FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Potential pathogenic bacteria in metalworking fluids and aerosols from a machining facility

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Received 28 April 2010; revised 20 August 2010; accepted 6 September 2010.
Final version published online 18 October 2010.

DOI:10.1111/j.1574-6941.2010.00976.x

Editor: Max Häggblom

Keywords
bioaerosols; metalworking fluids; bacterial load; 16S rRNA gene survey; *Mycobacterium immunogenum*; *Alcaligenes faecalis*.

Abstract

The metalworking and machining industry utilizes recirculating metalworking fluids for integral aspects of the fabrication process. Despite the use of biocides, these fluids sustain substantial biological growth. Subsequently, the high-shear forces incurred during metalworking processing aerosolize bacterial cells and may cause dermatologic and respiratory effects in exposed workers. We quantified and identified the bacterial load for metalworking fluid and aerosol samples of a machining facility in the US Midwest during two seasons. To investigate the presence of potentially pathogenic bacteria in fluid and air, we performed 16S rRNA gene surveys. The concentration of total bacterial cells (including culturable and nonculturable cells) was relatively constant throughout the study, averaging 5.1×10^8 cells mL^{-1} in the fluids and 4.8×10^5 cells m^{-3} in the aerosols. We observed bacteria of potential epidemiologic significance from several different bacterial phyla in both fluids and aerosols. Most notably, *Alcaligenes faecalis* was identified through both direct sequencing and culturing in every sample collected. Elucidating the bacterial community with gene surveys showed that metalworking fluids were the source of the aerosolized bacteria in this facility.

Introduction

The metalworking industry utilizes recirculating metalworking fluids to cool, remove metal fines, lubricate, and prevent corrosion during metal grinding and cutting procedures. High-shear forces and excess heat create metalworking aerosols during standard grinding, drilling, and cutting tasks (Simpson *et al*., 2003). Metalworking fluids are mixtures of oils, biocides, dissolved metals (e.g. chromium, nickel, cobalt), microorganisms, antifoaming agents, and other organic and inorganic constituents, which can be toxic to operators (Woskie *et al*., 1996; Kennedy *et al*., 1999; Gordon, 2004). Epidemiological assessments with machine operators in the metalworking industry have identified dermatologic effects (Godderis *et al*., 2008) and respiratory effects (Barhad *et al*., 1975; Woskie *et al*., 1996; Greaves *et al*., 1997; Kennedy *et al*., 1999; Woskie *et al*., 2003; Park *et al*., 2007; Park *et al*., 2008). Kennedy *et al*. (1999) reported an increase in bronchial responsiveness with symptoms of asthma in metalworking apprentices after 2 years of exposure, while a study by Park *et al*. (2007) showed a decrease in pulmonary function with daily shift progression among machining workers. Therefore, the National Institute for Occupational Safety and Health has recognized the adverse effects of metalworking fluid aerosols on machinists and has issued a recommended exposure limit of 0.4 mg m^{-3} thoracic particulate mass, which are inhalable particles that are capable of penetrating into the bronchial region of the lung (US Department of Health and Human Services, 1998).

The interest in bacterial exposures to metalworking personnel has intensified because an outbreak of hypersensitivity pneumonitis was linked to high concentrations of bacteria in metalworking fluid (Wallace *et al*., 2002). Hypersensitivity pneumonitis is a pulmonary disorder involving an immunologic reaction of the lung to inhaled sensitizing agents of organic origin, such as bacterial or fungal antigens within aerosols (Bernstein *et al*., 1995; Khalil *et al*., 2007). To cause such an immunological response, the

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bacterial cell does not have to be viable. *Pseudomonas* spp. has been discussed as the primary gram-negative culture in some metalworking fluids (Simpson *et al.*, 2003). Gram-positive bacteria are also abundant in metalworking fluids and the cell wall of, for example, mycobacteria have been found to induce an inflammatory response in humans (Falkinham, 2003). In fact, the gram-positive *Mycobacterium immunogenum* has been isolated in multiple metalworking facilities across North America and have been associated with at least 98 cases of hypersensitivity pneumonitis (Chang *et al.*, 2004). The relative abundance of *M. immunogenum* in metalworking fluid suggests that this organism is resistant to biocides commonly used in the metalworking industry (Wallace *et al.*, 2002; Selvaraju *et al.*, 2008). The thick hydrophobic cell wall of mycobacteria increases their resistance to conventional disinfectants, such as chlorine, monochloramine, chlorine dioxide, and ozone treatments, giving them a competitive edge in potable water (Carson *et al.*, 1978; Taylor *et al.*, 2000; Falkinham, 2003; Vaerewijck *et al.*, 2005).

Multiple control measures have been applied in metalworking facilities to reduce occupational exposure to aerosols. Machine enclosures, exhaust filters, and electrostatic precipitators have been added, while biocides and formaldehyde in fluids have been used to combat microbial growth (Linnainmaa *et al.*, 2003; Cohen & White, 2006). Here, we quantified and identified bacteria from air samples of *one* metalworking facility with machine enclosures and biocides within a sampling period of 6 days for two seasons. We investigated the presence of potential pathogenic bacteria with 16S rRNA gene sequence surveys. We also sampled metalworking fluids to ascertain whether the bacterial communities of the fluid and aerosol were similar. To our knowledge, this would be the second study to analyze communities for both metalworking fluid and aerosol samples. A recent study by Gilbert *et al.* (2010a, b) utilized gradient gel electrophoresis fingerprints to assess metalworking fluid communities; and herein, we used comprehensive gene surveys. We, therefore, did not rely on just culturing techniques that have been shown to only identify $< 1\%$ of the microorganisms in environmental samples (Amann *et al.*, 1995; Pace, 1997; Edwards, 2000) and 0.02–0.2% of the bacterial cells in aerosol samples (Angenent *et al.*, 2005). Furthermore, with a comprehensive gene survey, we also included nonviable bacteria other than specifically targeted genera of *Pseudomonas* and *Mycobacterium*.

Materials and methods

Facility details

The US Midwest metalworking facility that was sampled in this study employs ~100 workers. The computer numerical control area of the facility houses a total of 13 machines: five Makino A51 horizontal machining center units and eight other computer numerical control type units with metalworking fluids [the workshop floor plan (Fig. S1) with other details about this facility can be found in the online Supporting Information].

Sampling plan

Metalworking fluid, indoor metalworking aerosol, and outdoor aerosol samples were collected during this study for two seasons – winter and summer of 2008. For both seasons, we sampled for a total period of 6 days in two 3-day intervals (days 1–3 and days 4–6), which were separated by a 2-day weekend. We sampled two Makino A51 machines (machines A and B) in the computer numerical control area (Fig. S1), and because both of them had independent fluid recirculation tanks, the fluids were sampled and tested separately throughout the study. The indoor aerosol samples were collected adjacent to the work area of these two machines. Background air samples were collected outdoors and upwind from the facility. Quantitative culturing and microscopy methods were performed daily for all samples, while samples for DNA extraction were filtered and stored at $-80\,^{\circ}C$ after each sampling event.

