



## Microbial Sequencing Analyses Suggest the Presence of a Fecal Veneer on Indoor Climbing Wall Holds

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### Abstract

Artificial climbing walls represent a unique indoor environment in which humans interact closely with a variety of surface types. Climbing wall holds may mediate transmission of organisms between individuals, and yet there are no studies that identify microorganisms present on these surfaces. In the current study, the micro-organisms found on climbing wall holds were characterized by analysis of amplified SSU rRNA gene sequences. In contrast to many other studies of built environments, the majority of microorganisms on holds were most closely related to microbes annotated as being recovered from environmental sources, such as soil, with human skin also representing an important source. Regional patterns were evident as rRNA gene sequences from the marine cyano-bacterium *Prochlorococcus* were abundant in gyms found within 16 km of the ocean. Enterobacteriaceae were present on 100 % of holds surveyed, and the members detected are commonly associated with fecal matter.

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## Introduction

In Western countries, most people spend the vast majority of their lives indoors [33, 34]. Microorganisms within these built environments can be introduced by people, pets, food, water, and air [4], and recent studies have indicated that humans (particularly skin, nostrils, and hair) are the primary contributors to microbial communities in classrooms [28], kitchens [21], bathrooms [20], office buildings [26, 46], subway systems [47], shopping centers [52], and hospitals [27, 32]. Indeed, temporal changes in aerosolized microbial load have been correlated with human density in subway systems [16]. Similarly, human-associated bacteria were relatively more abundant in occupied versus unoccupied classrooms at the University of Oregon [38], emphasizing the importance of human presence in contributing to microbial dispersal in indoor environments.

Climbing gyms are a unique environment where climbers tightly grip handholds that generally have a rough

surface much like sandpaper. While the surface allows for better grip, it can also lead to tears in the skin. Such abrasions may allow an entry point for pathogens [51] or may make the transfer of blood borne pathogens a possible, albeit unlikely, concern [50]. Outbreaks of community-acquired bacterial infections have been previously associated with athletes and equipment used in athletic facilities [2, 6, 10–12, 42]. Possible vectors of transmission in these cases include skin-to-skin contact, abrasions/skin damage, respiratory pathways, sharing of equipment such as hygiene products and clothing, and contact with surfaces such as mats, pads, weights, and exercise equipment [12]. Both hands and surfaces have been implicated in transmission of potential pathogens such as methicillin-resistant [25] and/or methicillin-sensitive staphylococci [54], *Klebsiella* spp. [9], fecal bacteria [29], and viruses [24, 36, 37, 39]. Although there is debate in the literature regarding the relative role of skin-to-skin contact versus environmental surface contact in the transmission of infection in athletic settings [3, 5, 11], climbing holds may represent a potential reservoir for pathogens. However, we know almost nothing about dispersal of microorganisms from sources such as hands, climbing shoes, or air onto climbing holds.

## Materials and Methods

### Sample Collection

A total of 12 handholds were sampled across 4 climbing gyms (3 holds/gym) in March–June of 2011. Three climbing gyms are located within southern Massachusetts and Rhode Island (Y, H, and C), and all three are within 16 km of the ocean. In contrast, the fourth is located in the mountains of North Carolina (SLB), roughly 500 km from the ocean. Prior to sampling, all climbing gym establishments were made aware of the nature of the investigation through written and verbal consent, and it was agreed that the identities of the facilities would be kept anonymous. Handholds were selected based on the criteria that (1) each hold represented the beginning of a climbing route and (2) had not been cleaned by employees for at least 1 month. Handhold surfaces were thoroughly swabbed with sterile cotton-tipped swabs moistened with sterile phosphate buffered saline (PBS). Following sampling, each swab tip was aseptically placed in a sterile 2 ml cryogenic vial and stored on ice for temporary storage (<12 h), followed by –20 °C overnight storage.

