DOI 10.1099/mic.0.29009-0

Deinococcus radiodurans engineered for complete toluene degradation facilitates Cr(VI) reduction

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Received 17 March 2006
Revised 8 May 2006
Accepted 8 May 2006

Toluene and other fuel hydrocarbons are commonly found in association with radionuclides at numerous US Department of Energy sites, frequently occurring together with Cr(VI) and other heavy metals. In this study, the extremely radiation-resistant bacterium *Deinococcus radiodurans*, which naturally reduces Cr(VI) to the less mobile and less toxic Cr(III), was engineered for complete toluene degradation by cloned expression of *tod* and *xyl* genes of *Pseudomonas putida*. The recombinant Tod/Xyl strain showed incorporation of carbon from ^{14}C-labelled toluene into cellular macromolecules and carbon dioxide, in the absence or presence of chronic ionizing radiation. The engineered bacteria were able to oxidize toluene under both minimal and complex nutrient conditions, and recombinant cells reduced Cr(VI) in sediment microcosms. As such, the Tod/Xyl strain could provide a model for examining the reduction of metals coupled to organic contaminant oxidation in aerobic radionuclide-contaminated sediments.

INTRODUCTION

Contamination at US Department of Energy (DOE) waste sites comprises large inventories of organic, inorganic and radionuclide contaminants in the soil and vadose zones. The contamination is a result of past disposal of wastes directly to cribs and trenches, and also of leaking waste storage tanks (Macilwain, 1996; Riley *et al.*, 1992). The most common contaminants that have been found in combination in soils include: radionuclides, such as uranium, strontium and caesium; heavy metals, such as chromium, lead and mercury (Lovley & Coates, 1997; McCullough *et al.*, 2003); the fuel hydrocarbons benzene, toluene, ethylbenzene and xylenes (BTEX); and chlorinated hydrocarbons, such as trichloroethylene and polychlorinated biphenyls (Riley *et al.*, 1992). Metal reduction/immobilization and toxic organic compound degradation, carried out by metabolically active bacteria close to sources of contamination, where radionuclide concentrations can be very high, could prevent or minimize dissemination of contaminants before they become widely dispersed in the environment. For bioremediation to be effective in such areas, micro-organisms must be able to withstand cellular toxicity caused by heavy metals, solvents and chronic ionizing radiation. These requirements have not been met by any single known organism, nor are they likely to be achieved in the foreseeable future by engineering genetic components of radiation resistance into other bacteria that are radiation sensitive (Daly, 2000; Daly *et al.*, 2004; Ghosal *et al.*, 2005; Saier, 2005). Therefore, our approach has been to express cloned genes in the naturally radiation-resistant *Deinococcus* bacteria, extending their intrinsic metabolic functions (Brim *et al.*, 2000, 2003; Lange *et al.*, 1998).

Deinococcus radiodurans strain R1 (ATCC BAA-816) is the most well-characterized member of the radiation-resistant bacterial family *Deinococcaceae* (Makarova *et al.*, 2001; Omelchenko *et al.*, 2005). It is non-pathogenic, amenable to genetic engineering and historically best known for its extreme resistance to gamma radiation (Brim *et al.*, 2003; Daly & Minton, 1996; Daly, 2000; Daly *et al.*, 2004; Ghosal *et al.*, 2005; Lange *et al.*, 1998). Under complex nutrient conditions, the bacteria can grow and functionally express cloned foreign genes in the presence of 60 Gy h^{-1} (Brim *et al.*, 2000; Daly, 2000; Lange *et al.*, 1998). These

Abbreviations: BTEX, benzene, toluene, ethylbenzene and xylenes; DOE, US Department of Energy; TDO, toluene dioxygenase.

characteristics were the impetus for its genomic sequencing and annotation (White *et al.*, 1999), global proteome and transcriptome analyses (Lipton *et al.*, 2002; Liu *et al.*, 2003), and ongoing development for bioremediation (Brim *et al.*, 2000; Daly, 2000; Lange *et al.*, 1998). The isolation of *D. radiodurans* from highly radioactive sediments beneath a waste tank located on the DOE Hanford Site that had leaked high-level radioactive waste {depth, 84 ft (25·60 m); 21 μCi (777 kBq) ^{137}Cs [γ, β^{-}]^{E} (g soil)$^{-1}$; Fredrickson *et al.*, 2004} underscores the potential in further developing this species as described here.

Toluene, a fuel hydrocarbon, is a contaminant in hundreds of DOE mixed waste sites (Riley *et al.*, 1992). This contaminant is a growth substrate for a number of organisms, including *Pseudomonas putida* strains F1 and mt-2, for which the genetics and biochemistry have been studied in great detail (Aemprapa & William, 1998; Harayama & Rekik, 1990; Horn *et al.*, 1991; Timmis *et al.*, 1994; Wackett *et al.*, 1994; Zylstra & Gibson, 1989). However, *P. putida* is extremely sensitive to ionizing radiation (Daly, 2000; Daly *et al.*, 2004). *P. putida* F1 and mt-2 express *tod* and *xyl* genes, respectively, for the catabolism of fuel-derived aromatic hydrocarbons, and they represent two of the most proficient toluene-degrading micro-organisms yet reported. With respect to *P. putida* genes encoding degradation of toluene, our goal has been to construct a pathway in *D. radiodurans* that allows it to completely degrade this solvent. We note that growth of *D. radiodurans* in radioactive environments is dependent on the presence of a rich source of Embden–Meyerhof–Parnas substrates (e.g. fructose, glucose and maltose) and amino acids (Venkateswaran *et al.*, 2000). Thus, our objective, with respect to remediation of toxic metals and organic compounds at radioactive DOE waste sites, has been to expand the repertoire of metabolic functions of *D. radiodurans* under nutrient-rich biostimulated conditions (Venkateswaran *et al.*, 2000).