Aerosol sampling procedure

Aerosol samples were collected with liquid impingers (Biosampler®, AGI-30, SKC, Eighty-four, PA) at a rate of 12.5 LPM for 60 min, allowing a total sample volume of 0.75 m^3 to pass through each of three samplers (three samplers were run simultaneously at each location). Three flow meters (RMA-22 Dwyer, Michigan City, IN) were used to control the airflow rate (one flow meter for each Biosampler). The Biosamplers were positioned on a sampling stand 1.5 m from the floor and were filled with 20 mL of sterile phosphate buffer solution (PBS). After a 30-min sampling period, the volume of PBS for each sampler was topped off when necessary to ensure that the sampling solution was not lost to evaporation. At the end of the sampling period, this solution was combined from the three samplers and the final volume was recorded. A condensation particle counter (3760A, TSI, Shoreview, MN) was used for real-time analysis of the total concentration of particles $> 0.01\,\mu m$ in diameter at the same location as the aerosol sampler. The inlet air tube of the condensation particle counter was positioned at the center of the same sampling stand. Two high-volume vacuum pumps (2688VE44, 2669CE44, Thomas, Lake Zurich, IL) were connected to the condensation particle counter and the Biosampler flow meters with norprene tubing (Masterflex, Vernon Hills, IL).

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Bacterial quantification: colony counts, total bacterial counts, and quantitative PCR (qPCR)

To quantify heterotrophic bacterial growth, we inoculated duplicate, serial-diluted, aliquots of metalworking fluid, aerosols, and sterile PBS onto tryptic soy agar plates amended with 0.1 g L^{-1} cyclohexamide (C7698, Sigma, St. Louis, MO) to repress fungal growth. We used an Autoplate 4000 (Spiral Biotech, Norwood, MA) to plate these samples. All inoculated plates were incubated at 35 °C for 24 h before quantification by colony counts, which we expressed as CFUs. Total cell counts were conducted through the epifluorescence microscopy procedure: 0.5 mL of a 1/10th dilution of metalworking fluid or 20 mL of the collection solution was filtered through a 0.22-μm black polycarbonate filter (GE Water, Trevose, PA), stained with 1 μM of DAPI solution (Sigma), and rinsed. Cells were then counted from 20 fields of view using an epifluorescence microscope (Olympus BX41, Center Valley, PA). qPCR was used to determine the bacterial DNA load within each sample. Wells contained 2 μL of extracted DNA template and 23 μL of SYBR green master mix (ABgene, Rockford, IL) supplemented with 0.25 U UDP-*N*-glycosidase and 10 μM of universal bacterial primers (forward primer – 5′-TCCTACGGGAGG CAGCAGT-3′; reverse primer – 5′-GGACTACCAGGGTATC TAATCCTGTT-3′) (Nadkarni *et al*., 2002). Samples were analyzed with a Stratagene Mx3000P qPCR system (Cedar Creek, TX), using the program outlined, and verified by An *et al*. (2006) (40 cycles at 95 °C for 15 s, 1 min at 60 °C, 30 s at 72 °C with data collection temperatures of 85–88 °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 dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The standard curve of *E. coli* DNA ranged from 0.034 pg L^{-1} to 34.7 ng L^{-1} with an R^2 of 0.94.

DNA extraction and PCR amplification

For each day of sampling, we concentrated 40 mL of PBS collection solution (pooled from three samplers), before DNA extraction using an isopore membrane filter of 0.2-μm pore-size (GTTP02500, Millipore, Billerica, MA) mounted on polysulfone filter funnel stands (28144-700, Pall, Ann Arbor, MI) that were sterilized by a sequential treatment with bleach, DI water rinse, UV, and the autoclave. Samples were immediately stored at −80 °C until the filter was subjected to a bead-beating and phenol:chloroform extraction protocol (Angenent *et al*., 2005). The 16S rRNA genes were amplified using a 30-cycle touchdown PCR [initial 2-min denaturing step at 94 °C, followed by 20 cycles at 92 °C for 30 s, gradient 65–45 °C (−1 °C per cycle) for 90 s, 72 °C for 90 s; these touchdown cycles were followed by 10 cycles at 92 °C for 30 s, 45 °C for 30 s, 72 °C for 30 s, and a final 72 °C extension for 15 min]. The 50-μL solution contained 1.25 U of GoTaq (Promega Corp., Madison, WI), 0.4 pmol μL^{-1} forward and reverse primers (8F – 5′-AGAG TTTGATCCTGGCTCAG-3′; 1391R – 5′-GACGGGCGG TGWGTRCA-3′) targeting bacterial 16S rRNA genes (Lane, 1991), 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 mg mL^{-1} bovine serum albumin (BSA), and 2 μL of template. Control positive and negative reactions were included with each reaction set, while BSA was added to impede amplification inhibition. DNA from representative colonies grown on the tryptic soy agar plates were extracted and PCR amplified with universal bacterial primers (8F and 1391R) according to the same PCR protocol.

Cloning, sequencing, and analyses

16S rRNA gene surveys were performed for a pooled ($n = 6$) sample of metalworking fluid from each machine during each of two seasons (winter fluid machine A; winter fluid machine B; summer fluid machine A; and summer fluid machine B). The aerosol samples that were collected adjacent to both machines A and B (each machine had a separate sampling setup) were pooled (from three sampling days) into four samples based on season and sampling days 1–3 and days 4–6. An additional aerosol sample (days 4–6) from machine B collected during the winter sampling event was also surveyed (winter aerosol machine A and B days 1–3; winter aerosol machine A and B days 4–6; winter aerosol machine B days 4–6; summer aerosol machine A and B days 1–3; and summer aerosol machine A and B days 4–6). For each of these samples, PCR products were cleaned, concentrated (Montage PCR Cleaning Kit, UFC7PCR50, Millipore), and sent to the Genome Sequencing Center at the Washington University School of Medicine in St. Louis, for cloning, purification, and Sanger sequencing on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). The 16S rRNA gene PCR products from the single cultures grown on TSA were gel purified (Montage DNA gel extraction kit, LSKGEL050, Millipore). Purified 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 nt for these environmental isolates and, therefore, did not build a consensus sequence or use them for our phylogenetic analyses. These sequences were identified through RDP (Cole *et al*., 2007). The 16S rRNA gene sequences obtained directly from environmental samples were analyzed as described by Perkins *et al*. (2010). A circular neighbor-joining phylogenetic tree was created with the online INTERACTIVE TREE OF LIFE (ITOL) program (Letunic & Bork, 2007).

Statistical analysis

For each method of quantification, a statistical analysis was compiled using the R Project for statistical computing software (http://www.r-project.org).

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Nucleotide sequence accession numbers

Near-full-length 16S rRNA gene sequences were deposited in GenBank under accession numbers FJ657671–FJ658996. Sequences from the cultured plates were deposited in GenBank under accession numbers GQ463228–GQ463237.

Results

Bacterial quantification

Metalworking fluids

We had anticipated that the total bacterial load of the metalworking *fluids* for this study would not change much between the first (days 1–3) and the second sampling interval period (days 4–6) within one season, which was a correct assumption ($P = 0.88$). Therefore, we averaged our quantitative data acquired for each machine during the 6 days of sampling for each season ($n = 6$; Table 1). The total volumetric cell counts quantified through epifluorescence ranged from 3.91×10^7 to 1.55×10^9 cells mL^{-1} in the winter fluid from machine A and machine B, respectively. These counts for the summer samples were more similar to each other, ranging from 1.08×10^8 to 3.62×10^8 cells mL^{-1} for machines B and A, respectively, and were significantly higher than the PBS background (Table 1). The bacterial cells in the metalworking fluid were culturable at high concentrations exceeding 1×10^8 CFUs mL^{-1} for the winter fluid for machine A and the summer fluids for both machines. The concentration of culturable cells for the winter fluid sample from machine B was six orders of magnitude lower (9.50×10^2 CFUs mL^{-1}) with a CFUs/cells ratio of 2.43×10^{-5} (Table 1). Based on operator information we obtained at the factory, the biocides in both machines were the same. qPCR analysis further validated the considerable bacterial load for the metalworking fluids, with bacterial DNA concentrations ranging from 194 μg mL^{-1} in the winter fluid from machine A to 984 μg mL^{-1} in the summer fluid from machine B (Table 1). The summer fluids, which were higher in temperature (Table 1), contained a relatively higher DNA concentration than the winter samples. A three-way ANOVA between the season, machine, and quantification method determined all three to be significant factors ($P < 0.001$).

