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GOING EXTREME FOR SMALL SOLUTIONS TO BIG ENVIRONMENTAL CHALLENGES

Christopher E. Bagwell
U.S. Department of Energy, Savannah River National Laboratory, Department of Environmental Sciences and Biotechnology, Aiken SC USA

Prokaryotes are the most abundant and diverse life form on planet Earth, estimates of prokaryotic numbers and productivity approach 10^{30} $year^{-1}$ (Whitman et al., 1998). Prokaryotes are responsible for catalyzing important biogeochemical reactions and transformations that sustain the biosphere. Prokaryotes have been evolving for 3 – 4 billion years (Ernst, 1983) and during this time, have found ways to occupy every conceivable environment on the planet; including the most inhospitable habitats both nature and man have created.

'Microbial ecology is the study of microbial physiology under the worst possible conditions'
T.D. Brock, 1966

Most environmental microbiologists would agree that this popular quote by Thomas Brock straightforwardly applies to the maintenance of microbial diversity and ecosystem functions amidst a complex backdrop of ever changing biological, physical, and chemical conditions in terrestrial and aquatic biomes. But does it stop there, what about the de facto 'worst possible condition?'

Many of the environmental challenges outlined in this chapter are not exclusive to the United States but rather affect much of the industrialized world (Pedersen, 1999) because of past military activities and continued expansion of the military – industry complex. While not comprehensive, this chapter is devoted to the scale, scope, and specific issues confronting the cleanup and long-term disposal of the U.S. nuclear legacy generated during WWII and the Cold War Era. Furthermore, microbial interactions and metabolism in, around, and affecting existing and planned geological nuclear waste repositories are serious concerns for safe disposal, future planning, and reliable risk assessment for the environmental and human health.

Nuclear Legacy Waste

Extensive volumes and complex mixtures of nuclear waste are a lasting legacy of the Cold War Era. The U.S. began building the first atomic bomb in 1942. These efforts generated more than 36 million cubic meters of long-lived radioactive and toxic waste by the end of the Cold War in 1989. Irradiated fuels and past nuclear processing streams still await treatment and safe disposal in aged storage configurations. Mixtures of metals, radionuclides, hydrocarbons, and ions contaminate soils, sediments, and groundwater across the nuclear – industrial complex. The U.S. Department of Energy (DOE hereafter) is responsible for management, disposal, and long-term stewardship of this lasting legacy. Because of the prohibitive cost and inefficiencies of existing chemical and physical remedial strategies to address large volumes of contaminant mixtures, newly advanced technologies are greatly needed for reduction and treatment of nuclear legacy waste. Bioremediation is a potentially powerful and innovative technology for converting toxic pollutants to benign end products with a significant cost savings over conventional approaches. The reality, however, is that bioremediation is complicated by the unpredictability of natural ecosystems, complex interactions between co-contaminants and the environmental or containment matrix, and the uncertain behavior of bacterial populations to periodic shifts, and quite often arduous environmental conditions. Most industrial and DOE contaminated environments contain complex contaminant mixtures of varying concentrations. Successful application, management, and ultimate acceptance of bioremediation as a legitimate treatment strategy for nuclear legacy waste demands that a bioremedial candidate be robust; capable of maintaining acceptable rates of metabolism and growth on target pollutants in the presence of co-contaminant toxins,

salts, and radioactivity. Technological advances are greatly needed to generate novel solutions for legacy waste reduction, elimination, and stabilization.

High-level Radioactive Waste (HLW). During the Cold War, Pu^{239} production for national defense began by irradiating uranium or other target elements in a nuclear reactor. During reprocessing of spent reactor fuel, approximately 99% of U^{235} and Pu^{239} were reclaimed. All remaining radionuclides, fission products, fuel components, and nonradioactive chemicals used during reclamation made up the high-level waste stream. High-level waste currently resides at over 100 different sites across the contiguous U.S., but the majority of Cold War legacy HLW is located at Hanford (<65 million gallons), near Richland, WA U.S.A., and the Savannah River Site (roughly 35 million gallons), near Aiken, SC U.S.A. (Wicks and Bickford, 1989). The current national inventory of HLW has more than 1 billion curies of radioactivity. The Savannah River Site tank waste and much of the Hanford tank waste contains Fe, Al, Si, Ca, F, K, alkali cations, organic solvents, radionuclides, fission products, and other governmentally regulated metals. Underground HLW tanks have provided more than half a century of storage, though many of these tanks are well beyond their projected life span. The caustic and corrosive chemistry of HLW has caused some HLW tanks to leak. Many of the oldest single-shell tanks at Hanford have confirmed leaks. An estimated 1 million gallons of HLW has been released into the vadose zone at the Hanford site (Fredrickson et al., 2004).

Treatment and safe permanent storage of HLW is an ongoing priority not only for legacy materials, but for all countries that operate nuclear power stations. Approximately 37% of legacy tank waste by volume will enter the Defense Waste Processing Facility (DWPF, in operation at SRS and in construction at Hanford) for conversion to a safe and stable glass form. The balance of the waste volume will be addressed by additional low-level radioactive facilities. Characteristic organic constituents within the aqueous phase are especially problematic for separations and processing of high-level waste. Organic constituents exist in high level waste and in mixed waste in the form of complexants used during separations processes, radiolysis products from degradation of complexants and solvents, and from waste tank decontamination reagents. One of the preferred decontamination reagents was oxalic acid, which can create problems for storage and final disposal processes due to its unique solubility properties as a sodium salt. Additional reagents in use at SRS and Hanford include glycolic acid, citric acid, and formic acid. The potential for *in situ* removal (degradation) of organic constituents within HLW tanks and other storage configurations could greatly improve processing efficiency of HLW and other nuclear waste streams by established chemical methods.

