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# Co-selection of Mercury and Antibiotic Resistance in Sphagnum Core Samples Dating Back 2000 Years

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**Metal exposure might induce multiple drug resistance (MDR) in bacteria in environments devoid of antibiotics via the process of co-selection, but the extent is poorly known. Core samples from two sphagnum peat bogs in central Maine, USA, were analyzed for total Hg content and were radiocarbon dated. Culturable bacteria isolated from various core depths were assayed for antibiotic- and Hg-resistance and the presence of *merA* (mercuric reductase). Our results show that sphagnum peat bogs represent natural ecosystems that contain ambient levels of Hg that select for indigenous bacterial strains that are not only Hg resistant, but also possess the MDR phenotype.**

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**Keywords** antibiotic, Co-selection, mercury, resistance, sphagnum bog

## INTRODUCTION

True peatlands, including sphagnum bogs, are comprised of strictly autochthonous organic matter, partially decayed remains of plants and other organisms once living at the site, deposited layer upon layer in waterlogged situations that allow accumulation to exceed decomposition. In sphagnum bogs, permanent waterlogging occurs once the materials are buried to a depth

of about 20 cm (Moore and Webb 1978), at which time microbial degradation all but ceases. This produces a finely stratified record of vegetational, environmental, geochemical and chemical change that pollen records indicate displays no more than 25–40 years of vertical mixing of the record, well within the statistical uncertainties of radiocarbon dating techniques (Faegri and Iversen 1975; Moore and Webb 1978; Birks and Birks 1980; Blaauw et al. 2004)

Species of sphagnum moss are the main constituents of the primary mat of ombrotrophic bogs in New England, locally accompanied by hygrophilous shrubs of the family Ericaceae and sedges of the genera *Carex* and *Eriophorum* (Cyperaceae). An ombrotrophic bog is an isolated body of freshwater, with water and minerals introduced solely through atmospheric deposition. Until recently, little attention has been given to microbial communities of sphagnum bogs. Due to low pH of the substrate and the absence of an adequate nutrient supply, sphagnum moss has been previously considered inhospitable to bacterial growth (Painter 1991). Furthermore, the low buffering capacity in this acidic environment enhances the toxicity of metals that may be present in sphagnum bogs. However, recent studies have shown that bacterial communities indigenous to sphagnum bogs play crucial nutritional roles in this environment (Opelt and Berg 2004; Belova et al. 2006; Morales et al. 2006; Opelt et al. 2007).

Because sphagnum does not have xylem and has no contact with soil in a bog setting, free-living nitrogen-fixing organisms are essential for the nitrogenous requirements and growth of plants (Opelt et al. 2007). High population numbers of diazotrophic *Paenibacillus* species have been isolated from sphagnum bogs. In addition, *Rahnella* spp. have been shown to solubilize phosphates in the bog environment (Opelt et al. 2007). Although the role that bacterial communities play in sphagnum mats has been addressed recently, the influence of Hg in the sphagnum bog environment on the co-selection of Hg- and antibiotic-resistant strains among the indigenous bacterial populations remains unclear.

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Sphagnum moss has a unique ability to sequester divalent cations (Shotyk et al. 2005; Lodenius et al. 1983). Many heavy metals, including mercuric ion, exist in the divalent cationic state in the environment. Water input to the bog system as either rain or runoff does not cause flushing or leaching of these metals from the sphagnum substrate; instead they are sequestered tightly by the sphagnum, creating an environment rich in toxic and non-toxic metals.

Although Hg is pervasive environmentally, many bacterial species can adapt to its presence by invoking an efficient Hg-detoxification system (Barkay et al. 2003). The most common bacterial Hg resistance mechanism is reduction of mercuric ion [Hg (II)] to the elemental form [Hg (0)] catalyzed by mercuric reductase (Barkay et al. 2003). The gene encoding mercuric reductase, *merA*, is a part of the *mer* operon that is often located on mobile genetic elements such as transposable elements and plasmids, and is therefore readily transferred among bacterial species by horizontal gene transfer (HGT) mechanisms (Summers 2002; Barkay et al. 2003; McIntosh et al. 2008). The *mer* operon is closely linked genetically with antibiotic resistance genes (Wireman et al. 1997). Antibiotic resistance determinants are usually organized in gene cassette systems that contain genes conferring resistance to a wide variety of antibiotics (Rowe-Magnus and Mazel 2002).

The transfer of antibiotic resistance genes in antibiotic-rich environments such as hospitals, aquaculture and land-based agriculture, is a commonly documented phenomenon (Nester et al. 1999). However, antibiotic resistance genes are also being spread throughout bacterial communities in environments devoid of antibiotics (Baker-Austin et al. 2006). One proposed mechanism for the occurrence of antibiotic resistance in such environments is via co-resistance, which occurs when the genes encoding resistance phenotypes are linked together on the same mobile genetic element (Baker-Austin et al. 2006). This physical linkage allows for the co-selection of other genes located on the same genetic element. Associations between heavy metal exposure and specific patterns of antibiotic resistance have been reported (McArthur and Tuckfield 2000; Stepanauskas et al. 2006; McIntosh et al. 2008).

Linkage between Hg resistance and antibiotic resistance has been documented in a wide range of bacterial habitats, including oral and fecal microbial flora of primates (Wireman et al. 1997), oral microbial flora of patients with amalgam fillings (Summers et al. 1993), fish gastrointestinal tracts (Akinbowale et al. 2007), mine sediments (Nemergut et al. 2004), and freshwater microcosms (Stepanauskas et al. 2006). These bacterial isolates with plasmid-encoded *mer* operons are often shown to be associated with antibiotic resistance gene cassettes (Mazel et al. 2000). However, all of the above studies on co-selection of metal and antibiotic resistance involve environments either contaminated with toxic metals or were experimental studies in which metal exposure is directly manipulated to test for co-selection in bacterial communities.

