extracts did not result in DNA amplification.

To quantify bacterial populations, qPCR was performed on an ABI 7500 fast real-time PCR system (Applied Biosystems) using universal bacterial primers and TaqMan probes targeting the 331–797 E. coli numbering region of the 16S rDNA. Reaction mixtures, thermocycler conditions and standard curves using B. atrophaeus (ATCC 49337) genomic DNA were as described previously (Hospodsky et al., 2012). For presenting bacterial genome quantities, cycle threshold values were calibrated versus total bacterial genomes. The calibration accounted for the 10 rRNA operon copies in B. atrophaeus and the mean of four rRNA operon copies per genome for all bacteria (Lee et al., 2009). DNA extracts from water and buffers used for hand washes from gloves and Whirl-Paks prior to their use were run as qPCR negative controls. These extracts did not result in DNA amplification. To determine the genome copy number as a function of hand area, a surface area of 400 cm^2 was assumed. This value was calculated by the method from Hsu & Yu (2010) assuming a mean breath and width of 8.5 and 19 cm, respectively, for adults.

**Generation of phylogenetic libraries and data analysis.** Phylogenetic library preparation was conducted for all 44 hand-wash samples. rRNA-encoding genes were amplified for all US and Tanzanian hand washes using the 343F and 926R primers spanning the V3 to V5 regions of the 16S rRNA-encoding universal bacterial gene. The primers included sequencing adaptors on the forward primer, keys and multiplex identifiers (Liu et al., 2007). Amplicon preparation, sequencing and data analysis were conducted for all 44 samples as described previously (Hospodsky et al., 2012). Briefly, four 25 μl PCRs were produced for each sample and then pooled. Field blanks did not result in amplification and were not further considered in downstream amplicon processing. After desalting and primer removal using a Qiagen PCR purification kit, amplicons were visualized on a 1.2% agarose gel. PCR products of the desired hand length that were absent of visible short DNA fragments were quantified using Quant-IT PicoGreen kit (Invitrogen) and pooled at equimolar ratios. Amplicon pools were further cleaned utilizing the Agencourt AMPure PCR purification system (Beckman Coulter), DNA extracts from blank filters were used as negative controls and, as they did not result in amplicoms, were not further considered for sequencing preparation. Sequencing of the pooled amplicons was performed in duplicate at the Yale Center for Genome Analysis. Libraries were produced using Roche 454 pyrosequencing, and incorporated the GS-FLX sequencer and Titanium series chemistry.

Quantitative sequence analysis was performed using tools in the QIIME (Quantitative Insights Into Microbial Ecology) 1.6.0 package (Caporaso et al., 2010). Denoising was conducted using Titanium PyroNoise.
(Caporaso et al., 2010; Quince et al., 2009, 2011). Sequences were removed from the analysis if they were <200 bp in length, did not contain the barcode or primer sequence, or had any ambiguous nucleotides, or had a machine-based quality score <25. Sequences from samples that were prepared in duplicates were combined after denoising. All sequences were clustered subsequently into operational taxonomic units (OTUs) at a minimum identity of 97%. Representative sequences were aligned using PyNAST against the greengenes core set from February 2011 (DeSantis et al., 2006). Phylogenetic assignments were made using the naïve Bayesian classifier in the Ribosomal Dataset Project (Wang et al., 2007).

Within **QIIME**, Fast UniFrac (Hamady et al., 2010) was utilized to produce principal coordinate analyses (PCoA) for comparing the phylogenetic distances between pairs of the 44 samples. Phylotype assignment was made using the RDP classifier and greengenes core set as described above. For PCoA that used previously published datasets including soil samples, human microflora and freshwater samples (Costello et al., 2009; Lauber et al., 2009; Gilbert et al., 2010), the sequences were assigned to phylotypes by BLASTing against the greengenes database to identify their closest matching sequences (Hamady et al., 2010). To accomplish this, the greengenes database was formatted into the BLAST database using formatdb and the resulting tree was used to assess the phylogenetic relationships between all examined samples. Each sequence was assigned to its closest BLAST hit in the formatted greengenes database and clustered into phylotypes at 97% sequence similarity for input into Fast UniFrac. The biplot shown in Fig. 3 (below) was generated within **QIIME** using the default setting of the **make_3d_plot.py** command for generating biplots passing the assigned taxonomy file on the family level with -t and -n=7 to show the seven most prevalent taxa on that level. Within the script, the most abundant taxa (based on an averaging algorithm) are chosen and displayed within the 3D plot. Tag information and the unprocessed DNA sequences obtained in this study have been deposited in the MG-RAST database archive under accession numbers 4503472.3, 4503473.3, 4503474.3 and 4503475.3.

