Engineering radiation-resistant bacteria for environmental biotechnology

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Seventy million cubic meters of ground and three trillion liters of groundwater have been contaminated by leaking radioactive waste generated in the United States during the Cold War. A cleanup technology is being developed based on the radiation-resistant bacterium *Deinococcus radiodurans,* which is being engineered to express bioremediating functions.

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Abbreviations
DOE Department of Energy
TCE trichloroethylene

Introduction

Immense volumes of radioactive waste, generated from the production of over 46,000 nuclear weapons in the United States between 1945 and 1986 [1], were disposed of directly to the ground; this is a period when national security priorities often surmounted concerns over the environment. These wastes contain inorganic and organic contaminants that include radionuclides, heavy metals, acids/bases, and solvents [1]. In the United States, buried radioactive wastes (3×10^6 m^3) have contaminated about 7×10^7 m^3 of surface and subsurface soils and about 3×10^{12} dm^3 of groundwater [2••]. Environmental nuclear contamination is believed to be even more severe in the former Soviet Union [3]. With the end of the Cold War in the early 1990s, The United States Department of Energy (DOE) shifted its emphasis from nuclear weapons production to stabilization and cleanup of these waste environments. This remediation effort is now the largest program of its kind ever undertaken by the United States [2••].

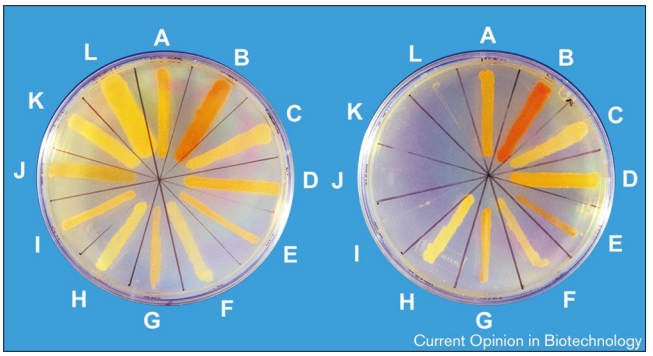
In 1992, the DOE surveyed 91 out of 3000 contaminated sites at 18 United States research facilities [1]. The most common contaminants from DOE wastes that have been found in ground and groundwaters include the radionuclides ^{235}uranium $(\gamma,\alpha)^E$, ^{238}plutonium $(\alpha)^E$, ^{99}technetium $(\beta^-)^E$, ^{90}strontium $(\beta^-)^E$, and ^{137}cesium $(\gamma,\beta^-)^E$, and the metals chromium, lead and mercury along with a myriad of toxic organic compounds (e.g. toluene and trichloroethylene [TCE]) [1]. One third of the 91 characterized sites are radioactive, with some reported radiation levels as high as 10 mCi/L within or close to the contaminating sources [1]. These high radiation levels, in combination with the chemical hazards, are extremely damaging to living organisms over extended periods, often resulting in cell death.

Of the 3000 waste sites disclosed by the DOE, the total cleanup cost, by methods that utilize costly pump and treat technologies and/or soil excavation and incineration, was estimated recently as being between $189 and $265 billion (see http://www.em.doe.gov/bemr96 for the 1996 *Baseline Management Report*). DOE budget projections for cleanup activities for the next 10 years exceed $60 billion [2••]. These vast waste sites are therefore potential targets for less expensive *in situ* bioremediation technologies utilizing specialized microorganisms that can detoxify both metallic and organic contaminants. However, the utility of microbiological methods for the primary treatment of highly radioactive environmental wastes will largely be determined by, firstly, the ability of microorganisms catalyzing the desired function(s) to survive and function under radiation stress, and secondly, the ability of basic research to produce bioremediation systems that do not cause undesired secondary effects that threaten the general public or further damage the environment.

Numerous bacteria (including *Shewanella* and *Pseudomonas* spp.) have been described and studied in detail for their ability to transform, detoxify, or immobilize a variety of metallic and organic pollutants [4–11]. Like most organisms, however, these bacteria are sensitive to the damaging effects of radiation (Figure 1j,l) [12], and their use in bioremediation will probably be limited to environments where radiation levels are very low. By developing microbiological techniques suitable for intervention in areas close to or within leaking sources where radiation levels are highest, stabilization/decontamination efforts could begin before the pollutants disseminate into the environment. Therefore, radiation-resistant microorganisms that can be used for environmental cleanup need to be found in nature or engineered in the laboratory to address this problem. Remarkably, highly radioactive DOE waste sites have not yet been surveyed for their microbial ecology, where natural selection may have already yielded bacteria with favorable bioremediating characteristics. The isolation of radiation-resistant bacteria is easy, even from non-extreme environments; one highly effective method is to select for growth on solid nutrient-rich medium incubated in the presence of chronic gamma radiation (6000 rad/hour). Numerous novel bacteria have been isolated this way including an example shown in Figure 1h. A collection of extremely radiation-resistant bacteria is being assembled at Uniformed Services University of the Health Sciences, and readers of this review are invited to participate in this global survey by sending soil samples (1 cm^3) to the author for screening.

