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Harnessing a radiation inducible promoter of *Deinococcus radiodurans* for enhanced precipitation of uranium

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ABSTRACT

Bioremediation is an attractive option for the treatment of radioactive waste. We provide a proof of principle for augmentation of uranium bioprecipitation using the radiation inducible promoter, P*ssb* from *Deinococcus radiodurans*. Recombinant cells of *D. radiodurans* carrying acid phosphatase gene, *phoN* under the regulation of P*ssb* when exposed to 7 kGy gamma radiation at two different dose rates of 56.8 Gy/min and 4 Gy/min, showed 8–9 fold increase in acid phosphatase activity. Highest whole cell PhoN activity was obtained after 2 h in post irradiation recovery following 8 kGy of high dose rate radiation. Such cells showed faster removal of high concentrations of uranium than recombinant cells expressing PhoN under a radiation non-inducible deinococcal promoter, P*groESL* and could precipitate uranium even after continuous exposure to 0.6 Gy/min gamma radiation for 10 days. Radiation induced recombinant *D. radiodurans* cells when lyophilized retained high levels of PhoN activity and precipitated uranium efficiently. These results highlight the importance of using a suitable promoter for removal of radionuclides from solution.

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1. Introduction

Bioremediation involves use of organisms for removal of toxic components present in the environment. This has been achieved both by using natural or genetically engineered strains to aid waste management (Valls and de Lorenzo, 2002). Choice of the appropriate organism depends on its ability to survive and efficiently express desired genes under conditions prevailing in waste sites. This necessitates use of radioresistant organisms for bioremediation of radioactive waste.

The Gram positive bacterium, *Deinococcus radiodurans* can tolerate high doses of ionizing radiation. Members of the Deinococcaceae family are vegetative, non-pathogenic, ubiquitous, and exhibit remarkable resistance to DNA damage caused by ionizing radiation, desiccation, ultraviolet radiation and electrophilic mutagens (Battista, 1997; Minton, 1994, 1996; Slade and Radman, 2011; Venkateswaran et al., 2000). *D. radiodurans*, which can survive 15 kGy ionizing radiation, is one of the most radiation-resistant members of this family. This has prompted its genetic engineering to express proteins capable of detoxifying organic solvents and heavy metals that prevail in radionuclide contaminated wastes (Brim et al., 2000, 2003; Lange et al., 1998). The organism has been extensively studied for its highly proficient DNA damage repair mechanisms that are aided by proteins rapidly induced upon irradiation (Misra et al., 2006; Zahradka et al., 2006). Radiation-enhanced gene expression in this organism appears to be regulated by transcriptional activation and may be exploited for enhanced expression of desired genes. This approach can be useful for radioactive waste management at radioactive storage sites, nuclear facilities and waste holding tanks.

One of the ways to augment levels of recombinant gene expression is to use appropriate promoters, which are recruited by the bacterium under specific growth conditions. Use of stress-inducible promoters provides a choice of gene expression control under defined stimuli. Bacterial promoters that are triggered by organic contaminants and regulate expression of proteins involved in degradation of the concomitant pollutants have found several applications (Diaz and Prieto, 2000; Girotti et al., 2008; Liu et al., 2010; Wise and Kuske, 2000). Likewise, promoters of genes involved in metal transport, sequestration or detoxification are induced in the presence of the target metal itself (Lloyd and Lovley, 2001; Summers, 2009; Yagi, 2007). To exploit the radiation resistant property of *D. radiodurans* for bioremediation, promoters that control expression of radiation inducible genes appear to be attractive candidates.

In bacteria, a majority of promoters possess consensus sequences of hexamer boxes, −10 (TATAAT) and −35 (TTGACA) with which the σ-factors interact and facilitate RNA polymerase

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binding (Burgess and Anthony, 2001) and transcription. In *D. radiodurans*, the upstream regions of 29 radiation-inducible genes often lack a consensus promoter sequence but contain a 17 bp palindromic sequence known as the Radiation/Dessication Response Motif (RDRM) (Makarova et al., 2007). The radiation inducible promoter for the *ssb* gene encoding the single stranded DNA-binding protein (SSB) harbors both consensus motifs and two RDRM sequences (Ujaoney et al., 2010). Upstream region of *groESL* gene, which codes for a chaperone protein and is not inducible by radiation, has the consensus promoter sequence and does not possess any RDRMs (Meima et al., 2001; Ujaoney et al., 2010).

In the past, *groESL* promoter, P*groESL*, from *D. radiodurans* was used to constitutively over-express *phoN* gene of *Salmonella enterica* serovar Typhi that encodes a non-specific acid phosphatase (NSAP) (Appukuttan et al., 2006). This enzyme can cleave a phosphomonoester substrate to release the phosphate moiety, which in turn can cause precipitation of metals such as uranium and cadmium from solutions (Misra et al., 2012). Recombinant *D. radiodurans* strain that carries a P*groESL*-*phoN* construct on plasmid pPN1 was shown to bring about metal precipitation even after being subjected to 6 kGy gamma radiation under non-growing conditions, while *Escherichia coli* cells carrying the same construct failed to do so (Appukuttan et al., 2006). Further, *Deinococcus* (pPN1) cells retained PhoN activity and uranium precipitation ability upon lyophilization and exhibited a shelf life of 6 months at room temperature with only 6% loss of activity (Appukuttan et al., 2011). PhoN was also expressed in *D. radiodurans* under regulation of the *ssb* gene promoter region containing two RDRMs (P*ssb*) that is inducible by radiation (Ujaoney et al., 2010). This recombinant strain, *Deinococcus* (pSN4), showed 6–8 fold higher PhoN activity upon irradiation, compared to unirradiated cells (Ujaoney et al., 2010).