Low-level contaminated soil and groundwater. Another major legacy of the Cold War is enormous volumes of contaminated soil, sediment, and groundwater across the DOE - industrial complex. Since long-term waste disposal issues were not fully recognized during the production era, nuclear processing streams were often routed to unlined storage configurations (i.e., landfills, trenches, basins) or simply disposed to the subsurface environment. Over time, contaminants have leached from reservoirs into the subsurface environment and groundwater transport has greatly exacerbated the extent of contamination. Many of the same waste elements and pollutant mixtures found in HLW are also present in the environment at many government, military, and industrial installations around the world, though in much lower concentrations. Many of these pollutants are extremely toxic and long-lived in the environment. Environmental and public exposure is a major risk driver; containment and remediation are an immediate priority. The scale of this issue is currently estimated at 79 million cubic meters of contaminated soil, nearly 2 billion cubic meters of contaminated groundwater, and several million cubic meters of buried waste distributed over more than 100 sites at approximately 20 DOE installations across the U.S. (Fioravanti and Makhijani, 1997; Riley and Zachara, 1992).

'Bioremediation holds great promise for some of our worst problems. There is no compound, man-made or natural, that microorganisms cannot degrade.'

Terry Hazen, in response to the Deepwater Horizon oil spill in the Gulf of Mexico, 2010.

Bioremediation of Nuclear Legacy Waste. *In situ* bioremediation describes the use of biological processes to convert toxic pollutants to safe end products within the contaminated environmental matrix. There are a number of established methods for doing this. Indigenous microorganisms can either be stimulated to boost rates of pollutant transformation (biostimulation), or if indigenous microbes cannot sustain desired activity, new beneficial populations can be introduced directly into the environment in order to achieve the desired outcome (bioaugmentation). Biological treatments can effectively prevent expansion of subsurface contaminant plumes (Major et al., 2002; Padmanabhan et al., 2003) and, in some cases, provide direct treatment of source zones (Adamson et al., 2003). Tremendous effort has been aimed towards *in situ* degradation of organic pollutants; though, bioremedial applications have recently been expanded to include metals and radionuclides (Lloyd and Macaskie, 2000). This approach utilizes bacteria that can support energetic metabolism by respiring redox sensitive metals or radionuclides, thereby changing their oxidation state from a water-soluble form to an immobile, mineral precipitate. However, the majority of DOE facilities are co-contaminated by complex mixtures of organic and inorganic compounds, and existing biological technologies proven successful for single classes of pollutants are often rendered ineffective under mixed waste conditions (Ruggeiro et al., 2005). As such, biological solutions have so far not been widely applicable for hazardous or mixed waste. High- and low-level nuclear waste is strictly processed by engineered chemical strategies. In certain instances biologically catalyzed remedies may be useful as a stand alone treatment process, but for more complex waste streams their utility may be restricted to pre- or post-treatments or as a polishing step. The utility and acceptance of bioremediation as a valid treatment strategy for nuclear waste necessitates that the biological candidate exert specific action against target compounds while withstanding the toxic effects of complex contaminant mixtures. New discoveries of naturally occurring ‘extreme’ bacteria could generate robust, cost-effective biological-based treatment strategies applicable to different nuclear waste categories, ranging from in tank pretreatment of HLW to the control and size reduction of contaminated soil and groundwater plumes.

The use of the term ‘extreme bacteria’ in this context is not particularly limited to conventional strains that establish a living at extremes in temperature, pressure, salt saturation, etc., but more precisely microbes that inhabit relevant ecosystems and display meaningful metabolic and / or physiological interactions with nuclear legacy components. Appropriate examples of microorganisms having bioremedial applicability in this context are provided in Table 1. The balance of this chapter will be split into two succinct sections for the presentation of original research aimed at teasing apart and going beyond to infer complex microbiological interactions with legacy waste materials generated by past nuclear production activities in the United States. The intended purpose of this research is to identify cost effective solutions to the specific problems (stability) and environmental challenges (fate, transport, exposure) in managing and detoxifying persistent contaminant species.

Table 1. Selected examples of microbial biotechnologies and applications for energy production and environmental restoration. 'Extreme' bacteria are defined here as microbes capable of withstanding excessively toxic environments and having remedial applicability for reducing volume and the inherent toxicity of legacy waste stockpiles and affected lands.

