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Potentially Pathogenic Bacteria in Shower Water and Air of a Stem Cell Transplant Unit[▽]

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Potential pathogens from shower water and aerosolized shower mist (i.e., shower aerosol) have been suggested as an environmental source of infection for immunocompromised patients. To quantify the microbial load in shower water and aerosol samples, we used culture, microscopic, and quantitative PCR methods to investigate four shower stalls in a stem cell transplant unit at Barnes-Jewish Hospital in St. Louis, MO. We also tested membrane-integrated showerheads as a possible mitigation strategy. In addition to quantification, a 16S rRNA gene sequencing survey was used to characterize the abundant bacterial populations within shower water and aerosols. The average total bacterial counts were 2.2×10^7 cells/liter in shower water and 3.4×10^4 cells/m^3 in shower aerosol, and these counts were reduced to 6.3×10^4 cells/liter (99.6% efficiency) and 8.9×10^3 cells/m^3 (82.4% efficiency), respectively, after membrane-integrated showerheads were installed. Potentially pathogenic organisms were found in both water and aerosol samples from the conventional showers. Most notable was the presence of *Mycobacterium mucogenicum* (99.5% identity) in the water and *Pseudomonas aeruginosa* (99.3% identity) in the aerosol samples. Membrane-integrated showerheads may protect immunocompromised patients from waterborne infections in a stem cell transplant unit because of efficient capture of vast numbers of potentially pathogenic bacteria from hospital water. However, an in-depth epidemiological study is necessary to investigate whether membrane-integrated showerheads reduce hospital-acquired infections. The microbial load in shower aerosols with conventional showerheads was elevated compared to the load in HEPA-filtered background air in the stem cell unit, but it was considerably lower than typical indoor air. Thus, in shower environments without HEPA filtration, the increase in microbial load due to shower water aerosolization would not have been distinguishable from anticipated variations in background levels.

Hospital water supplies are frequently inhabited with environmental waterborne microbes, including bacteria (*Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium avium*, *Stenotrophomonas maltophilia*, and *Achromobacter* spp.) and fungi (*Aspergillus* spp. and *Fusarium* spp.) (4, 13, 63). Although water storage tanks may be cleaned annually and residual chlorine levels of water contents maintained, these measures alone cannot prevent the formation of biofilms along inert surfaces of the tank and piping systems. Biofilms attached to living and inert surfaces consist of complex communities of microbes that produce glycocalyx polysaccharides, which protect bacteria from desiccation, chemical treatments, and immunologic attack (25). They can form quickly and have been found in dental water lines only a few weeks after installation (14). Finally, biofilms can harbor pathogens that are periodically released through sloughing of fringe layers (25, 70). Common point-of-use sources of potential exposure to waterborne microbes contained in biofilms in the health care setting include showerheads, water tanks, faucets, aerators, water fountains, and ice machines (2, 4, 13, 31, 34, 38, 68).

While microbes found in water usually pose no risk for healthy individuals, they can be opportunistic pathogens capable of causing serious and life-threatening infections in severely immunocompromised individuals. Patients with cancers of the blood, lymph, and bone marrow (leukemia, lymphoma, and myeloma) are frequently treated with intense chemotherapy, irradiation, and/or stem cell transplant, resulting in neutropenia and profound immunosuppression. Stem cell transplant patients are encouraged to bathe or shower daily before and after transplant to help maintain skin integrity; these patients also almost universally have a central venous access device for the administration of chemotherapy and other medications. Opportunistic microbes in shower water may contaminate the central venous catheter and provide a mechanism for bacterial invasion into the bloodstream (48). Once a patient has become infected, treatment of these organisms may be more difficult because of antibiotic resistance. Thus, severely immunosuppressed patients are at risk of significant morbidity and mortality from exposure to health care-acquired environmental pathogens.

Although there is no data on bacteria in hospital showers, the shower environment, particularly the aerosolized shower mist (i.e., shower aerosol), has long been suspected as a source of opportunistic pathogens (4, 10). Inhalation of aerosolized pathogenic bacteria in the shower may result in respiratory infections and dissemination of organisms from the lungs into the bloodstream. The advent of high-throughput sequencing now allows for qualifying the bacterial composition in aerosols

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(23, 28). Angenent et al. (5) investigated the aerosols generated from an indoor therapy pool environment in which multiple staff members contracted *Mycobacterium avium* infections and hypersensitivity pneumonitis (i.e., swelling of alveoli due to an immunologic reaction to airborne particles). A fraction (>30%) of the bacteria in pool water was identified as *M. avium*, which preferentially partitioned into the aerosol (>80%) (5). In a previous study conducted by Bollin et al. (10), *Legionella pneumophila* isolates were collected from shower and sink aerosols; however, to our knowledge, there are no published studies that document the effectiveness of membrane-integrated showerheads in decreasing the microbial load in indoor air.

We investigated whether shower stalls in a stem cell transplant unit in a hospital could be a source of potential pathogens by quantifying and qualifying the bacterial load, colony count, and bacterial DNA in shower water and air. An engineering control that consisted of a showerhead with an integrated 0.2-μm-pore-size membrane was utilized to ascertain whether conventional hospital showers aerosolize bacteria and therefore whether a significant increase in the bacterial load was observed compared to HEPA-filtered background air. A total of four shower stalls in individual rooms in a stem cell transplant unit were evaluated during two seasonal sampling periods (two stalls per season). Each shower stall was sampled for 3 days with a conventional showerhead in place and then for 3 days with a membrane-integrated showerhead installed in the same shower. Our goal was to determine the overall mitigation effectiveness of utilizing membrane-integrated showerheads in reducing the presence of potentially pathogenic bacteria from shower water and aerosols. Patient infections were not evaluated during the course of this study.

MATERIALS AND METHODS

Shower environment. Barnes-Jewish Hospital is a 1,228-bed tertiary care teaching hospital affiliated with the Washington University School of Medicine in St. Louis, MO. The stem cell transplant unit has 26 private rooms with positively pressured, HEPA-filtered air. Each room (54 m^3) has a private, attached bathroom (9.0 m^3) with a sink, commode, and curtain-closing shower stall (2.2 m^3). The hall door, internal restroom door, and shower curtains were kept closed during sampling. The rooms were occupied by patients, but the patients did not use the shower during the sampling process. Housekeeping personnel cleaned each restroom and shower daily before sampling. The stem cell transplant unit is supplied with water from six independent hot water risers with each riser feeding between four and six rooms; sample showers were selected from the different risers. The St. Louis City Water Division supplies the hospital water tanks with domestic water. Water storage tank temperatures are maintained between 49 and 52°C. Each tank is cleaned annually by hospital maintenance; however, chlorine levels are not managed by hospital maintenance personnel (based on historic records, the levels are ~0.1 mg/liter free chlorine and ~0.3 mg/liter total chlorine).