We previously reported that cultures of wild-type *D. radiodurans* can reduce Cr(VI) to the less mobile and less toxic Cr(III) oxidation state (Eary & Rai, 1987; Fredrickson *et al.*, 2000). Cr(VI) is a known human carcinogen, but its reduction to Cr(III) renders the metal non-mutagenic and non-carcinogenic (Sugden *et al.*, 2001). Constructing a *D. radiodurans* strain capable of mineralizing toluene and using energy derived from toluene catabolism to help fuel native (Fredrickson *et al.*, 2000) or cloned (Brim *et al.*, 2000) metal-reducing functions could be useful in remediating many radioactive waste sites. With this goal in mind, we cloned genes of the *P. putida tod* and *xyl* operons into *D. radiodurans* and evaluated the toluene-degrading and Cr(VI)-reducing capabilities of the engineered strain in minimal and rich nutrient medium, and in uncontaminated sediment samples obtained from the DOE Hanford Site, amended with toluene and/or Cr(VI). We report that *D. radiodurans* expressing *P. putida tod* and *xyl* operons is capable of mineralizing toluene and other fuel hydrocarbons, and that energy derived from toluene catabolism is coupled to its native Cr(VI)-reducing capabilities (Fredrickson *et al.*, 2000).

METHODS

***D. radiodurans* engineering strategy.** The entire *D. radiodurans* strain R1 (ATCC BAA-816) genomic DNA sequence (White *et al.*, 1999) was searched for similarity to the *P. putida tod* and *xyl* sequences using BLAST. With the exception of a homologue of *xylJ* in *D. radiodurans* (DRA0122) (38 % amino acid identity), no sequences were found to have significant similarity, either as DNA or as translated peptides, with *todC1*, *C2*, *B*, *A*, *D* and *E*, and *xylF*, *Q* and *K*. The integration vector pMD417 contains a 4 kbp *D. radiodurans* chromosomal segment (*bc*, Fig. 1), which contains a constitutively expressed deinococcal promoter (Daly & Minton, 1996). The *bc* segment allows the vector to recombine by a single crossover into the targeted *D. radiodurans* S11 chromosome sequence (Brim *et al.*, 2000; Daly & Minton, 1996) located on the 2·8 Mbp chromosome (Chromosome I) (White *et al.*, 1999; Brim *et al.*, 2000). Upon integration, sequences cloned within pMD417 become flanked by 4 kbp *bc* repeats. pMD858 (Fig. 1) is the product of cloning a 6 kbp *Eco*RI–*Nde*I (converted to *Bam*HI) fragment of pDTG351 (Horn *et al.*, 1991), containing *todC1C2BADE*, into the *Eco*RI–*Bam*HI site of pMD417K, which encodes Km resistance. Transformation of pMD858 into strain R1 with Km selection yielded MD859. pMD864 (Fig. 1) is the product of cloning the 5·5 kbp *Xho*I fragment (containing *xylFJQK*) from pTS66 (Harayama & Rekik, 1990) into the *Eco*RI–*Bam*HI site of pMD417C, which encodes Cm resistance. Transformation of pMD864 into strain R1 with Cm selection yielded MD883. Southern analysis with radiolabelled probes confirmed the predicted integration structures of MD859 and MD883, and the adjacent integration of the *tod* and *xyl* cassettes, in strain MD884 (Fig. 1c).

Toluene degradation by *D. radiodurans* under non-growth conditions (resting cells). *D. radiodurans* was initially grown in TYF broth [1 % Bacto Tryptone (Difco), 0·5 % yeast extract and 0·2 % fructose] and *P. putida* F1 was grown in L broth (with 0·1 % glucose; Hugouvieux-Cotte-Pattat *et al.*, 1990). Both strains were grown to mid-exponential phase (OD_{600} 0·5; approx. 1×10^{8} cells ml^{-1}). Cells were then centrifuged and washed three times with 25 mM sodium phosphate buffer, pH 7·2, containing 0·2 % fructose, and resuspended in 25 mM potassium phosphate, pH 7·2, containing 0·2 % fructose, to OD_{600} 2·75 (approx. 6×10^{8} cells ml^{-1}); these cells were termed 'resting cells'. Resting cells cannot grow because of the absence of amino acids and essential micronutrients (Venkateswaran *et al.*, 2000). Resting cells (10 ml; OD_{600} 2·75) were placed in sterile 250 ml biometers and 3 ml 1 M NaOH was added to each sidearm. Then, 250 μl [ring-UL-^{14}C]toluene [specific activity 56·2 μCi μmol^{-1} (2·08 MBq μmol^{-1}); 378 μM in *N*,*N*-dimethylformamide] was added to the cells in the biometer. The final concentration of [ring-UL-^{14}C]toluene was 9·22 μM. At various time intervals, an aliquot of the NaOH solution containing trapped CO_2 was transferred to a scintillation vial, purged vigorously for 2 min (in controls, this has been found to be sufficient to remove residual toluene) and mixed with 15 ml EcoLume scintillation fluid. After 12 h in the dark, a sample was removed and radioactivity was measured using a Beckman LS 3801 scintillation counter. A 25 μl volume of cell suspension was removed from the biometer and spotted onto a 1 × 1 cm silica TLC plate (see below). After drying, the plate was placed in a scintillation vial, along with 5 ml EcoLume, and non-volatile radioactivity was measured. The cells were pelleted at 4350 ***g*** for 10 min at 4 °C and washed three times with ice-cold 0·85 % NaCl. Fractionation into cellular components was accomplished as described by others (Hanson & Phillips, 1981).

