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Firing Range Soils Yield a Diverse Array of Fungal Isolates Capable of Organic Acid Production and Pb Mineral Solubilization

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Anthropogenic sources of lead contamination in soils include mining and smelting activities, effluents and wastes, agricultural pesticides, domestic garbage dumps, and shooting ranges. While Pb is typically considered relatively insoluble in the soil environment, some fungi may potentially contribute to mobilization of heavy metal cations by means of secretion of low-molecular-weight organic acids (LMWOAs). We sought to better understand the potential for metal mobilization within an indigenous fungal community at an abandoned shooting range in Oak Ridge, TN, where soil Pb contamination levels ranged from 24 to >2,700 mg Pb kg dry soil^{-1}. We utilized culture-based assays to determine organic acid secretion and Pb-carbonate dissolution of a diverse collection of soil fungal isolates derived from the site and verified isolate distribution patterns within the community by 28S rRNA gene analysis of whole soils. The fungal isolates examined included both ascomycetes and basidiomycetes that excreted high levels (up to 27 mM) of a mixture of LMWOAs, including oxalic and citric acids, and several isolates demonstrated a marked ability to dissolve Pb-carbonate at high concentrations up to 10.5 g liter^{-1} (18.5 mM) in laboratory assays. Fungi within the indigenous community of these highly Pb-contaminated soils are capable of LMWOA secretion at levels greater than those of well-studied model organisms, such as *Aspergillus niger*. Additionally, these organisms were found in high relative abundance (>1%) in some of the most heavily contaminated soils. Our data highlight the need to understand more about autochthonous fungal communities at Pb-contaminated sites and how they may impact Pb biogeochemistry, solubility, and bioavailability, thus consequently potentially impacting human and ecosystem health.

The U.S. Environmental Protection Agency (EPA) estimates that roughly 160 million pounds of lead (Pb) bullets find their way into the environment each year from recreational use alone (53). In addition, the U.S. Department of Defense (DoD) reports expending more than 2 million pounds of Pb across more than 3,000 active small arms firing ranges in the United States each year (24). Other countries, such as The Netherlands, Denmark, Canada, and England, report similarly high annual deposition rates of metallic Pb from hunting, angling, and recreational shooting (25, 26, 28, 34, 49, 54).

Metallic Pb is typically considered relatively insoluble in the soil environment due to complexation with various organic and inorganic soil colloids, sorption, and precipitation (57). Pb bullets most frequently weather to lead carbonate (10, 30, 31, 55), which also has very limited solubility. However, heavily contaminated soils, such as those found at outdoor shooting ranges, when weathered over many years may result in Pb saturation on binding sites, leading to Pb mobilization through the soil profile and into subsurface environments, including ground water (7, 28, 37). The primary cause of Pb mobilization in soils appears to be dissolution and oxidation; however, the rates of these processes and the resulting weathering products are highly variable and site specific.

Some of this site-specific variability may be explained by interactions of Pb with the indigenous soil microbial communities. Soil microbes are intimately associated with the biogeochemical cycling of metals, and even toxic metals, including Pb, may be directly or indirectly involved in, or affected by, aspects of microbial growth, metabolism, and differentiation (15). Microbial activities frequently result in immobilization or mobilization of metals, depending on the specific organisms and mechanisms involved, as well as the microenvironment in which these processes are taking place (16, 18, 19).

Soil fungi have been recognized recently as potentially significant contributors to the mobilization of heavy metals by means of organic acid production in contaminated soils (2, 3) and low-grade mining ore (36) in a process often termed bioleaching. Fungal production of low-molecular-weight organic acids (LMWOAs) has been almost exclusively studied in model organisms, such as *Aspergillus* spp. (2, 3, 16, 27, 36, 39, 56). Despite the fact that it is a process that may contribute significantly to Pb mobilization in soils, no thorough studies of these processes in indigenous soil fungi have been reported.

While *Aspergillus* spp. have been the primary focus for understanding such bioleaching processes to date, it is still unclear which soil fungi in natural systems may excrete LMWOAs into their environments and thereby contribute to soil Pb mobilization. Simply put, it is well established that model fungal organisms can secrete LMWOAs in laboratory or bioreactor settings, but virtually nothing is known about the capacity of native soil fungal populations from Pb-contaminated sites to produce acids and

Received 3 April 2012 Accepted 5 June 2012
Published ahead of print 22 June 2012
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Supplemental material for this article may be found at http://aem.asm.org.
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doi:10.1128/AEM.01091-12

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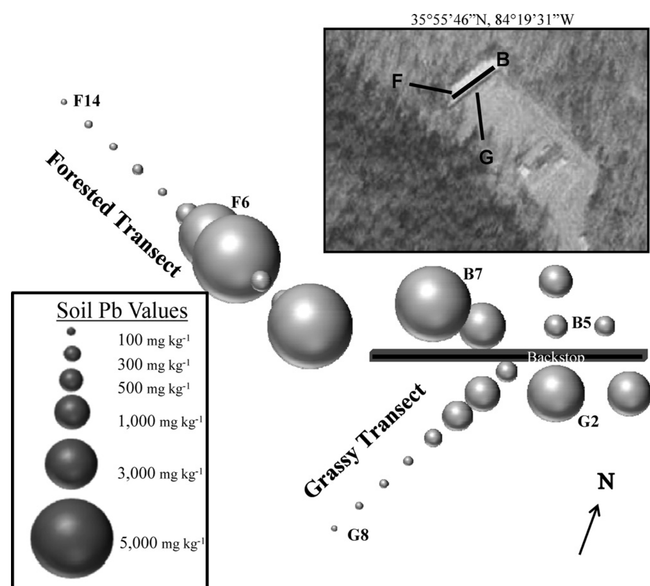

FIG 1 The inset is an aerial photograph of the firing range at the Oak Ridge National Laboratory where soil samples were collected on 2 July 2010. The sampling transects are indicated as follows: B, firing backstop; F, forested transect; G, grassy transect. The bubble plot gives soil Pb concentrations along each transect.

their potential to mobilize Pb in soils. Therefore, the aim of this study was to begin to elucidate this potentially important functional ability in soil fungal isolates originating from a heavily contaminated firing range soil. Our specific objectives were to (i) assay the soils of a Pb-contaminated shooting range for fungi capable of excretion of LMWOAs, (ii) quantify LMWOA secretion and Pb solubilization, and (iii) verify the presence and quantify the relative abundances of these organisms *in situ* at the site.

