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Pervasiveness of UVC_{254}-resistant *Geobacillus* strains in extreme environments

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Abstract

We have characterized a broad collection of extremophilic bacterial isolates from a deep subsurface mine, compost dumping sites, and several hot spring ecosystems. Spore-forming strains isolated from these environments comprised both obligate thermophiles/thermotolerant species (growing at > 55 °C; 240 strains) and mesophiles (growing at 15 to 40 °C; 12 strains). An overwhelming abundance of *Geobacillus* (81.3%) and *Bacillus* (18.3%) species was observed among the tested isolates. 16S rRNA sequence analysis documented the presence of 24 species among these isolates, but the 16S rRNA gene was shown to possess insufficient resolution to reliably discern *Geobacillus* phylogeny. *gyrB*-based phylogenetic analyses of nine strains revealed the presence of six known *Geobacillus* and one novel species. Multilocus sequence typing analyses based on seven different housekeeping genes deduced from whole genome sequencing of nine strains revealed the presence of three novel *Geobacillus* species. The vegetative cells of 41 *Geobacillus* strains were exposed to UVC_{254}, and most (34 strains) survived 120 J/m^2, while seven strains survived 300 J/m^2, and cells of only one *Geobacillus* strain isolated from a compost facility survived 600 J/m^2. Additionally, the UVC_{254} inactivation kinetics of spores from four *Geobacillus* strains isolated from three distinct geographical regions were evaluated and compared to that of a spacecraft assembly facility (SAF) clean room *Geobacillus* strain. The purified spores of the thermophilic SAF strain exhibited resistance to 2000 J/m^2, whereas spores of two environmental *Geobacillus* strains showed resistance to 1000 J/m^2. This study is the first to investigate UV resistance of environmental, obligately thermophilic *Geobacillus* strains, and also lays the foundation for advanced understanding of necessary sterilization protocols practiced in food, medical, pharmaceutical, and aerospace industries.

Keywords UV resistance · Extremophile · Ecology · *Geobacillus* · *gyrB*

Introduction

Despite ongoing advancements in antimicrobial technologies, human efforts to eradicate microbial life in controlled environments are constantly thwarted by terrestrial bacteria. Imperfect sterilization techniques negatively affect a wide array of industries (e.g., textile, food, medical, aerospace) and their associated consumers (Favero et al. 1966; Frederiksen et al. 2006; Heyndrickx 2011; Rylander and Lundholm 1978; Somers et al. 2001; Stenfors Arnesen et al. 2008; WHO 2014). In the aerospace industry, unchecked microorganisms could have uniquely disastrous effects. Specifically, terrestrial spacecraft could contaminate other cosmic bodies with Earthly microbes if the space-bound vehicles are not sufficiently sanitized before takeoff (forward contamination). Such contamination could disrupt the natural state of our solar system and potentially confound the results of future life-detection missions (Favero et al. 1966).

In the spirit of preserving the solar system in its natural state, the National Aeronautics and Space Administration (NASA) has developed precautions to minimize harmful contamination through the cosmos. To minimize bioburden onboard flight hardware, construction takes place in oligotrophic clean room facilities with filtered air, controlled humidity, and

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routine antimicrobial sanitation regimens (Crawford 2005; Newcombe et al. 2005). In preparation for delicate life-detection missions, NASA also has a history of chemically sterilizing space-bound equipment, then baking it with dry heat at 110 °C for an extended period (30 h in the case of the Viking landers) (Board 1992; Rummel 2001). When a spacecraft departs from Earth, the naturally biocidal conditions in outer space (e.g., extreme temperatures, vacuum, exposure to ionizing radiation, and heavy high-energy (HZE) particles) provide additional possible biocidal conditions to kill organisms on exposed surfaces. UV irradiation is a particularly important sterilizing agent in this process. It has been demonstrated as the most deleterious factor of the outer space environment, with UVC (200 to 280 nm) comprising the biocidal wavelength (Horneck et al. 2012; Horneck et al. 2001; Lindberg and Horneck 1991; Newcombe et al. 2005; Nicholson et al. 2005).

Microbes inside NASA clean rooms are known to display exceptional UV resistance, although they are not typically exposed to irradiation beyond normal lighting (Crawford 2005). The robustness of these organisms is largely attributed to endospore formation. That is, certain species of bacteria will differentiate from vegetative cells to metabolically dormant spores when faced with adverse conditions such as low nutrient abundance (as in clean room facilities) (Newcombe et al. 2005; Nicholson et al. 2000; Setlow 1995). These spores are inherently resistant to an assortment of sterilization techniques including chemical oxidizing agents, extreme desiccation, wet and dry heat, and UV and gamma irradiation, and they have been acknowledged as the "hardiest known form of life on Earth" (Nicholson et al. 2000).

To proactively avoid forward contamination and increase understanding of the molecular basis for UV resistance, it is crucial to seek out microbes that are likely to survive the antagonisms of interplanetary travel such as UV irradiation and use them as model specimen for developing advanced sanitation techniques. Previous studies in this area have centered on laboratory strains of *Bacillus subtilis* (Nicholson and Galeano 2003), as well as bacterial spores isolated directly from spacecraft assembly facilities (SAF) (Newcombe et al. 2005). This limited selection of test subjects leaves many terrestrial microbes unexplored, especially spore-forming clostridia and geobacilli, and their ability to survive and adapt to extraterrestrial conditions unknown.

The objective of the present study is to phylogenetically characterize thermophilic bacterial strains and their resistance to UVC_{254}. A large collection of extremophilic, spore-forming environmental isolates (252 strains) collected over 10+ years from eight distinct locations (ranging from deep subsurface to hot springs) scattered throughout the USA and India have been tested. Thermophilic *Geobacillus* and mesophilic *Bacillus* species were found to dominate each sampled location. Endospore-forming obligate thermophiles like *Geobacillus* are valuable UV resistance test subjects because endospores from thermophiles are considered to have superior wet heat resistance compared to their mesophilic counterparts (Condon et al. 1992; Gerhardt and Marquis 1989; Nicholson et al. 2000). The adaptations affording this hardiness are hypothesized to not only defend against wet heat but also increase UVC_{254} resistance.

Materials and methods

Sampling sites, sample collection, and isolation of thermotolerant strains

Sample sites

Water and soil samples were collected from various natural and anthropogenic sites in the United States of America (USA) and India (Fig. 1). The anthropogenic sites (four locations) are all located in the USA, and they include (a) Sanford Underground Research Facility (SURF), Lead, SD; (b) Washington State University Compost Facility (WSUCF), Pullman, WA; (c) Rapid City Landfill in Rapid City, SD; and (4) Edgemont disposal site, Edgemont, SD. The naturally occurring sites (three locations) targeted for sampling all reside within India, and they are (a) Manikaran Hot Spring, Manikaran, Himachal Pradesh; (b) Vashisht Hot Spring, Manali, Himachal Pradesh; and (c) Sohna Hot Spring, Gurgaon, Haryana. One oligotrophic SAF clean room of the Johnson Space Center (JSC) situated in Houston, TX, was also incorporated in this study to allow comparison between the environmental isolates (252 strains) and one isolate evolved in the spacecraft-associated environment (La Duc et al. 2007). These sites were chosen due to their extremophilic nature such as deep subsurface, compost site (aerobic), low nutrients, and hot geysers. Specific characteristics of each sampling location including latitude and longitude of the sites are outlined in Table 1. The temperature and pH of the sampling sites were measured, when possible, with the help of a handheld temperature and pH probe meter IQ170 (IQ Scientific, CA, USA).

SURF

SURF was formerly known as the Homestake Gold Mine. Throughout the nineteenth and twentieth centuries, it was the most active gold mine in the USA with the largest and deepest gold reserves. More recently, it has been repurposed as a unique underground research lab for physics, microbiology, astrobiology, geology, and geomicrobiology, among other fields (Heise 2015). In addition to the geothermal gradient (a universal phenomenon), the heat profile within SURF is influenced by autocompression and expansion of surface air.