Extreme condition/ phenotype	Representative organism(s)	Biotechnological Utility or Concern	Reference(s)
Acid (pH <3) Acidophiles	Thiobacilli, Nitrifying bacteria, *Arthrobacter, Bacilllus,* Acid Mine Drainage communities	Metal mobilization, corrosion, bioleaching, bioremediation	Diercks et al., 1991, Jonkers, 2008;Martinez et al., 2007
Alkalinity (pH >9) Alkaliphiles	*Alkaliphilus metalliredigens*	Metal/actinide mineralization	Roh et al., 2007
Salt (> 0.2 M NaCl) Halophiles	*Halomonas* sp. WIPP1A	Radionuclide mobilization	Francis et al., 2000
Radiation Resistant Bacteria	*Deinococcus radiodurans*	Bioremediation of radioactive sites	Brim et al., 2000; Fredrickson et al., 2000,
Acidophilic, Dessication Resistance	*Bacillus* spp., *Pseudomonas* spp., WIPP strains	Waste mobilization from solid formations, corrosion	Diercks et al., 1991; Francis et al., 1980a,b, 1990,1998; Horn and Meik, 1995,
Organic Solvent Resistance	*Arthrobacter* spp., *Clostridium thermohydrosulfuricum*	Soil and groundwater remediation, Energy production	Lovitt et al., 1984; Sardessai and Bhosle, 2002,
Metal Respiring (Reducing) bacteria	Geobacter spp.	Tc(VII), U(VI), etc. precipitation, Energy production	Anderson et al., 2003; Bond and Lovley, 2003; Liu et al., 2002; Lovley and Nevin, 2011
Metal Respiring Hyper-thermophile	*Geoglobus ahangari*	Bioremediation	Kashefi et al., 2002
Extreme metal resistance	*Arthrobacter spp.*	Bioremediation	Margesin and Schinner, 1996
Psychrotrophic Organic solvent	*A. chlorophenolicus* A6	Bioremediation	Backman and Jansson, 2004
Obligate Dehalorespiration	*Dehalococcoides ethanogenes*	Soil and groundwater remediation	Lendvay et al., 2003

I. High Level Waste Microbiology

Kineococcus radiotolerans was isolated within a shielded cell work area containing highly radioactive nuclear waste at the U.S. Department of Energy's Savannah River Site, SRS (Aiken, SC U.S.A.) (Phillips et al., 2002). *Kineococcus* is an orange pigmented, aerobic, nonsporulating actinomycete belonging to the *Kineosporiaceae* family. Consistent with its origins in a high-level nuclear waste environment, *K. radiotolerans* has proven to be exceptionally robust and to possess tremendous potential for bioremediation of hazardous and mixed waste environments where toxicity precludes efficient metabolism and survival for other bioremediation candidates (examples included in Table 1). While it is certain that *K. radiotolerans* survived the extremely harsh environment of high-level nuclear waste, the specific mechanisms that assured survival are unknown. The possibility that this bacterium may have catabolized organic components from nuclear waste for biomass conversion or maintenance energy is especially intriguing and may lend themselves for development of bioremediation technologies of organopollutants from various nuclear legacy waste classifications.

The primary challenges to any applied biotechnology for broad classifications of nuclear legacy waste for reduction and detoxification purposes include the ability to withstand organic and inorganic toxicity, and exposure to γ-radiation. Gamma radiation is one of the most energetic forms of electromagnetic radiation. Gamma rays penetrate tissues and cells, causing direct damage to DNA (namely double strand breaks, DSB), proteins, and membranes. The majority (80%) of the resultant damage caused by exposure, however, is indirect and caused by secondary reactions stemming from the ionization of water and formation of free radical species, primarily •OH. DNA lesions block gene transcription and genome replication, and if not correctly repaired, could introduce detrimental mutations or cell death. Relatively few DNA double strand breaks are lethal for most bacteria. *E. coli* succumbs to around 10 DSB and *Shewanella oneidensis* MR-1 dies after 1 DSB (based on calculations of 0.0114 DSBs / Gy / Genome; Daly et al., 2004). Remarkably, *Kineococcus radiotolerans* can accumulate more than 200 DSB (20kGy γ-radiation) and within 3 – 4 days all DNA and cellular damage is repaired and cell division resumes (Bagwell et al., 2008a). The cellular and biomolecular phenomena underlying the extreme radioresistance phenotype in *K. radiotolerans* are unknown, and have the subject of recent research efforts. Preliminary data indicates important differences compared to the current *Deinococcus radiodurans* model. These differences could suggest that independent evolutionary events are responsible for the radioresistance phenotype in these distinct bacterial lineages.

Reactive oxygen species (ROS) and oxygen free radicals are produced endogenously during aerobic metabolism as O_2 is reduced to H_2O, but are also formed during radiolysis of water. The latter is particularly problematic in radioactive environments given that a bacterial cell can be nearly 90% water. Pathways for cellular injury by exposure to ROS, chiefly •O, H_2O_2 or •OH, involves a number of subcellular cyclic reactions that generate additional reactive oxygen radicals. These highly reactive species oxidize unsaturated fatty acids in cell membranes, RNA, damage proteins, and generate DNA lesions (Marnett, 2000; Proctor and Reynolds, 1984). ROS and oxygen free radicals also react with certain transition metals (e.g., Fe^{2+}, Cu^{2+}) in the cell by Fenton or Haber Weiss reactions to produce damaging hydroxide radicals (Imlay, 2003). Thus, radiation resistance and oxidative defense pathways are intricately linked. Genetic experimentation and genome comparisons have quantified the involvement of conventional repair and recombination pathways as well as numerous functionally uncharacterized genes in extreme radioresistance; though the phenotype is not strictly encoded within the genome, a suite of reactive and stabilizing metabolites as well as unusual cellular biochemistry also play critical roles.