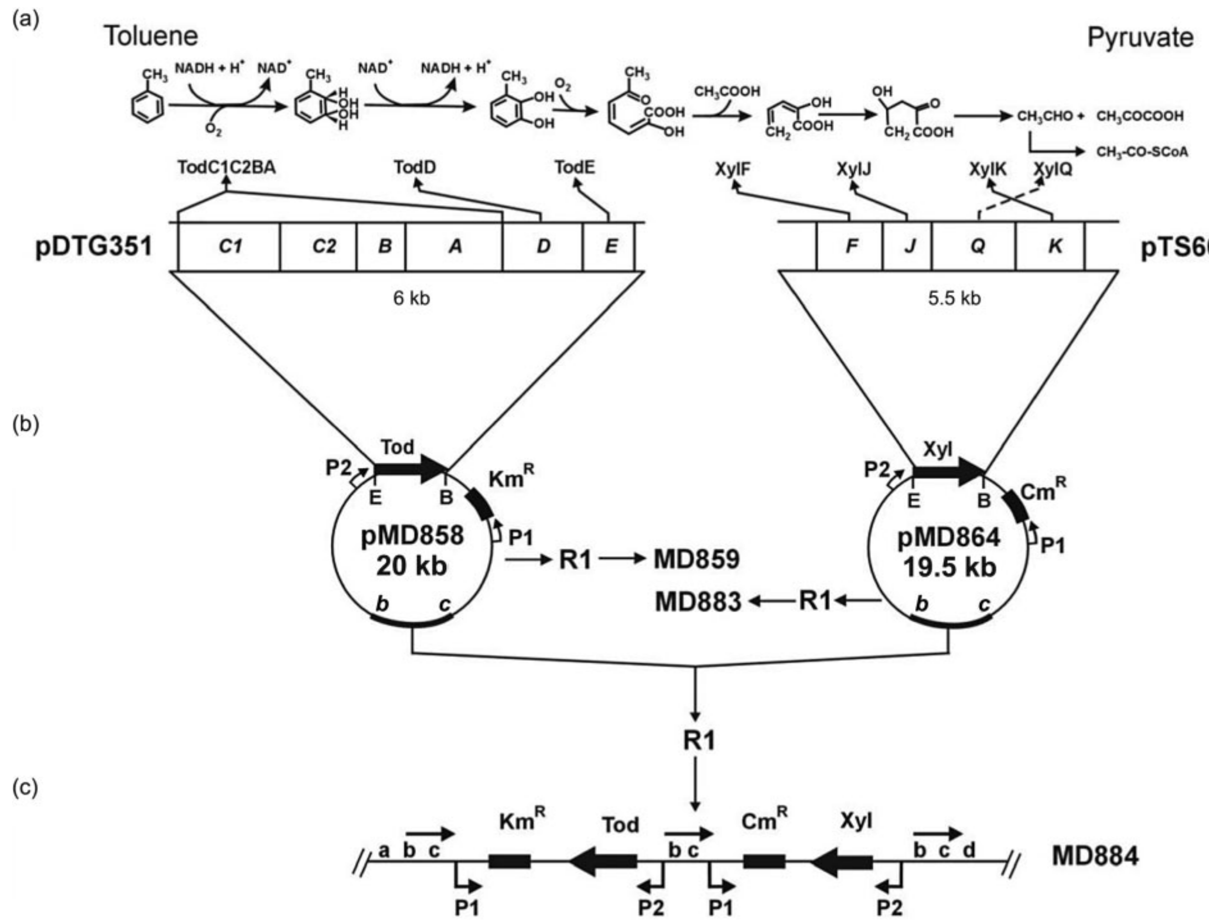

Fig. 1. Construction of toluene-mineralizing *D. radiodurans*. (a) Intermediates of toluene degradation encoded by the indicated genes. (b) Co-integration of the *tod* and *xyl* gene cassettes into *D. radiodurans* involved the construction of two different plasmids derived from the tandem duplication vector pMD417. Both constructions placed the two degradation cassettes downstream of a constitutively expressed promoter P2 that is distinct from the constitutive P1 promoter upstream of the resistance genes for Km (Km^R) and Cm (Cm^R). (c) Co-transformation of pMD858 and pMD864 into strain R1 with double Km and Cm selection yielded strain MD884. R1, *D. radiodurans* strain R1 (ATCC BAA-816).

Toluene degradation by *D. radiodurans* under growth conditions (rich medium). Two 20 ml vials were suspended using copper wire inside a 1 l glass bottle, which was closed with a butyl rubber septum. Stainless steel needles [8 in (20·32 cm), 22 gauge], connected to two-way stainless-steel Luer-lock valves, were fitted so that they passed through the septum and into the suspended vials. After autoclaving the outfitted bottle, 6 ml TYF broth, containing 250 μM toluene and 4·5 μM [ring-UL-^{14}C]toluene [specific activity 56·2 μCi μmol^{-1} (2·08 MBq μmol^{-1})], was inoculated with MD884 (*todC1C2BADE+xylFJQK*) at 5×10^7 cells ml^{-1} (mid-exponential-phase cells grown in TYF to OD_{600} 0·5) and added to one vial via its needle. A 1 ml volume of 2 M NaOH was added to the other vial in the same manner. The flask was incubated at 32 °C, at 200 r.p.m., to early stationary phase (OD_{600} 1·0; approx. 2×10^8 cells ml^{-1}), over a period of 1000 min. At various time points, aliquots of cell suspension, and aliquots of NaOH trap solution, were removed and tested for radioactivity associated with ^{14}C (see above).