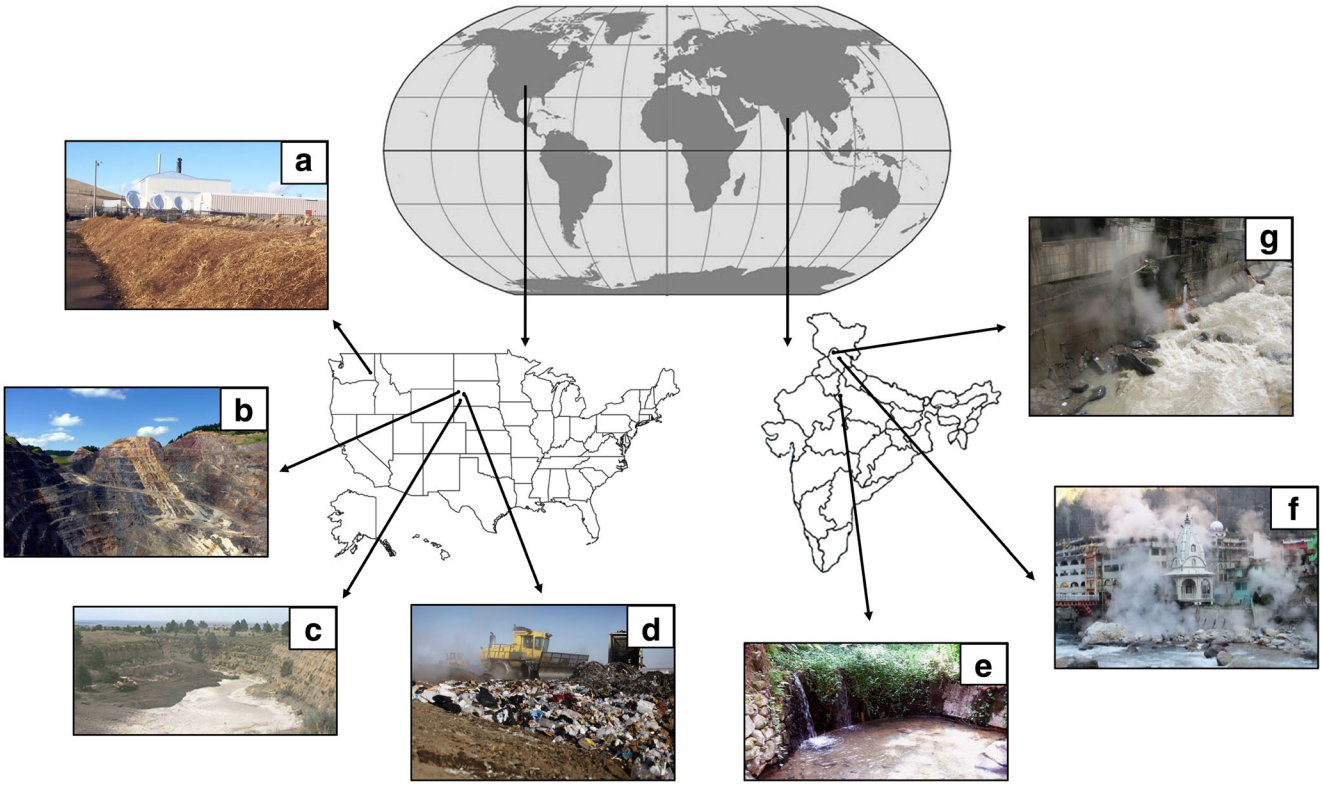

Fig. 1 Extreme environmental sampling sites studied. **a** Washington State University Compost Facility, Pullman, WA. **b** Sanford Underground Research Facility, Lead, SD. **c** Edgemont disposal site, Edgemont, SD. **d** Rapid City Landfill, Rapid City, SD. **e** Sohna Hot Spring, Gurgaon, Haryana, India. **f** Manikaran Hot Spring, Manikaran, Himachal Pradesh, India. **g** Vashisht Hot Spring, Manali, Himachal Pradesh, India

The surface air is autocompressed as it descends into the mine, resulting in conversion of the gas's potential energy to thermal energy. Then, the high temperature in the mine due to the geothermal gradient causes the air to expand, increasing the intermolecular separation and internal energy of the gas. Overall, this process comprises an adiabatic expansion, which raises the temperature as per the Joule-Thomson effect (Maurya et al. 2015). Samples were collected from SURF at a depth of 1478.28 m, where the recorded temperature was 55 °C.

WSUCF

WSUCF started its operation in 1994 to process organic waste generated from the Washington State University campus. It was the first compost facility in a university campus, and today it composts approximately 25,000 cubic yards annually. The output of this operation serves as a soil amendment and source of nutrients.

Rapid City Landfill

The Rapid City Landfill is a composting/landfilling facility. Composting is an aerobic process, resulting in CO_2 production. Landfilling, on the other hand, is an anaerobic process, causing CH_4 emission (Lindeberg and Radiwon 2017). This distinction between the processes should not be overlooked, as aerobic vs. anaerobic conditions hold significant influence over the microbial ecology in a given environment. In addition, the production of CO_2 in a compost pile could cause an increase in acidity due to carbonic acid formation. A shift in pH would further manipulate the microbial ecology. However, the bacterial samples collected for the present study originated from windrows at Rapid City Landfill, which are designed to facilitate regular aeration. Thus, calling these samples "landfill" isolates is actually a misnomer because the windrows function aerobically (as composting systems) and hence the microbial ecology is expected to facilitate many aerobes.

Edgemont disposal site

The Edgemont disposal site is approximately 85 miles from Rapid City. It was built to remediate a uranium mining site which was decommissioned in 1989 (Department of Energy 2013). The disposal site is built in a basin at the head of an ephemeral tributary to the Cheyenne River, holding four million tons (three million cubic yards) of contaminated material with a total activity of 527 curies of radium-226 in a specially designed cell to prevent its leakage into the water and land (Department of Energy 2013).

Table 1 Locations and characteristics of sampling sites

Name	Location	Coordinates	Local Characteristics	Sample Description	pH	Temp. (°C)	Sampling depth	Number of strains characterized	Reference
Sanford Underground Research Facility (SURF)	Yates Shaft, Lead, SD, USA	44.35° N, 103.76° W	Iron-rich metasedimentary deposit from the Paleoproterozoic period, with circulating groundwater	Soil and water	7.5	55	1480 m	125	Osburn et al. (2014)
Washington State University Compost Facility (WSUCF)	Pullman, WA, USA	46.73° N, 117.15° W	Compost piles of organic waste generated at Washington State University	Soil	9	50	Dug into compost piles until they were warm	57	McGee (2015)
Rapid City Landfill	Rapid City, SD, USA	44.03° N, 103.19° W	Standard open air windrows	Soil	Not measured	70	40 cm	20	This study
Edgemont disposal site	Edgemont, SD, USA	43.27° N, 103.79° W	Contaminated remnants from decommissioned uranium mine (total activity of 527 curies of radium-226)	Soil	6.9	28	Surface of soil	10	Department of Energy (2013)
Manikaran Hot Spring	Manikaran, Himachal Pradesh, India	32.03° N, 77.35° E	High salinity, sulfur, and dissolved hydrogen contents	Water	8.2	90	Surface of water	29	Cinti et al. (2009)
Vashisht Hot Spring	Manali, Himachal Pradesh, India	32.27° N, 77.19° E	Water emerges from granitic deposits	Water	8.8	55	Surface of water	8	Naresh, Ankusha et al. (2013)
Sohna Hot Spring	Gurgaon, Haryana, India	28.24° N, 77.03° E	High salinity, sulfur, and dissolved hydrogen contents	Water	9	52	Surface of water	3	Cinti et al. (2009)
Johnson Space Center (JSC)	Houston, TX, USA	29.55° N, 95.09° W	Outside the entrance of a class 5 K garment changing room	Wipe		Not controlled Floor outside garment changing room	Surface of floor	1	La Duc et al. (2007)

Indian hot springs

The samples collected from the Vashisht, Manikaran, and Sohna Hot Springs in India span a total distance of ~ 1000 km of water flow. The Vashisht Hot Spring is located elegantly above the Beas River near Manali, Himachal Pradesh. Here, water from the unconfined shallow sources and confined/semiconfined aquifers in the Vashisht valley descend deep down along the major structural faults and return to the surface after absorbing heat from the natural (and anomalously extreme; 96 °C) geothermal gradient (Cinti et al. 2009; Shanker 1988). The Manikaran Hot Spring, located in Himachal Pradesh, India, is situated in the Parbati River of Parbati valley, at an altitude of 1.76 km. It has existed since prehistoric times (Kumar et al. 2014), and its temperature is actually high enough for locals to cook vegetables and other foodstuffs (96 °C). Steam is visible on the terrace of the Parbati River in Manikaran due to supposed travertine deposits along the vent (Bhatia et al. 2015). The Sohna Hot Spring comes under the Sohna Fault Zone, which runs from Sohna to the Delhi Ridge, west of the town of New Delhi (Sharma et al. 2003). The spring emerges at the junction of alluvial deposits with Alwar quartzite. High H_2 content is observed as the result of a reaction between water and Si, and Si-O radicals (Cinti et al. 2009). Like the Vashisht Hot Spring, the cold water of Sohna flows to a deep descent under the effect of gravity, is heated (45 to 52 °C) by an anomalous geothermal gradient, and moves upward toward the surface.

Sample collection

Soil and water samples were collected aseptically from the aforementioned sites in 50-mL falcon tubes. Using sterile scoopers, soil samples were collected down to a 10-cm depth from the soil surface at SURF and the Edgemont disposal site. The Rapid City Landfill samples were collected from a 40-cm depth. At WSUCF, the compost piles were dug to a depth where the pile was warm for sampling. All of these soil samples were packed in anaerobic gas jars and transported to the South Dakota School of Mines and Technology (SDSMT) laboratory on ice packs and kept at − 80 °C until further analysis. The water samples from Indian hot springs were aerobically transported on ice packs to the SDSMT lab. Collection, transfer, and processing of samples were carried out aseptically using standard microbiological techniques (Rastogi et al. 2010b).