Metalworking aerosols

To investigate whether the bacterial load in the air of the metalworking facility remained similar during the sampling period of 6 days, we averaged the quantitative data from the indoor aerosol samples for each consecutive 3-day sampling interval period during each season (days 1–3 and days 4–6). We had not previously investigated the air mixing characteristics of the sampling area, and therefore, we also investigated the quantitative aerosol data separately for the samplers that were placed in close vicinity to machines A and B. The data from the outdoor aerosol samples were averaged for the entire 6-day sampling period during each season ($n = 6$). Next, a four-way statistical analysis with all quantitative aerosol data determined that there was a significant difference for the bacterial quantities between seasons or between quantification methods ($P < 0.001$). However, the analysis did not find a significant difference between the sampling interval days 1–3 and 4–6 ($P = 0.79$) or between machines ($P = 0.77$). Therefore, we report the average data for the entire 6-day sampling period for both indoor samplers (machines A and B combined). The averaged epifluorescence cell count for the indoor aerosols collected during the winter sampling event was 6.04×10^5 cells m^{-3} (Table 2). Compared with the indoor air, the background air sample taken outside the facility had a considerably lower average bacterial load of 1.21×10^5 cells m^{-3} (Table 2). This shows that metalworking activities raised the bacterial load in the indoor air. Compared with the winter indoor air samples, the summer epifluorescence cell counts from the indoor air were slightly

Table 1. Six-day average concentrations of total cells, CFUs, and DNA from 16S rRNA genes for the metalworking fluids collected from the recirculation tank of machines A and B and PBS background fluid during the winter and summer sampling event

Seasons	Machine	Temperature (°C)	pH	cells mL^{-1}*	CFUs mL^{-1}†	CFUs/cells	μg DNA mL^{-1}‡
Winter	A	30.0	8.6	$1.55 \times 10^9 \pm 0.42 \times 10^9$	$1.16 \times 10^8 \pm 1.79 \times 10^8$	7.53×10^{-2}	$1.94 \times 10^2 \pm 10.7 \times 10^2$
	B	25.3	9.3	$3.91 \times 10^7 \pm 1.31 \times 10^7$	$9.50 \times 10^2 \pm 0.13 \times 10^2$	2.43×10^{-5}	$5.89 \times 10^2 \pm 8.32 \times 10^2$
	PBS	–	–	$1.06 \times 10^2 \pm 3.98 \times 10^2$	BD	0	0.92 ± 1.70
Summer	A	35.3	8.2	$3.62 \times 10^8 \pm 2.11 \times 10^8$	$2.87 \times 10^8 \pm 2.00 \times 10^8$	7.93×10^{-1}	$8.25 \times 10^2 \pm 9.68 \times 10^2$
	B	31.7	8.1	$1.08 \times 10^8 \pm 0.83 \times 10^8$	$1.38 \times 10^8 \pm 1.06 \times 10^8$	1.29	$9.84 \times 10^2 \pm 0.03 \times 10^2$
	PBS	–	–	$4.13 \times 10^2 \pm 18.7 \times 10^2$	0.43 ± 1.12	1.05×10^{-3}	BD

*Total bacterial cell counts with direct microscopy.
†CFUs with heterotrophic plates.
‡Quantitative levels of 16S rRNA gene DNA with qPCR; ± SD with $n = 6$.
BD, data is below the limit of detection.

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Table 2. Six-day average concentrations of total cells, CFUs, particles, and DNA from 16S rRNA genes for the aerosols collected from two sampling locations ($n = 12$), and background outside air (upwind from the facility; $n = 6$) during the winter and summer sampling event

Seasons	Locations	cells m^{-3}*	CFUs m^{-3}†	CFUs/cells	Particles m^{-3}‡	ng DNA m^{-3}§
Winter	Inside	$6.04 \times 10^5 \pm 1.69 \times 10^5$	$5.58 \times 10^3 \pm 8.06 \times 10^3$	9.24×10^{-3}	$1.36 \times 10^{10} \pm 0.28 \times 10^{10}$	8.58 ± 3.97
	Outside	$1.21 \times 10^5 \pm 0.33 \times 10^5$	$3.86 \times 10^1 \pm 16.3 \times 10^1$	3.20×10^{-4}	$4.76 \times 10^9 \pm 2.79 \times 10^9$	1.11 ± 0.72
Summer	Inside	$5.90 \times 10^5 \pm 1.06 \times 10^5$	$2.59 \times 10^3 \pm 3.94 \times 10^3$	7.35×10^{-3}	$1.19 \times 10^{10} \pm 0.36 \times 10^{10}$	5.06 ± 2.46
	Outside	$9.79 \times 10^4 \pm 1.73 \times 10^4$	$3.01 \times 10^2 \pm 4.99 \times 10^2$	3.08×10^{-3}	$9.42 \times 10^8 \pm 7.18 \times 10^8$	0.63 ± 0.13

*Total bacterial cell counts with direct microscopy.

†CFUs with heterotrophic plates.

‡Quantitative levels of 16S rRNA gene DNA with qPCR.

§Particle concentration with a condensation particle counter; $\pm$SD with $n = 12$ for inside and $n = 6$ for outside.

lower (5.90×10^5 cells m^{-3}, Table 2). The background outdoor air samples collected in the summer were also slightly lower than the winter outdoor air sample (9.79×10^4 cells m^{-3}, Table 2). In accordance with the winter sampling event, the bacterial load in the summer indoor air was elevated considerably compared with the outdoor air. The concentration of culturable cells for the indoor aerosols was two orders of magnitude lower compared with the total cells present for the winter and the summer (5.58×10^3 and 2.59×10^3 CFUs m^{-3}, respectively) with a CFUs/cells ratio of 9.24×10^{-3} and 7.35×10^{-3}, respectively (Table 2). This ratio was slightly lower for the outside aerosols with 3.20×10^{-4} and 3.08×10^{-3} for the winter and summer, respectively (Table 2). The total particle counts (including both cells and inorganic particles) in the metalworking facility were similar during the winter and summer sampling events, and were four to five orders of magnitude higher than the epifluorescence cell counts (Table 2). Finally, quantitative DNA concentrations in the aerosol sample were slightly higher during the winter sampling event compared with the summer sampling event (Table 2).