A long standing paradigm in radiation biology was that radiation induced biological effects resulted from direct damage to DNA. This logic stemmed from the acknowledgement that radiation induced DNA lesions are toxic to living cells, particularly double strand breaks, and that DNA damage (and thus survivability) is radiation dose dependent. For decades, *Deinococcus radiodurans* has been studied for heritable trait(s) conferring extreme resistance and high fidelity DNA repair (Battista, 1997; Makarova et al., 2001) and while genes and gene products are clearly one important aspect to extreme radioresistance, our views are

expanding to include additional aspects of cell and molecular biology. Still, three DNA centric models have been proposed to explain the extreme resistance phenotype; 1) conventional enzymatic defenses operating at extraordinary efficiency, 2) the involvement of novel repair functions, and 3) a highly condensed, multigenomic nucleoid (Battista, 1997; Cox and Battista, 2005; Levin-Zaidman et al., 2003; Zimmerman and Battista, 2005). No single hypothesis explains in full the underlying genetic complexity of the extreme resistance phenotype (i.e., Udupa et al., 1994); however, preferential utilization of manganese is thus far the only biochemical strategy broadly conserved among a diverse collection of extreme-resistant bacteria (Daly 2004, 2007). The antioxidative capacity of Mn has been known for decades though with renewed 'discovery' comes an important paradigm shift in radiation biology because manganese, unlike iron, does not catalyze hydroxyl radical formation through Fenton / Haber-Weiss chemistry. Manganese may also mitigate protein oxidation by scavenging oxygen radicals (Daly et al., 2007). Elemental ratios of Mn:Fe have been proposed as a potentially reliable indicator of a cell's susceptibility to oxidative stress (Daly et al., 2004). While Mn accumulating bacteria accrue comparable levels of DNA damage as Fe-accumulating bacteria for a given dose of γ-radiation (Daly et al., 2004; Granger et al., 2011), manganese appears to quench secondary chemical reactions that produce reactive oxygen species; thus, cellular damage is minimized, critical enzymatic repair processes are protected and remain active to extend cell survivorship (Daly, 2009).

Kineococcus radiotolerans was evaluated for preferential utilization of Mn, or an analogous molecular role for alternative redox active metals that may similarly function as a cellular antioxidant or to minimize protein damage following environmental assaults (Bagwell et al., 2008b). In a reciprocal experimental design, colony formation during chronic irradiation (4 days at 60 Gy / hr) was measured in response to the addition of individual redox metals at a single concentration (100μM each of Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, and Mo^{2+}). Overall, the metal only treatments had very little effect on colony formation, and the irradiated, metal(s) minus control cultures consistently yielded between 50 – 100 colony forming units (CFUs). Interestingly, colony formation during chronic irradiation in the presence of either Fe^{2+} or Mn^{2+} was negligible compared to the controls; however, the addition of Cu^{2+} combined with chronic radiation resulted in a lawn of bacterial growth, as was observed for the no metal, nonirradiated control cultures. The hypothesized physiological role for Mn^{2+} accumulation for radioresistance of *Deinococcus* and other radiation resistant bacteria cannot explain this result, as Cu^{2+} does participate in Fenton or Haber-Weiss chemistry for the formation of reactive oxygen species (Letelier et al., 2005). This study marked the first documented case whereby bacterial growth was legitimately enhanced during chronic irradiation. Growth conditions that were expected to prompt copper catalyzed production of oxygen radicals actually promoted the growth of *K. radiotolerans*, and this response could not be duplicated by chronic irradiation or copper supplementation alone.

Copper is an essential cofactor for a variety of enzymes involved in aerobic respiration and energy production; however, only trace quantities are required and so, intracellular levels are tightly regulated by the cell (Rae et al., 1999). Yet, *K. radiotolerans* actively accumulates Cu^{2+} intracellularly, SEM/EDS spectra for copper were only detected from the cytoplasm of thin section preparations and the extent of accumulation is correlated with aqueous phase concentration. The consequences of copper accumulation are evident however, Cu^{2+} loaded cultures display increased sensitivity to peroxides and methyl viologen, and post-irradiation recovery is delayed (Bagwell et al., 2008b). These conditions, however, were intended to push the physiological limits for these stressors; copper accumulation did not interfere with cell growth during chronic irradiation and growth rate and biomass yields were unaffected by high levels of copper accumulation (Bagwell et al., 2010). These results imply that *K. radiotolerans* cultures are not burdened by copper accumulation; implying clear capacity for copper coordination and sequestration, and that oxidative defenses are responsive to this growth condition. A conventional Cop-type copper homeostasis pathway has not been deduced from the *K. radiotolerans* genome sequence; though we have partially characterized participatory metal sequestration systems involved in intracellular copper accumulation (e.g., glutathione) and coordinated expression of antioxidants (e.g., amino acids, carbohydrates, organic osmolytes) and enzymatic defenses that manage and maintain a proper intracellular environment (Bagwell et al., 2010). A systematic dose – response study has recently revealed that 2 specific concentrations of cupric sulfate (500

nM and 100 μM) significantly increase the rate of cell division and metabolic respiration in *K. radiotolerans* cultures. Note that 100 μM copper was the concentration used during chronic irradiation that stimulated cell growth and colony formation. We can surmise that perhaps the same phenomenon (i.e., boost in metabolic rate and / or efficiency) may also occur during chronic irradiation though the exact cellular mechanism and molecular function(s) of Cu^{2+} have not been elucidated. As an aside, we have demonstrated conservation of copper stimulated growth among diverse actinobacteria; for example, 40% of isolated soil actinobacteria from a single shallow subsurface site displayed increased growth rate by copper supplementation. Oxygenic respiration increased markedly in *Lechevalieria xinjiangensis* a novel actinomycete isolated from radiation polluted soil (Wang et al., 2007) at the following Cu^{2+} concentrations, 1 and 3 μM, 100, 250, 500 nM. The same phenomenon has also been documented at 35μM and 250nM Cu^{2+} for *Kineococcus auranticus* (Yokota et al., 1993), the closest known relative to *K. radiotolerans* which does not exhibit the extreme resistance phenotype. The relevance of copper metabolism to *K. radiotolerans'* survival in HLW is unclear; nonetheless, useful applications for this trait can be envisioned. Preferential uptake and intracellular accumulation of copper could be useful as a flow-through bio-filter for copper capture from radionuclide containing waste waters or perhaps some of these bacteria may be useful resources for bioleaching of copper or other precious metals from their ores.