Sample processing

Microbial species with heavy metal resistance (Rastogi et al. 2009) were isolated from the Edgemont disposal site, while species catalyzing lignocellulosic biomass hydrolysis (Rastogi et al. 2009) were isolated from SURF, WSUCF, Rapid City Landfill, Manikaran Hot Spring, Vashisht Hot Spring, and Sohna Hot Spring. Isolating the strains with these characteristics involved adding 10 g of soil samples or 10 mL of water to 95 or 90 mL of sterile water, respectively, to make a suspension. Serial dilutions of the suspensions were prepared to make a final dilution of 10^{-5}. Aliquots of 100 μL of the suspension from dilutions 10^{-3}, 10^{-4}, and 10^{-5} were spread plated on Luria Bertani (LB) agar plates and kept in an incubator at either 37 or 60 °C (depending on the sample origin) for 24 h. For isolation of aerobic thermophilic and thermotolerant bacteria, single colonies were picked with a sterile toothpick and added to 100 mL of LB broth in 500-mL Erlenmeyer flasks. The flasks were incubated at 60 °C and 200 rpm. Sample collection and processing to grow obligate thermophiles from the JSC clean room surfaces are described elsewhere (La Duc et al. 2007).

Molecular identification of spore-forming strains

Purification and 16S rDNA amplification of spore-forming bacterial strains

The archived microbial isolates were thawed from − 80 °C storage and streaked on tryptic soy agar (TSA) plates (Becton Dickinson and Co.). Depending upon the sampling origin, all 252 environmental strains were tested for their growth at either 55 °C (thermophilic/thermotolerant) or 37 °C (mesophilic). Single isolated colonies were selected for further experiments and archived in 20% glycerol suspensions (stored at − 80 °C). Prior to identifying the isolates, whole cells from cultures in the early log phase were used to inoculate polymerase chain reaction (PCR). When PCR failed, cells were subjected to freeze-thaw cycle, or DNA was extracted using an automated DNA extraction instrument (Mayer et al. 2016).

PCR amplification was performed with universal bacterial primers 27F and 1492R to amplify the near-complete 16S rRNA gene sequence for all 252 strains (Lane 1991). Thermal cycles for PCR were 94 °C initial denaturation for 10 min, 94 °C denaturation for 1 min, 55 °C annealing for 1 min, 72 °C extension for 1 min, for 34 cycles, 72 °C final extension for 10 min, and final hold at 4 °C. Small aliquots of the PCR products were loaded on agarose gel to verify the presence of 1.5-kbp fragments, and the remaining PCR products were cleaned up using an exonuclease I and Antarctic phosphatase digest (New England Biolabs).

The 16S rRNA PCR products were sequenced via single primer extension with universal bacterial primers 27F and 1492R (Macrogen, Rockville, MD). Sequencing chromatograms were manually inspected and trimmed using FinchTV (Smita et al. 2013). The sequences from both primer extensions were manually stitched together, thus yielding near-complete 16S rRNA gene sequences. When needed, in

addition to 27F and 1492R, universal primer 1100R was also used to fill the gap of the 16S rRNA gene sequences. With this method, the V4 hypervariable region (1100R sequences) of the 16S rRNA gene is sequenced, which has been shown to yield approximately equivalent species richness estimates as compared to sequencing the near-complete 16S rDNA (Youssef et al. 2009). FinchTV was used for manual trimming and quality control of these partial sequences. The 16S sequences obtained for all environmental isolates have been archived in the GenBank database (accession no. MF964944-MF965195).

Whole genome sequencing

To overcome possible species-level ambiguities associated with 16S rRNA gene sequencing, additional molecular techniques were used to validate or debunk the 16S-based identities. Nine environmental strains were selected for whole genome sequencing (WGS) analyses based upon their superior UV resistance (> 120 J/m^2 for vegetative cells; Table 2), distinct phylogenetic affiliations, and obligately thermophilic growth requirements. Seven of the selected strains belong to separate species isolated from the SURF location. One strain from WSUCF, as well as one from the Manikaran Hot Spring, were also included. Strains from the other environmental locations did not exhibit superior UV resistance (data not shown) and were therefore excluded. WGS of the JSC clean room isolate was also carried out and compared with WGS of the nine environmental strains (making a total of 10 strains subjected to WGS). All 10 of these strains were tested for their ability to grow at both 37 and 55 °C prior to WGS, and all of them were verified to grow only at 55 °C, indicating obligate thermophiles. Pure bacterial strains were cultured in 5 mL sterile tryptic soy broth (TSB) (Becton Dickinson and Co.) in 50-mL Falcon tubes, then allowed to grow overnight before extracting genomic DNA using an automated DNA extraction system (Maxwell 16; Tissue LEV Total RNA Purification kit; Promega). The final elution volume of genomic nucleic acid was 50 μL. A small aliquot was used to quantify DNA concentration with Quant-iT Assays and Qubit Fluorometer (Invitrogen) before proceeding for WGS. The WGS was carried out at Macrogen using an Illumina HiSeq2500 instrument with a paired-end module. The NGS QC Toolkit version 2.3 (Patel and Jain 2012) was used to filter the data for high-quality vector- and adaptor-free reads for genome assembly. High-quality vector-filtered reads were assembled with MaSuRCA (Zimin et al. 2013) under the default parameters predicted by the assembler for each genome, and annotated with the help of Rapid Annotations using Subsystems Technology (RAST) (Overbeek et al. 2014). The resultant WGS were deposited in the GenBank database (accession nos. NADP00000000-NADS00000000, NAGD00000000-NAGF00000000, and PIJF00000000-PIJH00000000).

gyrB amplification and sequencing

Additionally, for the same 10 strains selected for WGS, the *gyrB* gene was amplified as described previously (Yamamoto and Harayama 1995). The presence of *gyrB* amplicons (approx. 1.2 kb) was verified via 1% agarose gel electrophoresis. An exonuclease I and Antarctic phosphatase digest (New England Biolabs) was used to clean up the PCR products before performing single primer extension DNA sequencing with UP-1 and UP-2R (Macrogen, Rockville, MD). FinchTV was used to manually audit the quality of the sequences and stitch them together. Each of these gyrB sequences can be found in the GenBank database (accession nos. NADP00000000.1-NADS00000000.1, NAGD00000000.1-NAGF00000000.1, and PIJF00000000.1-PIJH00000000.1).

Phylogenetic analysis

Each gene sequence was subjected to similarity searches using the BLAST(N) program of the National Center of Biotechnology Information (NCBI) search engine. The searches were limited to sequences from type material, and a conservative cutoff of 98.65% similarity was used to minimize false positives in detecting candidates for novel taxa via 16S rRNA sequence analysis (Kim et al. 2014). For *gyrB* sequence analysis, 95% was considered the cutoff (Yamamoto and Harayama 1995).

Multilocus sequence typing

For all 10 strains with acquired whole genome sequences, multilocus sequence typing (MLST) analysis was carried out as described previously (Larsen et al. 2012). The *Bacillus* MLST scheme employed here uses seven housekeeping genes: *glpF* (glycerol uptake facilitator protein), *gmk* (guanylate kinase, putative), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (phosphoribosylaminoimidazolecarboxamide), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase) (Priest et al. 2004).

Digital DNA-DNA hybridization and average nucleotide index

Digital DNA-DNA hybridization (dDDH) was performed and pairwise average nucleotide index (ANI) was calculated for the WGS of the selected 10 strains. Pairwise ANI was calculated using an established algorithm (Goris et al. 2007). The dDDH analysis was performed using the Genome-to-Genome Distance Calculator 2.0 (GGDC 2.0) (Meier-Kolthoff et al. 2013) available on the website http://ggdc.dsmz.de/. Briefly, the genome sequences in FASTA format were submitted to GGDC 2.0 along with the sequences in FASTA format for the

Table 2 UV resistance characteristics of vegetative cells

Bacterial species[a]	Total strains	Number of strains that exhibited growth after exposure to UVC_{254}[b]		
		120 J/m^2	300 J/m^2	600 J/m^2
Spore-forming vegetative cells				
G. caldoxylosilyticus	1	1	1	
G. jurassicus	1	1		
G. kaustophilus	12	12	3	
G. stearothermophilus	4	4	(1)	1
G. subterraneus subsp. *aromaticivorans*	2	2	(1)	
G. thermocatenulatus	11	7 (3)	1	
G. thermodenitrificans subsp. *thermodenitrificans*	2	2		
G. thermodenitrificans subsp. *calidus*	5	3 (2)		
G. toebii	1	(1)		
G. zalihae	2	1 (1)		

[a] The species were determined via 16S rRNA gene sequence analysis

[b] Numbers indicated in parentheses represent strains that grew exclusively in liquid media

Geobacillus type strains: The results were obtained by comparing query genomes (10 environmental isolates) with reference genomes (21 validly described *Geobacillus* species) to calculate intergenomic distances. The results from the recommended calculation formula were chosen as final.