Sequence analysis

The samples were obtained during the winter and summer seasons and consisted of four metalworking fluid samples and five aerosol samples within the computer numerical control area of the facility (Fig. S1). Our statistical analysis had shown that the quantitative data for the metalworking fluids was significantly different between seasons and machines, but not between sampling intervals, which resulted in surveying the pooled interval samples for each season and machine separately (four samples). For the metalworking aerosols, our analysis did show a difference between seasons, but not between interval periods and sampling locations (machines). Even though we showed that the variability of quantitative aerosol data within the sampling event was not significant, we still surveyed the aerosol samples from the two interval periods separately to gauge the variability in community composition within the seasonal sampling period. The fact that no quantitative difference in aerosol counts between machines was shown possibly indicates that the indoor area air was well mixed and that the location of the sampler within the area did not affect the quantitative aerosol data. Based on this information, the air samples for machines A and B were pooled into four aerosol samples to differentiate just between season and interval period. We checked whether this assumption was valid with the 16S rRNA gene survey by comparing the community composition for a fifth aerosol sample from machine B during the winter sampling event for the second interval period (days 4–6), with a pooled sample from machine A and B for the same period.

We generated 1326 near-full-length, nonchimeric 16S rRNA gene sequences, which were distributed between a total of seven different phyla (Table 3). The most abundant phylum for the combined nine samples was *Proteobacteria* with $> 90\%$ of the total number of sequences (Table 3 and Fig. S2). Eight out of our nine surveyed samples consisted primarily of proteobacterial sequences with either the class *Alphaproteobacteria* or *Betaproteobacteria* as the most abundant group. There was one exception – the sequences for the winter metalworking fluid sample from machine B contained 89% of the phylum *Actinobacteria* and only 10% of the phylum *Proteobacteria* (Table 3). This was for the same fluid sample that was characterized with a considerably lower CFUs/cells ratio than the other three fluid samples (Table 1). Besides being abundant in the winter fluid sample for machine B, *Actinobacteria* was only found to be above detection in the winter aerosol machines A and B during days 4–6 and the summer aerosol machines A and B during days 1–3 (both at levels $\sim$1%, Table 3). Regardless of sample type, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were present in all samples. The phyla *Bacteroidetes* and *Firmicutes* were each found in seven samples between levels of 0.5–4%. Finally, the phyla *Fusobacteria*, *Chloroflexi*, and *Aminanaerobia* were each only detected in one sample at low abundance (0.5–1%) (Table 3).

We used a color-coded neighbor-joining phylogenetic tree (with all 1326 sequences) in which each of the nine colors represents a different sample type to show the

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Table 3. Phylum and class-level comparison of sequences from each metalworking sample

	Winter fluid(%)		Summer fluid(%)		Winter aerosol(%)			Summer aerosol(%)	
Phylum	Machine A	Machine B	Machine A	Machine B	Machine A and B Days 1–3	Machine A and B Days 4–6	Machine B Days 4–6	Machine A and B Days 1–3	Machine A and B Days 1–3
Alphaproteobacteria	14.2	1.4	13.0	55.2	78.0	34.0	20.6	32.6	36.4
Betaproteobacteria	82.7	1.4	78.3	31.0	16.7	63.5	73.5	52.4	38.8
Gammaproteobacteria	1.6	7.2	0.0	10.3	0.7	0.3	2.0	12.3	19.8
Firmicutes	1.6	0.0	0.0	3.4	0.7	0.7	2.9	0.9	4.1
Bacteroidetes	0.0	1.4	4.3	0.0	3.3	0.7	1.0	0.4	0.8
Actinobacteria	0.0	88.4	0.0	0.0	0.0	0.7	0.0	0.9	0.0
Fusobacteria	0.0	0.0	4.3	0.0	0.0	0.0	0.0	0.0	0.0
Chloroflexi	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0
Aminanaerobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0
Total sequences	127	138	23	29	150	288	102	227	242

The percentages of phylum or class distribution for samples from the metalworking fluids and aerosols. The phylum *Proteobacteria* was subdivided into *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*.

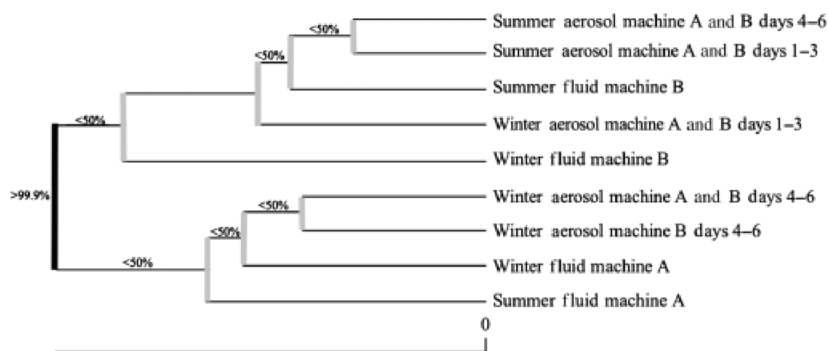

Fig. 1. Weighted normalized UniFrac analysis for differentiating each community in the fluid and aerosol samples.

considerable overlap of individual sequences for the metalworking fluid and aerosol samples between the two seasons (Fig. S2). This is also shown by a weighted environmental clustering test that further demonstrates the considerable overlap between the fluid and aerosol communities for both seasons because samples did not clearly cluster by sample type (fluid vs. aerosols – Fig. 1). From this information, it is clear that the fluids are the source of aerosols in the metalworking facility. Our weighted and normalized UniFrac clustering results also suggest that the air in the machining room was well mixed because the bacterial communities for the winter aerosol samples for days 4–6 that were taken close to machines A and B clustered closely with the sample that was taken close to machine B, even though the winter fluid sample from machine B was very different (the most separated sample with UniFrac) (Table 3 and Fig. 1). The UniFrac results were less clear in answering the question whether the communities for the aerosol samples varied between interval periods days 1–3 and days 4–6. The summer aerosol samples clustered together well for the two periods, but this was not observed for the winter aerosol samples (Fig. 1). Therefore, the abundance of different phyla was different between the interval periods for the winter aerosol samples, which is also apparently based on the phyla distribution results (Table 3).

Further phylogenetic analyses were performed to classify individual 16S rRNA gene sequences based on sequences in public databases. We found 200 different operational taxonomic units (OTUs) for the 1326 nonchimeric full-length sequences (Table 4). To investigate whether OTUs from different sample types were closely related, we performed a maximum likelihood analysis on the sequences that were identified within three bacterial phyla (Fig. 2). Again, sequences from winter fluid, summer fluid, winter aerosol, and summer aerosol were often closely related. For example, *Alcaligenes faecalis*, which is one of the most abundant species in our gene survey, was represented in all nine samples. Thus, in general, our survey supports the fact that the metalworking fluid is the likely source of the bacterial aerosols and that no obvious difference between seasons and machines was observed. However, as we had shown above,

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Table 4. Sequence details and potential pathogens found in each sample