Through a combination of genomics guided physiological experiments, we have sought to derive at an answer to the key question of whether *K. radiotolerans* can actively metabolize organic components of radioactive HLW, or whether it is simply able to survive extremes in environmental conditions. Inferring the potential for bacterial interactions with or metabolism of inorganic pollutants is more complicated as direct and indirect pathways for electron transfer are not strictly conserved within the genome. *K. radiotolerans* is an obligate aerobe but has not been thoroughly examined for specific interactions with or the ability to affect the solubility of metals or radionuclides. The genome of *K. radiotolerans* lacks strongly annotated orthologs for known degradation genes and pathways for pervasive environmental pollutants, aromatic hydrocarbons, petroleum derivatives, and volatile organic compounds found in HLW. In addition to many of these components, SRS HLW also contains low molecular weight organic complexants and decontamination reagents (i.e., oxalate, glycolate, citrate, and formate), which are noteworthy because they interfere with existing downstream processing of legacy HLW materials. None of these low MW organic compounds are suitable growth substrates for *K. radiotolerans*; however, formate and oxalate each sustained cell viability during periods of prolonged starvation (Bagwell et al., 2008a). The genome of *K. radiotolerans* encodes for a single formate dehydrogenase whose functionality has not be deduced; though in this capacity it may function to generate reducing equivalents for maintenance purposes by oxidizing formate to CO_2. A putative pathway for oxalate mineralization is unknown. It is conceivable that *K. radiotolerans,* and possibly other radioresistant microbes known to inhabit highly radioactive or mixed waste environments (e.g.,Fredrickson et al., 2004; Francis 1990; Wolfram et al., 1996), may be useful as a pretreatment for scrubbing metabolizable organic constituents from HLW, or other radioactive waste streams, to improve process efficiency and cost effectiveness of existing technologies. Expanded investigation of HLW microbial communities and explicit experimentation of promising microbial species with relevant nuclear waste streams is in large part precluded by cost; however this research area has the potential for a major return on that investment.

II. Bacteria Inhabiting Plutonium Laden Soils in the Unsaturated Subsurface. Plutonium is a rare naturally occurring metal on Earth, though its manufacture for military and civil applications has produced vast quantities of fission products whose disposition and ultimate disposal remain major challenges at nuclear production, testing, and waste disposal sites (Harley, 1980; Runde, 2000). More than 500 tons of plutonium has accumulated from spent nuclear fuel, along with lesser quantities of other actinides. Environmental contamination is widespread due to atmospheric nuclear detonations however levels are relatively low (< 0.4 pg / g). Few sites around the globe maintain high localized concentrations of actinides, including former production facilities and current repositories (U.S. Department of Energy complex, Waste Isolation Pilot Plant (WIPP), Carlsbad NM USA), mines, underground testing sites (e.g., Nevada Test Site

and Amchitka, USA) as well as sites of accidental releases (e.g., Chernobyl reactor, Ukraine) and natural disasters (Fukushima Daiichi nuclear plant, Japan). Plutonium is a long-lived radioisotope (λ = 24,000 years); thus major environmental issues concern the long-term fate and transport in soil and groundwater (or from storage structures), as well as the rate and extent of accumulation up through the food web (e.g., Au, 1974;Demirkanli et al., 2009; Kaplan et al., 2010; Thompson et al., 2009; Wicker et al., 1999).

In surficial sedimentary environments plutonium is highly immobile, Pu (III, IV) are the predominant oxidation states. Pu(IV) adsorbs strongly onto reactive mineral and cellular surfaces, though amorphous complexation (oxides, carbonates, hydroxides), colloidial formations, and oxidation can produce mobile forms of Pu. In fact, there are numerous examples of Pu being transported over large distances (Dai et al., 2002; Kersting et al., 1999; Ketterer et al., 2010, Novikov et al., 2006; Xu et al.,, 2008). Microbial metabolism can also directly (altering oxidation state, bioaccumulation, bioprecipitation, passive mobilization on bacterial surfaces) and indirectly (chemical complexation with metabolites or cell debris, pH, Eh) affect the solubility and/or mobility of plutonium. While none of these biological interactions have been evaluated under realistic or quasi-field conditions, we speculate that a bacterial role could be quantitatively significant to the fate and transport of Pu in the environment (Francis, 2001;Neu et al., 2005).