UV irradiation and microbial lethality

Prior to each microbial lethality assessment, pure cultures of the test strains were incubated overnight in TSB (Becton Dickinson and Co.) at 55 °C, under aerobic conditions. After UV exposure, the cell suspensions were incubated under the same conditions to assess survivability.

UVC_{254} resistance of vegetative cells

For 41 purified environmental strains, an appropriate aliquot (100 μL) of the overnight culture was diluted with 10 mL sterile phosphate buffered saline (PBS) to yield a final cell concentration of approximately $< 10^6$ cells/mL. In order to screen a large number of strains (41 isolates), we attempted to qualitatively test overnight cultures which might include both vegetative cells and matured spores. When these cultures were microscopically observed, no mature spores were noticed. The diluted culture was exposed to 2, 5, and/or 10 min of UVC_{254} at a dose rate of 100 μW/cm^2 while being continuously stirred in a glass petri dish with the lid open. At each time interval, aliquots of the UV-exposed cell suspension were dispensed onto one TSA plate and separately into TSB. The resultant TSA and TSB media inoculated with cultures were incubated overnight, and the growth of each strain was qualitatively recorded (Newcombe et al. 2005).

UVC_{254} resistance of spores

Vegetative cells of highly UV-resistant (> 300 J/m^2; 254 nm), obligately thermophilic (verified to grow only at 55 °C and not at 37 °C) strains isolated from SURF (two isolates), WSUCF (one isolate), and Manikaran Hot Spring (one isolate) were selected for spore purification and inactivation kinetics quantification. For comparison purposes, one strain isolated from JSC was also subjected to the same analysis. Overnight cultures (1 mL) were spread in duplicate onto petri dishes containing sterile Difco sporulation medium. The plates were allowed to sit for about 1 h at room temperature, then they were incubated at 55 °C for 6 days. At different time points, a loopful of culture grown on the sporulation medium was suspended in sterile water, and the cultures were inspected via phase-contrast microscopy to verify the presence of spores. If spores comprised > 99% of the observed culture, all materials grown on the sporulation medium were harvested in sterile water and purified via incubation with lysozyme and salt washes to eliminate cell debris (Kempf et al. 2005). After purification, the spores were again viewed under the microscope to ensure no vegetative cells remained and cell debris had been removed (Kempf et al. 2005). The density of the pure spores was quantified on TSA, then diluted to approximately $< 10^6$ spores/mL and used for UVC_{254} characterization. The physical setup for UVC_{254} exposure, including dose rate, did not differ from that described for vegetative cell characterization. Aliquots were taken from the UV-exposed spore samples after cumulative doses of 25, 50, 100, 200, 500, 1000, and 2000 J/m^2. These aliquots were appropriately diluted in sterile PBS and plated on TSA to measure colony counts at each time interval. Based on the colony counts, the inactivation kinetics of each strain was quantified for each strain.

Results

A total of 252 environmental bacterial isolates collected from extreme locations throughout the USA and India, as well as one JSC isolate, were analyzed in this study. Every isolate was grown on TSA at pH 7.0. Strains originating from environments with elevated temperatures (240 strains) were incubated at 55 °C, while those from mesophilic environments were incubated at 37 °C.

With the above cultivation scheme, strains growing at 55 °C were demonstrated to be thermotolerant, but narrowing in on obligately thermophilic strains with potentially interesting spore characteristics required additional testing. Therefore, 19 strains were semirandomly selected for assessment of their ability to grow at both 37 and 55 °C. Three criteria were factored into the selection process. First, the species-level identity (according to 16S rRNA identity) allowed us to select at least one strain from each of the 10 most frequently encountered environmental *Geobacillus* species. The second consideration was the geographical origin of the isolates. When possible, two strains belonging to each of the 10 most common *Geobacillus* species were selected—one originating from SURF, and the other originating from an Indian Hot Spring. For several of the *Geobacillus* species, multiple strains were isolated from each environment. In these situations, the third criterion dictated that the strain with the most UV-resistant vegetative cells was selected. For comparison, the JSC isolate *G. thermodenitrificans* T9a was also included in this selection of 19 strains. All 19 strains grew only at 55 °C and lacked growth at 37 °C; hence, they were confirmed as obligate thermophiles. Among 19 thermophiles, 10 strains were further selected and characterized for WGS, *gyrB*, spore purification, and inactivation kinetics under UVC_{254} bombardment.

Figure 2 provides a complete breakdown of all 252 strains (excluding the JSC strain) identified using 16S rRNA gene sequencing from each geographical location. The abundance of thermophilic/thermotolerant (Fig. 2a) and mesophilic species (Fig. 2b) is shown. According to 16S rRNA gene sequence analysis of all 252 strains, 24 different species and seven genera were represented (Fig. 2). Among various genera identified, *Geobacillus* species were discovered to be the most abundant, as 205 of the 252 (~ 81.3%) total isolates identified were members of this genus and the rest were of *Bacillus* or closely related species. The only non-*Bacillus* isolate was *Isoptericola hypogeus*, with only a single isolate of this species encountered. In contrast to all of the rod-shaped, endospore-forming *Firmicutes* found in this study, *I. hypogeus* is a coccoid and non-spore-forming actinobacterium. Considering 98.65% 16S rRNA gene sequence similarity to be the cutoff for distinct species (Kim et al. 2014), these results indicate each environmental isolate is indistinguishable from some previously identified species. However, because of the conservative 98.65% similarity cutoff value, it is worth noting that some of the isolates tentatively identified may actually represent novel species that are undetectable by 16S alone. More studies are warranted.

Geobacillus in natural ecosystems

All three natural sample sites yielded only thermophiles and thermotolerant organisms. Culture-based analysis of the samples from Manikaran Hot Spring resulted in the isolation of 29 bacterial strains. The isolates are all classified as *Firmicutes* and belong to the genus *Geobacillus* or *Bacillus*. Listed in descending order of abundance, the species obtained from this site were *B. licheniformis*, "*G. zalihae*," *G. kaustophilus*, *G. stearothermophilus*, *Bacillus glycinifermentans*, *G. subterraneus* subsp. *aromaticivorans*, and *G. vulcani*. Note "*G. zalihae*" is not yet a validly described species, so its name is placed inside quotation marks. Also, it is important to acknowledge the 16S rRNA gene sequence profiles of some strains isolated from Manikaran Hot Spring were phylogenetically affiliated to known mesophiles, *B. licheniformis* and *B. glycinifermentans*. Since these environmental strains were not tested for their growth at 37 °C and are not classified as *Geobacillus* species, they may be thermotolerant mesophiles and not obligate thermophiles. The eight Gram-positive isolates obtained from the Vashisht Hot Spring were similar to those from the Manikaran Hot Spring in that they were characterized as members of the phylum *Firmicutes* and genus *Geobacillus.* These include the species "*G. zalihae*," *G. vulcani*, *G. thermocatenulatus*, and *G. stearothermophilus,* (listed in descending order of abundance; Fig. 2a). *Firmicutes* also dominated the Sohna Hot Spring, as the three strains isolated there were identified as "*G. zalihae*" (two isolates) and *G. vulcani* (one isolate).

Geobacillus in anthropogenic ecosystems

The four anthropogenic sites contained not only thermophilic/thermotolerant species, but also mesophiles. The largest number of bacterial isolates (123 strains) originated from the Sanford Underground Research Facility (SURF), including both thermophilic/thermotolerant species and mesophiles. Listed in descending order of prevalence, the isolated species capable of growing at 55 °C were identified as "*G. zalihae*," *G. thermocatenulatus*, *G. stearothermophilus*, *Bacillus licheniformis*, *G. kaustophilus*, *G. thermodenitrificans* subsp. *thermodenitrificans*, *G. thermodenitrificans* subsp. *calidus*, *G. caldoxylosilyticus*, *G. subterraneus* subsp. *aromaticivorans*, *Aeribacillus pallidus*, *G. vulcani*, *Bacillus glycinifermentans*, *Brevibacillus formosus*,