TLC analysis. The cells were pelleted and 10 ml of the supernatant was added to 3 ml freshly prepared 1 mg 2,4-dinitrophenylhydrazine ml^{-1} in 2 M HCl, and the solution was incubated at 23 °C for 48 h. The solution was extracted with 3 vols ethyl acetate and the extract was dried. Authentic 2-hydroxypenta-2,4-dienoate, formed from allylglycine using L-amino acid oxidase (Collinsworth *et al.*, 1973), and 4-hydroxy-2-oxo-valerate, synthesized as described by Dagley & Gibson (1965), were derivatized in the same way. Extracts were dissolved in 200 μl ethyl acetate, and spotted onto a Silica gel 60 F_{254} TLC plate (EM Science), which was then run in *sec*-butyl alcohol. Afterwards, a known quantity of a non-volatile ^{14}C compound was spotted on the plate as an internal reference and standard. The radioactivity and location of spots on the plate were quantified using a Molecular Dynamics Storm 840 phosphorimager, and Adobe Photoshop imaging software. Derivatives of 2-hydroxypenta-2,4-dienoate and 4-hydroxy-2-oxovalate, as seen under visible or short-wavelength UV light, produced spots at R_F values of 0·54 and 0·36, respectively.

Irradiation assays under non-growth conditions. [^{14}C]Toluene was added to resting cells in 30 ml crimp-top glass vials, which were sealed immediately with a teflon/butyl rubber insert. For irradiation, samples were placed symmetrically in a J. L. Shepherd and Associates model 143-45 ^{137}Cs irradiator producing 22 Gy h^{-1}. At 28 h, cells were harvested, and washed two times with 25 mM sodium phosphate buffer, pH 7·2, containing 0·2 % fructose for *D. radiodurans*, or 0·85 % NaCl for *P. putida* F1. Small molecules were removed by three washes with ice-cold 10 % trichloroacetic acid and the remaining pellet of macromolecules was counted for ^{14}C incorporation (Hanson & Phillips, 1981).

GC-MS. Benzene, toluene, ethylbenzene or chlorobenzene (45 μM) or *p*-xylene (16 μM) was added to sealed vials containing 1 ml resting *D. radiodurans* cells concentrated to an OD_{600} of 7·5 (approx. $1·5\times10^9$ cells ml^{-1}). After 26 h shaking at 23 °C, samples were extracted and analysed by GC-MS on a Hewlett Packard 6890 gas chromatograph, as described previously (Lange *et al.*, 1998). In parallel experiments, *D. radiodurans* R1 was shown not to oxidize any of the substrates tested.

Growth with toluene and fructose. *D. radiodurans* cells were initially grown in 50 ml TYF broth at 21 °C to an OD_{600} of 0·5. After pelleting the cells, and washing them three times with 10 mM sodium phosphate buffer, pH 7·2, cells were resuspended in 1 ml of the same buffer. Resuspended cells (100 μl) were used to inoculate 25 ml basal minimal medium (Brim *et al.*, 2003; Daly *et al.*, 2004), or basal minimal medium supplemented with high concentrations of amino acids (rich minimal medium) (Venkateswaran *et al.*, 2000) and containing 0 or 0·5 % fructose. The medium was in 125 ml flasks, to which toluene was introduced via vapour bulbs (Gibson *et al.*, 1970) to maintain saturation. Flasks were shaken at 200 r.p.m. at 21 °C.

Preparation of sediment slurries. The most radioactive vadose sediments examined at DOE facilities have very low moisture contents (2–6 %) and are nutrient poor (Fredrickson *et al.*, 1993). Uncontaminated subsurface [81·0–81·5 ft (24·7–24·8 m)] sediments were obtained during coring of borehole 299-W22-48 in the 200 W area of the Hanford Site central plateau. Sediments were sieved to <2 mm, air-dried before use and prepared as slurries with *Deinococcus* minimal medium (Brim *et al.*, 2003). Vadose sediments and coring techniques have been described previously (Fredrickson *et al.*, 1993, 2004).

Cr(VI) reduction assays. Cr(VI) reduction was measured in suspensions of sediment. Samples were inoculated with cells to a final concentration of approx. 5×10^7 ml^{-1}. Sediment suspensions, consisting of 2 g sediment in 10 ml basal minimal medium (i.e. without fructose or other carbon source) (Brim *et al.*, 2003), in 25 ml glass pressure tubes sealed with Teflon septa, were treated with gamma irradiation (35 kGy) and shown to be sterile, and then inoculated with cells, amended with K_2CrO_4 to 50 μM and toluene (100 μM) or fructose (11 mM), where noted. The use of minimal medium enabled us to isolate the effects of carbon source on Cr(VI) reduction, while providing amino acids and micro-nutrients needed for survival (Venkateswaran *et al.*, 2000). Suspensions were incubated at 30 °C for the indicated times under static conditions. Cr(VI) reduction was determined by measuring the loss of Cr(VI) from solution with time by mixing filtrates (pore size, 0·2 μm) with *sym*-diphenylcarbizide reagent (0·25 % in acetone) and measuring the absorbance of solutions at 540 nm, as described previously (Fredrickson *et al.*, 2000).