In contrast to these metal polluted environmental test sites, sphagnum bogs provide a unique natural biological system for the investigation of Hg and antibiotic resistance co-selection. Hg in the sphagnum bog environment is present as a natural element of the earth's crust, and in the post Industrial Era, it is also deposited atmospherically as a pollutant from precipitation (Barkay et al. 2003). Because Hg is held tightly by the sphagnum substrate (Lodenius et al. 1983; Shotyk et al. 2005), it allows temporal trends in atmospheric deposition to be observed by examination of Hg levels within the column (Steinnes et al. 2005). Total Hg content analyses of bogs throughout northern Maine and Ontario (Norton et al. 1997; Givélet et al. 2003; Roos-Barraclough et al. 2006) have shown that spikes in Hg concentrations in samples from various core depths correspond to times when anthropogenic Hg emissions were high.

Cores taken from two bogs, Round Pond bog in Franklin County, and Hamilton Pond bog in Kennebec County, Maine, USA, have sphagnum mat depths that carbon-date back to approximately 2000 ybp (years before present). Total Hg analyses on these bogs revealed ambient Hg concentrations at all depths. Bacteria living deep within the core represent populations that originated significantly before the era of antimicrobial chemotherapy. Any selective pressures for antibiotic resistance exerted on microbes indigenous to the acidic environment of a bog that is 2000 years old, were most likely due to co-selection with other non-antibiotic agents such as Hg or other heavy metals. Other studies have been conducted on metal and antibiotic resistance co-selection within metal contaminated sites. Conversely, we report here the co-selection of Hg and antibiotic resistance in a natural environment. Furthermore, this environment contains sufficient Hg to exert selective pressure on indigenous sphagnum bog bacteria to produce the Hg- and antibiotic-resistant phenotypes observed.

## METHODS

### Sampling Site Description and Core Collection

Both Hamilton Pond (44°27'58"N, 69°50'15"W) and Round Pond (44°31'14"N, 70°05'22"W) are kettle basins in complex glacial esker systems, and originated shortly after deglaciation of the region about 14,000 calendar years ago. Round Pond bog has a small first-order outlet stream at the southern end; outflow of excess water from Hamilton Pond is likely subsurface, through adjoining esker sediments, into adjacent Stuart Pond, from which an outlet stream carries excess flow. Neither Hamilton Pond nor Round Pond have inlet streams.

A 2 m core from Round Pond bog and a 1 m core from Hamilton Pond bog were sampled in the fall of 2006 using a modified Livingstone Piston Corer. One cm slices of core sediments were removed aseptically using sterile spatulas from each core at five cm intervals and stored in sealed, sterile polypropylene tubes. Sample data were recorded and samples numbered beginning with the oldest (deepest) core samples as the lowest

numbered samples and proceeding sequentially to the top of the core. Representative samples were selected for evaluation from a range of depths in each core. From Hamilton Pond bog, slice numbers 1, 5, 9, 13, 16, and 20, and from Round Pond bog, slice numbers 1, 8, 17, 25, 31, and 36, were processed and used in this study. Cores were kept wrapped in foil, and both cores and samples were stored at 4°C. The two cores collected in 2006 were used for radiocarbon dating and isolation of metabolically active bacterial isolates. Both bogs were cored and re-sampled a second time in fall, 2007. Samples from the most recent cores were analyzed for total Hg concentration and used for isolation of endospore-forming bacterial isolates.

### Radiocarbon Dating

Sphagnum bog environments represent sites of slow but continuous surface accumulation of organic matter with negligible vertical mixing, so radiocarbon dating of thin slices of the organic mat (excluding any identifiable modern roots when present) is a standard geological technique for determining ages of deposits. Though recent papers have suggested <sup>14</sup>C dating problems with such ombrotrophic peats (e.g., Kilian et al. 1995; Shore et al. 1995; Nilsson et al. 2001), Blaauw et al. have been unable to corroborate those findings (Kilian et al. 1995; Shore et al. 1995; Nilsson et al. 2001; Blaauw et al. 2004). In our study, 5-mm-thick slices of pure sphagnum peat from approximately mid-level and the base of each core were oven-dried at 50°C and submitted for commercial AMS radiocarbon dating (Beta Analytic, Miami, FL, USA).

### Total Mercury Analysis of Cores

Total mercury concentration (total-Hg) was measured by thermal decomposition, amalgamation and atomic absorption spectroscopy using a DMA-80 mercury analyzer (Milestone, Inc.). Sphagnum samples from Round Pond bog (depths of 1, 6, 101, and 193 cm below the surface) and Hamilton Pond bog (depths of 1, 8.5, 50, and 94 cm below the surface) were analyzed. All sphagnum core samples were taken from the center of wet cores and dried at 40°C for at least 24 h (or until dry weight stabilized). Six replicate sample aliquots of approximately 0.1g (mostly leaf with some stem) were prepared from each core depth and site, and mean total-Hg concentration in µg/kg dry weight is reported in Table 3. Commercial greenhouse sphagnum was used for comparison, and a sample of plant material grown in Hg-free soil was also included as a control.

