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## Recombinant *D. radiodurans* cells for bioremediation of heavy metals from acidic/neutral aqueous wastes

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The stability and superior metal bioremediation ability of genetically engineered *Deinococcus radiodurans* cells, expressing a non-specific acid phosphatase, PhoN in high radiation environment has already been established. The lyophilized recombinant DrPhoN cells retained PhoN activity and uranium precipitation ability. Such cells also displayed an extended shelf life of 6 months during storage at room temperature and showed surface associated precipitation of uranium as well as other metals like cadmium. Lyophilized cells, immobilized in polyacrylamide gels could be used for uranium bioprecipitation in a flow through system resulting in 70% removal from 1mM input uranium solution and a loading of 1 g uranium/g dry weight cells. Compared with a batch process which achieved a loading of 5.7 g uranium/g biomass, the efficiency of the column process was low due to clogging of the column by the precipitate.

### Introduction

Bacteria like *Citrobacter* and *Pseudomonas* sp. have the ability to transform, detoxify or immobilize a variety of metallic and organic pollutants.<sup>1,2</sup> However, like most organisms, these bacteria are very sensitive to ionizing radiation, and their use for bioremediation in high radiation environments is very limited. Aqueous liquid nuclear waste generated during the reprocessing of spent fuel rods contains high levels of radioactivity.<sup>3</sup> Actinides present in this waste along with other fission

products emit highly damaging beta and gamma radiation. Use of radio-resistant microbes is an essential prerequisite for bioremediation in such an environment.

In recent years, the extremely radio-resistant Gram positive bacterium, *Deinococcus radiodurans*, has become an organism of choice for engineering a number of strategies for bioremediation of radioactive waste.<sup>4-6</sup> The genus, *Deinococcus* comprises of a large number of radioresistant species, many of which also display desiccation, temperature and metal tolerance (*D. deserti*, *D. geothermalis* and *D. indicus*).<sup>7-9</sup> The most studied, *D. radiodurans* strain R1 has a small 3.28 Mb genome comprised of two chromosomes (2.64 Mb and 0.41 Mb), a megaplasmid (0.18 Mb) and plasmid (0.045 Mb) and can withstand exposure to 5–6 kGy of ionizing radiation without significant loss of viability.<sup>10</sup> Such extreme radioresistance of the strain stems from its phenomenal DNA repair proficiency. Multiplicity of genome organized in a torroid ring-like structure, possession of novel genes for repair of damaged DNA (Extended Synthesis-Dependent Strand Annealing, PprA etc.), Mn(II) complexes which protect cellular enzymes from oxidative stress damage and efficient damage cleaning systems that restore homeostasis post-irradiation, all contribute to radioresistance.<sup>11-15</sup> Comparative genomes of at least three deinococcal strains has revealed commonality of genes, molecular mechanisms underlying radioresistance across the species.<sup>16</sup>

Attempts to introduce novel bioremediation capabilities into *D. radiodurans*

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have been successful. Strategies involving both chromosomal integration as well as vector based expression of foreign genes in trans have proved effective in this organism.<sup>4-6</sup> Explorations on the possibility of using *D. radiodurans* in high radiation environments also revealed the inherent organic solvent tolerance<sup>4</sup> and metal reduction ability<sup>17</sup> of this organism. Taken together, *D. radiodurans* has emerged as an organism which is easy to genetically manipulate and is also radiation resistant with reasonable inherent pollutant tolerance. These traits make this organism ideally suited for remediation of nuclear waste sites.

Recombinant strains of *D. radiodurans* expressing toluene dioxygenase (TDO) from *Pseudomonas putida* F1 could oxidize a variety of organic substrates, while strains carrying the *merA* operon could reduce toxic ionic Hg(II) to the less toxic elemental mercury Hg(0).<sup>4,5</sup> Importantly, the recombinant strains could grow in the presence of both the pollutant and  $\gamma$ -radiation at 60Gy/h. Improvements in the bioremediation capabilities of *D. radiodurans* were brought about by pyramiding different genes into a single strain to bring about complete, simultaneous degradation of organic pollutant (complete oxidation of toluene by expression of *tod* and *xyl* genes)<sup>18</sup> as well as to impart metal detoxification and organic pollutant degradation ability (toluene and mercury detoxification by expression of *tod* and *mer* operons)<sup>5</sup> for mixed waste.