The work described herein was part of a larger effort to define and measure the biogeochemical controls on plutonium fate and transport in the unsaturated vadose zone. Briefly, an open top lysimeter gallery was operated at the U.S. Department of Energy's Savannah River Site (located in Aiken, SC USA) for 11 years (1981 – 1991) to study the affects of natural environmental conditions and soil biogeochemical processes on Pu mobility and speciation (Kaplan et al., 2004, 2006). Lysimeters were backfilled with native vadose soil (kaolinite - Fe-oxide composition, 0.01% OM, 0.1 ppm TOC, low ionic strength, pH 5.5) and characterized sources of weapons grade $^{238, 239, 240}$Pu (III, IV, VI); leachate samples were collected regularly for Pu speciation. Consistent with expectations, Pu(IV) was the predominant oxidation state measured in the lysimeters, though a minor fraction of Pu(III) was also detected. Effectively 99% of the Pu mass moved only 1.25 cm from the source; however, the remaining 1% was transported 21.6 cm to the soil surface and 12 cm down through the lysimeter. The current conceptual model for downward transport suggests that Pu is most often reduced and largely immobile except for short-lived, but very important bursts of active transport when Pu(IV) oxidizes to Pu(V) (Demirkanli et al., 2007). Measured upward movement cannot be explained by the model and thus, is an important concern for risk assessment determinations.

Bacteria were grown directly from the Pu(IV) lysimeter soils by overlaying with molten dilute or groundwater plating medium. The majority of pure culture isolates were high % G+C *Actinobacteria*, though strains belonging to the *Gammaproteobacteria, Firmicutes, and Deinococcus-Thermus* lineages were also cultivated. Overlapping 16S rRNA genotypes (*Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria*) have been inventoried from SRS vadose soils, as well as from minerologically and chemically contrasting surface soils from Los Alamos National Laboratory, a former Pu production site in Los Alamos, NM USA (carbonate-smectite-biotite-vermiculite soil, 0.3% OM, high ionic strength, pH 8.5) (courtesy Cheryl Kuske). Recovered strains from many of these lineages was not entirely unexpected as they are each well adapted to low nutrient availability, frequent and prolonged wet-dry cycling, or display insensitivity to contaminant toxicity because of remarkable resistance, spore formation or inactive life stages. Consistent with Zhang et al. (1997) who projected that diversity and biological activity in shallow subsurface environments will be restricted most prominently by resource bioavailability, QPCR estimations yielded 1.6 $\pm$ 0.2 x 10^4 rrn gene copies per gram of SRS vadose soil. This baseline description of microbial diversity in relevant soils from 2 former Pu production sites helps to focus our attention to those bacterial lineages most likely to encounter and thus affect Pu, or other actinides, at these locations and quite possibly, long-term storage facilities and disposal sites of related geography or storage composition (e.g., WIPP, SRS, Hanford).

We postulate that some of the features that afford survival in the vadose zone may also provide protection against radiation exposure and metal toxicity; in turn, these biological responses have the potential to affect

the mobility of actinides in shallow subsurface environments. Representative bacterial strains obtained from the Pu(IV) lysimeter soils were characterized for resistance traits and pathways known to be involved in the complexation and speciation of Pu (Francis, 2001;Neu et al., 2005), results are summarized in Table 2. Here, we will pay particular attention to stress resistance and the production of bioactive metabolites.

Radiation and Desiccation Resistance. The vadose zone is typified by fluctuating moisture regimes but the mineralogy of the SRS vadose soils (sandy-clay, low organic content) exacerbates this condition; soils warm quickly and maintain low water retention capacity. Dehydration can produce DNA modification(s) and oxidative cellular damage analogous to radiation exposure (UV, IR); as such, a common suite of resistance pathways may provide cross-protection to both stressors. Causal linkages have been proposed for the observed covariance in resistance phenotypes (Mattimore and Battista, 1996; Shukla et al., 2007), however these hypotheses have not been broadly tested. Three of the most UV-C resistant strains (R2A5, R2A7, R2A9) maintained viability following 8 weeks of dehydration; though 2 of these strains were *Bacillus* spp. and survivability may rely on spore formation. Three isolates, from genera *Arthrobacter* and *Bacillus*, were unaffected by λ radiation or prolonged periods of desiccation exceeding 9 weeks. In general, resistance to λ radiation, but not UV-C, co-varied with desiccation resistance, with the exception of two *Streptomyces* related bacteria (GW1, GW2) which survived an 8 kGy dose of ionizing irradiation yet showed great sensitivity to desiccation. Isolates closely related by 16S rRNA gene sequence to *Streptomyces* and *Deinococcus* had no tolerance for dehydration longer than 1 week but showed resilience to high levels of λ radiation. Genera related to *Arthrobacter* and *Corynebacterium* showed limited survivability when irradiated yet were resilient to five weeks of desiccation.

Radiation Resistance, IR and UV-C. Upon exposure to 4 kGy γ -radiation from a ^{60}Co source, only 6 pure culture strains exhibited 100% survival; 4 strains survived 8 kGy γ-radiation (GW3, GW7, R2A5, R2A7; data not shown). All strains within our collection proved more resistant to germicidal UV-C than γ-radiation. Four strains (*Actinobacteria, Bacillus*) withstood the highest doses of UV-C and IR. It is plausible that spore formation among the *Bacillus* spp. may have exaggerated resistance estimations in spite of our best efforts to conduct experiments with freshly prepared vegetative cultures.

Table 1. Phylogenic identification and physiological stress resistance of bacterial isolates collected from vadose soil lysimeters at the U.S. DOE's Savannah River Site.