MATERIALS AND METHODS

Site description and experimental design. The study site (Fig. 1) is an abandoned small arms shooting range located in a cleared area once dominated by oak (*Quercus* spp.), maple (*Acer* spp.), and pine (*Pinus* spp.) trees near the Oak Ridge National Laboratory (ORNL) (35°55′46″N, 84°19′31″W) in Oak Ridge, TN. The area designated for target and shooting practice was cleared of trees when the firing range was first established circa 1950, after which the target area became dominated by typical old field grasses (*Festuca* spp.) and forbs (*Lespedeza* sp.). Initially, an earthen berm was utilized as a firing backstop, which to this day remains relatively devoid of vegetation. A more formal firing backstop consisting of tires was erected along the target area slightly south of the earthen berm sometime around 1982 (Jon Justice, personal communication). This resulted in three distinct vegetation types surrounding the backstop: forested areas north and west of the backstop, dominated by oak, maple, and pine trees; grassy areas south of the backstop, previously cleared for shooting and now dominated by grasses and forbs; and the backstop embankment, which remains mostly devoid of vegetation.

This firing range was previously part of a study by Moseley et al. (35) in which the soils (soils 7, 8, and 9) were reported to contain between 1,000 and 4,000 mg Pb kg^{-1}, an average of 1.63% total carbon, 1.31% total organic carbon, a pH of around 6, and an average of 20.6%, 31.7%, and 47% sand, silt, and clay, respectively. These soils were found to have high levels of Pb bioaccessibility ranging from 75.7% up to 95.7%, indicating that significant proportions of Pb in these soils are readily exchangeable and bioavailable despite their clay-rich texture and limestone mineral origin (35).

Soil analyses and sample collection. For our study on the soil fungi in Pb-contaminated soils, in July 2010, we established one transect through each area of the different vegetation types: forested, grassy, and backstop (which was mostly devoid of vegetation). Five to 12 points along each of the three transects were analyzed in the field for total soil Pb content using a handheld Niton XLi/XLt 700 Series X-ray elemental analyzer (XRF) (Niton LLC, Billerica, MA). Points along the grass-dominated transect were denoted with a G (G1 through G10), points along the forest-dominated transect were denoted with an F (F2 through F14), and points to the north of the backstop in the area devoid of vegetation were denoted with a B (B4 through B8). All soil measurements and samples were collected after having first cleared the transect point of vegetation and surface debris to expose bare soil. At each point along all transects, the X-ray analyzer was placed in contact with the soil surface, and measurements were taken according to the manufacturer's instructions for the device.

Based on these field measurements, we collected one representative "low-Pb" and one "high-Pb" soil sample from each transect. At each soil sample collection point, three soil cores were collected aseptically within 10 cm of each other to a depth of 15 cm with a standard 2.54-cm-diameter soil corer; these three cores were then composited in the field. Between sampling points, the soil corer was wiped clean of soil particulates, rinsed with a 10% bleach solution, subsequently rinsed with a 70% ethanol solution, and allowed to air dry. The single exception to this sampling procedure was when soils from two different "high-Pb" points (B7 and B8) on the backstop transect were consolidated into one sample. The soil samples were aseptically transferred directly from the soil auger into Ziploc baggies and stored on ice for approximately 2 h for transport back to the laboratory and further processing. At that point, each soil sample was thoroughly homogenized; sieved to 4 mm to remove rocks, intact bullet slugs, and large fragments; and partitioned into subsamples for subsequent analysis.

The gravimetric water content was determined on replicate 10-g subsamples dried at 65°C for 120 h. Soil pH was determined with a Corning pH/ion Analyzer 350 in a slurry of 5 g soil to 5 ml H_2O after shaking for 30 m and then centrifugation at 3,000 rpm for 10 min. The soil total metal content was again determined on replicate 2.0-g subsamples after the soils were homogenized, sieved, and dried, using the hand-held Niton XLi/XLt 700 Series X-ray elemental analyzer as described above.

Soil fungal cultivation and isolation. To determine total cultivable fungi on each of the soils collected, serial dilutions were performed on three replicate field-moist soil subsamples (5 g each) and plated, in replicate, onto either malt extract agar (MEA) (Difco Becton, Dickinson and Co., Sparks, MD) or Czapek Dox agar (CZA) (HiMedia Laboratories Pvt. Ltd., India) adjusted to pH 5 and 7, respectively. The ability of cultured fungi to lower the medium pH was visualized with the indicators bromothymol blue in the CZA and methyl orange in the MEA. The plates were incubated at 21°C for 72 h, at which point counts were determined for total and acid-producing organisms. Acid producers with distinct morphologies were further isolated using the same media on which they were originally cultivated. The isolates were then tested for acid production on the alternate media/pH, as well as phylogenetically identified and further characterized as described below. Cultures of each of these isolates are available from the CBS Fungal Biodiversity Centre in The Netherlands (CBS accession numbers 132844 to 132858).