Experimental design. On a daily basis, three background air samples, three shower aerosol samples, and one shower water sample were collected from each shower stall, during two seasons (two stalls in the winter and two stalls in the summer of 2007). Data were collected for 6 days from each shower stall: 3 days with a conventional showerhead in place, followed by 3 days with a Pall-Aquasafe water filter (AQF7S) (Ann Arbor, MI) showerhead installed, which has an integrated 0.2-μm-pore-size membrane (i.e., membrane-integrated showerhead). For each day of sampling, the three similar aerosol samples were pooled for daily quantitative culturing and microscopy methods, but all 3 days of collected samples were pooled prior to DNA extraction for the molecular biology techniques due to the low levels of DNA present in the air of the HEPA-filtered rooms. Likewise, bacteria in the water samples were quantified through culturing and microscopy on a daily basis, and samples were pooled prior to DNA extraction. Thus, quantitative PCR and 16S rRNA gene sequencing were combined samples from 3 days of sampling. For each method of quantification, a statistical analysis was performed using the R Project for statistical computing software (www.r-project.org). Three-way analysis of variance tests were used to determine the significance of season, filtration, and sample type for each method of quantification. A correlation analysis was also conducted to determine the correlation between the data from the total bacterial counts and quantitative PCR. For this analysis, it was assumed that each data set had a bivariate normal distribution.

Sampling conditions. Aerosol samples were collected with swirling aerosol collectors (SACs) (Biosampler, AGI-30; SKC, Eighty Four, PA) for 90 min, allowing a total sample volume of 1.125 m^3 to pass through each of three samplers that were run simultaneously. The SACs were filled with 20 ml of sterile phosphate-buffered saline (PBS) (Cellgro, Manassas, VA) and maintained on a sampling stand about 1.5 m from the floor. In addition to the SACs, three flow meters (RMA-22; Dwyer, Michigan City, IN) were used to control the flow rate pulled through the SACs. Two high-volume vacuum pumps (2688VE44 and 2669CE44; Thomas, Lake Zurich, IL) were connected to the flow meters and SACs with neoprene tubing (Masterflex, Vernon Hills, IL). This tubing ran under the hospital room and bathroom doors from the hallway to the shower stall. A daily site blank sample was collected by preparing one SAC on location with 20 ml of PBS and immediately pouring it out into the sampling vial. Daily background aerosol samples were collected from the shower stall prior to turning on the shower. Shower aerosol samples were taken at the same location while the shower was running at a water temperature of 33 to 43°C, which is the human comfort zone in terms of shower water temperature. One liter of shower water was collected for analysis while the shower was running during the shower aerosol sampling period.

DNA extraction and PCR amplification. One 100-ml shower water sample and a pooled 40-ml SAC fluid sample (from three samplers) were concentrated each day with 0.2-μm-pore-size, gamma-sterilized microfunnel filters (4803 Pall; Pall, Ann Arbor, MI). Samples were then immediately stored at −80°C until elution from the three daily filters (in one tube) with 2 ml of elution buffer (5). DNA was extracted from the elution wash using a bead-beating and phenol-chloroform extraction protocol (40). The 16S rRNA genes were amplified by a 30-cycle touchdown PCR. The 30-cycle touchdown PCR consisted of an initial 2-min denaturing step at 94°C, followed by 20 cycles, with 1 cycle consisting of 30 s at 92°C, 90 s at a temperature from 65°C to 45°C (temperature decreased 1°C per cycle), and 90 s at 72°C. These touchdown cycles were followed by 10 cycles, with 1 cycle consisting of 30 s at 92°C, 30 s at 45°C, and 30 s at 72°C, and a final extension step of 15 min at 72°C. The 50-μl solutions contained 1.25 units of GoTaq (Promega Corp., Madison, WI), 0.4 pmol/μl (each) forward and reverse primers (8F [5′-AGAGTTTGATCCTGGCTCAG-3′] and 1391R [5′-GACGG GCGGTGWGTRCA-3′]), 0.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 0.8 mg/ml bovine serum albumin, and 2 μl of template. Positive- and negative-control reactions were included with each reaction set. Shower aerosol and negative-control PCR products were ethanol precipitated and reamplified with an additional 20 cycles of PCR, with 1 cycle consisting of 30 s at 92°C, 30 s at 45°C, and 30 s at 72°C, with the same reaction solution. Negative controls were negative for the repeated data sets.

Bacterial quantification: colony counts, total bacterial counts, and qPCR. Microfil S filtration devices (0.22-μm pore size; Millipore, Billerica, MA) were used to filter 100 ml of each water sample. The filters were placed on heterotrophic tryptic soy agar plates and incubated at 35°C before quantifying the CFU. Direct cell counts were conducted by an epifluorescence microscopy procedure (5). In short, 45 ml of water or 20 ml of SAC fluid was filtered through 0.22-μm black polycarbonate filter (GE Water, Trevose, PA), stained with 1 μM 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO), and rinsed. Cells were then counted with an epifluorescence microscope (Olympus BX41, Olympus, Center Valley, PA). Quantitative PCR (qPCR) was used to determine the bacterial DNA load within each sample. Wells contained 2 μl of extracted template and 23 μl of SYBR green mix (ABgene, Rockford, IL) supplemented with 0.25 U UDP-*N*-glycosidase, and 10 μM (each) universal bacterial primers (forward primer [5′-TCCTACGGGAGGCAGCAGT-3′] and reverse primer [5′-GGACTACCAGGGTATCTAATCCTGTT-3′]) (47). Samples were analyzed with a Stratagene Mx3000P qPCR system (Cedar Creek, TX), using the program outlined by Nadkarni et al. (47) (i.e., 40 cycles, with 1 cycle consisting of 15 s at 95°C, 1 min at 60°C, and 30 s at 72°C with data collection temperatures of 85°C to 88°C and analysis of a final melting curve). To generate a standard curve for qPCR, DNA was extracted from *Escherichia coli* and quantified using a PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Eugene, OR). The standard curve of *E. coli* DNA ranged from 4.7 pg to 4.7 ng with an R^2 of 0.987.