**Statistical analysis.** ANOSIM (analysis of similarities), ADONIS (analysis of variance using distance matrices) and ANOVA tests on phylogenetic distances matrices were performed within the **QIIME** toolbox. P test significance estimations between each category of samples employing Monte Carlo simulations with 500 iterations were performed in Fast UniFrac (Hamady et al., 2010). All resulting P values obtained on phylogenetic data comparisons were Bonferroni-corrected to account for the number of comparisons performed. Comparisons between the two sets of qPCR data were made using an unpaired homoscedastic t-test.

**RESULTS**

**DNA sequencing results and microbial diversity estimates**

For the 44 samples, 108,302 high-quality sequences were produced with a mean of 2461 sequences per sample. Rarefaction curves and richness estimates were based upon OTUs defined at 97% similarity (Fig. S1). Observed OTUs continued to increase with increasing numbers of sequences, suggesting that the quantity of sequences produced did not define the full diversity. When rarefied for 850 sequences, the number of observed species (or unique OTUs) ranged from 180 to 300 (mean 234), which is within the range shown previously for human hands (Fierer et al., 2008). Based on Chao1 richness at an even sampling depth of 850 sequence, US (mean 1237) samples were higher in richness than Tanzanian samples (mean 883), although these differences were not statistically significant.

**Concentration of bacteria on human hands**

Fig. 1 provides a box-and-whisker graph of bacteria cell concentration on individual hands estimated from qPCR-based gene enumeration targeting universal bacterial regions. Populations within a specific country were the dominant source of concentration variation. Significant bacterial concentration differences were observed between the two countries ($P=0.002$). Mean cell concentrations on hands measured in numbers per square centimetre varied by a factor of 11 (US: 34,982 versus Tanzania: 386,753).

**Bacterial community variability across human populations**

Fig. 2 is a UniFrac PCoA-based comparison of microbial populations from hands, demonstrating that the Tanzanian and US communities group separately, and that these inter-country differences were significant. Pairwise significance testing in Fast UniFrac by country category (Tanzania and US) revealed that the UniFrac distances separating the microbial communities across the two countries were significantly different ($P<0.001$) (UniFrac distances shown in Fig. S2). ANOSIM and ADONIS computations within **QIIME** on country and hand-wash frequencies were run, and confirmed the result that country was the primary factor for microbial diversity differentiation ($P<0.001$). Hand-washing frequency (times per day) had no association with clustering ($P=0.12$). ANOVA testing that accounted for
family-level taxonomic abundance revealed that diversity differences were driven by *Propionibacteriaceae* (*P*<0.001, >40 times more abundant in the US than in Tanzanian samples), *Staphylococcaceae* (*P*<0.001; 7 times more abundant in US than in Tanzanian samples, respectively) and *Rhodobacteraceae* (*P*<0.001; 120 times more abundant in Tanzanian than in US samples, respectively). These results are highlighted graphically in the biplot in Fig. 3.

**Relative abundances**

The relative abundances based on phyla level and family level were calculated in QIIME, and are presented in Figs 4 and 5, respectively. The results shown in Fig. 4 readily reveal major differences in the microbial populations and demonstrate phyla that promote these differences among specific human populations in the two countries (minor taxa are shown in Fig. S3). Whilst the most abundant phyla in all countries were the same as those commonly considered on human skin (Grice *et al.*, 2009; Kong, 2011), typically *Actinobacteria*, *Firmicutes* and *Proteobacteria*, the relative proportions of each varied considerably from country to country with *Proteobacteria* being the most abundant in hand communities from Tanzania and *Firmicutes* being the most abundant phyla present on US hands. On a more detailed scale, Fig. 5 displays relative abundance based on the highest taxonomic resolution to which sequences could be assigned confidently. Many bacteria that are attributed commonly to human hands and skin (*Staphylococcaceae*, *Propionibacteriaceae* and *Streptococcaceae*) were found in high abundance in the US samples at (mean ± SD) 28 ± 20, 16 ± 16 and 13 ± 15 % relative abundance, respectively, and reflect taxa distributions described previously for human hands in prior US-based studies (Costello *et al.*, 2009; Fierer *et al.*, 2008). These common human skin taxa, however, were not as abundant in the Tanzanian samples, where the combined relative abundance of *Staphylococcaceae*, *Propionibacteriaceae* and *Streptococcaceae* was 6 ± 7 %, and approximately eight times less than the US hands.