Identity	Closest Match (%ID)	% Survival @ radiation (4kGy)	UV-C (mw/cm2)	Dehydration (wks)	Siderophore Production	Acid Production (pH6.84)	LA	AA	FA	PrA	PyA	UAdR	UAdR
GW1	*Streptomyces thermovioleus* S001573938 (79%)	100	>160	0	Fe Starvation	3.74	1.16	BDL	0.10	0.13	0.64		
GW2	*Xanthomonas campestris* S000639468 (97%)	0	120	4	ND	6.36							
GW3	*Kitasatosporia kifunense* U93322 (98%)	100	>160	0	ND	4.14	1.54	BDL	0.06	0.08	0.65		
GW4	*Dermacoccus nishinomiyaensis* X87757 (99%)	0	>160	5	ND	4.66	2.83	BDL	0.68	BDL	BDL		
GW6	*Dermacoccus nishinomiyaensis* AM992178 (95%)	0	80	2	ND	4.83	BDL	0.47	BDL	0.08	0.32	+	
GW7	*Micrococcus grandis* AY424359 (85%)	100	>160	1	TSA	6.4							
GW9	*Arthrobacter pascens* S001248364 (68%)	0	30	3	ND	5.56	0.98	0.46	0.13	0.17	1.44	+	+
GW11	*Arthrobacter* sp. S001020196 (83%)	0	>160	1	TSA	4.84	BDL	BDL	0.83	0.05	0.06	+	+
GW12	*Terrabacter* sp. S000717117 (94%)	0	50	0	Fe Starvation	4.89	1.75	0.58	BDL	BDL	1.26	+	+
R2A2	*Corynebacteriumaqueohexanicum* AB210282 (98%)	0	80	1	ND	5.05	BDL	1.18	0.41	0.08	BDL		+
R2A4	*Amycolatopsis schigenensis* AB248535 (99%)	0	50	2	Fe Starvation	7.66							
R2A5	Uncultured *Bacillus* sp. S001047854 (97%)	100	>160	8	TSA	4.44	BDL	0.84	0.36	0.02	0.01		+
R2A6	*Micrococcus* sp. S001020197 (94%)	0.01	50	5	TSA	5.42	BDL	2.58	BDL	0.03	1.43		+
R2A7	*Bacillus cereus* S001046766 (100%)	100	>160	>9	TSA	4.73	BDL	0.89	0.40	0.02	BDL		+
R2A8	*Micrococcus luteus* S001577226 (95%)	0	40	0	ND	7.87							
R2A9	*Arthrobacter* sp. S000368538 (98%)	100	75	>9	TSA	6.88							
R2A10	*Aeoplasma phagocytophilum* S001589792 (98%)	0.01	50	8	ND	5.4	BDL	BDL	BDL	0.19	BDL		+
R2A11	*Arthrobacter* sp. S001020203 (90%)	0	50	8	ND	7.43							
R2A12	*Agromyces* sp. S000489357 (88%)	0	75	8	TSA	4.86	2.48	1.25	BDL	0.07	0.02		+
R2A13	*Arthrobacter* sp. S001020203 (89%)	0	50	8	ND	7.06							
Deinococcus radiodurans		100	100	8									
E. coli K12		0	30	1									

Strain designations indicate primary enrichment on groundwater medium (GW) or solidified R2A. Siderophore production assays were prepared by transferring cultures onto CAS agar plates following Fe(III) starvation or directly from TSA plating medium. ND indicates that no halos were observed. Acid production shows the endpoint pH of weakly buffered grown medium after 5 days of incubation. Confirmed organic acids included lactic acid (LA), acetic acid (AA), formic acid (FA), propionic acid (PrA), pyruvic acid (PyA), and two unidentified compounds; BDL indicates peaks were below detection.

Heavy Metal Resistance. Plutonium is both chemically and radiologically toxic; however concentrations that inhibit bacterial growth (Ruggiero et al., 2005) greatly exceed relevant environmental concentrations. Therefore, toxicity response pathways, which may affect Pu mobility, are likely most relevant at micro-scale processes in heterogeneous environments (i.e., mineral surface associated biogeochemistry) or anthropogenic impacted environments where actinide levels can be quite high, such as nuclear waste disposal sites (e.g., Francis, 1985; Harley, 1980). The SRS lysimeter strains exhibited resistance to a variety of heavy metals that are 1) more toxic than most actinides, including Pu, 2) much more pervasive environmental contaminants than actinides and, 3) whose toxicity is exerted at much lower, and thus relevant concentrations. Briefly, growth was uninterrupted by Cu^{2+} ranging from 250 μM – 1 mM. All strains were exceptionally sensitive to Ni with the exception of strains related to genus *Arthrobacter*, which tolerated 750 μM doses. Growth was measured, though severely retarded, by 1 mM Al; conversely, all isolates grew uninterrupted in the presence of 2 mM Cr^{6+}. Half of the strains exhibited growth rate and/or biomass stimulation in the presence of 1 μM – 250 μM Cu^{2+}. Specific concentrations of Al and Cr^{6+} stimulated growth rates of bacteria exhibiting the high levels of desiccation tolerance. Stress resistance implies that the majority of recovered strains could survive, sustain metabolic activity if only intermittently, and perhaps propagate in metal contaminated environments or mixed waste sites.