Cloning and sequence analysis. 16S rRNA gene surveys were conducted for the pooled shower water samples that combined the first 3 days of sampling prior

TABLE 1. Summary of bacterial quantification results for bacteria in shower water and air samples from a stem cell transplant unit[a]

Season	Riser	Membrane-integrated showerhead	Shower water samples						Shower aerosol samples				
			No. of CFU/liter[b]	No. of cells/liter[c]	No. of cells/CFU	Amt of DNA (pg/liter)[d]	No. of sequences[e]	No. of OTUs[f]	No. of CFU/m^3[b]	No. of cells/m^3[c]	Amt of DNA (pg/m^3)[d]	No. of sequences[e]	No. of OTUs[f]
Summer	A	No	27,776	7.1×10^6	256	478.8	247	131	BD[g]	1.7×10^4	4.6	250	49
		Yes	6.0	9.3×10^4	15,500	3.6	NA[h]	NA	BD	1.2×10^4	1.2	NA	NA
Summer	B	No	639.4	2.0×10^7	31,279	98.2	181	85	BD	3.9×10^4	2.1	151	91
		Yes	3.3	5.1×10^4	15,455	BD	NA	NA	BD	4.6×10^3	1.2	NA	NA
Winter	C	No	857.8	1.1×10^7	12,824	285.7	80	53	BD	4.0×10^4	2.1	NA	NA
		Yes	3.0	5.5×10^4	18,333	9.1	NA	NA	BD	6.8×10^3	0.8	NA	NA
Winter	D	No	1,466.5	5.1×10^7	34,777	2181	45	35	BD	4.1×10^4	7.5	NA	NA
		Yes	4.9	5.5×10^4	11,224	5.2	NA	NA	BD	1.2×10^4	3.8	NA	NA
Total							553	304				401	140

[a] Colony counts, total cell counts, DNA levels, amounts of 16S rRNA gene sequences, and operational taxonomic units of bacteria in the shower water and shower air with conventional or membrane-integrated showerheads from four different hot water risers in a stem cell transplant unit during two seasons.
[b] Three-day averages for the number of CFU on heterotrophic plates.
[c] Three-day averages for bacterial cell counts by direct microscopy.
[d] Three-day averages for quantitative levels of bacterial DNA by qPCR.
[e] Sequencing results from pooled samples for the number of nonchimeric, nearly full-length 16S rRNA gene sequences.
[f] Sequencing results from pooled samples for the number of operating taxonomic units obtained per pooled sample.
[g] BD, data are below the limit of detection.
[h] NA, data are not available.

to installation of the membrane-integrated showerhead. Gene surveys were also performed for two pooled aerosol samples taken during the summer without the membrane-integrated showerhead in place. For these samples, PCR amplicons were gel purified (Montage DNA gel extraction kit, catalog no. LSKGEL050; Millipore, Billerica, MA) and sent to the Genome Sequencing Center, Washington University School of Medicine, for cloning, purification, and Sanger sequencing on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). 16S rRNA gene sequences were edited and assembled into consensus sequences using PHRED and PHRAP aided by XplorSeq (33). Bases with a PHRAP quality score of <20 were trimmed from the data set before they were aligned using the NAST online tool (19). Chimeras were detected using Bellerophon (37) and removed. Nonchimeric sequences were compared to the Greengenes public database and aligned with the computer application software ARB (43) to determine the rRNA secondary structure information with phylogenetic identification. A sequence identity of 95% or greater was considered to mean organisms of a similar phylum, whereas an identity of 97% or greater was considered to mean the same species. The generated distance matrices were used in the DOTUR program to assess the number of operational taxonomic units (OTUs) and cluster them by pair-wise identity (percent identity [ID]) with a furthest-neighbor algorithm and a precision of 0.01 (58). Assignment of the majority of sequences to phyla was based on their position after parsimony insertion to the ARB dendrogram in the Greengenes database (20). Phylum classifications were double checked, and the percent ID was calculated for nonchimeric sequences with the Ribosomal Database Project II (RDP) (16). Phylogenetic trees using a heuristic algorithm were created with PAUP (72). Finally, UniFrac (42) was used to compare the sampled environments.

DNA extracted from representative colonies grown on tryptic soy agar plates was PCR amplified with universal bacterial primers (8F and 1391R) and gel purified (Montage DNA gel extraction kit, catalog no. LSKGEL050; Millipore, Billerica, MA). The single culture 16S rRNA gene amplicons were sent to Retrogen Inc. (San Diego, CA) for DNA sequencing with 8F as the primer. We obtained a single sequence with a length of ~700 nucleotides for the environmental isolates and did, therefore, not build a consensus sequence or use them for our phylogenetic analyses. These sequences were identified through RDP (16).

Nucleotide sequence accession numbers. Nearly full-length 16S rRNA gene sequences were deposited in GenBank under accession numbers FJ381971 to FJ382924. Sequences from the cultured plates were deposited in GenBank under accession numbers FJ382925 to FJ382941.

RESULTS

Bacterial quantification. The number of culturable bacteria in shower water from a conventional showerhead and a membrane-integrated showerhead ranged from 639 to 27,800 CFU/liter and from 3 to 6 CFU/liter, respectively (Table 1). The levels in the shower aerosol samples, however, were below the limits of detection of our methods. We did obtain total bacterial counts for the shower aerosol samples because the total bacterial counts (culturable and nonculturable) found with an epifluorescence microscope are much higher than the number of culturable bacteria (for shower water, this was on average ~17,000 times higher [Table 1]). We observed between 4,600 and 41,000 cells/m^3 in aerosol samples from the shower stall, while there were between 5.1×10^4 and 5.1×10^7 cells/liter in shower water (Table 1). Our statistical analysis with data from Table 1 indicated a significant difference between sample type (water and aerosol samples; $P = 0.008$) and an effect of installing the membrane-integrated showerhead ($P = 0.008$), but there was not a significant seasonal effect ($P = 0.27$) or riser effect ($P = 0.51$). To further compare our quantitative data and to gauge the effect of a conventional showerhead versus a membrane-integrated showerhead, we averaged the data from the two seasons and the four different risers (Fig. 1). This resulted in an averaged 99.0% (±1.3% [standard deviation]) ($P = 0.0008$) decrease in the number of culturable bacteria in shower water after membrane-integrated showerheads were installed. Similarly, the averaged total bacterial counts in shower water decreased from 2.2×10^7 cells/liter ($\pm 1.6 \times 10^7$) to 6.3×10^4 cells/liter ($\pm 2.1 \times 10^4$) before and after installation of the membrane-integrated showerheads, respectively, which constituted a decrease of almost 3 orders of magnitude (99.6% [±0.36%]; $P = 0.02$) of bacterial cells in shower water