The relative abundances of taxa presented in Fig. 5 revealed additional insights into the potential sources of bacteria on hands from the specific human populations in Tanzania. Tanzanian samples demonstrated elevated relative abundances of bacteria associated typically with soil, including
Rhodobacteraceae, Nocardioidaceae, Bacillaceae, Bradyrhizobiaceae and Rhizobiaceae (Garrity et al., 2001; Kuykendall et al., 2001; Logan & DeVos, 2001). Weighted and unweighted UniFrac-based comparisons of all hand populations from this study versus a suite of soil microbial populations (Lauber et al., 2009), US human skin, gut and oral populations (Costello et al., 2009), and freshwater microbial populations (Gilbert et al., 2010) are presented in Fig. 6. Unweighted analyses best describe compositional differences in microbial communities, and here demonstrated that the US and Tanzania hand communities were typical of human bacterial communities (oral, nose, gut and skin) and different from fresh water and soil. The weighted UniFrac analysis in Fig. 6, which better accounts for differences in abundance distribution across compositionally similar communities, indicated that the US and Tanzanian hand communities are similar to previously described human skin communities.

**DISCUSSION**

**Differences in bacterial populations**

Of the two human populations considered, the bacterial populations found in the US hand-wash samples were the most consistent with previous descriptions of the hand microbiome. This result is likely influenced by the fact that all previous DNA-based hand microbiome studies analysed samples from the US (Costello et al., 2009; Fierer et al., 2008; Grice et al., 2009). Highly abundant taxa observed on US hands (Fig. 5) and important drivers (Fig. 3) of the clustering of US samples included the Firmicutes (Propionibacteriaceae) and the Actinobacteria (Staphylococcaceae and Streptococcaceae), and are similar to those found previously using culture-dependent and -independent methods (Costello et al., 2009; Grice et al., 2009; Leyden et al., 1987).

Predominant taxa in hand washes from the Tanzanian cohort (Figs. 4 and 5) allow for hypothesizing how human activity and environmental exposure may impact the hand microbiome. For the Tanzania participants, the most abundant taxa and those that drove clustering included environment-associated micro-organisms. Rhodobacteraceae and Nocardioidaceae are not believed to be typical colonizers of human skin; they are, however, found commonly in soil and aquatic environments (Garrity et al., 2001; Kuykendall et al., 2001; Logan & DeVos, 2001). Both weighted and unweighted UniFrac-based comparisons in Fig. 6 demonstrate the shorter UniFrac distances between Tanzanian hand-wash samples and a suite of soil samples, which is consistent with Tanzanians having more intimate contact with the local environment. However, we caution that UniFrac analyses cannot determine unambiguously a source; they only show similarities and differences in communities. The lifestyle differences between the US and Tanzanian participants are notable, with US participants spending the majority of time indoors in contact with dry surfaces and none of the US study participants being a caregiver for young children. In contrast, the mothers...
comprising the study population in Dar es Salaam, Tanzania lived in open-air dwellings and were typically outdoors whilst performing daily household activities such as laundry, sweeping, food preparation and dish washing that require frequent contact with soil, untreated water and outdoor surfaces (Pickering et al., 2011). The greater
concentration of bacteria present on the hands of the Tanzanian cohort also supports the notion of an ephemeral source of bacteria from the environment. Finally, we caution that pyrosequencing does not provide the taxonomic resolution to track unambiguously the sources of Rhodobacteraceae and Nocardioidaceae from soil to hands. Thus, whilst data on human activity and beta diversity sequence comparisons are consistent with the hypothesis that the microbiome of Tanzanian hands is influenced by interaction with soil, other sources including food or the fact that these organisms are indeed adapted to the skin habitat cannot be ruled out.

Finally, whilst this research demonstrated significant differences in the skin microbiome across disparate human populations, such findings are not without precedent. Biogeographic differences among countries have also been observed for human saliva (Lazarevic et al., 2010; Nasidze et al., 2009, 2011) and changes in biodiversity associated with urbanization have recently been shown to impact the diversity of Gammaproteobacteria on human skin (Hanski et al., 2012). Very significant differences in the human gut microbiome may reflect diet (De Filippo et al., 2010; Eckburg et al., 2005). Bacterial communities sampled from the forearm skin in subjects within the US and Amerindians in the Venezuelan Amazon demonstrated distinct microbial diversity differences, with the authors suggesting an association of cutaneous bacterial communities with lifestyle and/or geography (Blaser et al., 2013). Whilst many of the above microbial population differences are impacted by differences in microbial habitat and substrate sources, we hypothesize that the hand population differences observed here also indicate the importance of bacterial community structures that are controlled by contact with the environment. Lastly, we caution that the results presented here are limited to the observation that bacterial populations were different among the two specific human populations tested. Whilst the specific taxa contained in the microbial communities for each cohort offer insights to potential sources, the study design does not allow for determining whether these differences are due solely to culture, behaviour or genetics. Future work should investigate causal mechanisms influencing the hand microbiome with a focus on documentation of relative abundances of organisms that are derived from contact with the environment versus organisms that are part of the active microbial ecology. Additionally, researchers conducting human skin microbiome studies should consider sampling a participant’s direct environment to infer the taxonomy of temporal and permanent skin microbiomes. Controlling for genetics by documenting the microbiome on other skin areas that have less contact with the environment can also be beneficial for human hand microbiome studies.

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