Sample	Nonchimeric sequences	OTUs	Detection method	Matching sequences	RDP % match to known species	Potential pathogen
Winter fluid	127	22	Direct	81	97.5	*Alcaligenes faecalis*; DQ857898
Machine A			Cultured	–	97.8	*Alcaligenes faecalis*; DQ110882
			Cultured	–	> 99.9	*Morganella morganii*; AJ301681
Winter fluid	138	21	Direct	2	99.2	*Alcaligenes faecalis*; AY959943
Machine B			Cultured	–	97.8	*Alcaligenes faecalis*; DQ110882
			Direct	1	97.6	*Brevundimonas diminuta*; AY730717
			Cultured	–	> 99.9	*Morganella morganii*; AJ301681
			Direct	1	96.5	*Mycobacterium chelonae*; AJ419969
			Direct	84	99.5	*Mycobacterium immunogenum*; AJ812215
			Cultured	–	> 99.9	*Ochrobactrum anthropi*; AJ276036
			Direct	1	97.6	*Pseudochrobactrum asaccharolyticum*; AM180485
Winter aerosol	150	31	Direct	3	97.3	*Alcaligenes faecalis*; DQ857898
Machine A and B			Cultured	–	97.8	*Alcaligenes faecalis*; DQ110882
Days 1–3			Direct	34	> 99.9	*Brevundimonas diminuta*; X87274
			Cultured	–	> 99.9	*Morganella morganii*; AJ301681
			Direct	2	97.5	*Pseudochrobactrum asaccharolyticum*; AM180485
			Direct	1	97.9	*Wautersiella falsenii*; AM238673
Winter aerosol	288	40	Direct	132	98.5	*Alcaligenes faecalis*; DQ857898
Machine A and B			Cultured	–	97.8	*Alcaligenes faecalis*; DQ110882
Days 4–6			Direct	2	98.3	*Bordetella trematum*; AJ277798
			Direct	37	98.3	*Brevundimonas diminuta*; EF191247
			Direct	4	98.8	*Ochrobactrum anthropi*; AB120120
			Direct	3	98.5	*Pseudochrobactrum asaccharolyticum*; AM180485
Winter aerosol	102	19	Direct	58	99.7	*Alcaligenes faecalis;* DQ857898
Machine B			Cultured	–	97.8	*Alcaligenes faecalis*; DQ110882
Days 4–6			Direct	2	99.4	*Ochrobactrum anthropi*; AJ294349
			Direct	1	98.3	*Brevundimonas diminuta*; EF1911247
			Direct	2	98.0	*Pseudochrobactrum asaccharolyticum*
Summer fluid	23	9	Direct	14	98.5	*Alcaligenes faecalis*; DQ857898
Machine A			Cultured	–	97.7	*Alcaligenes faecalis*; DQ857898
			Direct	1	98.8	*Ochrobactrum anthropi*; AB120120
Summer fluid	29	11	Direct	3	99.0	*Alcaligenes faecalis*; DQ857898
Machine B			Cultured	–	97.7	*Alcaligenes faecalis*; DQ857898
Summer aerosol	227	24	Direct	77	96.8	*Alcaligenes faecalis*; DQ857898
Machine A and B			Cultured	–	98.8	*Alcaligenes faecalis*; DQ110882
Days 1–3			Direct	19	98.2	*Brevundimonas diminuta*; DQ857897
			Direct	1	98.0	*Corynebacterium xerosis*; X81914
			Direct	3	99.4	*Ochrobactrum anthropi*; AY730720
			Direct	2	98.7	*Pseudochrobactrum asaccharolyticum*; AM180485
			Direct	1	96.8	*Wautersiella falsenii*; AM238670
Summer aerosol	242	23	Direct	62	98.0	*Alcaligenes faecalis*; DQ857898
Machine A and B			Cultured	–	97.7	*Alcaligenes faecalis*; DQ857898
Days 4–6			Direct	6	99.0	*Brevundimonas diminuta*; EF191247
			Direct	1	97.0	*Citrobacter amalonaticus*; AF025370
			Cultured	–	> 99.9	*Ochrobactrum anthropi*; AB120120
			Direct	2	98.3	*Ochrobactrum anthropi*; AM114409
			Direct	2	98.5	*Staphylococcus epidermidis*; CP000029
			Direct	1	97.2	*Wautersiella falsenii*; AM238670
Total	1326	200*		646		

*Total number of distinct OTUs found among all 1326 sequences.

The total number of nonchimeric sequences collected, number of OTUs, method of detection, and number of sequences that matched with an identity > 97% to a known potential pathogen in the RDP database are given.

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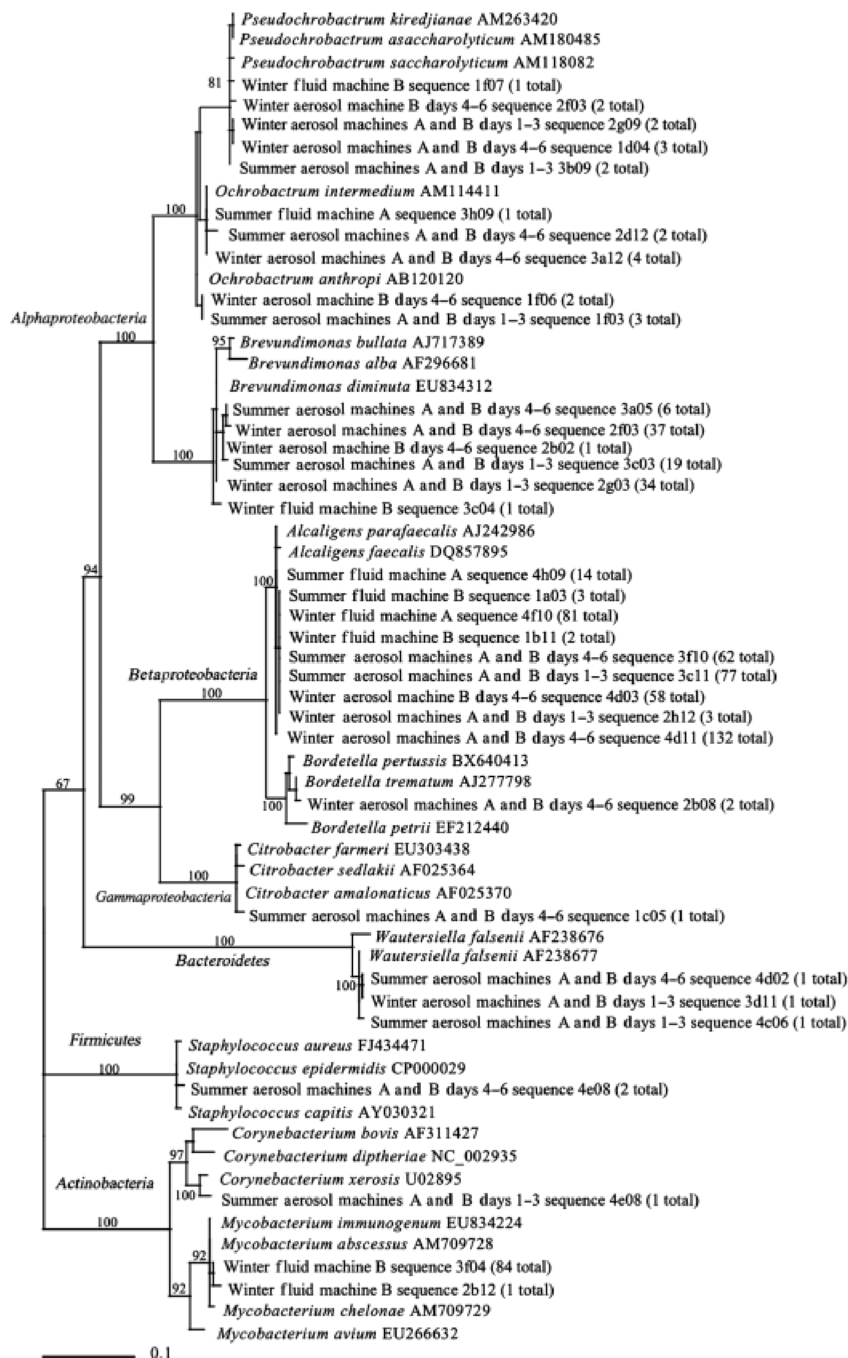

Fig. 2. Maximum likelihood phylogenetic distribution of potentially pathogenic 16S rRNA gene sequences collected in this study. Reference sequences from the NCBI database are included with their accession numbers. Individual metalworking fluid sequences are labeled with 'fluid' followed by their unique sequence identity. Metalworking aerosol samples are identified by 'aerosol' followed by their unique sequence identity. The total number of similar OTUs (based on a 3% cutoff) from this study is in parenthesis following the sequence identity. The scale bar represents base changes per site. The bootstrap numbers are based on the neighbor-joining method.

there is one exception – the winter fluid machine B was enriched with *Actinobacteria*. This enrichment did not result in considerably higher levels of these *Actinobacteria* in the indoor aerosol of the room (Table 3).