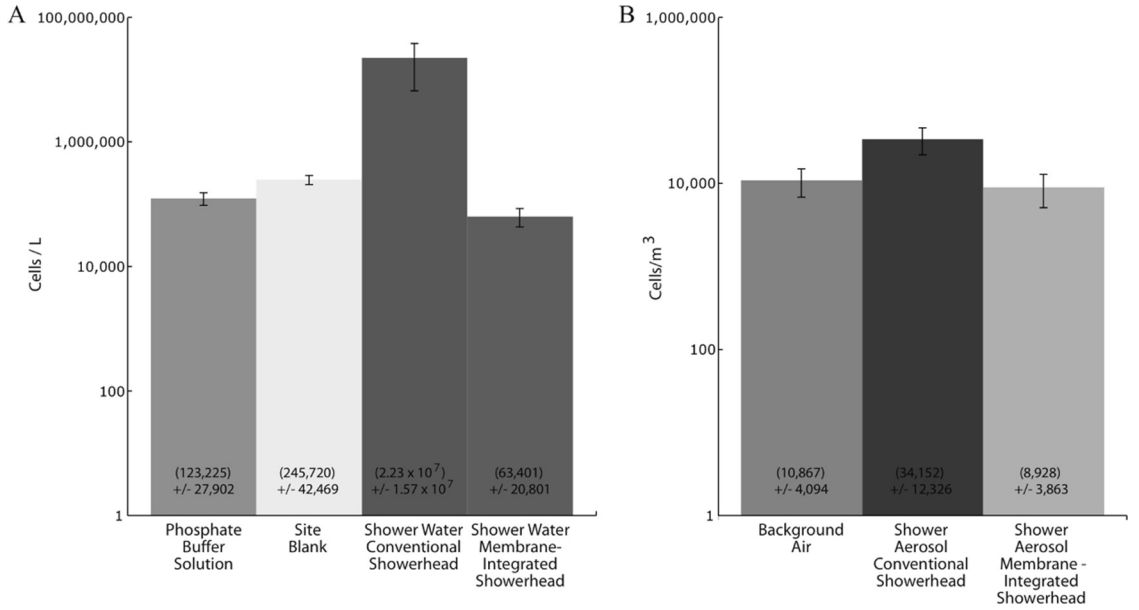

FIG. 1. Averaged total bacterial counts for shower water (A) and shower air (B) with conventional showerheads and with membrane-integrated showerheads. The total bacterial counts (number of cells per liter or per m^3) were determined by direct epifluorescence microscopy. Error bars indicate 95% confidence intervals for the combined summer and winter data.

(Fig. 1A). Using the PBS and the site blank as estimated backgrounds for the shower water, we found that the bacterial concentration in water collected with the membrane-integrated showerhead was lower than these background solutions (Fig. 1A). For the shower aerosol, the averaged total bacterial counts decreased from 3.4×10^4 cells/m^3 ($\pm 1.2 \times 10^4$) to 8.9×10^3 cells/m^3 ($\pm 3.9 \times 10^3$) before and after installation of the membrane-integrated showerheads, respectively (Fig. 1B), which is an 82.4% ($\pm 2.3\%$; $P = 0.07$) decrease. Furthermore, the averaged total bacterial counts for aerosols with the membrane-integrated showerheads were at the same level as the counts for the background aerosols (8.9×10^3 [$\pm 3.9 \times 10^3$] and 1.1×10^4 [$\pm 4.1 \times 10^3$], respectively; Fig. 1B). These reductions in total bacterial cells were comparable with qPCR for which the number of targeted amplicons decreased from an average of 7.6×10^2 pg DNA/liter to 4.5 pg DNA/liter in shower water and an average of 4.0 pg DNA/m^3 to 1.75 pg DNA/m^3 in aerosol samples before and after installation of the membrane-integrated showerhead, respectively (nonaveraged data are shown in Table 1). This corresponds to a 99.0% ($\pm 1.4\%$; $P = 0.01$) decrease in the shower water and a 70.5% ($\pm 16\%$; $P = 0.02$) decrease in the shower aerosol. Indeed, our statistical analysis showed a strong correlation between the data from epifluorescence microscopy and qPCR methods ($R^2 = 0.979$; $P < 0.0001$).

Sequence analyses. We characterized four shower water samples and two aerosol samples in shower stalls in the stem cell transplant unit by sequencing 954 nearly full-length 16S rRNA genes, which were acquired directly from the environment without a culturing step. These water and aerosol samples were pooled samples from three sampling days with a conventional showerhead. The DNA concentration for the winter aerosol samples and for all samples with the membrane-integrated showerhead was below the level required for a successful cloning step in the sequencing pipeline at the genome-sequencing center (even after two PCR amplification steps), and thus, we were not able to acquire gene surveys for these samples. All shower water samples were diverse in terms of their bacterial phylum composition (Fig. 2A, B, E, and F). Some similarities between the water samples were found—the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Clostridia*, and *Proteobacteria* were identified in all samples even though the percentage of each phylum was different for samples (Fig. 2). Several bacterial phyla were found only in a single sample: the phylum *Erysipelotrichales* (10%) in a summer water sample from riser A and TM7 (2%) in a winter water sample from riser D. A small number of nonclassifiable bacteria (2% to 9%) were found in each of the water samples (Fig. 2). Only bacteria from the phylum *Proteobacteria* were identified in the aerosol samples with *Alpha-*, *Beta-*, and *Gammaproteobacteria* in the summer aerosol sample from riser A, and *Alpha-* and *Betaproteobacteria* in the summer aerosol sample from riser B (Fig. 2C and D).

Further phylogenetic analyses were performed to classify individual 16S rRNA gene sequences. We found 444 different operational taxonomic units for the 954 nonchimeric sequences. The RDP algorithm was used to identify the sequences from shower water and aerosol samples from conventional showerheads with a specific interest to identify potentially pathogenic bacteria (Table 2). The most notable potential pathogens identified were *Mycobacterium mucogenicum* in shower water samples and *Pseudomonas aeruginosa* in the aerosol samples. To further verify the relatedness of these important potential pathogens to sequences in public databases, we performed maximum-likelihood, maximum-parsimony, and neighbor-joining analyses on the sequences that were identified within two groups of microbes: (i) the suborder *Corynebacterineae* of the phylum *Actinobacteria* to include