The type and quantity of organic acids secreted by each isolated fungus were determined by analyzing the broth supernatant by high-pressure liquid chromatography (HPLC). Broth tubes were inoculated by using a 5-mm plug of each isolate in 5.0 ml of 1% malt extract (ME) broth or 1% Czapek Dox (CZ) broth and incubated at 30°C for 120 h. After incubation, the tubes were centrifuged at 4,000 rpm for 10 min, and supernatant from each tube was filter sterilized to 0.2 μm. The sterile supernatant was acidified with 200 mM sulfuric acid and analyzed on a Waters 2707 Autosampler with a 2414 Refractive Index Detector and an Aminex HPX-87H column at a 0.60-ml min^{-1} flow rate at 35°C internal temperature. Standards included oxalic acid, citric acid, malonic acid, malic acid, lactic acid,

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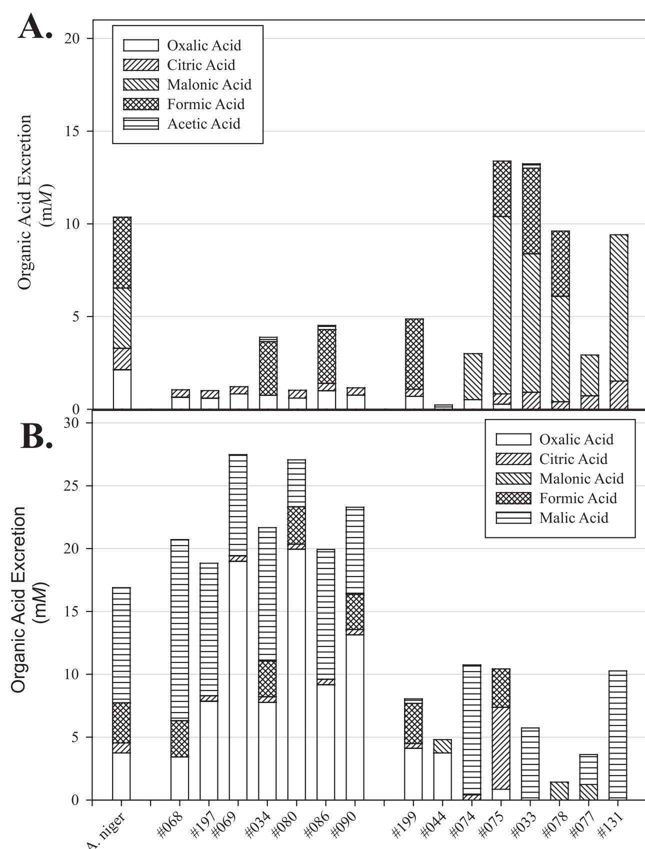

FIG 2 Organic acid secretion by *A. niger* and each soil fungal isolate into either 1% Czapek Dox broth (A) or 1% malt extract broth (B).

acetic acid, and formic acid at 2.5, 5.0, 10, 15, and 20 mM. Uninoculated broth of each type served as a medium control, and the values obtained for each isolate were adjusted to account for medium content.

To determine the ability of each isolated fungus to solubilize Pb at different concentrations, Pb-carbonate (Alfa Aesar, Ward Hill, MA) was incorporated into MEA at the equivalent of 2.5, 10.0, and 18.5 mM concentrations (1.5, 5.7, and 10.5 g liter^{-1}, respectively) and stirred continuously to maintain Pb-carbonate dispersal in the medium, and the mixture was then poured into standard 10-cm petri plates to a depth of roughly 5 mm. Three replicate Pb-carbonate plates at each concentration were each inoculated with a 7-mm-diameter plug of each isolate. The outward radius of hyphal growth and the radius of cleared Pb-carbonate were measured after 120 h of incubation at 21°C (Fig. 2). *Aspergillus niger* NRRL 3 (U.S. Department of Agriculture [USDA] ARS Culture Collection) was used as a positive control against which to measure both growth and the zone of clearing around the culture plug in the Pb-amended medium.

Isolate sequence characterization. Tissues from each isolated fungus found to acidify both media described above were aseptically collected from the surface of an agar petri dish and used in the MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions for 0.25 g soil. The extracted DNA was quantified with a Nanodrop ND-1000 spectrophotometer at 260 nm (Nanodrop Technologies, Wilmington, DE), and the large subunit of the fungal rRNA gene was amplified and sequenced using primers LR5R and LROF according to the methods of Castro et al. (8) for subsequent sequence analysis. For a more detailed description, see the supplemental material.

Partial sequences (400 to 500 bp) were then analyzed against the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) using BLASTN (1).

Soil fungal community pyrosequence analysis. The fungal-community composition of soils at the firing range was characterized by pyrosequence analysis as described by Gottel et al. (21) (for a full description, see the supplemental material). Raw fungal 28S rRNA gene sequences were trimmed and quality checked using MOTHUR (49a). MOTHUR was also used for preclustering at 2%, rarefaction curves, distance calculations, clustering, and further analysis based on operational taxonomic units (OTUs) (23). Fungal sequences were classified by their top BLAST hit compared against the SILVA LSU database in MG-RAST (34a) for each representative OTU. In order to determine the extent to which isolated fungal species were represented in the overall fungal community, the 52,069 LSU rRNA gene sequences from 454 analyses were formatted into a local BLAST database. The Sanger sequencing-generated LSU rRNA gene sequences from the top 15 Pb-solubilizing isolates were then compared to the 454 database using BLASTN (1).

Nucleotide sequence accession numbers. The edited sequences were submitted to GenBank and may be found under accession numbers JQ746543 through JQ746560.