We have earlier reported uranium bioprecipitation by genetically engineered *D. radiodurans* expressing a nonspecific acid phosphatase, PhoN. In *E. coli*, the enzyme resides in periplasm and cleaves a phosphomonoester to generate high, local concentrations of inorganic phosphate which can precipitate uranium (Fig. 1). The corresponding gene, *phoN* was cloned from *Salmonella enterica* serovar Typhi and expressed under the influence of a strong deinococcal *groESL* promoter ( $P_{groESL}$ ) in both *E. coli* and *D. radiodurans*.<sup>19,20</sup> The PhoN expression in recombinant bacteria was qualitatively ascertained on histochemical plates containing phenolphthalein diphosphate (PDP) and methyl green (MG). On such plates, colonies which are positive for acid

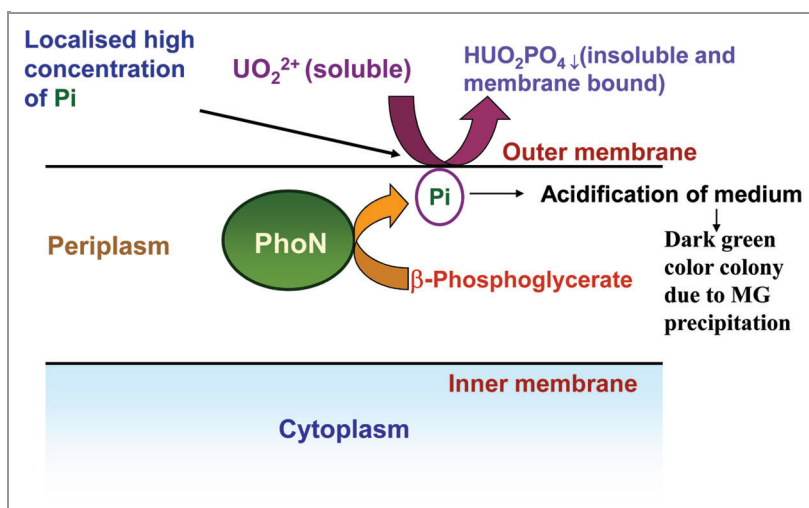
phosphatase appear dark green due to precipitation of methyl green at acidic pH caused by the release of inorganic phosphate ions (Fig. 1).<sup>21</sup> The liberated inorganic phosphate from a suitable substrate molecule like  $\beta$ -glycerophosphate, causes precipitation of metals as cell-bound metal phosphates and facilitates their easy removal from aqueous solution. Similar approach was also designed for uranium precipitation from alkaline solutions by cloning a novel, high specific activity alkaline phosphatase (PhoK) from *Sphingomonas* and overexpressing it in *E. coli*.<sup>22</sup> The recombinant *E. coli* overexpressing 55 times higher levels of PhoK, very efficiently precipitated uranyl carbonate from dilute solutions at pH 9.0. PhoK gene is currently being engineered into *D. radiodurans*. The mechanism for metal precipitation is essentially extracellular and de-coupled from growth. Therefore, the chemical toxicity of waste is not a deterrent in use of the recombinant strain.

### Application of DrPhoN Cells for Metal Removal

Our earlier studies using the recombinant *E. coli* (EcPhoN) and *D. radiodurans* (DrPhoN) cells expressing the *phoN* gene from the deinococcal  $P_{groESL}$  promoter showed that both the in-gel as well as the cell bound PhoN activities were higher in recombinant cells of *E. coli* than in

*Deinococcus*. A possible explanation for this may lie in the six layered cell wall which *D. radiodurans* is known to possess.<sup>23</sup> The precise localization of the PhoN enzyme among these six layers is not known since the periplasm is poorly defined in *Deinococcus*. But, this may limit access of substrate and its availability and result in lower activities.

The lower PhoN activity of the recombinant *Deinococcus* clones was compensated by using higher cell density to obtain the required activity.<sup>19</sup> When uranium precipitation was performed with EcPhoN and DrPhoN cells carrying equal PhoN activity ( $\sim 2000$  U as determined by *p*-nitrophenyl phosphate assays), the kinetics of uranium precipitation was nearly identical in both strains with  $>90\%$  uranium precipitated in approximately 3h (Fig. 2A). Appropriate controls were included to ensure that the observed precipitate was due to  $\beta$ -glycerophosphate dependent PhoN activity and not a result of spontaneous chemical precipitation under the experimental conditions used.<sup>19</sup> Controls were also included to correct for possible uranium loss due to non-specific binding to cells and to the container. Notwithstanding the difference between *E. coli* and *Deinococcus phoN* clones, equivalent PhoN activities from both the recombinant clones exhibited efficient and equal uranium precipitation capabilities (Fig. 2A). The chemical nature of the precipitate