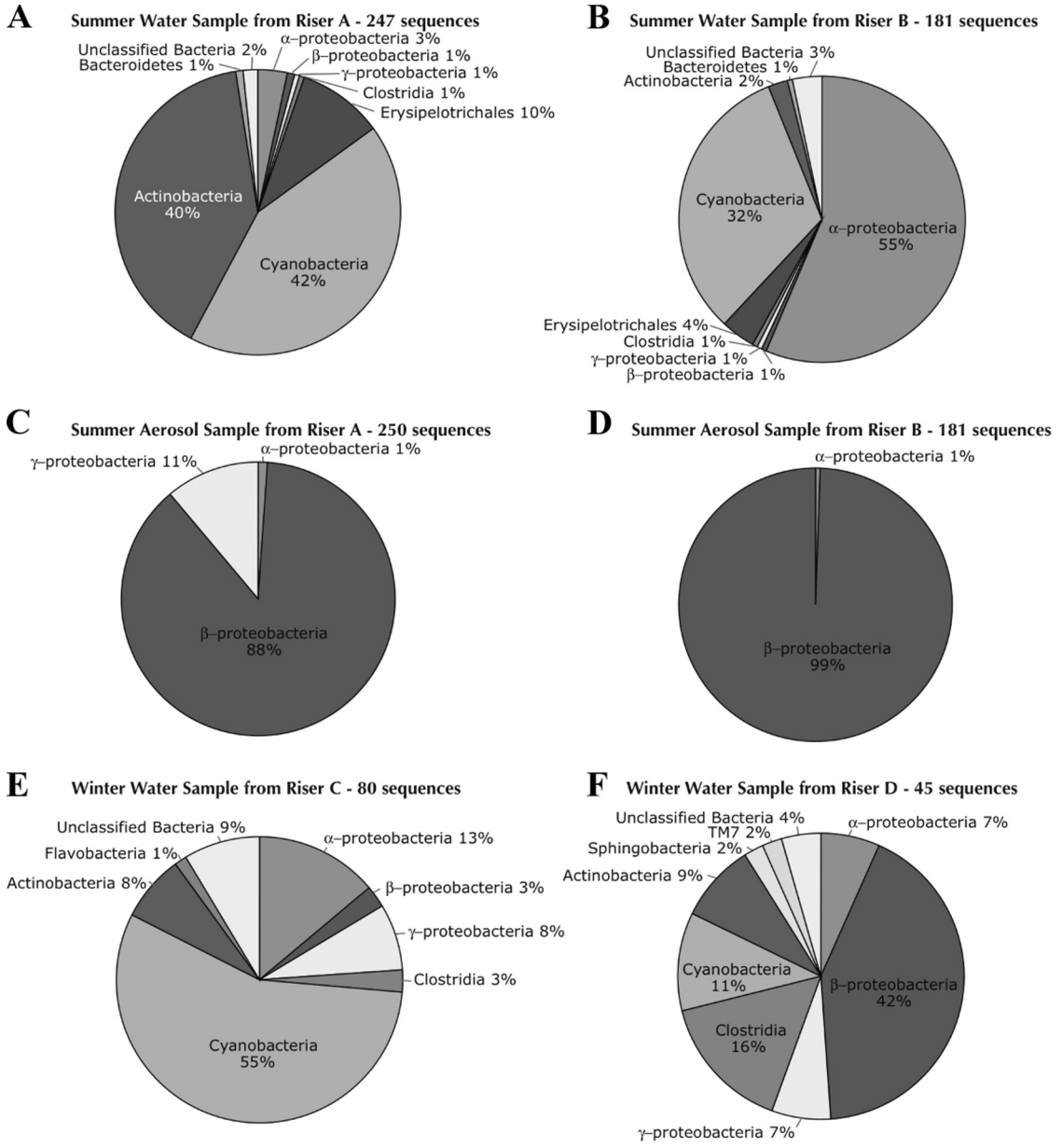

FIG. 2. Phylogenetic distributions of bacterial 16S rRNA gene sequences. The percentages of phylum or class distribution for samples from conventional showerheads are shown for the following samples: summer water samples from hot water riser A (247 sequences) (A), summer water samples from riser B (181 sequences) (B), summer aerosol samples from riser A (250 sequences) (C), summer aerosol samples from riser B (151 sequences) (D), winter water samples from riser C (80 sequences) (E), and winter water samples from riser D (45 sequences) (F). Phylum distribution was shown for all bacterial sequences except for the phylum *Proteobacteria*, which was subdivided into classes *Alpha*-, *Beta*-, and *Gammaproteobacteria*, and for the phylum *Bacteroidetes* (in panels E and F), which was subdivided into the classes *Flavobacteria* and *Sphingobacteria* (winter water samples).

closely related mycobacteria (Fig. 3B); and (ii) the class *Gammaproteobacteria* to include the *Pseudomonas* and *Legionella* genera (Fig. 3C). These analyses produced similar trees, with similar bootstrap support at resolved branches (data not shown). For the *Corynebacterineae*, 88 16S rRNA gene sequences from the summer water sample from riser A were most closely aligned to published *M. mucogenicum* and *Mycobacterium phocaicum* sequences (Fig. 3B). We also retrieved 18 sequences from colonies with different morphologies, which were grown from bacteria in shower water with conventional showerheads: from the summer water samples, we identified three sequences from riser A and three sequences from riser B, which were closely related to *M. mucogenicum* (≥99.5% ID). Therefore, this pathogenic strain was cultured under laboratory conditions and consisted of ~35% (88/247) of the directly retrieved sequences and 90% (9/10) of the cultured sequences from the summer water sample from riser A. Thus, this potential pathogen was present in the summer water samples at very high numbers, and at least some of the cells were viable. The other important potential pathogen identified was *P. aerugi-*

TABLE 2. Summary of potentially pathogenic bacterial sequences for bacteria in shower water and air samples from a stem cell transplant unit[a]

Season	Sample	Riser	No. of sequences[b]	Sequence ID	Method of detection	Class/phylum	% ID[c]	Nearest relative for potential pathogens
Summer	Water	A	88/247	4S_4g02	Direct extraction	*Actinobacteria*	99.5	*Mycobacterium mucogenicum*
			9/10	4S_isolate4	Culture	*Actinobacteria*	99.6	*Mycobacterium mucogenicum*
		B	19/24	11S_isolate3	Culture	*Actinobacteria*	99.5	*Mycobacterium mucogenicum*
	Aerosol	A	3/250	6S_3a07	Direct extraction	*Gammaproteobacteria*	99.3	*Pseudomonas aeruginosa*
		B	1/151	13S_2f07	Direct extraction	*Alphaproteobacteria*	97.6	*Bosea thiooxidans*
Winter	Water	C	2/8	4W_isolate2	Culture	*Firmicutes*	99.9	*Bacillus cereus*

[a] Summary of potentially pathogenic bacterial sequences for bacteria, their percent ID to a known sequence in a public database, and their relative abundance in shower water or shower aerosol samples from four different hot water risers in a stem cell transplant unit during two seasons.
[b] For sequences detected by direct extraction, the number of similar sequences to the total number of sequences assessed for each season and riser is shown. For sequences detected by culture, the number of similar CFU to the the total number of CFU isolated for each season and riser is shown.
[c] Percent ID values based on RDP sequence match near-neighbor identification.

nosa in the summer aerosol sample from riser A. This species was much less abundant compared to the *Mycobacterium* species when directly retrieved from the air sample (3/250 sequences) and was not identified in the water sample because of the presence of more abundant bacteria.