RESULTS

Soil chemistry. *In situ* field XRF analysis across the site clearly revealed increasing soil Pb content with decreasing proximity to the backstop (Fig. 1). Concentrations ranged from typical background levels for U.S. soils (~26 mg kg^{-1}) at locations furthest away from the backstop (sites G8 and F14) to around 3,000 mg Pb kg^{-1} at a location on the grassy transect closest to the backstop (site G2), near 5,000 mg Pb kg^{-1} at a location directly north of (behind) the backstop (site B7), and greater than 6,000 mg Pb kg^{-1} at several locations on the forested transect closest to the backstop (sites F2 and F5).

In order to obtain representative and somewhat comparable "low-Pb" and "high-Pb" samples from each vegetation type for microbial analysis, several soil cores were collected at sites G8, G2, F14, F6, B5, and B7 (Fig. 1).The total water contents of the soils collected were roughly similar in all samples and ranged from around 18% to just over 24%. Soil pH was lowest on the barren eroded soils behind the firing backstop, with values of 3.9 and 4.0 at sites B7 and B5, respectively, and the pH was highest on the forested transect at the location furthest from the backstop (site F14), with a value of 5.8. Laboratory analysis confirmed the pattern observed in the field of increasing Pb concentrations with decreasing distance to the firing backstop (Table 1 and Fig. 1). However, the highest values for soil Pb concentrations from laboratory analyses of homogenized, dried, sieved soils ranged up to roughly 2,800 mg kg^{-1} at site B5, followed by 2,600 mg kg^{-1} at site B7, 2,400 mg kg^{-1} at site G2, and 1,450 mg kg^{-1} at site F6. Other heavy metals are commonly found in firing range soils, including As, Cu, Ni, Sb, and Zn. However, Ni and Sb were not found in detectable concentrations at our site using the handheld XRF system employed, and As, Cu, and Zn were found in various concentrations ranging from typical background levels to slightly elevated levels at a few sites (Table 1). However, due to the low detection limits and higher variability associated with measuring lighter elements with the XRF system, their importance in these soils cannot be verified or discounted at this time.

Isolate identification. Forty morphologically distinct fungal isolates were obtained from the soils at the ORNL firing range that were capable of acidification of growth media in petri plates (Table 2). In total, 23 of the isolates represented the *Ascomycota*, including isolates with high similarity to GenBank representatives of the genera *Eupenicillium*, *Penicillium*, *Chromocleista*, *Neoseto-*

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TABLE 1 Laboratory-determined values for soil moisture, pH and heavy metals commonly found at shooting ranges for the homogenized and sieved soils collected from the abandoned ORNL firing range on 2 July 2010

Site ID	Vegetation cover	% H_2O	pH	Soil level (mg kg^{-1})[a]			
				Pb	As	Cu	Zn
F14	Forested	19.5	5.8	23.6 (15.7)	BD	BD	BD
F6	Forested	18.7	4.2	1,450 (50.0)	63.8 (37.8)	BD	BD
G8	Grassy	24.4	4.9	43.3 (17.9)	BD	BD	146 (53.3)
G2	Grassy	19.9	5.3	2,440 (47.1)	BD	72.5 (34.3)	66.6 (27.8)
B5	None	17.8	4.0	2,780 (83.7)	309 (65.3)	141 (59.4)	BD
B7	None	22.6	3.9	2,590 (92.0)	200 (70.6)	245 (76.7)	129 (58.6)

[a] Mean value (standard deviation). BD, below detection limit. Values for Ni and Sb were all below the detection limits.

phoma, *Diatrype*, and *Cladosporium*, with several isolates most closely sharing identity with previously unidentified soil fungal clones. The remaining 17 isolates represented the *Basidiomycota* and included isolates similar to *Phanerochaete flavidoalba*, as well as a number of organisms within the genera *Stereum*, *Peniophora*, *Trametes*, and *Kavinia*. Within these 40 isolates, there was considerable phylogenetic overlap (i.e., eight isolates with 100% identity to *Eupenicillium* sp. strain O1a_PD022). Consequently, only 15 isolates that were considered representative of the various groups were further characterized for acid production and Pb solubilization (Table 2). The notable exceptions to these criteria were the seven *P. flavidoalba* isolates. In order to assess the range of abilities

TABLE 2 Description of isolated fungi, including soil of origin, phylogeny, Pb-carbonate clearing, and organic-acid secretion