RESULTS

Construction of toluene-mineralizing *D. radiodurans*

Toluene-degradation functions encoded by *P. putida* (Aemprapa & William, 1998; Harayama & Rekik, 1990; Horn *et al.*, 1991) (Fig. 1a) were introduced into *D. radiodurans* (ATCC BAA-816) chromosomal pS11 locus (Brim *et al.*, 2000) as two distinct constitutively expressed gene cassettes (Fig. 1b, c). The integration of the *todC1C2BADE* genes within the *D. radiodurans* genome led to the construction of strain MD859, and a separate construction, which integrated the *xylFJQK* genes, yielded strain MD883. In strain MD884, both gene cassettes were present. It is well established that tandem duplication expression vectors inserted into the pS11 locus are stably expressed and maintained in *D. radiodurans* in the absence of antibiotic selection, even during chronic irradiation (Brim *et al.*, 2000; Lange *et al.*, 1998), or after high-dose acute irradiation (Daly & Minton, 1996). As expected, the presence of the *tod* and *xyl* cassettes (Fig. 1) did not affect resistance of *D. radiodurans* to irradiation (data not shown). In *P. putida* under aerobic conditions, TodC1C2BADE converts toluene to 2-hydroxy-6-oxo-2,4-heptadienoate (Zylstra & Gibson, 1989), and XylFJK converts 2-hydroxy-6-oxo-2,4-heptadienoate to acetate, pyruvate and acetaldehyde (Harayama & Rekik, 1990; Horn *et al.*, 1991); *xylQ* is present in the *xyl* cassette and encodes an acetaldehyde dehydrogenase that converts potentially toxic acetaldehyde to acetyl-CoA (Aemprapa & William, 1998).

Functionality of the *tod* and *xyl* cassettes in *D. radiodurans*

Incubating resting cells (see above) of *D. radiodurans* strain MD884 (*todC1C2BADE*+*xylFJQK*) or *P. putida* F1 with uniformly ring-labelled [^{14}C]toluene generated $^{14}CO_2$, with the yield from MD884 measured at approximately 60 % that of *P. putida* F1 (Fig. 2a). During co-incubation, or individual incubations of strains MD859 (*todC1C2BADE*) and MD883 (*xylFJQK*) (Fig. 1), no significant levels of $^{14}CO_2$ were produced in the presence of [^{14}C]toluene (Fig. 2a). These results indicate that strain MD884 functionally expresses the *tod* and *xyl* genes because $^{14}CO_2$ is predicted to be generated following toluene catabolism to acetate, pyruvate and acetyl-CoA, through which ^{14}C can enter native *Deinococcus* intermediary metabolic pathways (Ghosal *et al.*, 2005; Makarova *et al.*, 2001; Venkateswaran *et al.*, 2000). Other products of toluene biodegradation were also examined in engineered *Deinococcus* strains and *P. putida* F1 after incubating resting cells with labelled toluene. As expected, the ^{14}C-label accumulated in non-volatile products for strains MD884, MD859 and *P. putida* F1, but not for MD883 or wild-type *D. radiodurans* (Fig. 2c). Because XylF (Fig. 1a) removes acetate from 2-hydroxy-6-oxo-2,4-heptadienoate, a pathway truncated after XylF could result in some acetate-derived ^{14}C incorporation by the cell, $^{14}CO_2$ generation, and accumulation of 2-hydroxypenta-2,4-dienoate. Quantification of these radioactive intermediates using 2,4-dinitrophenylhydrazine derivation, and subsequent analysis via TLC and phosphorimaging, allowed comparison of their absolute radioactivities to the total radioactivity of the soluble degradation products. Of the total radioactivity derived from [^{14}C]toluene as degradation products generated during incubation with MD884 (Fig. 2a), 1·4 % ended up in the 2-hydroxypenta-2,4-dienoate derivative, while the