In the present study, the radiation inducibility of PhoN of *Deinococcus* (pSN4) strain was evaluated and optimized for uranium bioprecipitation in radiation environment. The results provide a proof of concept of enhanced bioremediation by exploitation of a radiation inducible promoter.

2. Materials and methods

2.1. Bacterial strains and growth conditions

D. radiodurans strain R1 transformed with plasmids pSN4 or pPN1 carrying the constructs P*ssb*-*phoN* or P*groESL*-*phoN* on pRAD1 plasmid respectively (Appukuttan et al., 2006; Ujaoney et al., 2010) were used. *D. radiodurans* cells carrying pRAD1 vector alone served as control. The strains were grown in TGY (1% Tryptone, 0.1% glucose, 0.5% yeast extract) medium supplemented with or without 3 μg/ml chloramphenicol (TGY-Cm) at 32 °C with agitation at 150 rpm. Bacterial growth was measured spectrophotometrically as turbidity (OD_{600}).

2.2. Radiation exposure and recovery

Recombinant *D. radiodurans* cells were exposed to gamma radiation from two ^{60}Co sources that permitted two different dose rates. Dose at the rate of 4 Gy/min (Gamma Chamber GC-220, Atomic Energy of Canada, Canada) and at 56.8 Gy/min (Gamma Chamber GC-5000, Board of Radiation and Isotope Technology, Mumbai, India) were applied to cell suspensions (OD_{600} of 5.0) in TGY medium. Unirradiated cells were kept at room temperature in dark during this period. Control and irradiated cells were subsequently inoculated (OD_{600} of 0.5) in TGY and allowed to recover at 32 °C with agitation at 150 rpm for up to 16 h. This period is referred to as post irradiation recovery (PIR). For studying dose-dependent induction of promoter activity, recombinant cells were irradiated at 56.8 Gy/min dose rate for 19 min to 3.16 h in order to deliver total gamma radiation doses between 1 and 10 kGy. *Deinococcus* (pSN4) cells, washed free of medium and re-suspended in distilled water were also irradiated at a dose rate of 0.6 Gy/min (Blood Irradiator, Board of Radiation and Isotope Technology, Mumbai, India) for 10 days and then used in uranium precipitation assay.

2.3. Assays for reporter gene activity

PhoN activity expressed from the P*ssb* and P*groESL* promoters was monitored in three ways. (a) Histochemical plate assay, briefly 5×10^6 cells/ml were spotted on TGY-Cm plates containing phenolphthalein diphosphate (PDP) substrate and methyl green (MG) pH indicator dye and incubated at 32 °C overnight. Green PhoN-expressing colonies on histochemical plates appeared as a consequence of precipitation of methyl green upon acidification caused by released phosphoric acid (Appukuttan et al., 2006); (b) zymogram analyses in gels for acid phosphatase activity, using NBT/BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate) as substrate (Appukuttan et al., 2006); (c) assay for cell bound PhoN activity by spectrophotometric (OD_{405}) measurement of *p*-nitrophenol (*p*NP) released from *p*-nitrophenylphosphate (*p*NPP) as described earlier (Bolton and Dean, 1972). Briefly, aliquots containing 5×10^6 cells/ml were used in 1.2 ml assay containing 100 mM acetate buffer pH 5.0. Reaction was stopped after half an hour by addition of 2 ml of 0.2N NaOH and OD at 405 nm was determined.

2.4. Western blot for PhoN detection

Whole cell protein extracts from recombinant *Deinococcus* cells were prepared by incubating cells at OD_{600} of 20 cells in Laemmli's buffer for 5 min in a boiling water bath. Fifty micrograms of protein from the soluble lysate was separated on 10% SDS–PAGE, followed by blotting on nitrocellulose membrane. Anti-PhoN polyclonal antibody (Rabbit IgG) was used at 1:500 dilution for detection by Anti-rabbit secondary antibody IgG conjugated to alkaline phosphatase (Sigma, St Louis, MO).

2.5. Uranium precipitation assay

Phosphatase mediated uranium precipitation was assessed as described earlier (Appukuttan et al., 2006). Briefly, irradiated recombinant *Deinococcus* cells, which had undergone PIR, were washed in distilled water and re-suspended in 2 mM acetate buffer, pH 5.0 containing uranyl nitrate and β-glycerophosphate, as specified in each experiment. Aliquots were removed at fixed intervals, subjected to centrifugation at 10,000 rpm for 3 min and the amount of uranium in the supernatant was determined using Arsenazo III reagent as described earlier.

For testing uranium precipitation using lyophilized cells, 80 mg of lyophilized powder was used in 50 ml solution containing 2 mM uranyl nitrate and 15 mM β-glycerophosphate in 2 mM acetate buffer (pH 5). Appropriate controls were included to rule out spontaneous precipitation of uranium, and to exclude biosorption of the metal to cells or sorption to the walls of the container, as described (Appukuttan et al., 2011).

2.6. Lyophilization

Irradiated cells after PIR and control cells grown for the same duration were rinsed twice with distilled water. Thick cell suspensions (~OD_{600} of 100) were poured into plastic Petri plates and frozen in liquid nitrogen. Frozen cells were lyophilized in a Lyospeed (Genevac, UK) at 0.07 mbar for 18 h (Appukuttan et al.,

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