Isolate ID	GenBank accession no.	Sampling site	Soil Pb content (mg kg^{-1})		Phylum	Closest BLAST match		% Identity
			Field	Laboratory		Strain	Accession no.	
002	JQ746543	B7	5,000	2,590	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
127	JQ746543	B5	450	2,780	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
128	JQ746543	B5	450	2,780	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
131	JQ746551	F6	3,860	1,450	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
181	JQ746543	B7	5,000	2,590	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
195	JQ746543	B5	450	2,780	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
207	JQ746543	B5	450	2,780	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
208	JQ746543	B7	5,000	2,590	*Ascomycota*	*Eupenicillium* sp. strain O1a_PD022	FJ800560.1	100
078	JQ746537	G8	27.4	43.3	*Ascomycota*	*Eupenicillium javanicum* AFTOL-ID 429	EF413621.1	99
129	JQ746539	F6	3,860	1,450	*Ascomycota*	*Penicillium simplicissimum* strain KUC5153	HM469430	99
122	JQ746560	F14	27.7	23.6	*Ascomycota*	*Penicillium variabile* strain KUC1476 18S	HM469398.1	94
130	JQ746550	F6	3,860	1,450	*Ascomycota*	*Penicillium variabile* strain KUC1476 18S	HM469398.1	94
033	JQ746556	F14	27.7	23.6	*Ascomycota*	*Penicillium* sp. strain JM-2008	EU500238.1	98
091	JQ746557	G8	27.4	43.3	*Ascomycota*	*Penicillium* sp. strain 6 JJK-2011	HM469409.1	99
198	JQ746549	F6	3,860	1,450	*Ascomycota*	*Penicillium daleae* strain NRRL 922	AF033442.1	99
092	JQ746545	F6	3,860	1,450	*Ascomycota*	*Chromocleista malachitea* isolate CBS	FJ358281.1	99
073	JQ746554	B7	5,000	2,590	*Ascomycota*	*Neosetophoma samarorum* strain CBS 139.96	GQ387579.1	99
043	JQ746546	F14	27.7	23.6	*Ascomycota*	*Diatrype disciformis* isolate AFTOL-ID 927	DQ470964.1	98
036	JQ746558	F14	27.7	23.6	*Ascomycota*	*Cladosporium uredinicola* strain CPC 5390	EU019264	99
066	JQ746535	F6	3,860	1,450	*Ascomycota*	Uncultured soil fungus clone FunCON4_10A	EU861775.1	99
067	JQ746535	F6	3,860	1,450	*Ascomycota*	Uncultured soil fungus clone FunCON4_10A	EU861775.1	99
077	JQ746536	B5	450	2,780	*Ascomycota*	Soil fungal sp. DM2-444	AB438921	100
034	JQ746553	F6	3,860	1,450	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
068	JQ746547	F14	27.7	23.6	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
069	JQ746553	F6	3,860	1,450	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
080	JQ746538	B7	5,000	2,590	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
086	JQ746538	B7	5,000	2,590	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
090	JQ746538	B7	5,000	2,590	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
197	JQ746547	F14	27.7	23.6	*Basidiomycota*	*Phanerochaete flavidoalba* Wu890805	GQ470667.1	99
194	JQ746540	F14	27.7	23.6	*Basidiomycota*	*Stereum rugosum* isolate TM02_369	EU522781	99
019	JQ746544	F14	27.7	23.6	*Basidiomycota*	*Stereum rugosum* isolate TM02_369	EU522781	98
081	JQ746548	F14	27.7	23.6	*Basidiomycota*	*Stereum rugosum* isolate TM02_369	EU522781	99
199	JQ746534	F14	27.7	23.6	*Basidiomycota*	*Stereum subtomentosum* EL11-97	AF506482.1	99
038	JQ746534	F6	3,860	1,450	*Basidiomycota*	*Stereum* sp. strain YMF1.1587	EF600046.1	99
044	JQ746552	2T	1,010	2,440	*Basidiomycota*	*Peniophora incarnata* NH10271	AF506425.1	99
075	JQ746541	B7	5,000	2,590	*Basidiomycota*	*Peniophora* sp. strain M48	HM595613	99
076	JQ746542	B7	5,000	2,590	*Basidiomycota*	*Trametes hirsuta* strain Wu 9408-41	AY351923.1	99
074	JQ746559	B7	5,000	2,590	*Basidiomycota*	*Trametes versicolor* isolate M126	HM595617.1	99
089	JQ746555	B7	5,000	2,590	*Basidiomycota*	*Kavinia* sp. strain FO25092	AJ406489.1	99

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within an isolate type, all of the *P. flavidoalba* types were carried forward for analysis. These isolates originated from soils associated with each of the different vegetation types and with a wide range of soil Pb concentrations.

Isolate acid secretion. Organic acid secretion by 15 soil isolates, as well as *A. niger*, in each type of broth at 120 h of incubation is shown in Fig. 2. Overall, isolates produced much lower levels of organic acids in CZ broth (Fig. 2A) (average, 4.7 mM) than in the ME broth (Fig. 2B) (14.8 mM). None of the *P. flavidoalba* isolates exceeded the positive control (*A. niger*) in terms of total organic acids produced while growing in CZ, but each produced small quantities of oxalic and citric acids (<1.0 mM each) (Fig. 2A). However, while growing in ME broth, all the *P. flavidoalba* isolates exceeded the acid production rate of *A. niger* (16.9 mM), with isolates 069 and 080 producing roughly 27 mM, of which nearly 20 mM was oxalic acid (Fig. 2B). Each of the other *Basidiomycota* isolates (isolates 199, 044, 074, and 075) also yielded an increased quantity and altered mixture of organic acids during growth in ME broth over growth in CZ broth (Fig. 2).

The isolates belonging to the *Ascomycota* exhibited a pattern contrasting with that of the isolates of *Basidiomycota* in the two growth media, and none produced detectable levels of oxalic acid in either medium. *Ascomycota* isolates produced roughly equivalent overall quantities of acids in each of the media; however, all four switched from a mixture of acids dominated by malonic acid with traces of citric acid in CZ (Fig. 2A) to a mixture dominated by malic acid with traces of malonic acid in ME (Fig. 2B). The *Penicillium* sp. (033) and *Eupenicillium javanicum*-like (078) isolates showed a more than 100% increase in overall acid production in CZ over ME broth. For a more detailed accounting of the mixtures of acids and changes by each organism in each medium, see the supplemental material.