### Bacterial Strain Isolation, Culture Conditions, and Stock Maintenance

First, 1.0 g of sphagnum sample was added to 9.0 ml of sterile phosphate buffer diluent (Hardy Diagnostics, Santa Monica, CA, USA). All samples were shaken for fifteen min, serially diluted and plated onto tryptic soy solid-plating media (TSA, Difco-BBL, Sparks, MD, USA). Culture plates were incubated at 20°C for 72 h. Individual colonies were selected from samples

representing the complete range of core depths, checked for purity and maintained on stock culture TSA slants. A total of 29 metabolically active, or readily culturable isolates were stored and maintained on TSA media.

Bog cores collected in October 2007 were used for recovery of bacterial isolates that were in the endospore stage of development at the time of sampling. A sample of 0.5 g of sphagnum sample from appropriate slices was added to 4.5 ml sterile water. Samples were mixed by vortexing for 5 min and placed in a waterbath at 70°C for 30 min in order to kill all metabolically active and non-sporulating bacterial cells. After appropriate dilution, samples were inoculated and spread plated onto TSA. Cultures were incubated at 20°C for 72 hours. Individual colonies were selected from each plate and inoculated onto stock culture TSA slants. A total of 25 endospore-forming isolates were maintained. All isolates were cryogenically stored at -80°C in a 50% mixture of glycerol and tryptic soy broth (TSB, Difco-BBL, Sparks, MD, USA).

### Hg Minimum Inhibitory Concentrations (MICs)

Hg minimum inhibitory concentration (MIC) assays were performed on each isolate according to the method of Wang, et al. (1989). Isolates were grown for 24–48 h at 20°C on TSA solid-plating media, and then resuspended a density equivalent to a 2.0 McFarland standard (Wang et al. 1989). After vortexing each cell suspension thoroughly, 5 µl of each was spotted onto TSA plates supplemented with 0 µM, 50 µM, 100 µM, and 250 µM HgCl<sub>2</sub>. Plates were observed every 24 h for 72 hours, and the MIC was recorded as the lowest concentration of HgCl<sub>2</sub> that inhibited bacterial growth.

### Antibiotic Susceptibility Testing

The activity of antimicrobial agents against all 54 isolates was assessed *in vitro*. Antibiotic MIC values for each isolate were determined on Sensititre<sup>®</sup> dried susceptibility panels GN2F (Gram-negative) and GPN2F (Gram-positive) (Trek Diagnostic Systems, Westlake, OH, USA). Quality control strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 were inoculated in parallel to the isolates as suggested by the manufacturer. The guidelines set by the National Committee for Clinical Laboratory Standards were followed, with the exception that the MIC panels were incubated for 72 h at 22°C.

Strains of *Rahnella aquatilis*, which were not culturable using the Sensititre<sup>®</sup> dried susceptibility panel culture system, were assayed using the antimicrobial disk susceptibility test method (Sensi-Discs; Becton, Dickinson and Co., Sparks, MD, USA) containing sulfisoxazole 2 mg, trimethoprim 5 µg, piperacillin 100 µg, amikacin 30 µg, tobramycin 30 µg, vancomycin 30 µg, kanamycin 30 µg, novobiocin 30 µg, penicillin 10 IU/IE/UI, streptomycin 10 µg, and tetracycline 30 µg. The 24-hour TSA plating cultures were resuspended to a 0.5 McFarland standard, and were spread by cotton swab evenly

over TSA plates. After the inocula absorbed into the media, Sensi-Discs were deposited aseptically onto the culture plates. Plates were incubated at 20°C for 2 d prior to measuring zones of inhibition according to the manufacturer's instructions.

### Antibiosis Assays

All metabolically active bacterial isolates were tested for their ability to produce compounds capable of inhibiting growth of 4 test bacterial strains, (*Pseudomonas aeruginosa* ATCC7853, *Enterococcus faecium* ATCC51559, *Bacillus subtilis* 168 ATCC23857, and *Escherichia coli* K12 ATCC29425). The 24-hour cultures were streaked in 2 parallel straight lines across TSA solid-plating media. Plates were incubated at 22°C for 3 d. Each of the 4 test strains were resuspended in sterile water to a 2.0 McFarland standard and streaked perpendicularly from the edge of the plate in the direction of, but not touching, the isolate growth. Plates were incubated at 22°C for 2 d and isolates were assessed for antimicrobial activity as visualized by inhibition of growth of test strains within 5–10 mm of the experimental culture streaks.

### Bacterial Isolate Identification (16S rRNA Gene Sequencing)

Bacterial strains were grown at 20°C for 1–2 d on TSA. Total genomic DNA was extracted from all bacterial strains using the Colony Fast-Screen™ kit (Epicentre Technologies, Madison, WI, USA). A single colony was selected from each plate and suspended in 50 µl of PCR-lyse™ buffer (Colony Fast-Screen™ kit, Epicentre Technologies, Madison, WI, USA). The samples were incubated in an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) at 99°C for 10 min and were then cooled to 4°C prior to PCR amplification. PCR amplification cycles conducted with 27f and 1392r primers (Table 1) (Lane 1991) were as follows: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 2 min, followed by a final extension step of 72°C for 7.5 min prior to storage at 4°C.

16S rRNA gene sequencing was performed to confirm the identity of all strains after cleaning the PCR products (E.N.Z.A. Cyclepure Kit; Omega Bio-tek, Doraville, GA, USA). A region, approximately 1.5 kb, of the 16S rRNA gene was amplified in all isolates using the 27f and 1392r primers (Table 1). Amplicons were sequenced using the Big Dye® Terminator v3.1 Cycle Se-

quencing Kit and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). GenBank database searches were carried out for all sequences using the National Center for Biotechnology Information basic local alignment search tool (BLAST) web server (<http://www.ncbi.nlm.nih.gov/BLAST>).