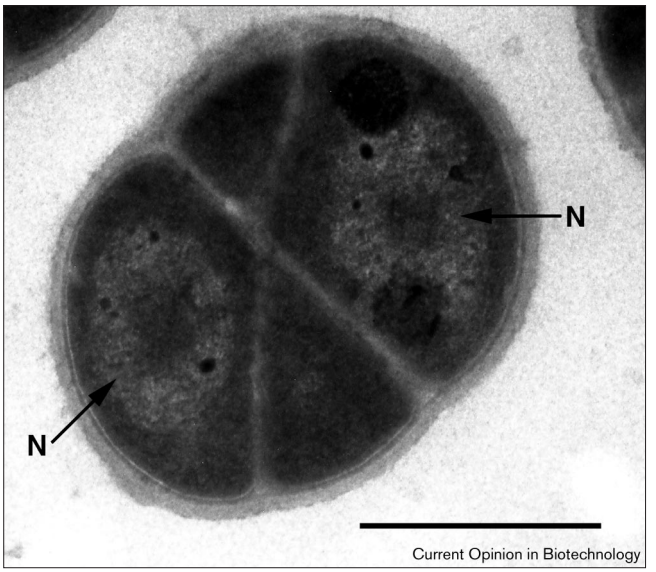
Most radiation-resistant bacteria that have been reported are spore-formers and are not remarkably radiation

Figure 1



Growth of bacteria in the presence of chronic irradiation (6000 rad/hour). Left, control plate incubated in the absence of radiation. Right, plate incubated in the presence of 6000 rad/hour gamma radiation (^{137}Cs). **(a)** *Deinococcus radiodurans*; **(b)** *Deinococcus radiopugnans*; **(c)** *Deinococcus grandis*; **(d)** *Deinococcus proteolyticus*; **(e)** *Deinococcus murrayi*; **(f)** *Deinococcus geothermalis*; **(g)** *Deinococcus radiophilus*; **(h)** novel deinococcal species isolated from an elephant's trunk (National Zoo, Washington DC); **(i)** *D. radiodurans* rec30 (*recA*$^-$); **(j)** *Shewanella oneidensis* (MR-1); **(k)** *Escherichia coli* (strain BL308 containing the *merA* operon); **(l)** *Pseudomonas putida* (KT2440).

Figure 2



Electron micrograph of a *D. radiodurans* tetracoccus showing wall structure and nucleoid morphology. Note that the nucleoids (**N**) are ring shaped. Bar = 1 μm.

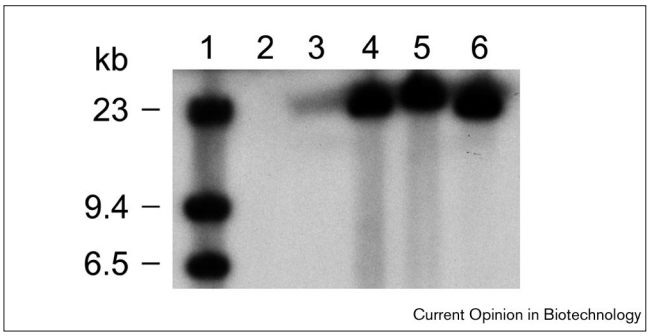
resistant when growing vegetatively; many of them are pathogens and most lack a developed system for genetic manipulation [12,13]. A few vegetative bacteria, such as *Enterococcus faecium* and *Alcaligenes* spp., show high resistance to irradiation, but often these too are pathogenic [13] and, therefore, would not be suitable for bioremediation. Bacteria belonging to the family *Deinococcaceae* are distinctly unusual [14,15•]. Not only are they the most radiation-resistant organisms discovered, but they are vegetative, easily cultured, and nonpathogenic. Despite ubiquitous distribution and ancient derivation, only seven species of the family *Deinococcaceae* have been described [15•,16]. Of these species, *Deinococcus radiodurans* is the only one for which a system of genetic transformation and manipulation has been developed [17–20].