Isolate Pb solubilization. The growth and Pb solubilization capabilities of each soil isolate and *A. niger* in multiple media at multiple Pb levels are shown in Fig. 3A to C, and photos of the seven *P. flavidoalba* isolates, as well as the *A. niger* control, are shown in Fig. S1 in the supplemental material. All isolates were capable of growth, in several cases even greater than that of the positive-control *A. niger*, at all tested concentrations of Pb. Notably, however, only the *P. flavidoalba* isolates were capable of Pb solubilization at all tested concentrations. At the lowest level of Pb (2.5 mM) (Fig. 3A), *A. niger* showed similar growth but greater effectiveness than *P. flavidoalba* in Pb solubilization. This was demonstrated by a zone of clearing (ZOC) of roughly 30 mm from the inoculating plug of *A. niger* after 120 h of incubation, exceeding hyphal growth by at least 10 mm. At the intermediate concentration of Pb in the medium (10.0 mM) (Fig. 3B), the average growth of the *P. flavidoalba* isolates actually increased over their growth rate at the lowest level of Pb (from 21.4 to 22.7 mm at 2.5 and 10 mM, respectively). The ZOC associated with these isolates was also greater than at the lowest level of Pb (10.4 and 15.4 mm at 2.5 and 10 mM, respectively). Even at the highest level of Pb in the medium (18.5 mM) (Fig. 3C), the average growth of the *P. flavidoalba* group of isolates remained undiminished at 19.7 mm and the ZOC at 13.5 mm. The greatest rate of growth and most effective Pb solubilization among all the isolates at all tested levels of Pb was demonstrated by isolate 080. At every Pb level, *P. flavidoalba* isolates, except 034, exhibited a growth rate greater than or equal to that of the positive-control *A. niger* (Fig. 3).

Among the remaining isolates, *Basidiomycota* and *Ascomycota* exhibited roughly similar growth rates at different Pb concentrations in the medium. Only one isolate (*Trametes versicolor*, 074) exhibited the capacity to solubilize Pb in the medium, and only at the 10.0 mM concentration, where the ZOC was roughly 11 mm. Within each phylum, the isolate growth rate was generally lower for the isolates originating from lower or background level Pb soils than those originating from intermediate or high-level Pb soils (Table 2 and Fig. 3A to C).

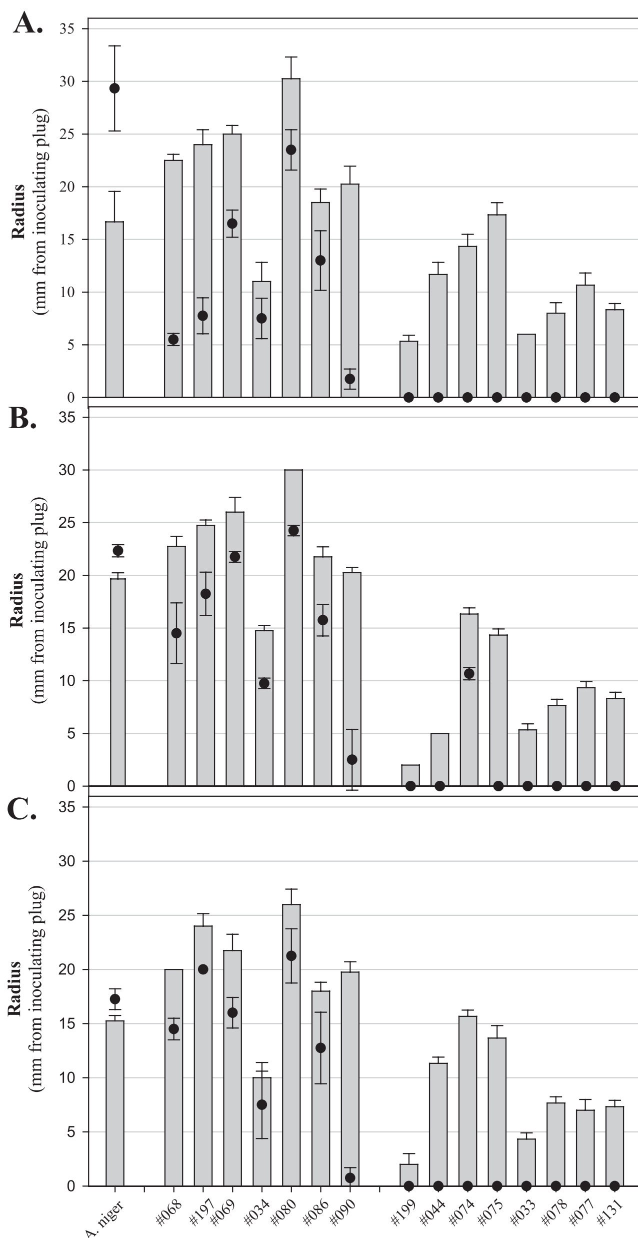

FIG 3 Hyphal growth (bars) and zone of clearing (points) for each isolate grown on malt extract agar with Pb-carbonate incorporated into the medium at 2.5 mM (A), 10.0 mM (B), or 18.5 mM (C). The error bars indicate standard deviations.

Presence and relative abundance of isolated organisms within the soil fungal community. Sequences with high identity to each of the cultured isolates were also found to be present in the soil pyrosequence libraries at various levels. Overall, OTUs with

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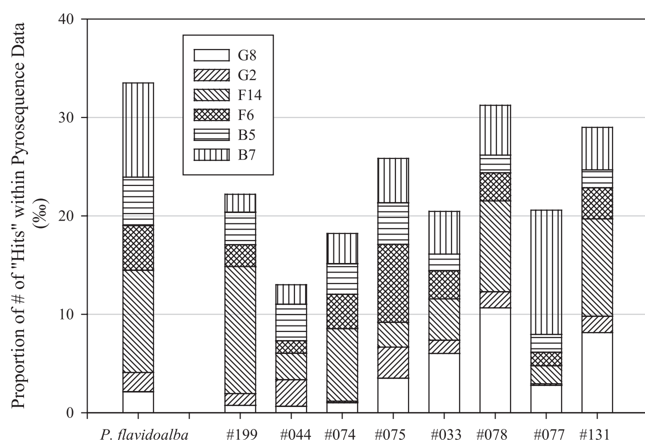

FIG 4 Proportion (‰) of hits for each isolate's LSU rRNA gene sequence within the 454 pyrosequence community data from each soil sample (≥97% identity), calculated as the number of hits for an isolate in a sample over the total number of fungal sequences for that soil sample.