### DNA Extraction, PCR, and DNA Sequencing

Each swab tip was transferred with sterile tweezers to a bead tube and DNA extracted from each swab using the

Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH) following manufacturer's protocol, except that bead tubes were heated at 65 °C for 10 min, prior to bead beading [18]. PCR reactions were carried out for a total of 32 cycles with a total reaction volume of 30, 2 µl of which was DNA template. Reagents and final concentrations were 1 × Phusion HF polymerase (2X mastermix; Finnzymes) with 8 % v/v DMSO and 0.5 µM forward and reverse primers in the final reaction volume. PCR thermal cycling conditions consisted of an initial denaturation and enzyme activation at 98 °C for 1 min, followed by 22 cycles of 3-step PCR: denaturation (98 °C for 5 s), annealing (touchdown 68–58 °C at 1 °C/cycle for 10 s for the initial 10 cycles and remaining 12 cycles held constant at 58 °C), and elongation (72 °C for 7 s). Touchdown PCR was employed in order to minimize primer-dimer formation. The last 10 cycles consisted of a 2-step PCR with denaturation (98 °C for 5 s) and annealing/elongation (72 °C for 21 s). PCR products were quantified using the PicoGreen dsDNA reagent (Invitrogen) and were gel purified using the Montage Kit (Millipore). Modified small sub-unit (SSU) rRNA gene primers 515f-927r were incorporated with adapter sequences for the GSFLX-Titanium platform of the Roche 454 Pyrosequencing technology as described previously [41]. The modified SSU rRNA gene primers were as follows: 515f-modified, 5'-GTGYCAGCMGCCG CGGTAA-3' and 927r-modified, 5'-CCGYCAATT CMT TTRAGTTT-3'. Additionally, the relative abundance of members of the family *Enterobacteriaceae* was verified for hold SLB2 using protocols adapted from [19]. In this case, amplicons from three separate PCR reactions using the 27f and 338r primers [18] were pooled and cloned using a TOPO TA *pcr*<sup>®</sup>2.1 vector kit (Invitrogen, Carlsbad, CA). Approximately, 150 colonies were sent to Beckman-Coulter Genomics (Danvers, MA) to be purified and sequenced using a Sanger platform. Taxonomic analyses for the Sanger sequences were conducted using the RDP classifier.

### Data Analysis and Quality Control

Sequences generated from pyrosequencing of SSU rRNA gene amplicons were binned by barcode and quality filtered using the “split\_libraries.py” script in the Quantitative Insights Into Microbial Ecology (QIIME v1.5) software [8]. Discarded sequences included those with errors in the barcode or primer, shorter than 250 nt, longer than 600 nt, with a quality score <50, with a homopolymer run greater than 6 nt, and those which contained ambiguous base calls. Flowgrams for remaining sequences were denoised using DeNoiser version 1.3.0-dev by [44]. Chimeric sequences were identified using UCHIME under reference mode (Edgar et al. 2011). Given that chimeric sequences were