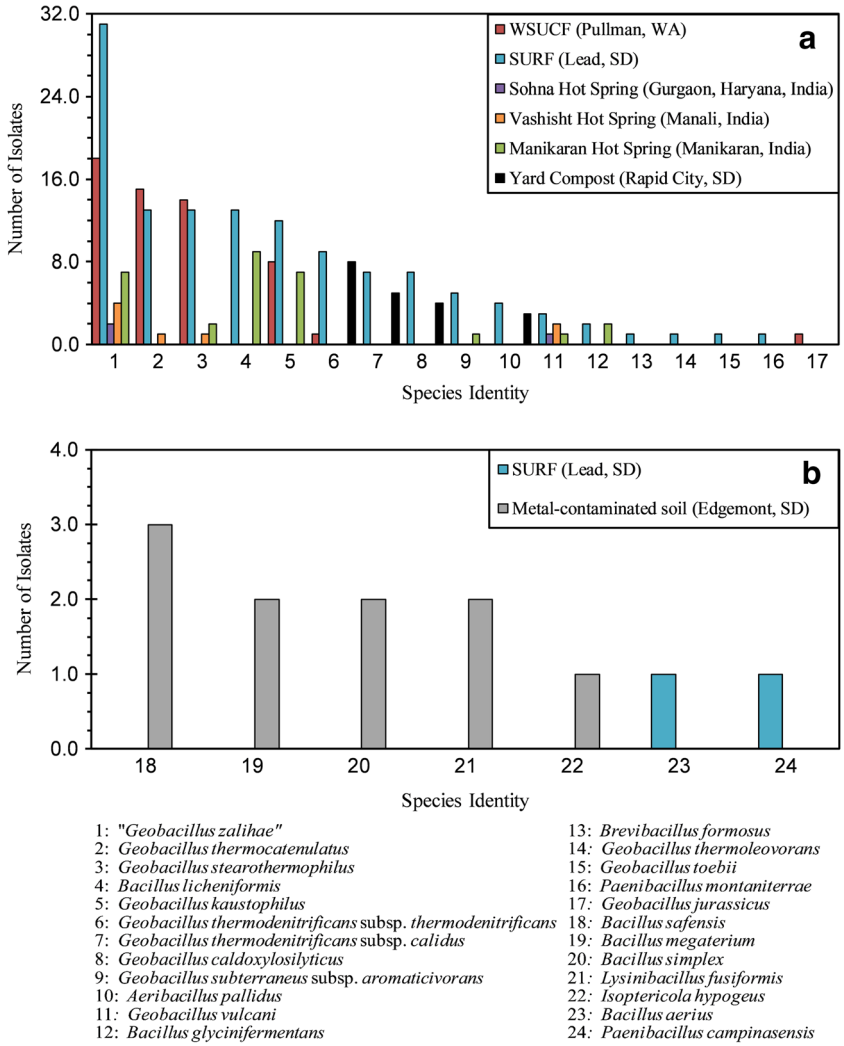

Fig. 2 Incidence of **a** thermophiles and **b** mesophiles isolated from various locations. Identity of the isolates was based on 16S rRNA gene sequence similarities. Arabic numerals along the X-axes of both graphs correspond with the identities of species encountered. The key is provided at the bottom

G. thermoleovorans, *G. toebii*, and *Paenibacillus montaniterrae*. The mesophilic species were identified as *Bacillus aerius* and *Paenibacillus campinasensis* (only one strain of each encountered). A separate collection of samples from the Washington State University Compost Facility (WSUCF) yielded 57 *Geobacillus* strains, which were identified as "*G. zalihae*," *G. thermocatenulatus*, *G. stearothermophilus*, *G. kaustophilus*, *G. thermodenitrificans* subsp. *thermodenitrificans*, and *G. jurassicus* (listed in descending order of abundance). Also, 20 thermophilic/thermotolerant strains were isolated from the Rapid City Landfill in Rapid City, SD. Listed in descending order of abundance, the species found at the landfill are *G. thermodenitrificans* subsp. *thermodenitrificans*, *G. thermodenitrificans* subsp. *calidus*, *G. caldoxylosilyticus*, and *Aeribacillus pallidus*, all of which belong to the phylum *Firmicutes*. Finally, 10 mesophilic strains were isolated from the Edgemont disposal site samples. Members of the genus *Geobacillus* were not obtained from this uranium-contaminated site. Instead, the species uncovered here included spore-forming *Bacillus safensis*, *Bacillus megaterium*, *Bacillus simplex*, *Lysinibacillus fusiformis*, and a non-spore-forming actinobacterium (*I. hypogeus*).

Cultivable thermophiles

As shown in Fig. 2a, 240 strains capable of growing at 55 °C were identified to a species level via 16S rRNA sequence analysis. "*G. zalihae*" was by far the most prevalent species among these isolates (62 total strains encountered) and was isolated from five of the six elevated temperatures (except Edgemont disposal site) sampling locations. Furthermore, "*G. zalihae*" was the most abundantly detected *Geobacillus* species in SURF, WSUCF, Vashisht Hot Spring, and Sohna Hot Spring. Although "*G. zalihae*" did not dominate the Manikaran Hot Spring, it was only slightly less prevalent than the most abundant species (seven strains encountered, as compared to nine strains of the preponderant species). The second most prevalent species across all of the thermophilic samples

was *G. stearothermophilus* (30 total strains encountered), which was isolated less than half as often as "*G. zalihae*." To underline the severity of this gap between the number of "*G. zalihae*" and *G. stearothermophilus* strains, abundance of the third most common species must also be considered. The disparity between the second and third most common species (*G. thermocatenulatus*) was minimal (29 total strains encountered). All other species outlined in Fig. 2a were present in incrementally lesser amounts. It is possible that the "*G. zalihae*" clade consists of several other closely related species that could not be distinguished by 16S rRNA gene sequencing, and hence "*G. zalihae*" isolates were observed to be disproportionately prevalent. In-depth WGS is required to definitively delineate the species.

Cultivable mesophiles

The mesophiles in this study (12 total strains identified to a species level) were identified as *Bacillus* and closely related species. Almost all of the mesophiles stemmed from the Edgemont disposal site, and two strains originated from SURF (Fig. 2b). Among these, *B. safensis* was the most common (three total strains encountered). Three other strains were present in the second most common frequency—these include *B. megaterium*, *B. simplex*, and *Lysinibacillus fusiformis* (two strains of each species encountered). Compared to the thermophilic samples, the distribution of mesophiles had a less evident bias toward one dominating species. However, this lack of visible trends may be a mere symptom of the limited sample set. In other words, the collection of samples was likely too small to show statistically significant trends.

Molecular identification of *Geobacillus*

It is well known that *Geobacillus* taxonomy is in a state of flux. In this study also, the 16S rRNA sequence-based phylogeny is not enough to differentiate *Geobacillus* species. Furthermore, even *gyrB*-based phylogeny that has been useful to delineate *Bacillus* species (La Duc et al. 2004a) is not able to differentiate *Geobacillus* species. Thus, whole genome-based MLST analyses were carried out. Coupling *gyrB* and WGS with the already completed 16S rRNA analyses was intended to provide greater species-level resolution and uncover secluded novel species among the *Geobacillus* strains isolated in this study (Supplementary Table S1). However, all approaches yielded different results, nullifying the anticipated benefits of *gyrB* analysis and reinforcing the need for WGS-based phylogeny to avoid confusion when characterizing *Geobacillus* isolates. Several strains isolated from SURF that had been identified via 16S rDNA analysis as distinct *Geobacillus* species (seven isolates), plus one isolate from WSUCF, and another one from Manikaran Hot Spring were included in the *gyrB* analysis. In addition to the environmental strains, the JSC SAF-associated strain was also included in the *gyrB* analysis (Supplementary Fig. S1). When a cutoff value of 95% *gyrB* sequence similarity was implemented to differentiate new bacterial species (Yamada et al. 1999), all but two strains (SURF-114 and SURF-189) aligned with the already established species.

The 10 strains selected for the *gyrB* analyses were also subjected to WGS, and their dDDH (Supplementary Table S2) and ANI values are depicted (Supplementary Table S3). To date, WGS of 14 other *Geobacillus* species are available in the public database. Based on dDDH (> 70% similarity) and ANI values (> 95% index), four of the *Geobacillus* SURF strains were identified as known species and the JSC T9a strain was identified as *G. thermodenitrificans.* The other five strains belong to three novel *Geobacillus* species that were yet to be described (Supplementary Table S2, Table S3). These novel strains from SURF were either closely associated with the "*G. zalihae*" clade (strains SURF-114 and SURF-189) or distinctly novel (strain SURF-46C-IIa). The third novel species was represented by one isolate from Manikaran Hot Spring (strain Manikaran-105) and another from WSUCF (strain WSUCF-18B), exhibiting high similarities compared to each other but constituting a novel species. However, the dDDH and ANI values of the five strains belonging to three novel species indicated disparity between 16S-, *gyrB*-, and WGS-based identities (Supplementary Table S1). To further investigate the phylogeny and novelty of these strains, sequences of seven housekeeping genes used for MLST of *Bacillus* species were retrieved from the WGS of the 10 selected strains, and a phylogenetic tree was constructed (Fig. 3). The MLST tree confirms the presence of three novel species belonging to the "*G. zalihae*," *G. subterraneous*, and *Geobacillus* sp. JF8 clades, thus agreeing with the WGS analyses, but a polyphasic taxonomic approach including phenotypic characterization is necessary to define the novelty of these isolates. The description of the novel *Geobacillus* species is not within the scope of this investigation.