### PCR amplification of *merA*

Degenerate primers (Table 1) (Vetriani et al. 2005; Ni Chadhain et al. 2006) in 2 sequential PCR experiments were used to probe all isolates for the *merA* gene. Genomic DNA was extracted as previously described. The first PCR (primers A5 and A2NF; Table 1) generated an amplicon of approximately 1200 bp that was not visible on an agarose gel. Cycling conditions were as follows: 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 30 sec; 55°C for 30 sec; 72°C for 1 min, followed by an extension step of 72°C for 7 min prior to storage at 4°C. PCR using the previous amplicon as a template, and A5 and A1SF primers (Table 1), resulted in a 288 base-pair product, which was confirmed using agarose gel electrophoresis. Cycling conditions for the second *merA* PCR were as follows: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 30 sec; 56°C for 20 sec; 72°C for 1 min, followed by an extension step of 72°C for 7 min prior to storage at 4°C.

### RESULTS

Radiocarbon age determinations on submitted core samples show clearly that both the Hamilton Pond bog and Round Pond bog cores penetrated to sediment depths predating European influence (Table 2). The pollen record from our cores also indicates minimal mixing at most, and is consistent with these radiocarbon age determinations, indicating that the cores extend to well before European contact (Clark CK, Krumdieck NW, Littlefield EF, Nelson RE. 2007. Pre- and Post-European Forest Composition and Bog Flora Changes Documented by Pollen from Maine Sphagnum Cores. Geol Soc Am Abstracts with Programs 39(6), 401.).

Round Pond bog sphagnum at 175 cm depth was deposited between 325 BCE (before the common era) and 125 BCE, while sphagnum at 90 cm depth in Hamilton Pond bog was living on the bog surface about 260 AD. Average sphagnum accumulation rates were thus 1.2 mm/yr at Round Pond bog and 0.56 mm/year at Hamilton Pond bog.

TABLE 1  
PCR Primers used in this study

Target	Primer	Sequence	Reference
16S rRNA	27F	5' AGAGTTTGATCCTGGCTCAG 3'	(Lane, 1991)
	1392R	5' GACGGCGGTGTGTAC 3'	
<i>merA</i> (mercuric reductase)	A5	5' ACCATCGTCAGGTAGGGGACCAA 3'	(Ni Chadhain et al., 2006; Vetriani 2005)
	A2NF	5' CCATCGGCGGCWSYTGCGTSAA 3'	
	A1SF	5' TGGGCAAGTNGCVACBGTTNGG 3'	

TABLE 2  
Radiocarbon dates for core samples from Round Pond and Hamilton Pond Bogs

Sample Site	Depth (cm)	<sup>14</sup> C Ages (years before present)	Average Calendar Year
Round Pond Bog	88	520 +/- 40	1430 AD
	175	2160 +/- 40	325 BCE or 125 BCE
Hamilton Pond Bog	50	1140 +/- 40	950 AD
	90	1820 +/- 40	260 AD

Total Hg concentration analysis of samples from both bogs revealed that detectable levels of total Hg were present at all depths in both Hamilton Pond and Round Pond bogs (Table 3). Total Hg levels of Round Pond bog ranged from 8.7 parts per billion (ppb) (at a depth of 193 cm) to 44.9 ppb (at a depth of 6 cm). The Hg concentration range was slightly higher at Hamilton Pond bog where total Hg levels were between 13.1 ppb (at the sphagnum mat surface) and 88 ppb (at a depth of 90 cm).

Of the 29 bacterial isolates assayed for antibiotic producing activity, only 3 strains inhibited growth of test bacterial strains (Table 4). Both *Pseudomonas putida* HB11, isolated from a core depth of 55 cm, and *Paenibacillus* spp. HB26, isolated from a core depth of 20 cm, in Hamilton Pond bog core samples, inhibited growth of the test organism *B. subtilis* 168. The *Pseudomonas putida* HB11 strain also inhibited growth of *E. faecium*. *Bacillus weihenstephanensis* RP7, isolated from a core depth of 30 cm in Round Pond bog inhibited growth of *E. faecium* and *E. coli* K12 (Table 4).

TABLE 3  
Total Hg concentration of dry leaf and stem Sphagnum core samples

Sample Site	Depth (cm)	Hg concentration ( $\mu\text{g}/\text{kg}$ )	Standard error
Control 1*	N/A	59.7	$\pm 17.8$
Control 2 <sup>†</sup>	N/A	2.6	$\pm 1.1$
Round Pond Bog	1	44.7	$\pm 4.9$
	6	44.9	$\pm 3.7$
	101	17.6	$\pm 2.0$
	193	8.7	$\pm 2.0$
	Hamilton Pond Bog	1	13.1
	8	88	$\pm 1.9$
	50	34.5	$\pm 3.8$
	94	30.3	$\pm 3.0$

Depths noted represent the distance below surface in which cores were sampled. For each sample, n = 6. \*greenhouse *Sphagnum*; <sup>†</sup>*Hordeum vulgare*, barley in non-Hg soil.