Metabolites and Organic Ligands. Microbial metabolites and ligands can complex with and affect the environmental mobility, and potentially the bioavailability, of Pu in soil (John et al., 2001; Kauri et al., 2006; Roberts et al., 2008; Thompson et al., 2009). Four isolates reacted positively on CAS-agar plates prepared for colorimetric assay of iron chelation and nearly half of the strains produced sufficient organic acids to significantly decrease the pH of their growth environment (Table 2). These Fe-binding siderophores were not chemically or structurally defined; however, because of the similarity in charge-to-ion radius, specific siderophores and transport proteins may not be able to distinguish between Fe(III) and Pu(IV). Uptake and accumulation of Pu(IV)-desferrioxamine-B complexes has been demonstrated for the soil bacterium *Microbacterium flavescens* (John et al., 2001) and corn plants (Demirkanli et al., 2009; Thompson et al., 2009); though desferrioxamine siderophores are less effective at promoting dissolution of Pu(IV) hydroxide (solubilized 7 μmol g^{-1} aged PuO_2) compared to other organic chelators (including organic acids) in simple solution systems. Therefore, biotic systems for the acquisition of Fe may inadvertently mobilize soluble or complexed Pu; which could have contributed to the upward mobility of Pu already in soil solution in our lysimeter system. Siderophores appear less likely to control the dissolution of tightly sorbed or aged Pu from mineral complexes.

The spent medium from organic acids producing strains was analyzed by ion chromatography, the composition of those mixtures are shown in Table 2. Two product peaks were consistently detected from numerous acid producing isolates but could not be confidently identified. We have been able to eliminate anions (Cl^-, SO_4^{2-}, NO_3^-, PO_4^-), amino acids, metabolic intermediates (glucose-6-phosphate, α-ketoglutarate) and the following organic acids: citric acid, isocitric acid, oxalic acid, succinic acid, fumaric acid, butyric acid, gluconic acid, glycolic acid, malic acid, caproic acid, valeric acid, and carbonic acid (and bicarbonate). All of the acid producing strains demonstrated the ability to withstand medium acidification during prolonged incubation, which could be ecologically relevant for vadose soils or a potentially effective strategy for mitigating cellular toxicity by increasing metal solubility. In 2-staged experiments we have confirmed that bacterial growth and excretion of organic acids significantly contributes to dissolution of aged Pu(IV)-soils, thus increasing aqueous phase concentration (0.6 – 0.9 %), and that organic acid mixtures strongly complex soluble Pu(V) (98% mass balance), 2-50% of the total Pu complexed with each of the individual compounds. It is interesting to note that approximately 1% Pu solubilization was achieved here with active bacterial cultures in 5 days; the same percent release was measured in open top lysimeters after 11 years. Likewise, soil humic acids have been shown to be effective at remobilizing Pu from soils; as much as 1.2% Pu can be released by fulvic acids (Santschi et al., 2002). Viewed from this perspective, these results are not insignificant. The quantity of labile carbon used in these experiments was excessive (50 mg-C / L) compared

to typical levels present in SRS vadose soils but are not unrealistic for certain waste repositories where carbon bioavailability supports high levels of bacterial metabolism (Francis, 2001;Gillow et al., 2000).

Because of the long half-life associated with most isotopes of Pu, slow but persistent biogeochemical processes are critically important to the long term fate and transport of this actinide. The vadose zone is almost always of direct concern, because this is where most accidental releases occur, tremendous volumes of legacy materials are buried in shallow storage configurations, Pu can have a long residence time in the vadose, and finally, this zone marks the transition between sensitive surface receptors and subsurface aquifers. These results are preliminary but are intended to help provide a clearer picture of how microbes that are able to persist in low nutrient, dry sedimentary conditions could potentially affect the long-term fate and stability of plutonium. These pathways need better definition and quantitative measurements are essential for accurate reactive transport models which are relied upon for environmental management, remediation, and long term stewardship of low-level burial sites.

CLOSING COMMENTS

The Cold War legacy persists in tanks, trenches, casks, and the environment and future generations must continue to confront the challenges of safe, permanent storage and disposal. Today, nuclear power is the safest, cleanest, and most cost effective source of energy and increased production will likely become necessary for future generations throughout the developing world, but the reality is that nuclear waste will accumulate and future nuclear disasters and accidental releases are inevitable. At the time of this writing the Fukushima Daiichi nuclear plant disaster is unfolding in Japan following an 8.9 magnitude earthquake and damaging tsunami. We can look to the past for examples of the lasting affects of nuclear production. While it can be inherently difficult to quantify precisely the long term impacts of nuclear exposure on humans and other mammals, genetic and molecular adaptations caused directly by the Chernobyl disaster and chronic exposure to radioisotopes are evident (e.g.,Kovalchuk et al., 2004, Vornam et al., 2004, Zhdanova et al., 2004). Heavily degraded lands and those impacted by long lived pollutants (e.g., the exclusion zones around Chernobyl and very likely the Fukushima reactor, Japan) will be of little to no value for future generations. Stabilization, remediation, and long term stewardship of these lands will not be a trivial task; inorganic pollutants are only affected, and thus controlled, by complex biogeochemistry which dictates solubility and reactivity, and unlike organic pollutants, safe removal by decomposition pathways is not an option. Microbes do have an important role to play but these processes are difficult to parameterize; we lack the fundamental understanding of the complex and often slow reactions that can occur with many inorganics over the life cycle of these materials. Microorganisms will interact with and microbial catalyzed processes will affect nuclear waste. Only through continued investigation will we be able to decipher the magnitude and outcome of these interactions, but through this effort we will also establish our ability to harness and control these activities in order to achieve a desirable end state.

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