>97% identity to *P. flavidoalba* were found in the greatest proportion in the soils of sites F14 and B7 at 10.3‰ and 9.56‰, respectively (Fig. 4). Roughly half that level (~5‰) of *P. flavidoalba* was found in the soils of sites F6 and B5 and minimal levels (~2‰) in both the samples from the grassy transect (G8 and G2). The remaining *Basidiomycota* isolates were found in the greatest proportions in the soils of sites along the forested transect (F6 and F14) (Fig. 4). *Stereum subtomentosum* (199) and *T. versicolor* (074) exhibited patterns similar to that of *P. flavidoalba*. While *Peniophora incarnata* (044) was found in the highest proportion at site B5, the proportion at which the isolate was found in each of the sites was minimal (0.65 to 3.72‰). The other *Peniophora* sp. (075) was found in the greatest proportions at site F6 (7.90‰), at roughly 4‰ in sites B5 and B7, and at only around 3‰ at G8 and G2.

Overall, the *Ascomycota* isolates had a greater proportion of hits in the soils at G8 than did the isolates of the *Basidiomycota*, and the *Ascomycota* isolates did not have many hits within the soils of G2 (Fig. 4). *Penicillium* sp. (similar to isolate 033) and *E. javanicum*-like sequences (similar to isolate 078) were both found in the greatest proportion (6.01‰ and 10.6‰ for 033 and 078, respectively) at site G8, but 033 was only at minimal levels in the other soils (1.33 to 4.33‰), while 078 was also found (9.22‰) at site F14. Conversely, the undescribed fungal clone (077) had the greatest proportion of hits at site B7 (12.6‰), which was also the highest proportion of hits for any one sample for the isolates of the *Ascomycota*. *Eupenicillium* sp. (131) was found in the greatest proportions in the "low-Pb" samples F14 (9.89‰) and G8 (8.14‰).

For a more complete description of the soil fungal-community pyrosequence analysis, phylogenetic breakdown, and community diversity assessment, see Fig. S2 and S3 in the supplemental material.

DISCUSSION

Soil Pb contamination at outdoor shooting ranges may select for distinct soil microbial communities, and these altered communities may have the potential to produce significant changes in the biogeochemistry of metals at the site, creating potential risks to humans and ecosystem functions. We examined soil from an abandoned firing range for cultivable fungi, their secretion patterns of LMWOAs, and potential for Pb-carbonate dissolution, as well as the abundance of OTUs similar to our isolates within these highly Pb-contaminated soils. We combined selective and differential culture-based techniques to target fungi capable of acid production and subsequently identified several fungi in this ecosystem that were capable of solubilizing Pb-carbonate, one of the most abundant Pb minerals commonly found in firing range soils (4, 30, 31). Pyrosequence analysis of fungal LSU rRNA genes from these soils verified the presence of organisms sharing genetic identity with our acid-producing isolates and revealed that soil fungal diversity varied with the soil Pb content and sampling method.

Of the more than 300 fungal isolates obtained, roughly 40% were able to acidify either CZA or MEA medium, and around 13% could acidify both medium types, which differed in pH, as well as C and N sources. Of these acid-producing isolates, 8 organisms (20%) from within the *Basidiomycota* exhibiting similarity to representative organisms from the genera *Phanerochaete* and *Trametes* were also able to alter Pb solubility in our plate assay. While many of these isolates appeared to display highly similar OTUs, the 15 distinct isolates examined for production of LMWOAs secreted a mixture of acids that differed depending on the culture media. Most of our environmental isolates originating from highly Pb-contaminated soils produced large quantities of mixed acids (≥10 mM). This high level of organic acid production, as well as variable compositions of the acid mixture, has been documented in the model organisms *A. niger* and *Penicillium bilaiae* due to varying substrate C and N sources (3, 36, 39).

The well-characterized fungal model species *A. niger* has been repeatedly tested, with mixed results, for organic acid secretion, bioleaching, and remediation potential for heavy metals from various substrates, including municipal waste and fly ash (5), low-grade mining ores (6, 36), and a number of soils (3, 6, 39, 44). *A. niger* is reported to produce organic acids in large quantities under specific laboratory culture conditions, with as much as 90% of the total mass of acids produced consisting of oxalic and citric acids (39). These two acids have been found to be the most powerful LMWOAs in terms of Pb release from minerals and Pb-contaminated soils (9). *A. niger* culture exudates have been applied to a variety of substances to affect metal leaching, and in the case of Pb, this has resulted in concentrations as high as 35 mg liter^{-1} of soluble Pb in leachates (39) and up to 52% of the total mass of Pb removed from the solid phase (5).

Oxalic acid overexcretion and the role it may play in toxic metal mineral transformations were also recently explored in the model entomopathogenic ascomycete species *Beauveria caledonica* (14). This organism can produce up to 2 mM oxalic acid in liquid culture after 10 to 20 days and produced a zone of clearing of 50 to 60 mm on 15 mM Pb-carbonate after 14 days of growth (14). In contrast, the *P. flavidoalba* obtained from Pb-contaminated soils in our study produced >27 mM oxalic acid and a zone of clearing of ~20 mm on 18.5 mM Pb-carbonate after just 5 days (120 h). A notable aspect of the *B. caledonica* study is that the authors were able to determine that it was, in fact, the overexcretion of oxalic acid by the fungus (rather than a mixture of LMWOAs or other metabolic by-products) that was having the observed effects on the various metals tested, and the tested metal minerals were transformed into metal oxalates (14).