TABLE 4  
Antibiosis assay of Hamilton Pond and Round Pond isolates producing antimicrobial compounds *in vitro*

Isolate	Test Species Inhibited
<i>Pseudomonas putida</i> HB11	<i>Enterococcus faecium</i> , <i>Bacillus subtilis</i> 168
<i>Paenibacillus</i> sp. HB26	<i>B. subtilis</i> 168
<i>Bacillus weihenstephanensis</i> R7	<i>E. faecium</i> , <i>E. coli</i> K12

Hg minimum inhibitory concentration (MIC) assays revealed that all metabolically active isolates were resistant to levels of at least 50  $\mu\text{M}$  HgCl<sub>2</sub> (Table 5). Two isolates of *Pseudomonas putida* (RP23 and RP25) collected from the deepest depth of the Round Pond bog core showed much higher resistance to Hg. *P. putida* RP23 grew on TSA supplemented with 100  $\mu\text{M}$  HgCl<sub>2</sub>, and *P. putida* RP25 was resistant to 250  $\mu\text{M}$  HgCl<sub>2</sub>. Regarding the endospore-forming isolates that were obtained after sphagnum sample heat treatment, resistance to Hg was not as high (Table 6). Seventeen of the 25 endospore-forming strains were inhibited by a concentration of 50  $\mu\text{M}$  HgCl<sub>2</sub>. All other isolates were inhibited by 100  $\mu\text{M}$  HgCl<sub>2</sub>.

The antibiogram showing only maximal MIC's for each antimicrobial compound tested on each isolate is illustrated in Tables 5 and 6. Regarding those strains that were immediately culturable from the sphagnum samples, termed the metabolically active isolates, multiple drug resistances were observed in each sample taken throughout the depth of the core. Bacterial resistance to antimicrobials in the  $\beta$ -lactam, aminoglycoside, cephem and folate pathway inhibitor families was prevalent in both bog core samples. Overall, the endospore-forming isolates were more susceptible to antibiotics than the metabolically active isolates; 26% of the endospore-forming strains were susceptible to all antibiotics tested, and 21% isolates were maximally resistant to only 1 antibiotic (Table 6).

Fifty-three of 54 bacterial isolates were identified using NCBI BLASTn, and the results are illustrated in Tables 5 and 6. Forty-five of the isolates generated BLASTn homologs allowing identification at the species level. Sequencing of 16S rRNA gene amplicons revealed high species diversity in each bog. *Rahnella*, *Paenibacillus*, *Pseudomonas* and *Bacillus* were the most highly represented genera of the metabolically active isolates. Eleven of the 29 metabolically active isolates were identified as *Rahnella aquatilis* and 7 were identified as *Paenibacillus* spp. As expected, all endospore-forming isolates were Gram-positive. Eleven of the 25 endospore-formers belong to the genus *Paenibacillus*; other representative genera capable of sporulation include *Bacillus*, *Lysinibacillus*, *Cohnella* and *Sporosarcina* (Table 6).

Amplification of *merA* using published primer sets produced the expected product of 288 base pairs (data not shown). Of the

TABLE 5

Characterization and antibiogram of metabolically active bacterial strains isolated from Round Pond and Hamilton Pond bogs

Isolate	Core Depth (cm)*	HgCl <sub>2</sub> ( $\mu$ M)	Maximum Antibiotic Resistance	mer A
<i>Paenibacillus xylanllyticus</i> HB22	0	100	OXA+, STR, SYN	Y
<i>Lysinibacillus sphaericus</i> HB23	0	100	CIP, STR	Y
<i>Pseudomonas migulae</i> HB24	0	100	AMP, A/S2, AXO FAZ, FOX, FUR, NIT, POD, SXT, TANS, TIM	N
<i>Rahnella aquatilis</i> HB 19	0	100	NOV, PEN, SUL, TMP	N
<i>Rahnella aquatilis</i> HB 30	20	100	NOV, PEN, SUL, TMP	Y
<i>Rahnella aquatilis</i> HB 29	20	100	NOV, PEN, SUL, TMP	Y
<i>Rahnella aquatilis</i> HB 27	20	100	NOV, PEN, SUL, TMP	Y
<i>Paenibacillus sp.</i> HB 26	20	100	AMP, AXO, CIP, OXA+, STR, SYN	Y
<i>Pseudomonas sp.</i> HBd	20	100	AMP, AZT, A/S2, FAZ, TANS, FUR, NIT, FOX, SXT, TIM, POD	Y
<i>Rahnella aquatilis</i> HB 18	35	100	NOV, PEN, SUL, TMP	Y
<i>Rahnella aquatilis</i> HB 17	35	100	NOV, PEN, SUL, TMP	Y
<i>Bacillus weihenstephanensis</i> HB 15	35	100	AMP, FAZ, OXA+, PEN	N
<i>Rahnella aquatilis</i> HB 9	55	100	NOV, PEN, SUL, TMP	Y
<i>Pseudomonas putida</i> HB 11	55	100	AMP, A/S2, FAZ, FUR, NIT, POD, SXT, TANS, TIM	Y
<i>Rahnella aquatilis</i> HBb	75	100	NOV, PEN, SUL	N
<i>Rahnella aquatilis</i> HB 5	92	100	NOV, PEN, SUL	N
<i>Rahnella aquatilis</i> HB 4	92	100	NOV, PEN, SUL	N
<i>Pseudomonas putida</i> HB 1	92	100	AMP, AZT, FAZ, FOX, FUR, NIT, POD, SXT, TANS, TIM	Y
<i>Paenibacillus sp.</i> RP1	0	100	AMP, AXO, CIP, CLI, OXA+, SYN	Y
<i>Pseudomonas putida</i> RP3	0	100	AMP, A/S2, FAZ, FEP, FOX, FUR, NIT, POD, SXT, TAZ, TIM	Y
<i>Pseudomonas putida</i> RP4	0	100	AMP, A/S2, AXO, AZT, FAZ, FEP, FOX, FUR, NIT, POD, TANS, TAZ, TIM	Y
<i>Rahnella aquatilis</i> RP5	30	100	AZT, FEP, TANS, AXO, TAZ, FUR, FOX, POD	Y
<i>Paenibacillus pabuli</i> RP8	30	100	AMP, AXO, OXA+, STR	N
<i>Bacillus weihenstephanensis</i> RP7	30	100	AMP, FAZ, OXA+, PEN, SXT	Y
<i>Paenibacillus pabuli</i> RP13	110	100	AXO, CLI, OXA+, STR, AMP, SYN	N
<i>Paenibacillus sp.</i> RP14	140	100	AXO, CLA, CLI, FRY, LZD, OXA+, PEN, STR, SYN, VAN	N
<i>Paenibacillus pabuli</i> RP16	140	100	AXO, CIP, CLA, CLI, ERY, LZD, OXA+, PEN, STR, SYN, VAN	Y
<i>Pseudomonas pabuli</i> RP23	180	>250	AMP, A/S2, AZT, FAZ, TANS, FUR, TAZ, NIT, FOX, SXT, TIM, POD	Y
<i>Pseudomonas patida</i> RP25	180	250	AMP, A/S2, AXO, AZT, FAZ, FEP, FOX, FUR, NIT, POD, TANS, TAZ	Y