Using the RDP algorithm, we identified 646 full-length sequences (out of a total of 1326 full-length sequences) to be > 97% identical to known potential pathogens (Table 4). Six of the potentially pathogenic bacteria were found in multiple samples (*A. faecalis*, *Ochrobactrum anthropi*, *Pseudochrobactrum asaccharolyticum*, *Brevundimonas diminuta*, *Wautersiella falsenii*, *Morganella morganii*). The maximum likelihood phylogeny identified and verified the relatedness of all the potential pathogens in our samples to cultured sequences in public databases (Fig. 2). Besides the six pathogens that were found in more than one sample, five sequences that were closely related to potential pathogenic bacterial species were found in only one sample (*Bordetella trematum*; *Citrobacter amalonaticus*; *Staphylococcus*

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epidermidis; *Corynebacterium xerosis*; and *M. immunogenum*) (Fig. 2). The enrichment of *M. immunogenum* in the winter fluid machine B is of importance because it is this actinobacterial species that caused the winter fluid sample for machine B to be an outlier compared with the other samples. Interestingly, *M. immunogenum* was not present in any other metalworking fluid or aerosol samples (not even in the other samples with *Actinobacteria*) (Fig. 2).

Discussion

Consistently high bacterial concentrations were found for the metalworking fluids

There are no suggested maximum containment levels specified for bacterial concentrations in metalworking fluids; however, numerous studies have shown the propensity of metalworking fluids to foster microbial growth. The facility assessed in this study had expressed concern regarding biomass accumulation in the fluid of multiple machines and had observed a decreased air quality in the computer numerical control area of the facility. In our analysis, the concentrations of bacteria (culturable and nonculturable) in the fluids were relatively constant throughout the study (averaging $5.1 \times 10^8 \pm 7.0 \times 10^8$ cells mL^{-1} with epifluorescence microscopy and $1.4 \times 10^8 \pm 1.2 \times 10^8$ CFUs mL^{-1} with culture quantifications). These findings are in accordance with previous metalworking fluid studies. Using fluorescence microscopy, Thorne *et al.* (1996) found the total (culturable and nonculturable) bacterial concentration of metalworking fluids to range between 1.5×10^7 and 1.7×10^8 organisms mL^{-1}, while other studies have shown culturing analyses of metalworking fluids to yield an average of 10^7 CFUs mL^{-1} (Mattsby-Baltzer *et al.*, 1989; Sandin *et al.*, 1991; Thorne *et al.*, 1996). The CFUs to cells ratios for three of the metalworking fluid samples were between 0.075 and 1.29 (Table 1), which shows that 7.5–100% of all counted cells were culturable in our lab. Thorne *et al.* (1996) and Abrams *et al.* (2000) found very similar percentages of 4–100%, respectively. However, the winter fluid sample collected from machine B showed that only 0.002% of the bacterial cells were cultured in our lab (Table 1). Veillette *et al.* (2004) also noted relatively low percentages for their metalworking fluid characterization. We showed that $> 60\%$ of the sequences from our winter fluid for machine B sample were identified to be *M. immunogenum* at the species level ($> 97\%$ identical) (Table 4), while Veillette *et al.* (2004) also detected *M. immunogenum* in their metalworking fluid samples with selectively designed PCR probes. Therefore, the relatively low CFUs/cells ratio may be a marker for *M. immunogenum* presence partly because of the slow growth rate of *Mycobacterium* spp. (Falkinham, 2003; Veillette *et al.*, 2008). However, this should be further verified with more metalworking fluid samples from additional locations.

Metalworking bioaerosol concentrations were comparable to other studies

The concentration of total bacterial cells, including culturable and nonculturable cells, within the facility averaged $4.8 \times 10^5 \pm 1.4 \times 10^5$ cells m^{-3} or a potential inhalation dose of $1 \times 10^6 \pm 3 \times 10^5$ microorganisms h^{-1} for staff, assuming an average inhalation rate for moderate activity to be 2.1 m^3 h^{-1} (EPA, 1997). This concentration is slightly lower than the Abrams *et al.* (2000) assessment, which ranged from 8.7×10^5 to 2.7×10^6 total thoracic bacteria m^{-3} within a metalworking facility. An aerosol sample at an indoor hospital therapy pool contained nearly the same concentration of cells (10^6 cells m^{-3}) compared with our study (Angenent *et al.*, 2005), while a lower concentration of slightly $< 2.1 \times 10^5$ cells m^{-3} was found inside a single-family home (Fabian *et al.*, 2005). The average outdoor aerosol sample collected in this study ($1.1 \times 10^5 \pm 0.2 \times 10^5$ cells m^{-3}) was similar to outdoor samples collected in Salt Lake City, UT (10^5–10^7 cells m^{-3}) (Radosevich *et al.*, 2002) and East St. Louis, IL (1.5×10^5 cells m^{-3}) (Rauer, 2005).

Potentially pathogenic bacteria were found in the metalworking fluid and aerosol samples

Typical culture analyses of metalworking fluids and aerosols have shown high concentrations of clinically significant *Pseudomonas* spp. and *Mycobacterium* spp. (Lewis *et al.*, 2001). The findings presented here did not yield significant quantities of either genus: 2% of the sequences in this study were from the genus *Pseudomonas* (30/1326) and one fluid sample contained sequence from the genus *Mycobacterium*. However, other organisms previously cultured in metalworking fluid samples were found during this analysis. *Acinetobacter* spp., *Alcaligenes* spp., *Morganella* spp., *Ochrobactrum* spp., and *Staphylococcus* spp. have each been isolated from metalworking fluids (Mattsby-Baltzer *et al.*, 1989; Sandin *et al.*, 1991; Laitinen *et al.*, 1999; Bracker *et al.*, 2003), while *Brevundimonas* spp., *Corynebacterium* spp., and *Ochrobactrum* spp. have been identified in metalworking aerosols (Gorny *et al.*, 2004). 3.3% (30/907) of the aerosol sequences identified here were from the genus *Acinetobacter*, but none were identical at the species level ($> 97\%$) to a species of *Acinetobacter* in the RDP database. Over 40% of the sequences (546/1326) in this study were from the genus *Alcaligenes* (Table 4). In particular, *A. faecalis* was found within both the cultured and the directly extracted sequences of all metalworking fluid and aerosol samples. *Alcaligenes faecalis* is typically nonpathogenic, but under certain circumstances, it has been identified in conjunction with bone abscesses (Fenollar *et al.*, 2006).