**Figure 1.** Schematic representation of a periplasm-located phosphatase-mediated bioprecipitation of uranium and the histochemical approach for detection of phosphatase-expressing bacteria.

was revealed by powder X-ray diffraction (XRD) studies to be uranyl hydrogen phosphate ( $\text{HUO}_2\text{PO}_4$ ) (Fig. 2B). Further, when recombinant EcPhoN and DrPhoN strains were subjected to very high doses of ionizing  $^{60}\text{Co}$ -gamma radiation (1 to 6 kGy), followed by challenge with 1mM uranyl nitrate, DrPhoN cells far outperformed the corresponding EcPhoN cells. The recombinant DrPhoN strain retained its uranium precipitation ability even after 6 kGy of  $^{60}\text{Co}$ -gamma irradiation. In contrast, EcPhoN cells showed severe inhibition of uranium precipitating ability at doses greater than 1 kGy.<sup>19</sup>

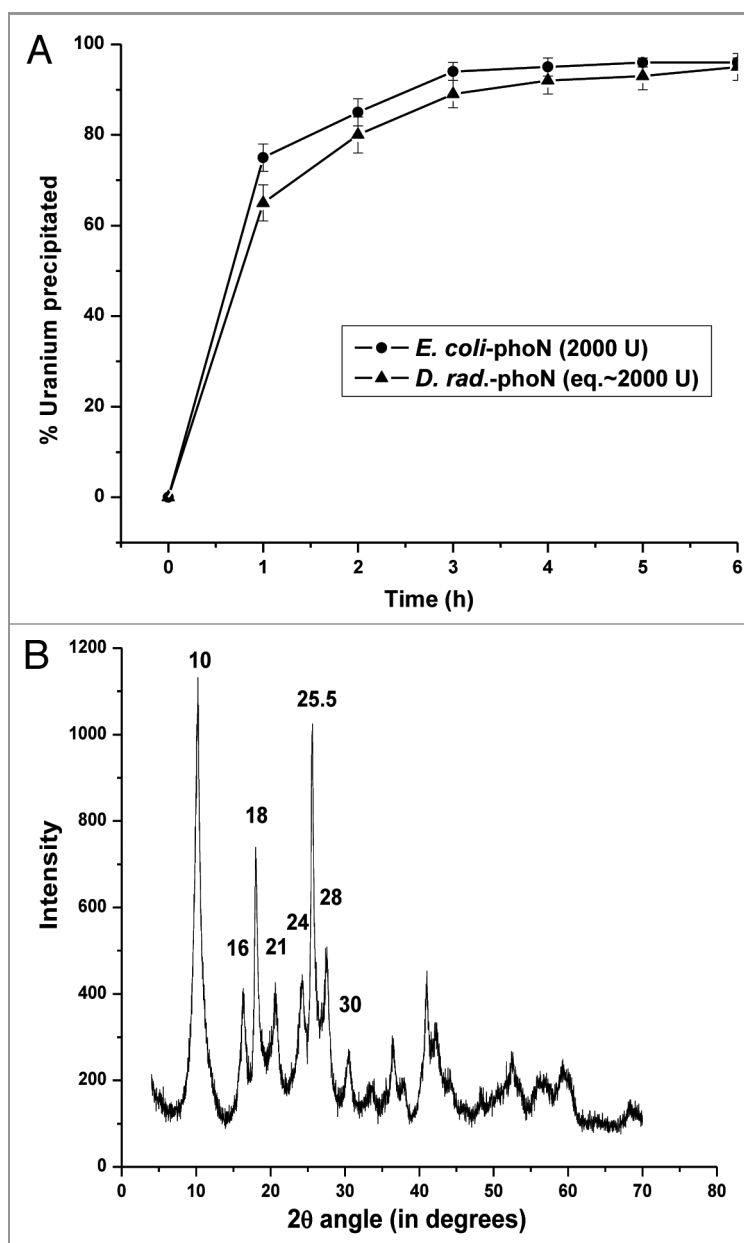
### Surface Bioprecipitation of Metals Circumvents Metabolic Toxicity of Metals