Antibiotic MIC values for each isolate were determined on Sensititre<sup>®</sup> dried susceptibility panels GN2F (Gram-negative) [amikacin (AMI), ampicillin (AMP), aztreonam (AZT), cefazolin (FAZ), cefepime (FEP), cefotetan Na (TANS), ceftriaxone (AXO), ceftazidime (TAZ), ceftoxitin (FOX), cefuroxime (FUR), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IMI), gatifloxacin (GAT), meropenem (MERO), piperacillin (PIP), nitrofurantoin (NIT), piperacillin/tazobactam constant 4 (P/T4) ticarcillin / clavulanic acid constant 2 (TIM2) tobramycin (TOB), trimethoprim/Sulfamethoxazole (SXT), cefpodoxime (POD), ampicillin/sulbactam 2:1 ratio (A/S2)] and sensititre disks sulfisoxazole (SUL) 2 mg, trimethoprim (TMP) 5  $\mu$ g, piperacillin (PIP) 100  $\mu$ g, amikacin (AMK) 30  $\mu$ g, tobramycin (TOB) 30  $\mu$ g, vancomycin (VAN) 30  $\mu$ g, kanamycin (KAN) 30  $\mu$ g, novobiocin (NOV) 30  $\mu$ g, penicillin (PEN) 10 IU/IE/UI, streptomycin (STR) 10  $\mu$ g, and tetracycline (TET) 30  $\mu$ g.

\*Core depth in centimeters below surface.

TABLE 6

Characterization and antibiogram of endospore-forming bacterial strains isolated from Round Pond and Hamilton Pond bogs

Isolate	Core Depth (cm)*	HgCl <sub>2</sub> ( $\mu$ M)	Maximum Antibiotic Resistance	<i>mer A</i>
<i>Bacillus cereus</i> SH1	5	50	AMP, FAZ, OXA+, PEN	N
<i>Bacillus sp</i> SH2	5	50	AMP, FAZ, OXA+, PEN	N
<i>Paenibacillus turicensis</i> SH3	5	50	CIP	N
<i>Paenibacillus borealis</i> SH4	5	50	NMR	Y
<i>Bacillus weihenstephanensis</i> SH5	5	100	AMP, FAZ, OXA+, PEN, SXT	N
<i>Lysinibacillus fusiformis</i> SH6	5	50	AXO, CIP, CLI, OXA+	N
<i>Lysinibacillus fusiformis</i> SH7	5	100	CIP	N
<i>Bacillus sp.</i> SH8	5	50	AMP, FAZ, OXA+, PEN	N
<i>Bacillus weihenstephanensis</i> SH9	15	100	AMP, FAZ, OXA+, PEN, SXT	Y
<i>Paenibacillus pabuli</i> SH10	15	50	AXO, CIP, CLI, OXA+, STR	N
<i>Paenibacillus amylolyticus</i> SH12	15	50	AXO, CIP, CLI, OXA+, STR	N
<i>Paenibacillus glycanilyticus</i> SH11	85	50	AXO, CIP, CLI, OXA+, STR	Y
<i>Bacillus weihenstephanensis</i> SH13	85	100	AMP, FAZ, OXA+, PEN, SXT	Y
<i>Sporosarcina globispora</i> SH14	85	50	NMR	N
<i>Paenibacillus anaericanus</i> SH15	85	100	CIP	Y
<i>Cohnella panacarvi</i> SH16	95	100	NMR	N
<i>Lysinibacillus fusiformis</i> SH17	95	50	AXO, CIP, OXA+	N
<i>Paenibacillus pabuli</i> SR1	10	50	AXO, CIP, CLI, OXA+, STR	N
<i>Bacillus sp.</i> SR2	10	100	AMP, FAZ, OXA+, PEN	N
<i>Bacillus cereus</i> SR4	10	100	AMP, FAZ, OXA+, PEN	Y
<i>Paenibacillus sp.</i> SR5	10	50	STR	N
<i>Paenibacillus glebae</i> SR6	30	50	NMR	N
<i>Paenibacillus glebae</i> SR7	30	50	CLI, OXA+	N
<i>Paenibacillus pabuli</i> SR8	190	50	CLI, OXA+, STR	Y