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Wautersiella falsenii and *P. asaccharolyticum* were identified in both our fluid and aerosol samples, but are the first potential pathogens (Kampfer *et al*., 2006a, b) in this study, that had not been previously isolated from metalworking fluids or aerosols. From these analyses, it is evident that potentially pathogenic bacteria in metalworking fluids were aerosolized into the machining workplace to possibly cause health concerns for severely immunocompromised metalworking personnel.

Abundant *M. immunogenum* in metalworking fluid did not result in aerosol enrichment

Even though *M. immunogenum* had become enriched in the winter fluid machine B, no mycobacteria were found in the aerosol samples (both the pooled aerosol sample and the aerosol sample taken in the vicinity of only machine B). This is in contrast to Shelton *et al*. (1999), who found high concentrations of *Mycobacterium* spp. in air samples collected near mycobacteria-contaminated machines. Aerosolization of *Mycobacterium* spp. has long been associated with pool and whirlpool environments where a 'bubble burst' mechanism at the water surface is the primary droplet source (Schafer *et al*., 2003; Angenent *et al*., 2005). The hydrophobic mycobacterial cells, which may become enriched around the bubble (and therefore may become enriched in aerosols), are ejected in water droplets after which they become bioaerosols when the water bubble dries up (Vaerewijck *et al*., 2005). Here, we did not find this phenomenon possibly because aerosol formation through metal grinding has been explained by a different mechanism – a combination of 'jet mist' and 'shear force' mechanisms, which generated a bimodal particle distribution in metalworking aerosols (Heitbrink *et al*., 2000; Wang *et al*., 2007). Similarly, no *Mycobacterium* spp. were found in shower aerosols generated by a jet mist mechanism, while they were found at high concentrations in a shower water sample (Perkins *et al*., 2009). Instead, *Proteobacteria* was, by far, the most abundant phylum in the aerosols, which supported the work of three out of the seven published outdoor air surveys and an indoor air survey that showed *Proteobacteria* to be exceeding 60% of the bacterial composition (Maron *et al*., 2005; Baertsch *et al*., 2007; Fierer *et al*., 2008). Furthermore, our work agrees with that of Gilbert *et al*. (2010a, b), who found an overall lack of anticipated *Mycobacterium* spp., with only two of the 44 sites being positive for mycobacteria in the fluids and none in the aerosols. However, we cannot rule out that the discrepancy between our study and that of Shelton *et al*. (1999), who did find aerosolized *Mycobacterium* spp. in the metalworking facility, can also be explained due to different mycobacterial fluid concentrations, machine designs, machining operations, room temperatures, and relative humidity levels (Bracker *et al*., 2003).

Understanding the microbial ecology within the metalworking fluids and aerosols will help guide interventions to prevent fluid overgrowth

The considerable presence of *A. faecalis* in the metalworking fluid and aerosol samples was a significant finding. The abundance of this bacterium could be one reason why *Pseudomonas* spp. and *Mycobacterium* spp. were not the most important pathogens to inhabit these metalworking fluids. Now, with the knowledge that *Alcaligenes* spp. is the abundant bacterium in the fluids and aerosols, a more targeted engineering control (e.g. biocide) can be developed. It needs to be realized, however, that a diverse community of bacteria inhabit the metalworking fluid, and that controlling one group of microorganisms may stimulate another group, and that, therefore, a comprehensive gene survey, which we performed in this study, is necessary to ascertain the real effects of mitigation measures.

Acknowledgements

The financial support for S.D.P. was through a 2005 graduate fellowship from the National Science Foundation. We would like to thank the personnel of the metalworking facility for their assistance in this study. We would like to acknowledge Elizabeth Campbell and Matt Sculnick for their assistance in lab analyses and anonymous reviewers for excellent comments.

References

Abrams L, Seixas N, Robins T, Burge H, Muilenberg M & Franzblau A (2000) Characterization of metalworking fluid exposure indices for a study of acute respiratory effects. *Appl Occup Environ Hyg* **15**: 492–502.

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.

An HR, Mainelis G & White L (2006) Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. *Atmos Environ* **40**: 7924–7939.

Angenent LT, Kelley ST, St Amand A, Pace NR & Hernandez MT (2005) Molecular identification of potential pathogens in water and air of a hospital therapy pool. *P Natl Acad Sci USA* **102**: 4860–4865.

Baertsch C, Paez-Rubio T, Viau E & Peccia J (2007) Source tracking aerosols released from land-applied class B biosolids during high-wind events. *Appl Environ Microb* **73**: 4522–4531.

Barhad B, Pilat L & Teculescu D (1975) Recent progress in the study of occupational lung diseases in Romania. *Brit J Ind Med* **32**: 164–168.

Bernstein DI, Lummus ZL, Santilli G, Siskosky J & Bernstein IL (1995) Machine operator's lung. A hypersensitivity

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pneumonitis disorder associated with exposure to metalworking fluid aerosols. *Chest* **108**: 636–641.

Bracker A, Storey E, Yang C & Hodgson MJ (2003) An outbreak of hypersensitivity pneumonitis at a metalworking plant: a longitudinal assessment of intervention effectiveness. *Appl Occup Environ Hyg* **18**: 96–108.

Carson LA, Petersen NJ, Favero MS & Aguero SM (1978) Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. *Appl Environ Microb* **36**: 839–846.

Chang S-C, Rihana A, Bahrman S, Gruden C, Khijniak A, Skerlos S & Adriaens P (2004) Flow cytometric detection and quantification of mycobacteria in metalworking fluids. *Int Biodeter Biodegr* **54**: 105–112.

Cohen H & White EM (2006) Metalworking fluid mist occupational exposure limits: a discussion of alternative methods. *J Occup Environ Hyg* **3**: 501–507.

Cole JR, Chai B, Farris RJ *et al.* (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* **35**: D169–D172.

Edwards C (2000) Problems posed by natural environments for monitoring microorganisms. *Mol Biotechnol* **15**: 211–223.

EPA (1997) Inhalation. *Exposure Factors Handbook*. *Vol. 1*, pp. 1–26. U.S. Environmental Protection Agency, Washington, DC.

Fabian MP, Miller SL, Reponen T & Hernandez MT (2005) Ambient bioaerosol indices for indoor air quality assessments of flood reclamation. *J Aerosol Sci* **36**: 763–783.

Falkinham JO III (2003) Mycobacterial aerosols and respiratory disease. *Emerg Infect Dis* **9**: 763–767.

Fenollar F, Roux V, Stein A, Drancourt M & Raoult D (2006) Analysis of 525 samples to determine the usefulness of PCR amplification and sequencing of the 16S rRNA gene for diagnosis of bone and joint infections. *J Clin Microbiol* **44**: 1018–1028.

Fierer N, Liu Z, Rodriguez-Hernandez M, Knight R, Henn M & Hernandez MT (2008) Short-term temporal variability in airborne bacterial and fungal populations. *Appl Environ Microb* **74**: 200–207.

Gilbert Y, Veillette M & Duchaine C (2010a) Metalworking fluids biodiversity characterization. *J Appl Microbiol* **108**: 437–449.

Gilbert Y, Veillette M, Meriaux A, Lavoie J, Cormier Y & Duchaine C (2010b) Metalworking fluid-related aerosols in machining plants. *J Occup Environ Hyg* **7**: 280–289.

Godderis L, Deschuyffeleer T, Roelandt H, Veulemans H & Moens G (2008) Exposure to metalworking fluids and respiratory and dermatological complaints in a secondary aluminium plant. *Int Arch Occ Env Hea* **81**: 845–853.