most likely among the less abundant sequences, the abundant sequences of cluster sizes >20 that were flagged as chimeras were hand checked against the NCBI nt database using BLAST [1]. Query sequences with mismatches on either end compared to a reference sequence were split at the point where reference and query sequences appeared to diverge. Each end of the split sequence was re-BLASTed, and sequences that did not demonstrate taxonomic consistency were discarded. The remaining sequences (23,985 total) were processed in Mothur [48] as outlined by [49, SOP version date 2/15/2013]. Briefly, we aligned to the SILVA bacterial reference alignment using NAST [13]; those sequences that did not begin at the V4 region and terminate at the end of the V5 region were discarded from further analysis. Vertical gaps were removed from the alignment, and leading and trailing columns were discarded such that no sequences had terminal gaps (vertical = T and trump = ., options in Mothur). Taxonomic classification of all remaining sequences was implemented using the RDP Classifier [53] at 80 % bootstrap confidence. The RDP Classifier was trained with the greengenes SSU rRNA database (DeSantis et al. 2006). Sequences were clustered into operational taxonomic units (OTUs) at 97 % identity using the average neighbor method (Mothur) with pairwise distances calculated from the multiple sequence alignment described above. The consensus taxonomy was assigned to OTU clusters using the classify.otu command in Mothur. Singletons were also discarded for downstream analysis. A phylogenetic tree was constructed from the filtered alignment using FastTree. Unweighted UniFrac, weighted UniFrac, and Bray-Curtis distance matrices were calculated and were visualized using principal coordinates analysis (<http://www.r-project.org>). Beta diversity metrics were derived from a rarefied OTU table at a depth of 461 sequences, corresponding to the sample with the lowest library size, H3 [22]. Phylogenetic analyses were conducted by first generating alignments using the online SILVA aligner [43]. Dendrograms were constructed using neighbor-joining and maximum-likelihood methods in the PHYLIP software package [17]. Exploration of potential sources for handhold-derived sequences was accomplished by recruiting sequences recovered in this study to annotated sequences in the Silva SSU\_Ref111 SSU rRNA gene database. The “isolation source” field in SSU\_Ref111 was used to find reference sequences putatively derived from soil, skin, or marine environments. Our sequences were then BLASTed against SSU\_Ref111 sequences annotated as being isolated from “soil”, “skin”, or “marine” environments to determine the isolation source of the closest reference match. Sequences that did not have a reference match within 95 % sequence identity were categorized as “no blast hit”. Sequences were deposited into the BioSample database under project

**Table 1** Phylum-level representation of sequences found on climbing holds

Phylum	Percentage of sequences
<i>Proteobacteria</i>	40.8
<i>Firmicutes</i>	21.9
<i>Cyanobacteria</i>	12.7
<i>Bacteroidetes</i>	8.0
<i>Actinobacteria</i>	6.9
<i>Acidobacteria</i>	1.8
<i>Planctomycetes</i>	1.7
<i>Armatimonadetes</i> (formerly OP10)	0.65
<i>Deinococcus-Thermus</i>	0.63
<i>Chlorobi</i>	0.45
<i>Spirochaetes</i>	0.38
<i>Fusobacteria</i>	0.27
Other Phyla (<0.1 %)	2.1
Unclassified	1.5

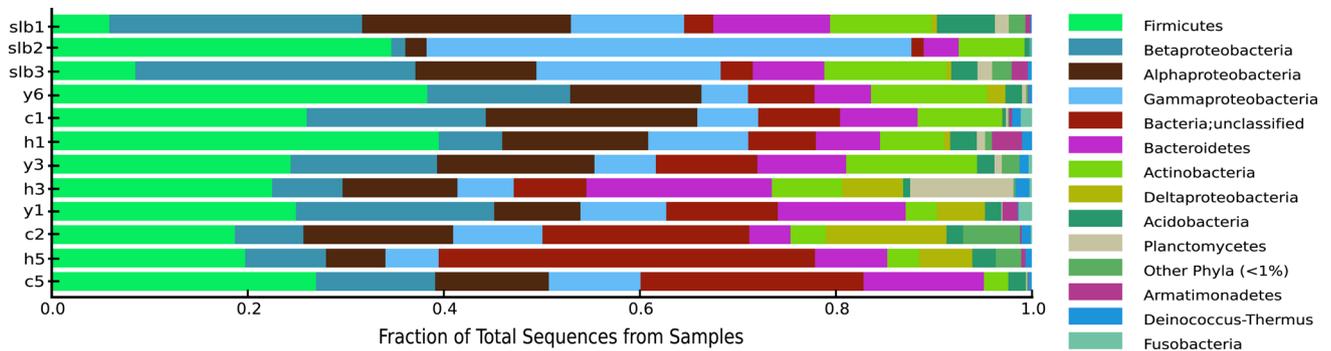
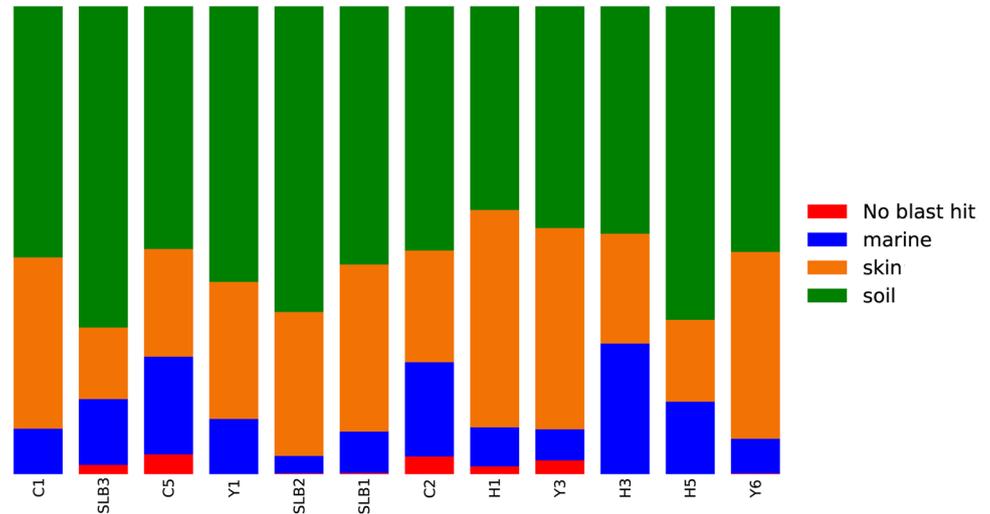
identification number PRJNA207668. The top 15 OTUs as well as OTU 271 and OTU 763 were given accession numbers SAMN02296807-SAMN02296824.