Antibiotic MIC values for each isolate were determined on Sensititre<sup>®</sup> dried susceptibility panels GPN2F (Gram-positive) [erythromycin (ERY), clarithromycin (CLA), vancomycin (VAN), quinupristin/dalfopristin (SYN), clinadamylin (CLI), cefazolin (FAZ), tetracycline (TET), ampicillin (AMP), gentamicin (GEN), levofloxacin (LEVO), linezolid (LZD), ceftriaxone (AXO), streptomycin (STR), penicillin (PEN), rifampin (RIF), gatifloxacin (GAT), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (SXT), oxacillin + 2% NaCl (OXA+)] (Trek Diagnostic Systems, Westlake, OH) or sensititre disks sulfisoxazole (SUL) 2 mg, trimethoprim (TMP) 5  $\mu$ g, piperacillin (PIP) 100  $\mu$ g, amikacin (AMK) 30  $\mu$ g, tobramycin (TOB) 30  $\mu$ g, vancomycin (VAN) 30  $\mu$ g, kanamycin (KAN) 30  $\mu$ g, novobiocin (NOV) 30  $\mu$ g, penicillin (PEN) 10 IU/IE/UI, streptomycin (STR) 10  $\mu$ g, and tetracycline (TET) 30  $\mu$ g. # NMR: no maximal resistance observed. \*Core depth is measured in cm below surface.

29 metabolically active isolates from Round Pond bog, *merA* was detected in 73% of the isolates. The percentage of *merA*-positive isolates found at Hamilton Pond bog was comparable at 67% (Table 5). There were markedly fewer endospore-forming isolates that had detectable *merA* gene amplicons. The percentage of *merA* positive endospore-forming isolates was 28% for Round Pond bog and 29% for Hamilton Pond bog. Table 6 shows that the distribution of detectable *merA* amplicons in the sporulating sample set does not correlate with bog depth.

## DISCUSSION

Separate studies have been conducted focusing on sphagnum's sequestration properties for Hg (Lodenijs et al. 2003; Shoty et al. 2005) and the characteristics of sphagnum bogs' di-

verse indigenous bacterial species (Opelt and Berg 2004; Belova et al. 2006; Morales et al. 2006; Opelt et al. 2007). However, this study incorporates both of the above research areas in order to investigate the characteristics of the bacterial response to sequestered Hg in the sphagnum environment. We show that sphagnum bogs represent a novel system in which to study the co-selection of antibiotic and Hg resistant bacterial strains that colonize this unique environment.

The bacterial species isolated from Round Pond and Hamilton Pond bogs show high diversity. *Pseudomonas*, *Rahnella* and *Paenibacillus* are the most common genera observed in the readily culturable or metabolically active strain set (Table 5). Previous studies on sphagnum bogs have shown high population levels of *Rahnella* spp., and these species are postulated to play a role in the solubilization of phosphates (Opelt et al. 2007).

As free-living diazotrophs, *Paenibacillus* spp. fix atmospheric nitrogen that is critical for the growth of the moss and other plants indigenous to the ombrotrophic sphagnum bog system (Opelt et al. 2007).

Not unexpectedly, *Pseudomonas* spp. were prevalent at all depths of the core samples because *Pseudomonas* spp. are ubiquitous environmentally by virtue of their catabolic diversity. Previous studies on microorganisms commonly associated with sphagnum bogs have shown that these species predominantly attach to plant surfaces, and as a consequence do not migrate vertically in sphagnum bog columns (Hasebe et al. 2003). Bacterial communities living in aquatic or water-logged environments like sphagnum bogs tend to form biofilms, which enable a more efficient and protected static mode of cellular growth in comparison to planktonic cells in these environments (Davey and O'Toole 2000).

Mercury analysis in the current study revealed detectable levels of total Hg at all depths of both sphagnum cores (Table 3). The Hg ranges observed at Round Pond (8.7–44.9 ppb) and Hamilton Pond (13.1–88 ppb) bogs are consistent with total Hg concentrations found in other sphagnum bogs located within the same geographic range (Norton et al. 1997; Givélet et al. 2003; Roos-Barraclough et al. 2006). Although Hg is currently considered a common environmental contaminant, it is also a heavy metal that exists as an element in the earth's crust (Barkay et al. 2003). Because Hg is prevalent throughout the bog core samples, and is held tightly in its divalent cationic form by sphagnum substrate (Lodenijs et al. 2003; Shoty et al. 2005), sphagnum bogs represent an environment that is relatively high in biologically available Hg. Because of mercury's inherent toxicity, bacterial species indigenous to this environment must adapt to the presence of this metal as a means of survival.

Round Pond bog and Hamilton Pond bog are both ancient sphagnum environments. Radiocarbon dating (Table 2) of selected samples from these two bogs shows average annual growth of 1.2 mm/yr at Round Pond bog and 0.56 mm/yr at Hamilton Pond bog. Radiocarbon dates suggest that sphagnum moss deep below the surface of the bog is contained in an ancient environment, yet we observed that bacterial strains isolated at these depths exhibited multiple drug resistance. Dates for deposition of sphagnum at the base of cores taken from Round Pond bog and Hamilton Pond bog, 325BCE and 260AD (Table 2) respectively, are well before the application of antimicrobial chemotherapy in the 1940s. Therefore, high levels of antibiotic resistance observed in bacterial populations far below the bog surface are not a result of selection that was influenced anthropogenically, since these bacteria have not likely been exposed to high levels of manufactured antimicrobial compounds.