Cell surface association of the bioprecipitated uranium was confirmed in DrPhoN cells by scanning electron microscopy wherein the uranyl phosphate precipitate appeared as small needle like structures covering the entire cell surface.<sup>24</sup> It has been hypothesized that cell associated metal precipitation is initiated at nucleation sites present on the cell surface.<sup>25</sup> In *Citrobacter*, the high content of phosphates in extracellular polysaccharide acts as complexation sites for the incoming metal ion and the initial nucleation site is consolidated by continuous addition of phosphate ligand generated by the enzymatic process.<sup>25,26</sup> The fact that a variety of organisms tested so far, including *E. coli*,<sup>27</sup> *D. radiodurans*<sup>19</sup> and *Sphingomonas*<sup>22</sup> could bring about cell bound metal precipitation indicates that the cell surface structures required for metal precipitation are not very specific but are of a more general character, across different bacteria and sufficient for efficient metal precipitation and loading.

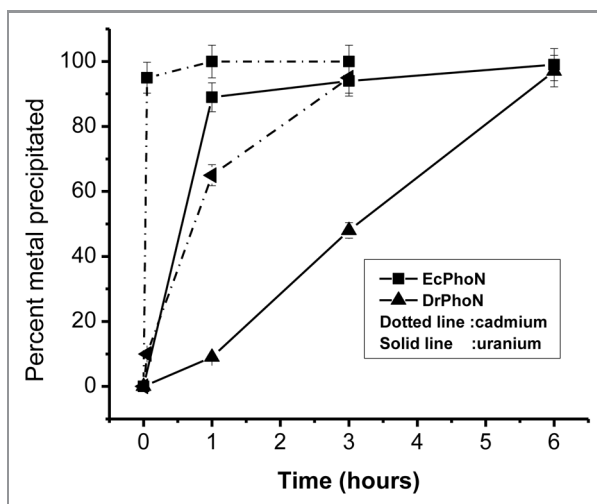
Phosphatase mediated bioprecipitation can be applied to a wide range of metals which form insoluble phosphates. Compared with uranium, EcPhoN and DrPhoN cells could precipitate 90% of 1mM cadmium from solution much more rapidly; in 3 h (Fig. 3). This is probably because of the lower solubility product of cadmium phosphate compared with uranyl phosphate. An important observation of this experiment was that the toxicity of

the metal to the organisms per se, did not affect their corresponding precipitation efficiency. *D. radiodurans* is a metal sensitive bacterium with a MIC of 0.018 mM for cadmium compared with 0.5–1 mM for *E. coli DH5 $\alpha$* .<sup>28</sup> However, both organisms could precipitate out the metal at comparable efficiencies, commensurate

with their phosphatase activity. This is an indication that in spite of the high metal sensitivity of *D. radiodurans*, its potential for phosphatase mediated bioprecipitation of metals is not affected. Perhaps extracellular precipitation shields the bacterium from metabolic ill effects of metals.



**Figure 2.** Uranium precipitation by PhoN expressing recombinant bacterial strains. (A) Comparison of uranium precipitation by EcPhoN and DrPhoN cells possessing equivalent PhoN activities. Cells possessing equivalent activity were incubated with 1 mM uranyl nitrate and 5 mM  $\beta$ -glycerophosphate in 2 mM acetate buffer (pH 5.0) at room temperature under static condition and loss of uranium from test solution was determined by Arsenazo-III reagent. (B) X-ray diffraction (XRD) analysis of the precipitate obtained in the cell pellet fraction after uranium precipitation assay with DrPhoN cells. The precipitate formed was identified as uranyl hydrogen phosphate ( $\text{HUO}_2\text{PO}_4$ ) by comparison with standard database.



**Figure 3.** Kinetics of bioprecipitation of uranium and cadmium by EcPhoN and DrPhoN cells. DrPhoN and EcPhoN cells (O.D<sub>600nm</sub> 1.0) were used to precipitate 1 mM cadmium chloride or uranyl nitrate from solution using 5 mM  $\beta$ -glycerophosphate in 2mM acetate buffer (pH 5.0). Timed aliquots were taken, cell suspension was subjected to centrifugation and the metal remaining in the supernatant was determined. Arsenazo-III reagent was used for estimation of uranium, while cadmium was estimated using Atomic Absorption Spectrophotometer.