Gordon T (2004) Metalworking fluid – the toxicity of a complex mixture. *J Toxicol Env Heal A* **67**: 209–219.

Gorny RL, Szponar B, Larsson L, Pehrson C, Prazmo Z & Dutkiewicz J (2004) Metalworking fluid bioaerosols at selected workplaces in a steelworks. *Am J Ind Med* **46**: 400–403.

Greaves IA, Eisen EA, Smith TJ *et al.* (1997) Respiratory health of automobile workers exposed to metal-working fluid aerosols: respiratory symptoms. *Am J Ind Med* **32**: 450–459.

Heitbrink WA, D'Arcy JB & Yacher JM (2000) Mist generation at a machining center. *AIHAJ* **61**: 22–30.

Kampfer P, Avesani V, Janssens M, Charlier J, De Baere T & Vaneechoutte M (2006a) Description of *Wautersiella falsenii* gen. nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. *Int J Syst Evol Micr* **56**: 2323–2329.

Kampfer P, Rossello-Mora R, Scholz HC, Welinder-Olsson C, Falsen E & Busse HJ (2006b) Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Micr* **56**: 1823–1829.

Kennedy SM, Chan-Yeung M, Teschke K & Karlen B (1999) Change in airway responsiveness among apprentices exposed to metalworking fluids. *Am J Resp Crit Care* **159**: 87–93.

Khalil N, Churg A, Muller N & O'Connor R (2007) Environmental, inhaled and ingested causes of pulmonary fibrosis. *Toxicol Pathol* **35**: 86–96.

Laitinen S, Linnainmaa M, Laitinen J, Kiviranta H, Reiman M & Liesivuori J (1999) Endotoxins and IgG antibodies as indicators of occupational exposure to the microbial contaminants of metal-working fluids. *Int Arch Occ Env Hea* **72**: 443–450.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acids Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. Wiley, New York.

Letunic I & Bork P (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* **23**: 127–128.

Lewis DM, Janotka E, Whitmer MP & Bledsoe TA (2001) Detection of microbial antigens in metal working fluids. *Int Biodeter Biodegr* **47**: 89–94.

Linnainmaa M, Kiviranta H, Laitinen J & Laitinen S (2003) Control of workers' exposure to airborne endotoxins and formaldehyde during the use of metalworking fluids. *AIHA J* **64**: 496–500.

Maron PA, Lejon D, Carvalho E, Bizet K, Lemanceau P, Ranjard L & Mougel C (2005) Assessing genetic structures and diversity of airborne bacterial communities by DNA fingerprinting and 16S rDNA clone library. *Atmos Environ* **39**: 3687–3695.

Mattsby-Baltzer I, Sandin M, Ahlstrom B *et al.* (1989) Microbial growth and accumulation in industrial metal-working fluids. *Appl Environ Microb* **55**: 2681–2689.

Nadkarni MA, Martin FE, Jacques NA & Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**: 257–266.

Pace NR (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.

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Park D, Chin K, Kwag H *et al.* (2007) Effect of metalworking fluid mist exposure on cross-shift decrement in peak expiratory flow. *J Occup Health* **49**: 25–31.

Park DU, Jin KW, Koh DH, Kim BK, Kim KS & Park DY (2008) Association between use of synthetic metalworking fluid and risk of developing rhinitis-related symptoms in an automotive ring manufacturing plant. *J Occup Health* **50**: 212–220.

Perkins SD, Mayfield J, Fraser V & Angenent LT (2009) Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. *Appl Environ Microb* **75**: 5363–5372.

Perkins SD, Woeltje KF & Angenent LT (2010) Endotracheal tube biofilm inoculation of oral flora and subsequent colonization of opportunistic pathogens. *Int J Med Microbiol* **300**: 503–511.

Radosevich JL, Wilson WJ, Shinn JH, DeSantis TZ & Andersen GL (2002) Development of a high-volume aerosol collection system for the identification of air-borne micro-organisms. *Lett Appl Microbiol* **34**: 162–167.

Rauer D (2005) Characterization and monitoring of ambient biological PM-2.5 at the St. Louis-Midwest Supersite. MSc Thesis, Washington University in St. Louis, St. Louis, MO.

Sandin M, Mattsby-Baltzer I & Edebo L (1991) Control of microbial growth in water-based metal-working fluids. *Int Biodeterior* **27**: 61–74.

Schafer MP, Martinez KF & Mathews ES (2003) Rapid detection and determination of the aerodynamic size range of airborne mycobacteria associated with whirlpools. *Appl Occup Environ Hyg* **18**: 41–50.

Selvaraju SB, Khan IU & Yadav JS (2008) Differential biocide susceptibility of the multiple genotypes of *Mycobacterium immunogenum*. *J Ind Microbiol Biot* **35**: 197–203.

Shelton BG, Flanders WD & Morris GK (1999) *Mycobacterium* sp. as a possible cause of hypersensitivity pneumonitis in machine workers. *Emerg Infect Dis* **5**: 270–273.

Simpson AT, Stear M, Groves JA, Piney M, Bradley SD, Stagg S & Crook B (2003) Occupational exposure to metalworking fluid mist and sump fluid contaminants. *Ann Occup Hyg* **47**: 17–30.

Taylor RH, Falkinham JO III, Norton CD & LeChevallier MW (2000) Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. *Appl Environ Microb* **66**: 1702–1705.

Thorne PS, DeKoster JA & Subramanian P (1996) Environmental assessment of aerosols, bioaerosols, and airborne endotoxins in a machining plant. *AIHA J* **57**: 1163–1167.

US Department of Health and Human Services (1998) Criteria for a recommended standard: occupational exposure to metalworking fluids. NIOSH, Cincinnati, OH.

Vaerewijck MJ, Huys G, Palomino JC, Swings J & Portaels F (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* **29**: 911–934.

Veillette M, Thorne PS, Gordon T & Duchaine C (2004) Six month tracking of microbial growth in a metalworking fluid after system cleaning and recharging. *Ann Occup Hyg* **48**: 541–546.

Veillette M, Page G, Thorne PS & Duchaine C (2008) Real-time PCR quantification of *Mycobacterium immunogenum* in used metalworking fluids. *J Occup Environ Hyg* **5**: 755–760.

Wallace RJ, Zhang YS, Wilson RW, Mann L & Rossmoore H (2002) Presence of a single genotype of the newly described species *Mycobacterium immunogenum* in industrial metalworking fluids associated with hypersensitivity pneumonitis. *Appl Environ Microb* **68**: 5580–5584.

Wang H, Reponen T, Lee SA, White E & Grinshpun SA (2007) Size distribution of airborne mist and endotoxin-containing particles in metalworking fluid environments. *J Occup Environ Hyg* **4**: 157–165.

Woskie SR, Virji MA, Hallock M, Smith TJ & Hammond SK (2003) Summary of the findings from the exposure assessments for metalworking fluid mortality and morbidity studies. *Appl Occup Environ Hyg* **18**: 855–864.

Woskie SR, Virji MA, Kriebel D *et al.* (1996) Exposure assessment for a field investigation of the acute respiratory effects of metalworking fluids. I. Summary of findings. *Am Ind Hyg Assoc J* **57**: 1154–1162.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Machine layout and sampling locations within the metalworking facility.
Fig. S2. Neighbor-joining phylogenetic distribution of all 16S rRNA gene sequences isolated in this study.

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