The individual sequences, their evolutionary relationship, and sample distribution are visualized in a comprehensive phylogenetic tree to identify commonalities between samples (Fig. 3A). Related sequences were found in the shower water and shower aerosol samples within the class *Betaproteobacteria*, the class *Gammaproteobacteria*, the order *Rhizobiales* (*Alphaproteobacteria*), and the genus *Sphingopyxis* (*Alphaproteobacteria*) (Fig. 3A). However, none of the related sequences in either water or aerosol samples were >97% identical (i.e., from a single species) to each other. This is illustrated in Fig. 3C for the class *Gammaproteobacteria* for which the sequences in the water samples were closely related to the genera *Acinetobacter*, *Legionella*, *Rickettsiela*, and *Aquicella*, while the aerosol samples were closely related to *P. aeruginosa*. We also found that the species composition in the water samples varied considerably between seasons (Fig. 3A) and that the summer water samples were more similar to each other than to the winter water samples (Fig. 4). Clustering of the samples based on the OTUs in the community also verified that the shower water

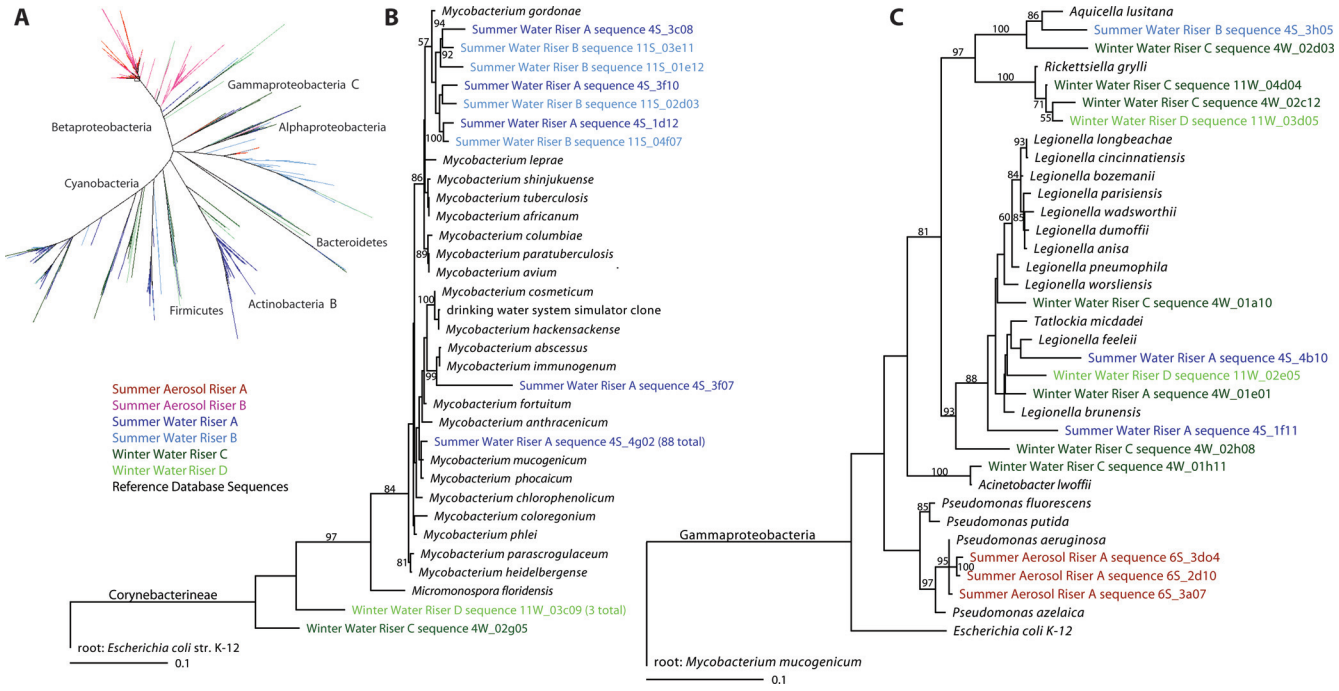

FIG. 3. Phylogenetic relationships of bacterial sequences from shower water and shower aerosol samples for four hot water risers in a stem cell transplant unit during two seasons. (A) Unrooted phylogenetic tree showing all sequences and their origin; (B) maximum-likelihood phylogenetic tree of selected sequences from the subclass *Corynebacterineae* originating from our environmental samples and from the Greengenes database; and (C) maximum-likelihood phylogenetic tree of selected members of the class *Gammaproteobacteria* originating from our environmental samples and from the Greengenes database. The bootstrap numbers were generated with a neighbor-joining analysis in panels B and C. The scale bar represents base changes per site in panels B and C.

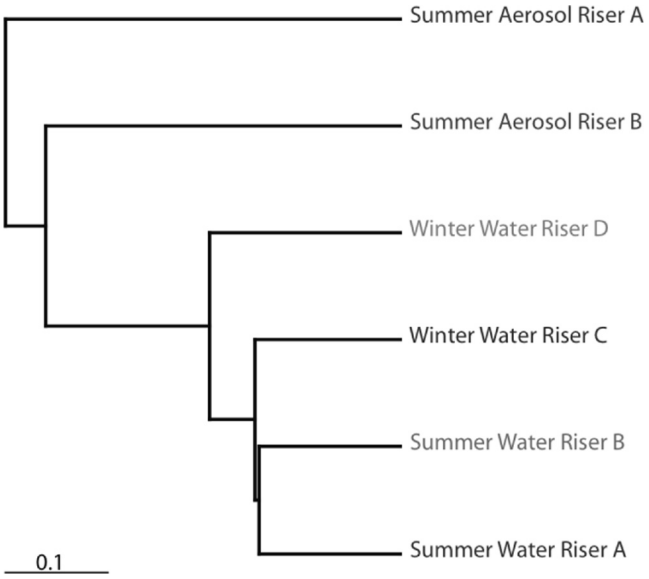

FIG. 4. Clustered sample sets with weighted and normalized UniFrac analysis. The scale bar represents branch length units.

and aerosol communities were more different from each other than the bacteria in different water samples regardless of the sampling season (Fig. 4).