UVC_{254} resistance of vegetative cells

In total, 41 strains belonging to 10 different *Geobacillus* species (according to 16S analysis) were tested for UVC_{254} resistance. These included the eight most abundant *Geobacillus* species and two arbitrarily chosen, less abundant *Geobacillus* species. Table 2 outlines the results for the qualitative analysis of vegetative cells with UVC_{254}. Cumulative doses of 120, 300, and 600 J/m^2, respectively, correspond with 2, 5, and 10 min of UVC_{254} exposure at a dose rate of 100 μW/cm^2. Most of the tested strains (34 out of 41) survived the minimum dosage (120 J/m^2), with the greatest fraction of killed strains belonging to the *G. thermocatenulatus* and *G. thermodenitrificans* subsp. *calidus* species. The

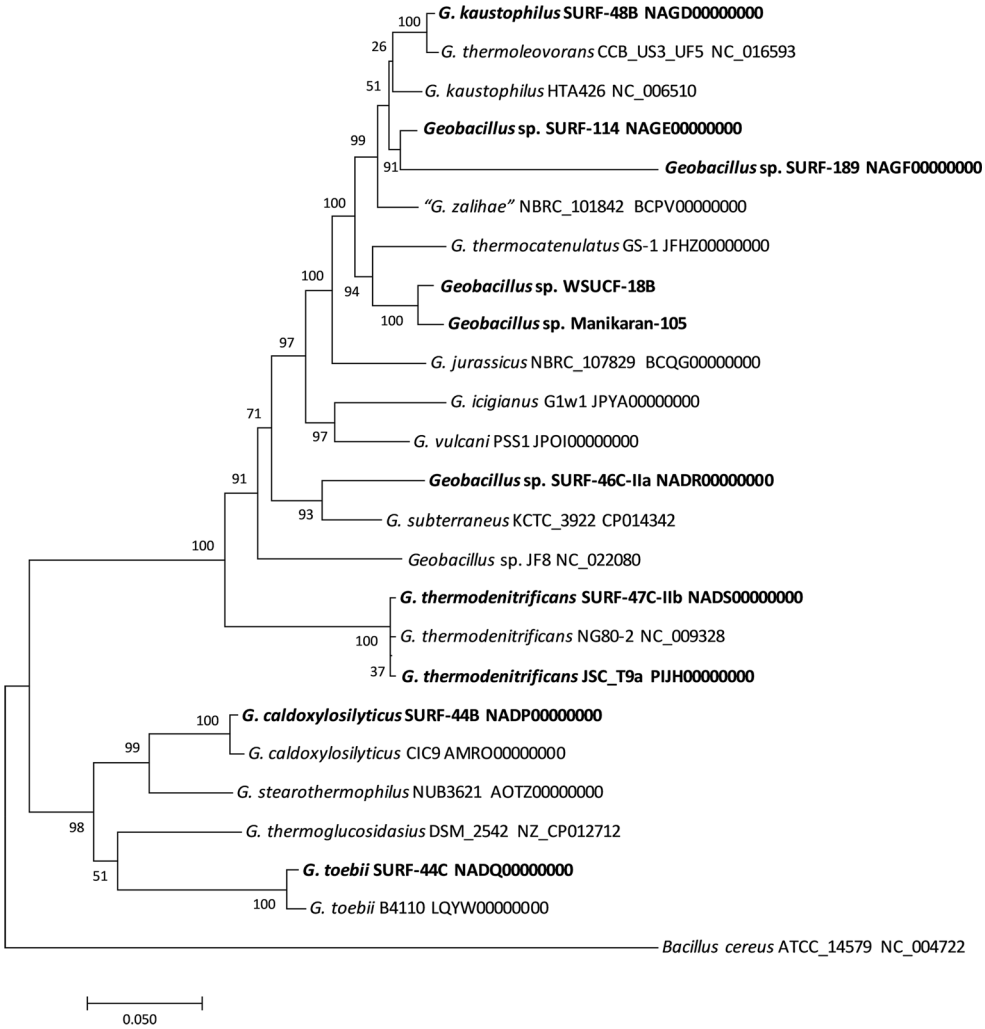

Fig. 3 Evolutionary relationships of *Geobacillus* species isolated during this study using MLST analysis. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.30143649 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 2677 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

penultimate dosage of UVC_{254} exposure (300 J/m^2) left a significantly smaller proportion of surviving strains (7 out of 41). These survivors included one strain of *G. caldoxylosilyticus*, three strains of *G. kaustophilus*, one strain of *G. subterraneus* subsp. *aromaticivorans*, and one strain of *G. thermocatenulatus.* Not surprisingly, the maximum cumulative dose of UVC_{254} exposure (600 J/m^2) proved to be the most lethal. Only a single strain of a novel *Geobacillus* sp. (WSUCF strain 18B) survived the UVC_{254} exposure 600 J/m^2.

UVC_{254} resistance of purified spores

UVC_{254} inactivation kinetics of spores purified from five thermophilic *Geobacillus* strains (four environmental strains, one strain isolated from JSC clean room) were tested. The criteria for selecting these strains included species identity, sampling origin, and/or demonstrated UV resistance of vegetative cells. Strain WSUCF-18B, which originated from the thermophilic WSUCF, was chosen for this test because the survival of its vegetative cells to UVC_{254} was superior to that of all the other tested strains (600 J/m^2). Strains SURF-44B and SURF-48B both originated from the hot, dark, deep subsurface environment of SURF, and they were chosen for their resistance to 300 J/m^2 UVC_{254}. The remaining two strains, Manikaran-105 and JSC_T9a, had not been previously tested for UVC_{254} resistance. Strain Manikaran-105, a Manikaran Hot Spring isolate, was selected because *gyrB* and WGS sequence analyses revealed that it belongs to the same species as the highly UVC_{254}-resistant strain WSUCF-18B, although it originates from a different sampling location. Strain JSC_T9a, on the other hand, was originally isolated from a clean room facility within the Johnson Space Center. It was chosen to provide insight to the comparative properties of environmental *Geobacillus* species and those evolved under the selective pressures of a manmade clean room. Figure 4 delineates the observed inactivation kinetics where N/N^0 is the fraction of revivable spores at each time point as compared to the initial concentration of viable spores. All five tested strains adhered to classical inactivation kinetics, characterized by an initial resistance to the UV irradiation, or "shoulder," followed by an exponential decrease in surviving spores. The LD_{90} for

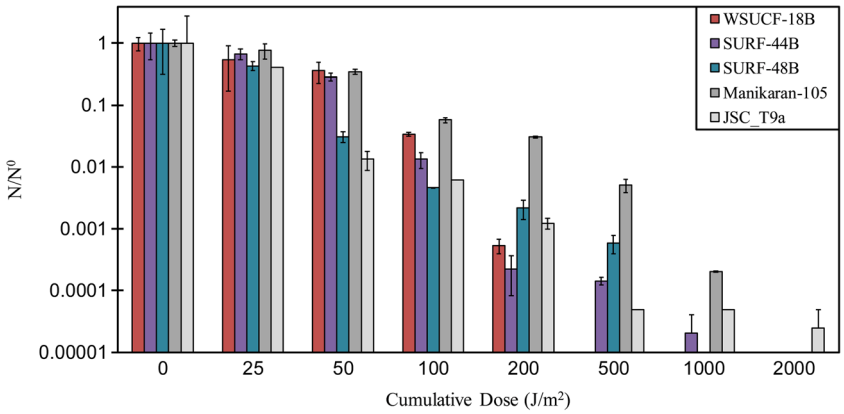

Fig. 4 UVC_{254} inactivation curves of *Geobacillus* spores. The dose rate was 100 μW/cm^2 and the isolates were collected from Washington State University Compost Facility (WSUCF), Sanford Underground Research Facility (SURF), Manikaran Hot Spring, and NASA Johnson Space Center (JSC) clean room facility

[a]Microbial characteristics of the strains involved in this experiment are summarized below. All species-level identities are given from the WGS analysis results. Presence of "-" under Vegetative cell UV resistance indicates strains that had not ben evaluated for UV resistance prior to the trials with purified spores.