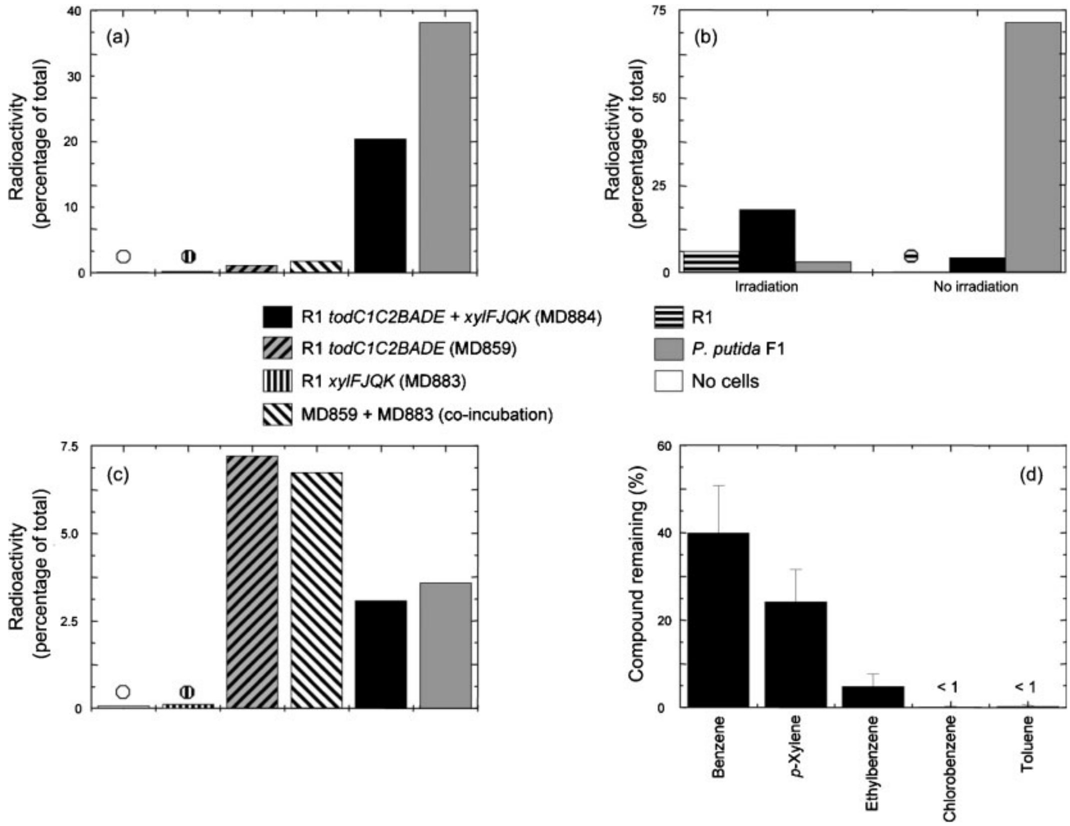

Fig. 2. Fate of [^{14}C]toluene in engineered *D. radiodurans* (resting cells). (a) Generation of $^{14}CO_2$. (b) Incorporation into macromolecular cellular components with and without ionizing irradiation (^{137}Cs [γ, β^-]^E, 23 Gy h^{-1}). (c) Production of non-volatile radioactive products. (a) and (c) Cells were adjusted to an OD_{600} of 5·0 and assayed at 48 h. (b) Cells were adjusted to an OD_{600} of 3·5, [^{14}C]toluene added to 4·1 μM, and assayed at 28 h. (d) Transformation, assayed at 26 h, of BTEX and chlorinated hydrocarbons by MD884 relative to *D. radiodurans* R1. Error bars indicate the standard deviation of three trials; (a), (b) and (c) a single replicate was made for each experimental point. When a column value is low, see inset circle for pattern designation and correspondence to key.

4-hydroxy-2-oxovalerate hydrazone contained 0·1 %. These findings indicate that XylJ is functional, since the next intermediate in the pathway, 4-hydroxy-2-oxovalerate, was detected. The relatively small amount of 4-hydroxy-2-oxovalerate in the resting cell medium suggests that 4-hydroxy-2-oxovalerate is a transient product, and that the entire pathway is functional. Incubation of non-irradiated MD884 or *P. putida* F1 with [^{14}C]toluene yielded ^{14}C incorporation into cell material, but no ^{14}C incorporation was detected in wild-type *D. radiodurans* (Fig. 2c). For non-irradiated MD884 incubations with [^{14}C]toluene, substantial ^{14}C incorporation was detected in purified preparations of DNA, RNA, protein, lipids, and non-volatile small molecules (Table 1). In the presence of irradiation (^{137}Cs [γ, β^-]^E, 22 Gy h^{-1}), [^{14}C]toluene incorporation in *P. putida* F1 cellular components was reduced by 96 %. In contrast, strain MD884 showed a 60 % increase in ^{14}C-incorporation compared with non-irradiated MD884 cells (Fig. 2b). Radiation-stimulated incorporation of [^{14}C]toluene into MD884 may be the result of radiation-induced oxygenation of toluene, which can produce *o*-, *m*- and *p*-cresols (Albarran & Schuler, 2002). The three isomeric cresols were detected by GC-MS in irradiated buffer controls containing toluene (data not shown), consistent with oxygenation via

Table 1. Amount of ^{14}C incorporated into cellular fractions following incubation of resting cells (non-irradiated) with 9·2 μM [ring-UL-^{14}C]toluene [specific activity 56·2 μCi μmol^{-1} (2·08 MBq μmol^{-1}); $3{\cdot}2\times10^6$ c.p.m.]

Results are for a single sample.

Cell fraction	Radioactivity (percentage incorporation)		
	D. radiodurans R1	MD884	*P. putida* F1
DNA	0·01	0·20	0·30
RNA	0·08	0·80	0·40
Protein	0·02	2·2	0·90
Lipids	0·01	0·20	0·20
Small molecules	0·01	0·30	0·50

radiation-derived radicals, and they appear to be assimilated by MD884. It is possible that the cresols are oxidized to catechols, thereby becoming substrates for both cloned and naturally encoded catabolic genes (Lange *et al.*, 1998). Thus, co-introduction of the *tod* and *xyl* cassettes (Fig. 1) into *D. radiodurans* imparts the ability to mineralize toluene, and to utilize carbon derived from its catabolism for biosynthetic purposes, in the presence or absence of chronic radiation. GC-MS analysis confirmed the ability of MD884 to degrade toluene and the other prevalent fuel hydrocarbons benzene, ethylbenzene and *p*-xylene, as well as the chlorinated hydrocarbon chlorobenzene (Fig. 2d). A 255 μM (approx. 24 mg l^{-1}) toluene aliquot was rapidly metabolized by MD884 growing exponentially in rich medium, with concomitant generation of toluene-derived CO_2. From a starting concentration (255 μM toluene) comparable to the highest reported in DOE mixed waste sites, about 50 % of the toluene was removed in 15 h (Fig. 3). Thus, both resting (nutrient-depleted) MD884 (Fig. 2) and exponentially growing (nutrient-rich) MD884 (Fig. 3) are capable of utilizing toluene because the Tod and Xyl proteins are constitutively expressed in the engineered *Deinococcus* (Fig. 1).