The bacterium *D. radiodurans* is probably the first polyextremophile to be identified, showing remarkable resistance to a range of damage caused by ionizing radiation, desiccation, ultraviolet radiation, oxidizing agents, and electrophilic mutagens [15•,21,22]. It is an aerobic, large tetrad-forming soil bacterium (Figure 2) [14] that is most famous for its extreme resistance to ionizing radiation; it not only can survive acute exposures to gamma radiation that exceed 1,500,000 rad without lethality or induced mutation, but it can also grow continuously in the presence of chronic radiation (6000 rad/hour; Figure 1a) without any effect on its growth rate or ability to express cloned genes [23••]. For comparison, vegetative cells of *Bacillus* spp. cannot grow at 6000 rad/hour and *Bacillus* spores show five orders of magnitude decreases in viability following an acute exposure to 200–1000 krad [12]. Adding to the growing resource of genetic technologies available for *D. radiodurans* is the recent whole-scale sequencing, annotation and analysis of its genome [24,25,26•]. This combination of factors has positioned *D. radiodurans* as an excellent candidate for development of safe microbiological treatments of contaminants present at many DOE facilities. By exploiting functional genomics and the genetic engineering technologies, it should be possible to engineer *D. radiodurans* for both metal remediation and organic toxin degradation within these radioactive sites.

Engineering *Deinococcus radiodurans* for bioremediation

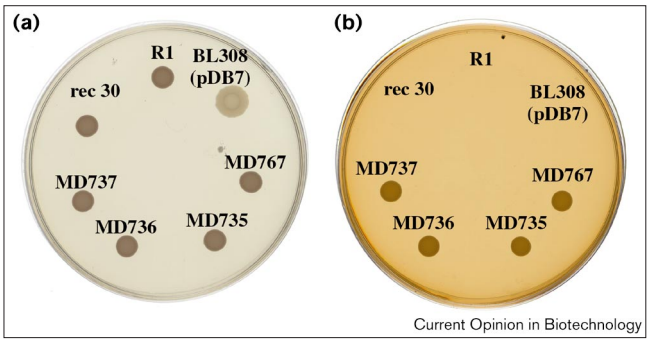
Research aimed at developing *D. radiodurans* for bioremediation began in 1997 with the demonstration that it can grow in the presence of ionizing radiation at 6000 rad/hour [23••], comparable to the most radioactive DOE waste sites. In fact, all reported members of the *Deinococcaceae* can grow at this dose rate (Figure 1a–g) and are poised to contribute their individual characteristics to this developing technology. For example, *Deinococcus geothermalis* (Figure 1f) grows optimally at ~50°C [16], and recently it has been shown that the expression systems developed for *D. radiodurans* work in this thermophile (Figure 3). As such, it is probable that the genetic technology being developed for *D. radiodurans* will be readily transferable to *D. geothermalis*, which could be useful in thermally insulated radioactive environments (e.g. within or beneath leaking tanks) where temperatures can be elevated due to radioactive decay. Initially, growth of the *Deinococcaceae* during high-level chronic irradiation exposure was thought to be unlikely as it had been reported

Figure 3



Transformation of *D. geothermalis* with an autonomously replicating 26 kilobase plasmid (pMD66) designed for *D. radiodurans* [18]. pMD66 contains a *D. radiodurans* origin of replication and two distinct deinococcal promoters for expressing cloned genes; these are functional in *D. geothermalis* growing at 50°C. DNA prepared from the indicated strains was digested with *Eco*RI, electrophoresed, and subjected to Southern blotting with a radiolabeled pMD66 probe. Lanes: 1, λ phage cut with *Hind*III; 2, *D. geothermalis* (wild type); 3, *D. radiodurans* R1 (wild type); 4, *D. geothermalis* + pMD66; 5, *D. radiodurans* + pMD66; 6, purified pMD66 cut with *Eco*RI.

Figure 4



Effect of continuous exposure to gamma radiation and mercury(II) on the growth of strains containing different copy numbers of the *mer* operon [34••]. 1 x 10^5 cells of each of the indicated strains were spotted onto **(a)** a nutrient agar plate and **(b)** a second nutrient agar plate containing 30 μM Hg(II) (Merbromin). Following plate inoculation, plate (b) was placed into a ^{137}Cs irradiator (6000 rad/hour) for incubation for five days. The control plate (a) was incubated at the same temperature in the absence of radiation for the same time. Strain identification: R1, wild type *D. radiodurans*; BL308, wild-type *E. coli* with the mercury-resistance plasmid pDB7; MD767, R1 with 10 *merA* chromosomal copies; MD735, R1 with 1 *merA* copy present on pMD66; MD736, R1 with 20 *merA* chromosomal copies; MD737, R1 with 150 *merA* chromosomal copies; rec30, *recA*$^-$ *D. radiodurans*.

that DNA replication in *D. radiodurans* ceases upon DNA damage [14,15•]. As manifested by their growth in a ^{137}Cs irradiator, however, these bacteria (Figure 1a–g) are proficient at simultaneous semi-conservative DNA replication and homologous recombination, as modeled previously [27]. *D. radiodurans* has recently been tested with a variety of cloned bioremediating gene functions that can be expressed during growth at 6000 rad/hour, and these engineered strains are being used in the design of more complex bioremediation systems.