## Results and Discussion

### General Findings

After removing sequences of insufficient quality or length, 20,500 sequences remained with an average length of 373 bp. Overall, an average of 1,708 (range 461–3,951) sequences was obtained for each of the 12 climbing holds sampled. A total of 546 OTUs, with an average of 158 (range 63–271) operational taxonomic units (OTUs), were obtained from each handhold at a 97 % cutoff. Sequences were distributed across five to seven main phyla (defined as phyla representing more than 1–5 % of the population): *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes* (Table 1). At the phylum level, climbing gym handholds appeared to harbor more diversity compared to human hands, which are dominated by three to four phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* [14, 18]. The greater phylum-level diversity obtained here is not due to greater sequencing depth, since the Fierer et al. [18] study had greater sequencing depth than our study (an average of 3,251 sequences per palm versus an average of 1,708 sequences per handhold in our study). Instead, the source of this phylum-level diversity likely reflects contact with soil on climbing shoes and exposure to dust from the air and other environmental sources. For example, climbing holds contained more than twenty-five

**Fig. 1** Exploration of potential sources for handheld-derived sequences. Sequences were recruited to sequence groups annotated as being obtained from the following environments: soil, skin, marine, or other environments (no blast hit). *C*, *H*, and *Y* represent the Boston-area gym samples, and SLB represents samples from the rural area gym. Numbers indicate the swab number that was sequenced (out of six collected)



**Fig. 2** Distribution of sequences (by phylum) across all 12 handholds. Taxonomic classification was assigned using the RDP classifier and retrained with the Greengenes taxonomy

phyla (data not shown), and at least two of the main phyla (represented by  $>1\%$  of sequences) are uncommon on hands: *Acidobacteria*, which are common in soil environments and *Planctomycetes*, which are common in soils and aquatic environments (Table 1; Fig. 2). In contrast, the nine additional phyla commonly detected on skin include *Cyanobacteria*, *Chlamydiae*, *Deinococcus*, *Deferribacteres*, *Thermus*, *Fusobacteria*, *Spirochaetes*, *Verrucomicrobia*, and the candidate phyla TM7 and SR1 [14].