Strain #	Species-level identity	Origin	Vegetative Cell UV Resistance
SURF-44B	*G. caldoxylosilyticus*	SURF	5 min
SURF-48B	*G. kaustophilus*	SURF	5 min
WSUCF-18B	*Geobacillus sp.*	WSUCF	10 min
Manikaran-105	*Geobacillus sp.*	Manikaran Hot Spring	-
JSC_T9a	*G. thermodenitrificans*	JSC	-

strains SURF-48B and JSC_T9a were both observed to be in the range of 25–50 J/m^2. The other three strains, WSUCF-18B, SURF-44B, and Manikaran-105, all exhibited LD_{90} in the rage of 50–100 J/m^2. However, in this case, the LD_{90} does not accurately reflect the ability of the strains to withstand UV exposure for an extended period. In fact, strain JSC_T9a exhibited one of the lowest LD_{90} doses, but ultimately survived the greatest cumulative dose (2000 J/m^2). This cumulative dose is equivalent to the record of the most UV-resistant strain found to date, *B. pumilus* SAFR-032. The survivability of strain JSC_T9a was rivaled by strains Manikaran-105 and SURF-44B, which both maintained viability up to the second greatest cumulative dose (1000 J/m^2). Interestingly, strains isolated from different environments but identified as the same *Geobacillus* sp. (WSUCF-18B and Manikaran-105) as per MLST analysis did not show similar UV resistance profiles. While spores of the strain WSUCF-18B were completely inactivated at 500 J/m^2, spores of Manikaran-105 were considerably hardier, showing resistance up to a cumulative dose of 1000 J/m^2 and requiring 2000 J/m^2 UVC_{254} dosage to become inactivated.

Discussion

This study uncovered a prevalence of UVC-resistant *Geobacillus* species across a diverse panel of extreme environments, showing that these resilient thermophiles are ubiquitous, albeit low in numbers, in nature. Given the characteristics of the sampling locations (hot, dark, deep subsurface, heavy metal-contaminated, and clean), a high fraction of the environmental isolates cultured at an elevated temperature was anticipated to be spore-forming bacteria. However, the overwhelming dominance of *Geobacillus* (~ 81% of the strains) was somewhat unexpected and one of the most notable outcomes of this investigation. Indeed, a previous culture-independent study of samples from the SURF reported members of *Firmicutes*, but incidence of *Geobacillus* species was assumed to be present due to the limitations of the probe sets involved in PhyloChip analysis (Rastogi et al. 2010a). This suggests *Geobacillus* strains may have been resident in low numbers, but particular enrichment culture conditions or deep sequencing approaches are required to exhume their presence (Zeigler 2014). In addition, it has been previously demonstrated that a considerable fraction of the total bioburden can never be cultivated on any given medium (Kieft 2000; Stackebrandt and Embley 2000), leaving numerous organisms undiscovered in any given study.

Members of the *Firmicutes* phylum are well adapted to hot spring environments, and our discovery of these microbes throughout the Indian hot springs agrees with previous reports (Narayan et al. 2008; Sharma et al. 2009). The Manikaran and Vashisht Hot Springs are subject to exceptionally high temperatures, produced by unique geographical phenomena. Specifically, temperatures of 96 °C can be observed in the spring water along the 1.5-km linear zone of the geothermal field in the Parbati valley. This intense heat is influential enough to exclusively facilitate the survival of thermophilic species such as *Firmicutes* when they are in spore form. Several past studies pertaining to the microbiology of hot

springs reported the isolation of *Firmicutes*, dominated by *Bacillus* and *Geobacillus*. Higher incidence of endospore-forming Gram-positive *Firmicutes* in the hot springs at Manikaran (Bhatia et al. 2015), *Geobacillus* sp. dominance in the hot springs in Soldhar, Uttarakhand, India (Sharma et al. 2009), and a protease-producing *Bacillus* sp. from the hot springs of Odisha, India, (Panda et al. 2016) were reported. Furthermore, hot springs in the Philippines (45 to 80 °C) were also shown to be largely populated with *Firmicutes*, and *Geobacillus* constituted the most abundant genus among all the isolates (Daupan and Rivera 2015). The present study provides additional testimony for the prevalence of *Firmicutes* and *Geobacillus* sp. in hot springs, but it is distinct from the others mentioned here inasmuch as it provides the first report of hot spring-associated *Geobacillus* sp. spores exhibiting UVC_{254} resistance to 1000 J/m^2 (strain Manikaran-105; Fig. 4).

Compared to the hot spring environments, SURF has been shown to harbor much richer microbial diversity (Osburn et al. 2014). Specifically, PhyloChip analyses of SURF samples targeting both dead and live cells have revealed the greatest number of species belonging to the *Proteobacteria* phylum, followed by *Firmicutes* (Rastogi et al. 2010a). Yet, in the present study, cultivable analysis paired with 16S rRNA sequencing pointed to a predominance of *Firmicutes* and a high representation of *Geobacillus* sp. To understand the disparity between these findings, consider the fact that species flourishing in deep subsurface mines must be able to withstand hostile life conditions such as high temperature, pressure, extreme pH, oligotrophic environments, metal reduction, and metal resistance (Rastogi et al. 2010a). It is doubtful whether members of the *Proteobacteria* present in SURF could withstand the harsh culture conditions (growing at 55 °C) employed during this study. The reported high incidence of proteobacterial signatures in previous studies might have originated from dead cells, whereas *Firmicutes* are better suited to such antagonisms (Vos et al. 2011) when grown at a high temperature (> 55 °C), as shown in this study. Hence, appropriate cultivable analyses might be needed to understand the existence of targeted living organisms. Although the characterization of *Geobacillus* strains isolated from SURF was limited (Bergdale et al. 2014), this is the first study reporting taxonomic profiles of several hundred *Geobacillus* strains isolated from SURF. Documentation of UVC_{254} resistance in spores of two SURF strains—*G. thermoleovorans* SURF-48B and *G. caldoxylosilyticus* SURF-44B—which respectively maintained viability up to 500 and 1000 J/m^2, is noteworthy (Fig. 4).

In concordance with the findings at the other elevated temperature sampling locations, the majority of the species observed in WSUCF compost belonged to *Firmicutes*. The dominance of this phylum has been validated in past microbiological studies within compost facilities (Chandna et al. 2013) and empty fruit bunches and palm oil mill effluent compost (Baharuddin et al. 2010). Thus, it is evident that the presence of organic material in compost favors the existence of species related to *Firmicutes*. The composting process typically involves changes in temperature at different stages, with an initial rapid increase in temperature, a period of sustained high temperature, and finally, a slow cooling of the compost (Dees and Ghiorse 2001). The WSUCF samples yielded only thermophiles (*Geobacillus* species), and mesophiles were not isolated. Among these thermophiles, a novel thermophilic *Geobacillus* sp. was isolated (strain WSUCF-18B). This strain is significant not only for its novelty, but also because its vegetative cells exhibited higher UVC_{254} resistance (600 J/m^2) than all other extreme environmental *Geobacillus* strains tested. In general, vegetative cells are killed at 25 to 50 J/m^2 of UVC; however, higher UVC_{254} resistance has been documented in the vegetative cells of several environmental Actinobacteria, *Firmicutes*, and Gram-negative bacteria isolated from oligotrophic clean rooms (Osman et al. 2008).

Overall, most of the thermophilic/thermotolerant species were found across multiple sampling locations (12 out of 17 species), and the fraction of *Geobacillus* species isolated multiple times from distinct locations was especially high (10 out of 12 species). Such overlap of microbial residents among these geographically separated environments is consistent with previous studies indicating *Geobacillus* spores gradually disperse themselves around the globe (Zeigler 2014). As evidence of their mobility, members of this genus have been isolated from all seven continents on Earth, the Pacific Ocean, the Mediterranean Sea, and various altitudes above and below the surface of Earth, including the upper troposphere at approximately 10,000 m (DeLeon-Rodriguez et al. 2013; Zeigler 2014). Even in locations that never reach the minimum temperature required for *Geobacillus* species growth (~ 40 °C on average), they have been found in high frequencies ranging from $1.5–8.9 \times 10^4$ g^{-1} depending on the sampling site (Ahmad et al. 2000; Bryanskaya et al. 2015; Caccamo et al. 2000; Cihan et al. 2011; Coorevits et al. 2012; Kuisiene et al. 2004; Marchant et al. 2002; Nazina et al. 2001; Nazina et al. 2005; Nicolaus et al. 1996; Poli et al. 2012; Poli et al. 2011; Poli et al. 2006; Rahman et al. 2007; Sung et al. 2002). Extreme longevity of *Geobacillus* endospores is thought to enable this migrant behavior. In unfavorable conditions, the spores lay dormant for extended periods of time while drifting from one environment to another (Zeigler 2014). Consequently, it is reasonable to infer that the overlap of identified species among these distinct geographies is not a coincidence, but the effect of long-term spore migration. This logic also implies that spores with superior longevity have the greatest aptitude to accumulate in high numbers around the globe. Thus, the inordinate abundance of "*G. zalihae*" species encountered in the present study may be partially credited to special adaptations of the spores of

"*G. zalihae*," allowing for long-term survival while migrating through harsh environments. Further studies are required to validate this speculation and evaluate the spores' durability in the face of relevant, naturally occurring antagonisms (e.g., extreme temperatures, pH, osmotic and chemical stress).