Metal remediation

The ability of a microorganism to resist the toxic effect of metals is frequently associated with its ability to transform those metals to less toxic chemical states [4,5,8,9,28,29]. A variety of metal resistance/reduction genes are being examined in *D. radiodurans* to determine if they confer resistance to common metallic waste constituents, as well as their ability to transform those metals. Generally, the solubility of metals is reduced at lower oxidation states, and enzymes catalyzing metal-reducing functions are becoming important components of metal immobilization strategies [4,30]. The radiation-sensitive bacterium *Shewanella oneidensis* (formerly *S. putrefaciens*) strain MR-1 (Figure 1j), which is highly effective at reducing soluble U(VI), Cr(VI), and Tc(VII) to insoluble U(IV), Cr(III), and Tc(IV) precipitates [31,32], respectively, has been subjected to whole-scale genomic sequencing (JF Heidelberg, J Eisen, C Fraser, abstract 13, 7th Conference on Small Genomes, 13–17 November 1999, Arlington, VA) and could contribute to the array of genes targeted for expression in *D. radiodurans*. A second approach to designing *D. radiodurans* for metal remediation is to expand on any natural metal-remediating capabilities it may have. Surprisingly, anaerobic cultures of *D. radiodurans* can reduce U(VI) and Tc(VII) in the presence of humic acids, and Cr(VI) can be reduced in the absence of humic acids [33]. Using its genomic sequence as a guide to manipulation, it may be possible to modify and enhance these functions by genetic engineering.

Mercury, chromium, and lead are among the most prevalent heavy-metal contaminants in DOE wastes and a series of genetic vectors that encode resistance to these metals have been constructed and are being examined in *D. radiodurans*. For example, the highly characterized *merA* locus from *Escherichia coli* has been cloned into *D. radiodurans* [28]. *merA* encodes mercuric ion reductase (MerA), which reduces highly toxic, thiol-reactive mercuric ion, Hg(II), to much less toxic and nearly inert elemental and volatile Hg(0). Four different *D. radiodurans* expression systems were developed and used to regulate *merA* expression by varying its cellular gene dosage [34••]. In short, *D. radiodurans* strains expressing MerA during growth at 6000 rad/hour were, firstly, resistant to the bacteriocidal effects of ionic Hg(II) at concentrations (30–50 μM) well above the highest concentration reported for mercury-contaminated DOE waste sites (10 μM) [1] (Figure 4), and secondly, able to efficiently reduce toxic Hg(II) to Hg(0). Other metal reducing/resistance functions that have been cloned into *D. radiodurans* and are being studied include genes from the following organisms that are specific for the indicated metal ions: *Desulfovibrio vulgaris* (*cyt*c3), U(VI); *Ralstonia eutrophus* CH34 (*czc*), Cd(II), Zn(II), and Co(II); and *Bacillus thuringiensis*, Cr(VI).

Toxic organic compound degradation

The use of *in situ* bioremediation for organic toxin-contaminated soils and groundwaters also poses as a viable alternative to conventional physicochemical treatments. Typical organic solvents present in mixed DOE wastes — benzene, toluene, ethylbenzene, and xylenes (collectively called BTEX) — are known growth substrates for some organisms such as *Pseudomonas putida*, of which the genetics and biochemistry have been studied in great detail [7,8,10,11], aided recently by the whole-scale sequencing of its genome (ER Heim, ERB Moore, M Stratz, KN Timmis, abstract 37, 7th Conference on Small Genomes, 13–17 November 1999, Arlington, VA). Furthermore, it has been shown that co-contaminating haloorganic solvents, such as TCE, are biotransformed (co-oxidized) during aerobic metabolism of certain aromatic compounds (e.g. toluene). This is a result of the broad-specificity of oxygenases from the toluene catabolic pathways that can, typically, co-oxidize TCE [7]. With respect to DOE facilities, there has been no adequate method for microbiological treatment of contaminated waste sites containing both hazardous organic and radioactive components because organisms such as *Pseudomonas* spp. are very radiation sensitive [12] (Figure 1l).