### Environmental Signatures

Many sequences obtained in this study appear to have come from non-skin sources; moreover, the majority of these sequences matched sequences obtained from soil (Fig. 1). For example, some of the most abundant OTUs, such as those assigned to *Comamonadaceae* or *Ralstonia* (Fig. 2; Table 2), matched sequences detected in soils or marine sediments. Further, many of the sequences assigned to *Acidobacteria* shared high identity (99–100%) with

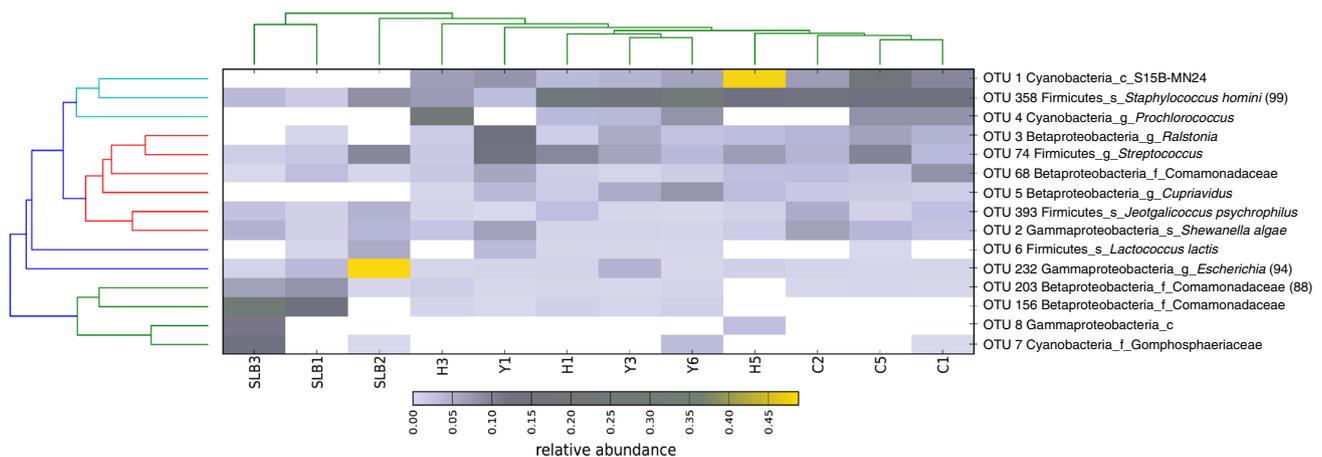
sequences from soil. For example, OTU 271 shared 99% identity with grassland soil clone SEG 08 030 [Accession HQ597748, 40]. It is likely that soil-related organisms may have been transferred to the climbing wall from the shoes of climbers. This is in contrast to studies of indoor environments which generally find an abundance of skin-related sequences [see review by 31] and is more in line with studies of outdoor air [7].

Additionally, sequences assigned to the cyanobacterial family *Gomphosphaeriaceae*, which are found primarily in freshwater habitats [45], were also more abundant (up to 9%) in two of the three inland (non-coastal) gym samples compared to 0.1–3% in two of the nine Boston, MA-area samples. Even more apparent was the prevalence of marine *Prochlorococcus* sequences in samples from the coastal (Boston, MA-area) climbing gyms representing up to 20% of sequences on 6 of the 9 holds, compared to the inland gym samples where this OTU was not detected (OTU 4, Figs. 3, 4). These data corroborate data from a recent study of bacterial diversity in office spaces that found an