Growth studies with *D. radiodurans* strain MD884 (*todC1C2BADE+xylFJQK*)

MD884 grew at a similar rate, and to a final cell density, as did wild-type *D. radiodurans* R1, in TYF and minimal medium (Brim *et al.*, 2003), and minimal medium supplemented with high concentrations of amino acids (enriched minimal medium) (Venkateswaran *et al.*, 2000), with fructose as the carbon source. Without fructose, *D. radiodurans* MD884 inoculated into enriched minimal medium plus saturating toluene, supplied via vapour bulbs (Gibson *et al.*, 1970), failed to grow (Fig. 4). When fructose was added to minimal medium, the rate and extent of growth were similar in both the presence and absence of toluene (Fig. 4). No significant lag phase was observed under the conditions used. In separate experiments without fructose, no growth was observed with toluene supplied to minimal medium cultures of strain MD884 using vapour bulbs, or via continuous sparging with toluene at approximately 1 % of its vapour pressure at room temperature (data not shown).

Chromate reduction by MD884 in toluene-supplemented sediment slurries

Previously, *D. radiodurans* R1 has been shown to be capable of reducing toxic Cr(VI) to non-toxic Cr(III) under anaerobic or aerobic conditions coupled to the oxidation of lactate or pyruvate (Fredrickson *et al.*, 2000). To isolate any toluene-derived benefit, we evaluated MD884 for its ability to aerobically reduce Cr(VI) to Cr(III) in slurries of natural sediment suspended in minimal medium. For Cr(VI)-amended sediment samples, to which toluene was added, only those containing MD884 facilitated Cr(VI) reduction (Fig. 5). In parallel experiments, Cr(VI) was not reduced by MD884 in the absence of toluene. The amount of Cr(VI) reduced in the presence of toluene was comparable with that previously obtained with 10 mM lactate (Fredrickson *et al.*, 2000).

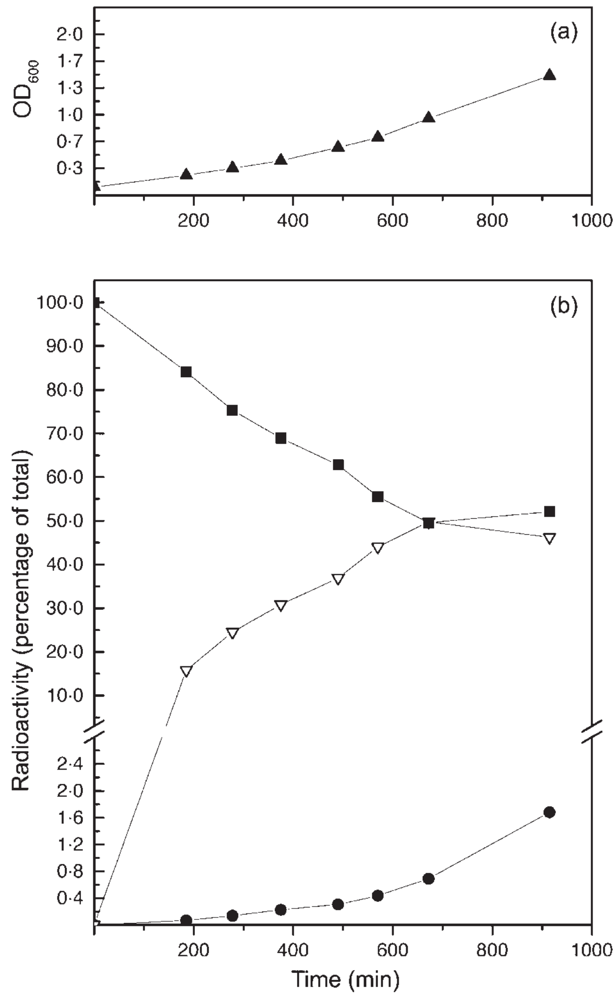

Fig. 3. Fate of [^{14}C]toluene in engineered *D. radiodurans* strain MD884 growing in TYF. (a) Growth; (b) $^{14}CO_2$ generation (●), non-volatile products formed (▽) and [^{14}C]toluene remaining (■). A single replicate was made for each time point.

DISCUSSION

We have previously expressed toluene dioxygenase (TDO) from *P. putida* F1 in *D. radiodurans*, and shown that engineered cells incubated with ^{14}C-labelled toluene in rich medium under chronic radiation yield toluene *cis*-dihydrodiol (Lange *et al.*, 1998); however, no $^{14}CO_2$, or intermediates that could be assimilated into cellular carbon, could be generated by this strain, and TDO did not confer the ability to derive energy from toluene oxidation. Rather, the TDO reaction itself consumed metabolic energy to produce toluene *cis*-dihydrodiol. Our goal of functionally coupling toluene oxidation with energy generation and biosynthetic processes of *D. radiodurans* was not achieved