DISCUSSION

The membrane-integrated showerhead reduced the microbial load in shower water and aerosols to background water and air microbial concentrations. The observed numbers of culturable bacteria in the shower water samples from the conventional showerhead were lower than the CDC's recommended maximum containment level of 500 CFU/ml (67), while total bacterial counts were within published findings for stored tap water cell counts (10^7 to 10^8 cells/liter) (57). Therefore, the bacterial levels in the shower water in this stem cell transplant unit with conventional showerheads were within a typical range for a hospital. The installation of the 0.2-μm-pore-size membrane-integrated showerhead lowered the microbial load considerably in the shower water to background water and air microbial concentrations that were similar to or lower than our lab-filtered buffer solutions (Fig. 1A).

In contrast, the microbial loads in the shower aerosols were much lower than typical indoor and outdoor environments due to the use of pressurized HEPA-filtered air in the rooms in the sampled stem cell transplant unit. The shower aerosol from the conventional showerhead contained an averaged total bacterial count of 3.4×10^4 cells/m^3 (~17 microbes per breath—assuming a tidal volume of 500 ml), which is approximately 1 order of magnitude lower than an aerosol sample taken in a single-family home (2.1×10^5 cells/m^3; 10^5 microbes per breath) (27); 2 orders of magnitude lower than in an indoor hospital therapy pool (10^6 cells/m^3; 500 microbes per breath) (5); 1 to 3 orders of magnitude lower than an outdoor aerosol sample collected in Salt Lake City, UT (10^5 to 10^7 cells/m^3; 50 to 5,000 microbes per breath) (52); and 1 order of magnitude lower than the outdoor air in East St. Louis, IL (1.5×10^5 cells/m^3; 75 microbes per breath). The latter total bacterial count is of interest because it allows us to compare our findings with the outdoor air of St. Louis, MO. During a year-long, daily sampling campaign of the outdoor air in East St. Louis, IL, which is seven miles east of the hospital, Rauer (53) measured an arithmetic average total bacterial count of 1.5×10^5 ($\pm 1.1 \times 10^4$) cells/m^3. He also found a seasonal effect on the total bacterial counts with higher counts (1.8×10^5 [$\pm 1.8 \times 10^4$]) cells/m^3 during the summer months. This would have resulted in an inhalation of ~90 microbes per breath on an average summer day. Thus, the microbial load in the indoor environment of our stem cell transplant unit (17 microbes per breath when the conventional shower was running) was considerably lower than that of the outdoor environment seven miles east of Barnes-Jewish hospital, in East St. Louis, IL.

In addition, the culturable bacterial levels in the ambient air of the stem cell transplant unit were also lower than in typical indoor environments, but the levels were similar for other stem cell transplant units. Angenent et al. (5) found the ratio of total bacterial cells to the CFU count from air to be between 500 and 5,000, while Radosevich et al. (52) reported a ratio of 1,250 for air. Our ratios in shower water were even higher than that (Table 1). Others have discussed a ratio higher than 100 for soil and water (3, 49). Therefore, due to the low sample volume and a relatively high ratio of total bacterial cells to CFU in our study, it is not a surprise that the CFU counts in our aerosol samples were below detection. On the basis of a ratio of 1,000 for air, which is in agreement with previously published studies (5, 52), we estimate the CFU counts of our aerosol samples to be 34 and 10 CFU/m^3 before and after installation of the membrane-integrated showerhead, respectively. This estimate is similar compared to the value for rooms in a stem cell transplant unit in Taiwan ($\leq$32 CFU/m^3) (41), but it is much lower than in a Polish pneumonia ward (296 to 530 bacterial and fungal CFU/m^3) (6). Thus, even with a conventional showerhead, the culturable bacterial levels in the shower aerosols from shower stalls in the stem cell transplant unit was very low.

The installation of the membrane-integrated showerhead decreased the microbial load to background levels (10^4 cells/m^3) of the indoor air of the stem cell transplant unit during a showering event (four to six microbes per breath). The relatively low background levels of bacteria in the indoor air were helpful in this study because we were able to show a relatively small increase of $\sim 2.5 \times 10^4$ cells/m^3 in microbial load for the shower aerosol when the conventional showerhead was on. Such an increase would not have been statistically significant for a study of a typical indoor air environment with 10^5 to 10^6 cells/m^3. This may explain why, to our knowledge, no other studies with a significant increase in shower aerosol bacterial levels were found in the literature.

Potentially pathogenic bacteria were present in shower water and aerosol samples from the stem cell transplant unit. The most notable potential pathogens in shower water were the relatively high numbers of *M. mucogenicum* sequences in the summer. However, no mycobacteria were detected in the winter water samples. Species within the *Mycobacteriaceae* family are known for their robustness due to a waxy outer membrane layer, which protects them from disinfectants, such as chlorine in domestic water (24, 29). *M. mucogenicum* was isolated from the water system in a French

hospital, where it was linked to two terminal infections in immunocompromised patients (2). In addition, *Mycobacterium* spp. in air have caused aerosol-related health problems (5, 28, 71), however, no mycobacterial sequences were identified in our summer aerosol samples. Other sequences in the shower water samples were identified as *Mycobacterium* spp., including *Mycobacterium gordonae* (98.5% ID). Identification of *Mycobacterium* spp. through 16S rRNA gene surveys is difficult because they are very closely related to one another with only 0- to 7-bp differences within the 16S rRNA gene between species, while in most other microbes this difference is between 5 and 15 bp (65). Therefore, Hussein et al. (37a) required a ≥99% homology to database sequences before species identification in their study of nontuberculosis mycobacteria in hospital waters. Even with such close to perfect homology, our maximum-likelihood phylogenetic tree showed that the sequences were indistinguishable between *M. mucogenicum* and *M. phocaicum* due to a 100% identical 16S rRNA gene sequence (Fig. 3B). *M. phocaicum* is also pathogenic and is associated with chronic pneumonia (1). Here, we identified our sequences as *M. mucogenicum* because RDP matched our directly extracted and cultured gene sequence closest to this species. However, we realize that for a true identification between *M. mucogenicum* and *M. phocaicum*, other genes or enzymes must be targeted.