**Table 2** Identification of the 15 most abundant OTUs found on climbing wall holds

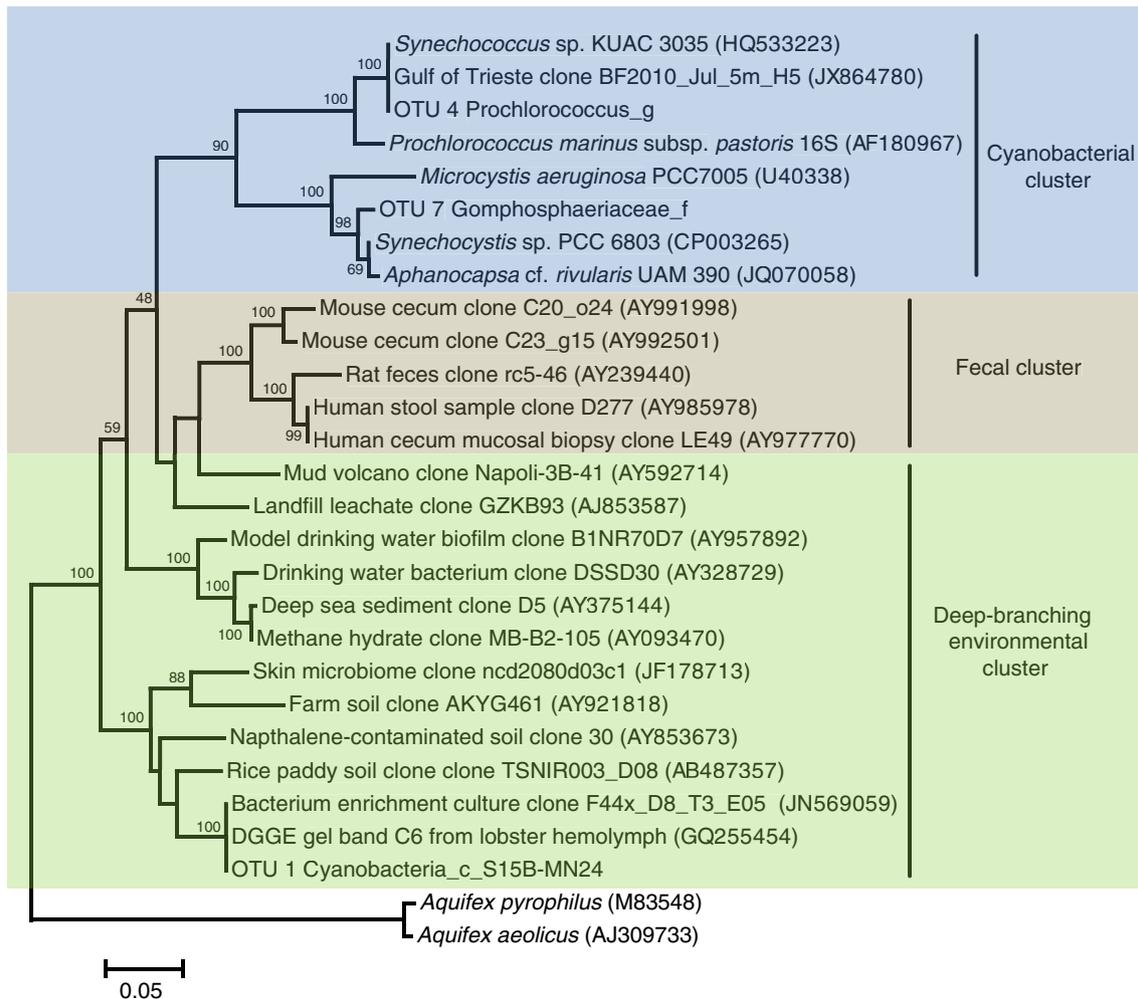
Taxon	Abundance (%)	OTU	Top BLAST hits (Accession, % ID)
<i>Escherichia_g</i>	9	232	<i>Shigella flexneri</i> strain CCAM 090021 (KC429777, 100 %) <i>Escherichia coli</i> O157:H7 str. Sakai strain (NR_074891, 100 %) <i>E. coli</i> O169:H-associated w/traveler's diarrhea (AB604197, 100 %)
<i>Staphylococcus_s</i>	8	358	<i>Staphylococcus warneri</i> strain Lad_25 K (KC354484, 100 %) <i>Staphylococcus epidermidis</i> strain BY3 (KC331168, 100 %)
<i>Streptococcus_g</i>	5	74	<i>Streptococcus sp.</i> THG-M4 (JX981959, 100 %) <i>Streptococcus oralis</i> strain HV 0s122 (JQ782211, 100 %)
<i>Cyanobacteria_c</i>	4	1	Enrichment culture clone F44x_D8_T3_E05 (JN569059, 100 %) DGGE gel band C6 lobster hemolymph (GQ255454, 100 %)
<i>Comamonadaceae_f</i>	4	156	Water-flooding petroleum reservoir clone S6B_101 (JQ433727, 100 %) Skin microbiome clone ncd365c05c1 (HM313304, 100 %)
<i>Shewanella_s</i>	3	2	<i>Shewanella algae</i> strain CD13 (KC210859, 100 %) Roach intestine clone Z2_S_KL_412 (KC337222, 100 %)
<i>Ralstonia_g</i>	2	3	Hydrothermal chimney clone P5-b58 (FR853020, 100 %) <i>Ralstonia solanacearum</i> strain in4ss52 (from soil) (JQ655458, 99 %)
<i>Prochlorococcus_g</i>	2	4	Gulf of Trieste clone BF2010_Jul_5m_H5 (JX864780, 100 %) <i>Synechococcus sp.</i> KUAC 3035 (HQ533223, 100 %)
<i>Comamonadaceae_f</i>	2	68	Lake bacterioplankton DGGE gel band FSW-Lt05-15 (GU734074, 100 %) Amazon River clone 4P_002a_H02 (JX672510, 99 %)
<i>Jeotgalicoccus_s</i>	2	393	Air sample clone XXM_1_036 (JX559182, 99 %) Skin microbiome clone ncd2808e06c1 (JF240832, 99 %)
<i>Comamonadaceae_f</i>	1	203	Salton Sea clone DSFBPENV11bac_1D (KC465634, 99 %) Soil clone GYn3-13 associated with Mn-Fe nodules (JX493061, 99 %)
<i>Cupriavidus_g</i>	1	5	<i>Cupriavidus sp.</i> MA09 (Atlantic forest soil) (KC111958, 99 %) <i>Cupriavidus sp.</i> CHP-YG38 (from soil) (JX965398, 99 %)
<i>Lactococcuslacti_s</i>	1	6	<i>Lactococcus lactis</i> subsp. <i>lactis</i> II1403 (KC429785, 100 %) Wastewater treatment clone SludgeG_upper_64 (AB515615, 100 %)
<i>Gomphosphaeriaceae_f</i>	1	7	<i>Synechocystis sp.</i> PCC 6803 (freshwater) (CP00326, 98 %) <i>Aphanocapsa cf. rivularis</i> UAM 390 (freshwater) (JQ070058, 98 %)
<i>Gammaproteobacteria_c</i>	1	8	Geothermal water clone Tat-08-009_43_53 (GU437483, 100 %) Coastal microbial mat clone clone GBII-44 (GQ441315, 100 %)