When all of the mesophilic strains in this study are considered (Fig. 2b), it is evident there is no overlap of species between the two mesophilic sampling locations (Edgemont disposal site and Rapid City Landfill). Furthermore, the mesophilic species did not overlap with the thermophilic/thermotolerant species. Another noteworthy finding among the mesophilic samples is the incidence of *I. hypogeus* in a sample from the heavy metal-contaminated Edgemont disposal site. This species was originally reported from a tufa sample collected in the Roman catacomb of Domitilla (Groth et al. 2005), and there have been no additional reports on *I. hypogeus* since the establishment of its novel taxonomy. Other members of the *Isoptericola* genus have been isolated from various environments including the termite hindgut, mangrove soil samples, and the Arabian Sea, and they are known for their cellulolytic and xylanolytic abilities (Kaur et al. 2014; Santhi et al. 2014; Shivlata and Satyanarayana 2015; Stackebrandt et al. 2004; Tseng et al. 2011). To our knowledge, the present study is the first to find an association between any *Isoptericola* species and radioactive materials. The uranium resistance of this particular *I. hypogeus* strain remains to be tested.

The environmental strains examined during this study generally displayed impressive robustness compared to typical dosimetry microbes. Vegetative cells of most tested strains (34 out of 41) survived the minimal dosage of UVC_{254} (120 J/m^2). For comparison, vegetative cells of the standard dosimetric strain *B. subtilis* 168 have previously shown their inability to be revived after a cumulative dose of only 50 J/m^2 under the same conditions (Newcombe et al. 2005). Moreover, the hardiness of the environmental spore formers can increase drastically after sporulation takes place, as dormant endospores are 10 to 50 times more resistant to UVC_{254} than vegetative cells suspended in water (Nicholson et al. 2000; Setlow 1988). The lesser UVC resistance of vegetative cells is at least partially explained by the lifecycle of α/β-type small acid soluble proteins (SASP), which are synthesized during sporulation and degrade when spore germination initiates (Shure et al. 1977). Without these proteins, spore DNA is significantly less protected from wet heat, dry heat, and UV irradiation. Spore-specific protein repair enzymes also play a crucial role in enhancing general toughness, but these are less involved in defending against UV irradiation because UV specifically targets DNA damage (Nicholson et al. 2000). Clearly, spores are better equipped than vegetative cells to withstand UV bombardment.

The UVC_{254} resistance of spores from thermophilic environmental *Geobacillus* strains demonstrated in this study was similar to previously studied spacecraft and clean room-associated *Bacillus* strains. Several *Bacillus* spores isolated from the surfaces of Mars Odyssey, X-2000 (avionics), the International Space Station, and their associated assembly facilities exhibited survival at a cumulative dose of 1000 J/m^2 (Newcombe et al. 2005). In the present study, similar UV resistances between environmental and clean room-associated strains are also observed in the spore inactivation kinetics (Fig. 4). *G. thermodenitrificans* JSC_T9a (isolated from JSC clean room) survived a cumulative dose of 2000 J/m^2 UVC_{254}, and environmental strains SURF-44B (from SURF, ~1.5 km below the ground surface) and Manikaran-105 (from Manikaran Hot Spring) survived the second greatest dosage of 1000 J/m^2. These results imply that NASA clean room environments are equally if not more "extreme" than the natural deep subsurface and hot spring sampling environments. However, none of these sampling locations are actually subject to intense UV exposure—the JSC clean room is illuminated with normal lighting, the deep subsurface of SURF is starkly dark, and the Manikaran Hot Spring is exposed only to natural light. Although UVA and UVB radiation is present in natural light, the Earth's ozone layer effectively filters out UVC rays (Rai and Srinivas 2007). So, a probable explanation for the extraordinary UVC_{254} resistance in these *Geobacillus* spores is that they are adapted to other physical antagonisms, and the benefits of their adaptations happen to be multifactorial. Strain JSC_T9a from the JSC clean room ought to be well suited to oligotrophic, minimal moisture, and chemically (cleaning reagents) harsh conditions, while strains SURF-44B and Manikaran-105 should be adapted to wet and dry heat. α/β-type SASP, which have been reported to provide an effective defense against all of these physical attacks, might be playing an important role in UV resistance. They are known to not only protect against UV rays but also resist DNA damage by desiccation and heat, and reduce reactivity to chemical treatments (Setlow and Setlow 1995; Setlow 1992; Setlow 1994; Setlow 1995).

In addition to α/β-type SASP, DNA repair enzymes may serve a crucial purpose in spore revival during germination and outgrowth (Nicholson and Fajardo-Cavazos 1997; Setlow 1995). Some of these enzymes are commonly found in both spores and vegetative cells, but there is at least one DNA repair protein that is exclusively present in spores, and its role is germane to UV resistance. This protein repairs the major spore-associated UV photoproduct, 5-thyminyl-5,6-dihydrothymine (Nicewonger and Begley 1997; Nicholson et al. 2000; Varghese 1970). Since the strains in this study did not originate from locations with regular UV exposure, it is presumed that they would not hone exceptional adaptations of this DNA repair enzyme. The WGS datamining of *Geobacillus* strains should be carried out to draw connections about the molecular mechanisms behind the UV resistance.

Although they may not contain extraordinary DNA repair enzymes, the particular microbes selected for this study—namely, *Geobacillus* and *Bacillus* strains—are highly significant for the interests of the aerospace industry. Both genera have been previously found in NASA clean rooms, thus reinforcing the value of these specimens for forward contamination studies (Mahnert et al. 2015). In past reports where *Geobacillus* was not encountered in spacecraft assembly clean rooms, it is reasonable to speculate the cultivation methods were simply inappropriate for isolating thermophiles. For example, cultivable analysis targeting mesophiles yielded Gram-positive and spore-forming *Bacillus* species (Venkateswaran et al. 2001). Likewise, a series of microbial surveys investigating 125 clean room-associated aerobic microbial strains also discovered no *Geobacillus*, but reported that *B. licheniformis* was the most prevalent species (25%), and *B. pumilus* was the second most prevalent (16%) (Kempf et al. 2005; La Duc et al. 2004a; La Duc et al. 2004b; Newcombe et al. 2005; Venkateswaran et al. 2003a; Venkateswaran et al. 2003b). Simply because *Geobacillus* was not observed in all of these studies, it does not mean it is not present. The hardiness of *Geobacillus* spores and their ubiquity in nature renders them likely inhabitants of most imaginable terrestrial environments, including clean rooms (Zeigler 2014). Hence, it is possible that these thermophilic isolates could realistically latch onto space-bound hardware and pose problems as contaminants and perhaps compromise life-detection missions.

Another valuable attribute of *Geobacillus* strains is their necessity to produce spores under unfavorable conditions (Zeigler 2001). The specific strains in this study are especially valuable, as the elevated temperatures, minimal humidity, and/or chemical harshness of their original environments provide selective pressure toward extreme microbial resiliency. Such organisms with a survival-driven need to create hardy spores can provide insight to the limits of terrestrial organisms' survivability under inhospitable conditions. This knowledge benefits not only NASA but also the Department of Homeland Security and other organizations (Anonymous 1980; Anonymous 1989; DeVincenzi et al. 1996; Hartley and Baeumner 2003; Horneck et al. 1994; Pierson 2001; Rummel 1989; Titball et al. 1991). All things considered, characterizing the UV resistance of these organisms will ameliorate human efforts to eradicate hardy microorganisms and enhance sterilization procedures in controlled environments.

Previous UV resistance studies have focused on less hardy model dosimetric strains, leading to the conclusion that spores of microorganisms cannot withstand more than approximately 200 J/m^2 UVC$_{254}$ (Nicholson et al. 2002). Standard sterilization techniques have been shaped around these studies, which is precisely why enhanced sterilization procedures are needed. For example, a cumulative dose of 400 J/m^2 is the standard dose for sterilizing drinking water (Link et al. 2004). However, both past and present studies have demonstrated the ability of spore-forming bacteria to drastically exceed this dose. Spacecraft-associated clean rooms are host to several notably hardy strains, as the most UV-resistant strain to date (*B. pumilus* SAFR-032) was isolated from the JPL-SAF (Newcombe et al. 2005). Also, a strain shown in the present study to possess comparable resistance to SAFR-032 (*G. thermodenitrificans* JSC_T9a) was isolated from the NASA JSC clean room. Both SAFR-032 and JSC_T9a can survive more than 2000 J/m^2 UVC$_{254}$, amounting to tenfold greater resistance than that of standard dosimetric strains. These UV resistance "champions" are also accompanied by exceptionally robust environmental strains capable of surviving at least 1000 J/m^2 UVC$_{254}$, as demonstrated in the present study.

This is the first report of thermophilic *Geobacillus* spores exhibiting UVC resistance, and further characterization of these thermophiles is necessary to unearth the molecular mechanisms affording the observed adaptations to environmental stressors including UV irradiation. Isolation of these exceptionally UV-resistant *Geobacillus* strains from a natural hot spring (Manikaran-105), compost facility (WSUCF-18B), and deep subsurface ecosystem (SURF-44B) is unprecedented, and more detailed UV screening of all 252 environmental isolates discussed in this study should be attempted in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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