### Uranium Precipitation: Batch vs. Flow-Through Process

With the objective of simplifying environmental application of recombinant PhoN expressing bacteria for metal bioremediation, cells were subjected to lyophilization. Lyophilized EcPhoN and DrPhoN cells retained phosphatase activity as well as uranium precipitation ability for up to six months of storage at room temperature.<sup>24,27</sup> Further, such lyophilized cells could be immobilized in polyacrylamide gels and packed into columns to construct a flow-through system for uranium precipitation. When gravity based flow-through column was used for uranium precipitation, a loading of 0.73 g uranium/g dry weight of biomass was achieved. An improvement in operation of the column was attempted by using a bigger column (2.5 cm I.D  $\times$  50 cm H, 90 ml void volume) and passing the assay solution upwards using a peristaltic pump. The results indicated that lyophilized cells (immobilized in acrylamide) remained stable throughout the operation of the column and over a long period of time. Nearly 70% removal of the input uranium concentration could be achieved when the column was operated at a flow rate of 38 ml/h. With time, the uranium

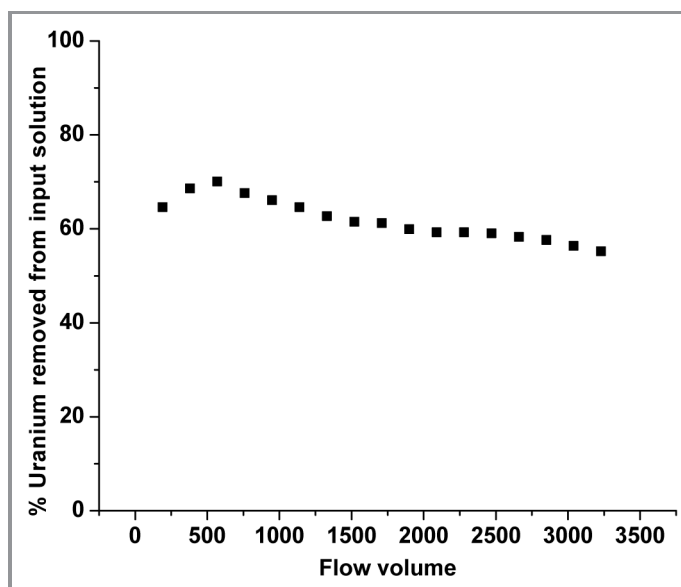
removal efficiency decreased since the column started to clog and resist flow of solution (Fig. 4). A maximum loading of 1 g uranium/g dry weight biomass could be achieved in the flow through process.

Our studies showed that batch operation was more suitable than column

operation of this system, especially since over a period of time the column was clogged by the precipitated uranium. Compared with a loading of 5.7 g uranium/g dry weight biomass achieved in a batch process, this may not seem attractive, however, the flow-through system has the advantages of continuous operation and ease of handling. More porous matrices are being explored to circumvent problems related to clogging of the column.

### Conclusion

The superior uranium precipitation ability of DrPhoN cells in high radiation environments had already been established. The ability to precipitate other toxic metals like cadmium, amenability for use in batch and continuous process and improved shelf life and ease of application achieved through lyophilization have been recent value additions to this strain. A number of further improvements are desirable to bring PhoN based metal precipitation technology to its full potential. These include (a) alternative, cheap substrate for phosphatase in place of  $\beta$ -glycerophosphate in order to make the process economically viable, (b) engineering



**Figure 4.** Column-based uranium removal from aqueous flow by immobilized DrPhoN cells. DrPhoN cells were immobilized in polyacrylamide gel and loaded into a column of bed volume, 90 ml. A solution containing 1 mM uranyl nitrate and 5 mM  $\beta$ -glycerophosphate in 2 mM acetate buffer was passed upwards through the column using a peristaltic pump at a flow rate of 38 ml/h. The uranium remaining in the flow through was estimated to determine percent uranium removed.

the phosphatase to localize closer to the cell surface especially in an organism like *D. radiodurans* where multi-layered cell walls may seriously limit the substrate availability and metal access to the enzyme and (c) recombinant *Deinococcus* cells also need to be tested for a wide array of metals for even non-nuclear applications, such as in nickel cadmium

battery waste clean-up. These possibilities are currently being investigated.

Application of metal bio-precipitating *Deinococcus* strains for in situ bioremediation would require investigation on (a) survival and stability of such strains in actual waste sites, (b) better insight into the precise metal-microbe interaction in cells, (c) scaling up of such cells for

large scale use and (d) improvements in their bioprecipitation efficiency. Although *D. radiodurans* is not a human pathogen, the concerns related to use of genetically engineered microbe are relevant and will need to be adequately addressed by ensuring its environmental biosafety and ecological safety for application.

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