In our study, we found environmental isolates similar to the

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basidiomycete white-rot fungi *P. flavidoalba* and *T. versicolor* capable of Pb-carbonate dissolution. The two *P. flavidoalba* isolates with the greatest capacity to dissolve Pb-carbonate (069 and 080) not only produced the greatest quantities of oxalic acid (>27 mM), but also originated from sites F6 and B7, with the highest levels of Pb contamination (Table 2 and Fig. 2).

Organic acid secretion by white-rot fungi is thought to function to free lignicolous substrates for degradation, acting in concert with extracellular enzymes that may also dramatically alter the soil or wood substratum in which they grow (41). Saprotrophic basidiomycetes, including the organisms identified in our work, which are classically termed "white-rot" fungi and may often be thought of as wood associated due to their fruiting patterns, can also be major agents of decomposition of a wide variety of lignocellulosic substrates in soils, including wood, leaf litter, grass straw, and other humic substances (51). The ability to utilize such a wide variety of substrates and to form a mycelial network interconnecting resource patches at the soil/litter interface, sometimes penetrating much deeper into the soil, can make these fungi ubiquitous in surface soils (51, 52).

Similar to the environmental isolates in our study, another example of a white-rot basidiomycete, *Abortiporus biennis*, has recently been reported to overexcrete oxalic acid and consequently to dissolve a variety of heavy metals, including Cu, Al, Zn, Cd, and Mn (22). Oxalic acid production in general has been observed in a variety of different wood-rotting fungal model strains (50). Oxalic acid production in white-rot fungi is thought to aid lignocellulose depolymerization by producing radical species, buffering the microenvironment of the fungus, facilitating oxidative enzyme activity, and aiding metal chelation (11) and tolerance (17). *P. flavidoalba* has not specifically been reported to produce LMWOAs but has been studied for bioremediation of olive mill wastewater through decolorization and depolymerization of various toxic components in wastewater streams (13, 32, 45). *T. versicolor* has also been studied for the same purpose (12), as well as for bioremediation of polycyclic aromatic hydrocarbons (46). A recent study of *T. versicolor* examined the organism's capacity to form calcium oxalate and indicated even higher levels of oxalic acid than were observed in our study (23).

A previous study by Sayer et al. (47) compared several fungal species and screened their capacities to solubilize insoluble metal compounds, including ZnO, $Zn_3(PO_4)_2$, and $Co_3(PO_4)_2$. The environmental isolates were obtained from garden soils, nickel-contaminated soils, and Pb-contaminated soils and were capable of solubilizing one or all of the tested metal minerals. However, the quantity or quality of organic acids secreted by each of the isolates was not determined, and their capacity to solubilize Pb was not tested, so direct comparison to the results of the current study was not possible.

OTUs similar to all the soil isolates examined for organic acid secretion and Pb solubilization in this study were also found in all samples in the pyrosequence analysis (at a ≥97% identity cutoff) (20, 33). None of our isolates were dominant in these soils; however, they were present at levels greater than 1%, which is not necessarily uncommon for fungi (42, 48). The fact that the organisms that produced the greatest levels of organic acids and had the greatest potential for Pb solubilization (*P. flavidoalba*) were found in fairly high proportions in some of the most heavily contaminated soils (B7) may indicate the possibility of bioleaching of Pb in those soils and should be the target of more detailed future studies at such sites.

While there are a number of studies examining the effects of mixed heavy metal contamination on soil microbial community structure and function, there are only a limited number of studies specifically targeting fungi. The majority of the previous work in this area, such as that of Pan and Yu (40) or Nakatsu et al. (38), is focused on more general soil microbial characteristics or process level functional responses. Studies such as these have shown reduced microbial biomass and enzyme activities and drastically altered soil microbial community structure in response to Pb contamination (29, 43). Even a recent study within the ectomycorrhizal (EcM) component of the fungal community at a shooting range in Finland revealed differential structure within the EcM community at Pb-contaminated sites without a significant decrease in overall fungal diversity but revealed nothing about the function of the fungal community with regard to Pb solubility.

Conclusions. In fact, the high incidence of model organisms (e.g., *A. niger* and *B. caledonica*) capable of acidifying media by means of organic acids is well known and established. Notably, however, the occurrence of such fungi cultured directly from Pb-contaminated soils capable of this level of acid hyperexcretion has not been previously established. Our results indicate that even within a single fungal species, different conditions, such as the specificity of C and N sources within the soil profile, may result in different levels and mixtures of organic acids. In our study, the undefined medium (malt extract), which is arguably more representative of the variety of C sources found in a soil system, resulted in much greater acid production (4- to 20-fold) than the defined medium (Czapek Dox), which has an inorganic N source and sucrose as the sole C source. These data highlight the need to understand more about autochthonous fungal communities at contaminated sites and how the selective pressures of heavy metal contamination may impact metal permanence and solubility in soils. These reciprocal biogeochemical impacts have important implications for trace metal bioavailability and overall ecosystem health.

ACKNOWLEDGMENTS

We thank T. Mehlhorn, S. Carroll, and M. Kerley for assistance in field and laboratory studies.

This study was funded by the Strategic Environmental Research and Development Program (SERDP), a joint partnership between the Department of Defense, the Department of Energy, and the Environmental Protection Agency. ORNL is managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract DE-AC05-00OR22725.

The article was written by a contractor of the U.S. Government under contract DE-AC05-00OR22725.

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