Three sequences out of a total of 250 sequences (~1%) in summer aerosols from riser A were identified as *Pseudomonas aeruginosa* (99.3% ID). *P. aeruginosa* is commonly linked with ventilator-associated pneumonia, as biofilms with *P. aeruginosa* grow on endotracheal tubes (7, 30). The other pathogen from a summer aerosol sample (from riser B) was identified as *Bosea thiooxidans* (98.6% ID), which is a member of the *Alphaproteobacteria* initially isolated from agricultural soil (62). Recently, this species has been identified as a close relative to multiple pathogenic aquatic organisms isolated from hospital water supplies in French and Swiss hospital water systems (39, 64). Finally, a strain of *Bacillus cereus* (99.9% ID) was cultured from winter water samples (riser C). *B. cereus* strains are associated with food-borne illnesses and *Bacillus anthracis*-related virulence genes that can be detrimental to immunocompromised individuals (61). Dohmae et al. (22) identified hospital towels and laundering facilities, including the rinse water, to be sources of *B. cereus*. Even though we sequenced up to ~250 clones from our samples, the diversity in environmental samples is high enough that we identify only the most abundant microbes. Less prevalent microbes have the ability to infect patients but will be identified only when the number of sequences per sample increases. Here, the less prevalent *B. cereus* was found in the water sample by culturing even though it was not identified with the 16S rRNA gene survey, because we enriched for this viable organism by the growth conditions used in our lab. The phenomenon of enrichment by culturing has been described in detail previously (56).

***Legionella* sequences were found in the shower water.** The threat of *Legionella* species as environmental contaminants has come to the forefront since it was first isolated in July 1976 (21). To date, there are over 70 members of the genus *Legionella* that inhabit natural aquatic environments as intracellular parasites to protozoa (12). Thus far, ~40% of the identified *Legionella* species are human pathogens, and *L. pneumophila* has been isolated in over 90% of culture-confirmed cases of legionellosis. Another 9% of legionellosis cases have been caused by *Legionella* spp., such as *Legionella longbeachae*, *L. bozemanii*, *L. feeleii*, *L. dumoffii*, *L. wadsworthii*, and *L. anisa*, and by *Tatlockia micdadei* (46). A total of six water sequences (two from summer water samples from riser A, three from winter water samples from riser C, and one from a winter water sample from riser D) were identified as *Legionella* (Fig. 3C). None of these were positively identified (>97% ID) to a known *Legionella* species in RDP, and they did not align closely to any *Legionella* species in the Greengenes database (Fig. 3C). This genus is ubiquitous in natural and man-made aquatic environments (9, 11, 12, 15, 17, 21, 35, 46), and finding sequences of this genus does not automatically indicate pathogenesis. To conclude whether the identified *Legionella* species in the shower water poses a threat to hospital patients, isolation (by culturing) and characterization of the strain are necessary.

Cyanobacterial sequences in the shower water are a marker for St. Louis drinking water. The concept of an endosymbiotic origin of chloroplasts within plants has been accepted (54). In this study, 51% (110/214) of the cyanobacterial sequences found in the water samples were further identified as 16S rRNA genes from chloroplasts of eukaryotic algae. Algae are ubiquitous in surface waters that supply drinking water treatment facilities (36, 69), and therefore, the presence of these cells in the hospital water is not surprising and represents an environmental marker for drinking water in St. Louis, MO. In the summer shower water sample from riser A, 95% (101/106) of cyanobacterial sequences were from chloroplast of the genus *Bacillariophyta* (diatoms) and matched closest to an environmental uncultured sequence collected from an Indian wetland (98.9% ID). Previous studies have reported similar findings in freshwater rivers and estuaries (18).

Proteobacterial sequences were common in water and aerosol samples. The phylum *Proteobacteria*, especially the classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*, is the dominant microbial group that was identified in bulk water distribution systems and drinking water biofilms (9, 26, 45, 59). In the present study, 28% of all the water sequences were *Proteobacteria* (160/553), and 78% of these were *Alphaproteobacteria* (124/160). The nitrifying organisms present within *Proteobacteria* are of particular interest because nitrification along with extended residence times, such as in storage tanks, are known to deplete chloramine residuals and ultimately lead to increased microbial growth (9, 60), resulting in an increase in growth of potentially pathogenic organisms in biofilms. Nitrifying organisms comprised 5% (29/553) of the identified water sequences in this study. We were, therefore, not surprised to find *Proteobacteria* sequences in the shower water. However, we had not anticipated the *Proteobacteria* to overwhelm the community in the summer aerosol samples, especially since the source water samples from the summer showed an abundance of *Mycobacteriaceae* sequences. Species in the family *Mycobacteriaceae* are known to selectively partition out from standing source water (e.g., pool water) into the aerosol by the "bubble burst" mechanism from the water film due to their hydrophobic cell membranes (5, 50). Here, we did not find this phenomenon, possibly because shower aerosol formation can be explained by a different mechanism, a mechanism similar to "jet

mist" generation. Our work verified the work of other gene surveys of air, which found *Proteobacteria* to be the most common phylum. Specifically, three out of the seven published outdoor air surveys showed *Proteobacteria* to be exceeding 60% of the bacterial composition (8, 32, 44). In addition, the indoor air of a shopping mall showed similar percentages of *Proteobacteria* (>60%) (66), while the indoor air in modern Finnish houses consisted of 44 to 50% *Proteobacteria* (55). Research is necessary to understand the mechanisms of proteobacterial enrichment in air.

Should membrane-integrated showerheads be used to protect extremely immunocompromised patients? We have shown that membrane-integrated showerheads in shower stalls in a stem cell transplant unit reduced the microbial load in water and aerosol. However, does this warrant the investment of ~$30 for each membrane-integrated showerhead per week (they must be replaced every 7 days) and will they prevent infections in the stem cell transplant patient population? Most of the potentially pathogenic sequences (88/247) in our 16S rRNA gene survey (out of 92/954) were found in one shower water sample (summer water sample from riser A). This potential pathogen, which was identified as *M. mucogenicum*, was also cultured from these summer shower water samples (with a conventional showerhead), and therefore, at least some of the *M. mucogenicum* bacteria were viable. Considering the average total bacterial count of 2.2×10^7 cells/liter in the shower water from the conventional showerhead, membrane-integrated showerheads in stem cell transplant units may prevent the transmission of waterborne pathogens from showerheads to extremely vulnerable patients. A further study is needed to determine the incidence of waterborne infections with and without the membrane-integrated showerhead and therefore to firmly conclude whether recommendation for this engineering control is warranted. Conversely, it is unlikely that these showerheads will be able to reduce the occurrence of infections from shower aerosols in the immunocompromised population due to a combination of the low microbial load of shower air (with a conventional showerhead) and the relatively low occurrence of potentially pathogenic bacterial species in the shower aerosol samples.

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