Many antimicrobial compounds, e.g., antibiotics, although commonly associated with use in the medical and veterinary industries, are produced as secondary metabolites by microorganisms in the environment. An antibiosis assay was performed on all readily culturable or metabolically active strains isolated from Round Pond and Hamilton bogs. Only 10% of these iso-

lates were able to produce compounds such that growth was inhibitory to the antibiosis test bacterial strains (Table 4). Studies previously conducted on bacterial communities of sphagnum bogs have found similar low incidences of antimicrobial producing bacteria (Opelt et al. 2007). A low incidence of bacteria able to produce antibiotics in the bog environments suggests that not only is the deep sphagnum substrate free of anthropogenic antibiotic influence, but it also does not contain high levels of natural antibiotics. Accordingly, bacterial isolates from deep subsurface sphagnum do not have a functional need for genetic resistances to broad classes of antibiotics because of the apparent absence of selection pressure exerted by antibiotics in this environment.

Despite the apparent absence of natural and anthropogenic antibiotic selective pressures, MDR bacteria were isolated from all depths of the sphagnum cores in both bogs (Tables 5 and 6). Broad maximal resistances to antibiotics in the  $\beta$ -lactam, aminoglycoside, cephem and folate pathway inhibitor families were observed. *Pseudomonas* spp. demonstrated the greatest antibiotic resistance with *P. putida* strains RP 4 and RP 5 possessing maximal resistance to 13 of the 23 tested antibiotics. Of the gram-positive isolates, *Paenibacillus* sp. RP 16 exhibited the greatest antibiotic resistance to 11 of the 19 tested antibiotics (Table 5). Only 3 gram-positive metabolically active isolates had resistance to the fluorquinolone, ciprofloxacin (Table 5). However, endospore-forming isolates possessed ciprofloxacin resistance (Table 6). In addition, *Paenibacillus* spp. RP14 and RP 16 show resistance to the macrolides erythromycin and clarithromycin as well as the glycopeptide vancomycin (Table 5). When many diverse species of bacteria isolated from the same environment have similar multiple drug resistance phenotypes, resistance genes are commonly found to be encoded on mobile genetic elements such as plasmids and transposable elements (Guerra et al. 2001; Rowe-Magnus and Mazel 2002; Tennstedt et al. 2003).

Also located on plasmids and certain transposable elements are genes for Hg resistance, including the structural genes located in the *mer* operon (Hobman et al. 2002; Barkay et al. 2003). Interestingly, the metabolically active bacterial strains exhibited high levels of Hg resistance, suggesting that ambient concentrations of Hg in the sphagnum bogs were at sufficiently high levels to function as a selective agent in this environment (Table 5). The Hg-resistance genotype, as detected by the presence of *merA* amplicons, was observed in the majority of the metabolically active bacterial isolates (73% in Round Pond bog and 67% in Hamilton Pond bog). Though it is likely the remaining metabolically active isolates also carry *merA* based on phenotypic observation of mercury resistance, the high diversity of *merA* confounds efforts to design PCR primer sets to universally amplify the gene (Barkay et al. 2003). Bacterial strains possessing the *mer* operon were distributed evenly at all depths of both cores, which suggests that biologically available Hg concentrations throughout the sphagnum cores were sufficient to influence selection of Hg-resistant bacterial strains.

The observation of the presence of *merA* amplicons in the majority of the isolates from Round Pond and Hamilton Pond bogs, both of which are environments with apparently low levels of antibiotics, provides support for the co-selection hypothesis. In these locations sphagnum-sequestered Hg is selecting for bacterial strains possessing the *mer* operon (McIntosh et al. 2008). Because of the mechanism of co-resistance in which *mer* operons and antibiotic resistance gene cassettes are linked proximally on transposable elements and plasmids (Mazel et al. 2000; McArthur and Tuckfield 2000; Nemergut et al. 2004), Hg in these bog environments would also provide selection pressure for bacterial strains with multiple drug resistance determinants.

The endospore forming bacterial isolates provide additional support for the co-selection of Hg and antibiotic resistant strains in the sphagnum bog environment. Certain Gram-positive bacterial species have the ability to develop into a dormant state with the formation of endospores in highly stressed environments or in conditions not conducive to vegetative growth (Driks 2002). The endospore forming strains isolated from both Hamilton Pond and Round Pond bogs show generally low resistances to Hg, with the majority of isolates having MIC's of 50  $\mu\text{M}$   $\text{HgCl}_2$  (Table 6). The frequency of *merA* amplicons detected in sporulating strains (29% of Hamilton Pond bog isolates and 28% of Round Pond bog isolates) in comparison to the readily culturable and metabolically-active isolates was markedly less.

Correspondingly, overall antibiotic resistance levels were also lower in the endospore-forming isolates (Table 6). Four isolates showed no maximal resistance to any of the clinical antibiotics tested, and four were maximally resistant to only one antibiotic. These findings are in support of the co-selection of strains possessing the Hg and antibiotic resistance phenotype, because both types of resistance were observed to be at reduced levels among the sporulating isolates. Additionally, the low abundance of *merA* positive isolates suggests that Hg was acting as the selective agent in the bogs and could be inducing sporulation in these strains.

## CONCLUSIONS

This is the first report describing the sphagnum bog environment as a unique natural system in which to study Hg's influence on the co-selection of indigenous bacterial strains that possess both Hg and multiple drug resistances. *merA* genes and the multiple drug resistance phenotype were found in an environment apparently devoid of antibiotics from anthropogenic and natural sources. The multiple antibiotic resistances observed in these indigenous bacterial strains was most likely due to pressure exerted by an indirect selective agent such as a metal. In the sphagnum bog environment, mercuric ion is sequestered by the sphagnum substrate. This sequestration applies a selective pressure on bacterial populations, and because of co-resistance of these genetic determinants, also selects for multiply drug-resistant bacteria. Hence, the sphagnum bog environment represents a natural habitat in which ambient Hg levels are sufficient

to co-select indigenous bacterial strains possessing Hg- and multiple drug resistance phenotypes.

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