Taxonomic classification was assigned using the RDP classifier and retrained with the Greengenes taxonomy. The highest level of classification for each OTU is indicated as follows: c = class, f = family, g = genus, and s = species. Abundance was calculated as a percentage of the total sequences meeting the quality standards (20,500)



**Fig. 3** Heat map demonstrating the relative abundance of the 15 most abundant OTUs. Sequences were acquired from climbing wall holds sampled in gyms in either a rural setting (SLB) or the greater Boston, MA-area (C, Y, and H). Both axes are clustered based on the Bray-Curtis dissimilarity metric. Taxonomic identification begins with phylum-level (or class level for *Proteobacteria*) classification

followed by the highest-level taxonomic classification for each OTU (c = class, f = family, g = genus, s = species), assigned using the RDP classifier and retrained with the Greengenes taxonomy. All taxonomic assignments by the RDP classifier indicate 100% confidence unless otherwise stated



**Fig. 4** Neighbor-joining dendrogram showing representative cyanobacterial sequences (OTU 1, OTU 4, and OTU 7) found on climbing wall holds in this study and sequences from *top* blast hits compared to other cyanobacterial sequences in the literature, including deep-branching clades of sequences detected in either fecal/colon/cecal

samples, skin samples, or environmental samples. Alignments were generated using SILVA [43], and dendrograms were calculated using PHYLIP [17]. *Bootstrapping values* are shown only for nodes that were supported by both maximum-likelihood and neighbor-joining analyses. *Aquifex* spp. were used to create the outgroup

abundance of cyanobacterial sequences in offices in the two coastal cities, New York, NY and San Francisco, CA and virtually absent in office spaces in the inland city, Tuscon, AZ [26].