Using an engineering approach similar to that described above for the construction of Hg(II)-remediating strains, organic toxin degrading *D. radiodurans* strains have been constructed. The toluene dioxygenase genes (*todC1C2BA*) of *P. putida* were functionally expressed in *D. radiodurans* [23••]. During chronic irradiation, these strains were able to oxidize toluene, chlorobenzene, and 3,4-dichloro-1-butene. In the presence of toluene, *D. radiodurans* containing *todC1C2BA* produced toluene-*cis*-dihydrodiol, which was further metabolized to 3-methylcatechol by a native non-specific dehydrogenase. Once formed, catechols readily polymerize to form insoluble polymers, which turn dark brown in the presence of toluene over the course of several days to weeks, and this has been observed in *tod*-engineered *D. radiodurans* strains. Other *Pseudomonas* catabolic genes that convert 3-methylcatechol to pyruvate have been introduced into *todC1C2BA*-containing *D. radiodurans*, which might yield a strain that is able to mineralize toluene and related compounds.

Genome flexibility

D. radiodurans shows remarkable genome plasticity. It is able to maintain, replicate and express extremely large segments of foreign DNA inserted into its genome by tandem duplication [17,34••]. This capability has been exploited recently to show that it can accommodate and functionally express highly amplified DNA duplication insertions encoding bioremediation functions. A strain was constructed expressing >100 copies per cell of both the *mer* and *tod* operons described above [34••]. This strain supports >2,000,000 basepairs of foreign DNA and it can metabolize toluene or chlorobenzene while at the same time resisting and reducing toxic ionic Hg(II) to volatile elemental Hg(0). There are good prospects, therefore, for introducing into a single *D. radiodurans* host the many different bioremediating gene systems that will be necessary for cleanup of heterogenous radioactive waste environments.

Physiology, radiation resistance and bioremediation

Adding to the challenge of surviving the harsh radioactive, metallic, and organic properties of DOE waste sites is the probability that *D. radiodurans* might be limited by several inherent physiologic constraints. For example, genomic informatics shows that the amino acid biosynthetic pathways for serine, cysteine and lysine are incomplete in wild-type *D. radiodurans*. One major thrust of current research involves characterization of its physiology and optimization of the external parameters for growth and survival in adverse radioactive environments. For example, under optimal growth conditions *D. radiodurans*' DNA repair capabilities are extremely well suited to survive either acute or chronic irradiating exposures [19,23••]; however, *D. radiodurans* is unable to grow and is rapidly killed in certain nutrient poor radioactive environments that support luxuriant *D. radiodurans* growth when radiation is absent. This phenotypic reversal from radiation resistance to sensitivity is of great interest and concern as it questions the suitability of *D. radiodurans* as a bioremediation host in radioactive waste sites. A combination of growth studies and analysis of the complete *D. radiodurans* genomic sequence has identified several defects in *D. radiodurans*' global metabolic regulation that limit carbon, nitrogen and DNA metabolism [35]; this was not necessarily unexpected given that *D. radiodurans* strain R1 has been maintained on synthetic nutrient rich medium for the past forty years. In nutrient-restricted conditions, DNA repair was found to be limited by this organism's metabolic capabilities and not by any nutritionally induced defect in genetic repair. This information has been used successfully as a guide to identify amino acids and vitamins that restore luxuriant growth of *D. radiodurans* in nutritionally restricted radioactive environments. Analyses such as these, coupled to the possible correction of genetic defects, will facilitate the design of *in situ* bioremediation protocols for this organism.

Conclusions

Physicochemical cleanup technologies that could be used to decontaminate the immense volume of soils, sediments, and groundwaters at DOE facilities are prohibitively expensive and dangerous. The use of microorganisms to stabilize and/or detoxify these waste environments is a viable alternative. A bioremediation strategy based on the radiation-resistant bacterium *D. radiodurans* is being developed for possible intervention within or close to the contamination sources where radiation levels are highest and where the use of radiation-sensitive organisms is precluded. A genetic system for *D. radiodurans* that is suitable for expression of proteins in

highly radioactive environments has been successfully tested using metal reducing and organic compound degrading genes. The physical, chemical, and physiologic constraints imposed on this bacterium by DOE wastes are being addressed incrementally in a coordinated way by genetic engineering. To date, the proposed use of *D. radiodurans*, and possibly *D. geothermalis*, for treatment of environments where radiation is the principle factor limiting microbial survival and function appears to be a realistic approach given these early data.

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