Interestingly, the most abundant cyanobacterial sequence (OTU 1) clustered closely with sequences of environmental origin [Accession AY328729, 55] and were distinct from those of fecal or cecal [Accession AY991998, 35] origin (Fig. 4). This sequence also shared 100 % identity (over ca. 374 nt) to a sequence obtained from lobster hemolymph (Table 2) and was abundant in all nine Boston-area holds (2–40 %), but completely absent from all three inland holds (Fig. 3), suggesting that this OTU may be marine derived.

#### Skin, Oral, and Fecal Indicators

A modest skin signature was apparent. For example, several of the bacterial groups found to be abundant on hand surfaces (Fierer et al. 2008) were also abundant on climbing holds including *Streptococcaceae*, *Burkholderiales*, and *Staphylococcaceae*, while other groups that are rare on hand surfaces were abundant on climbing holds such as *Halomonadaceae*, *Veilonellaceae*, and *Enterobacteriaceae*. More importantly, three of the top 15 most abundant OTUs had top Blast hits that are skin associated: (1) OTU 358, representing 8 % of sequences, was 100 % identical to *Staphylococcus epidermidis* strain BY3, (2) OTU 156, representing 4 % of sequences, was 100 %

identical to Skin microbiome clone ncd365c05c1, and (3) OTU 393, representing 2 % of sequences, was 99 % identical to Skin microbiome clone ncd2808e06c1 (Table 2).

Sequences assigned to the genus *Escherichia* (OTU 232) represented 9 % of the total sequences retrieved and were detected on all 12 holds (Figs. 2, 3; Table 2). This OTU represented up to 43 % of sequences found on hold SLB2, suggesting that proportionately large numbers of enteric bacteria can be found on climbing holds (Fig. 2; Table 2). Hold SLB2 not only had the highest DNA yield of all the holds (ca. 4 µg), but additionally an independent primer set revealed that *Enterobacteriaceae*-related sequences represented approximately 23 % of the total sequences on this hold. In the pyrosequencing analysis, *Shigella/Escherichia* spp. related sequences represented at least 13 % of the total sequences across all 12 holds or roughly 56 % of the sequences on hold SLB2. This is an important finding given that some pathogenic enterics, such as O157:H7 [23] and *Shigella* [30], have a low infectious dose [15, 27]. It is unclear whether the *Escherichia* spp. sequences obtained here are of human or animal origin, but at least one other study has found an abundance of fecal indicator bacteria from household pets (dogs) in outdoor air [7], corroborating the hypothesis that fecal bacteria [also known as a “fecal veneer” 15] may be prevalent in human environments. Finally, roughly 5 % of the sequences detected across all holds were potentially of oral origin including OTU 74 (1,018 sequences; detected on all 12 holds), which was 100 % identical to *Streptococcus oralis* strain HV 0s122 and OTU 763 (2 sequences), which was 100 % identical to the common oral microbe *Porphyromonas gingivalis*.

## Conclusions

Our results indicate dispersal of microorganisms from climbing shoes, hands, and environmental sources, with less input from human sources on climbing holds compared to other built environments. Because human-associated microbial communities typically contain a higher abundance of human pathogens and/or opportunistic pathogens [32], one might infer that, due to the greater environmental signature detected here, climbing holds potentially pose less of a health risk compared to other indoor environments. In summary, all twelve holds examined contained evidence of microorganisms associated with fecal material. Sequences associated with fecal microorganisms represented roughly 9 % of all sequences, including as many as 43 % of the sequences associated with a single climbing hold. While this does not necessarily represent a significant risk, climbers should take precautions, for example, by washing their hands both before and after climbing.

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