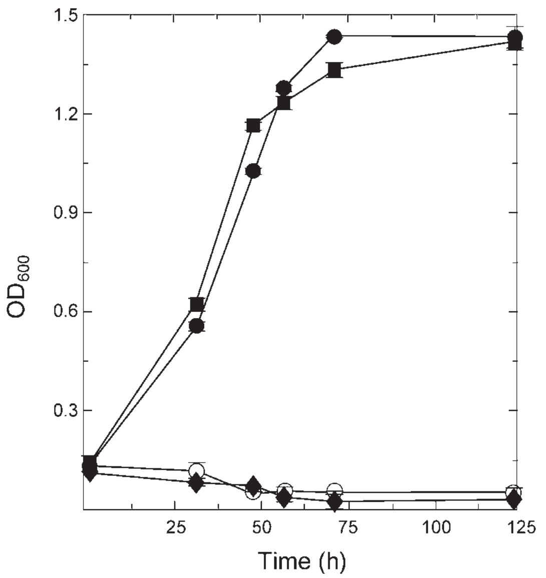

Fig. 4. Growth of MD884 with toluene and fructose. MD884 cells were inoculated into rich minimal medium with various combinations of fructose and toluene. Bars indicate the range of duplicate samples. ●, Saturating toluene+0·05% fructose; ■, no toluene+0·05% fructose; ○, saturating toluene, no fructose; ◆, no toluene, no fructose.

because of uncertainties in design/engineering strategies relating to the metabolic configuration of *D. radiodurans*. Since then, we have shown that *D. radiodurans* has a functional TCA cycle, containing an active glyoxylate bypass needed for growth on acetate as a sole carbon/energy source (Ghosal *et al.*, 2005; Lipton *et al.*, 2002; Liu *et al.*, 2003), that could be integrated with toluene oxidation. Importantly, pyruvate and acetate, the final products generated from the *P. putida* Tod and Xyl hybrid enzyme pathway (Harayama & Rekik, 1990; Zylstra & Gibson, 1989) selected for expression in *D. radiodurans* (Fig. 1), were shown to support moderate growth of *D. radiodurans* in minimal medium (Ghosal *et al.*, 2005).

We constructed a *D. radiodurans* strain that constitutively expressed the genes *todC1C2BADE* and *xylFJQK* integrated into the main chromosome (MD884, Fig. 1). Functional analysis of strain MD884 showed that it was able to mineralize toluene, and use carbon derived from its catabolism for cellular biosynthesis in the presence and absence of high-level chronic radiation (Fig. 2) of the type (^{137}Cs [γ, β^-]^E) that predominates at DOE waste sites (Fredrickson *et al.*, 2004; Riley *et al.*, 1992); in contrast, wild-type *D. radiodurans*, and engineered *D. radiodurans* strains containing either the *todC1C2BADE* or xyl*FJQK* genes (Fig. 2a, b, c), could not. Furthermore, strain MD884 could degrade benzene, ethylbenzene, *p*-xylene and chlorobenzene (Fig. 2d). These results are another step towards the goal of broad-based utilization of engineered *D. radiodurans* strains for select waste environments, since toluene and related compounds inventoried in 1992 persist at hundreds of DOE radioactive waste sites (Riley *et al.*, 1992).

As a minimum practical requirement for bioremediating a radioactive waste site with engineered *D. radiodurans*, the toluene-degrading functions (Fig. 1) need to function under complex nutrient conditions that support growth under chronic radiation (Venkateswaran *et al.*, 2000). *D. radiodurans* strain MD884 exceeded this requirement for the following reasons. (i) Resting cells (Fig. 2), as well as cells growing in rich medium (Fig. 3), showed efficient degradation of toluene, and high levels of incorporation of carbon from [^{14}C]toluene into cellular macromolecules; in comparison, toluene catabolism is strongly repressed in *P. putida* until other growth substrates are exhausted (Finette & Gibson, 1988). (ii) In slurries of sediment in minimal medium, only MD884 reduced Cr(VI) when toluene was added as the sole carbon/energy source (Fig. 5). These results demonstrate that strain MD884 may have utility for the reduction of metals coupled to organic contaminant oxidation in aerobic radionuclide-contaminated sediments, with or without the addition of growth-promoting nutrients (biostimulation) (McCullough *et al.*, 2003). The microbial enzymic reduction of multivalent metals and radionuclides can profoundly diminish their solubility and toxicity. Thus, *D. radiodurans* strain MD884 provides a possible microbiological mechanism for detoxifying radioactive waste sites co-contaminated with Cr(VI) and toluene (Riley *et al.*, 1992; McCullough *et al.*, 2003).

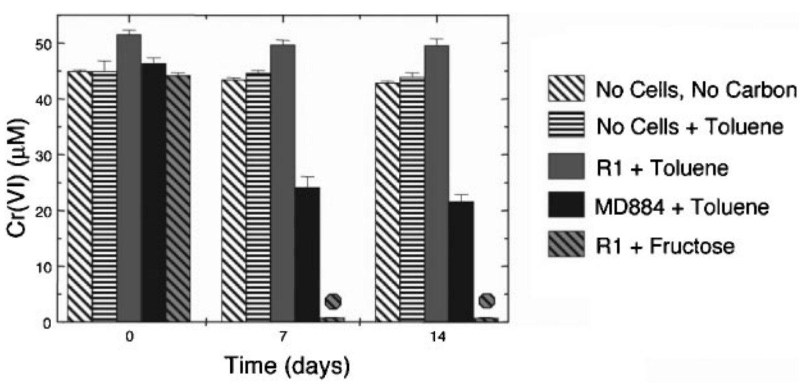

Fig. 5. Cr(VI) reduction by *D. radiodurans* strain MD884 in sediment suspensions. Error bars indicate the standard deviation of three trials.

The failure of strain MD884 to grow on toluene as the sole carbon/energy source (Fig. 4) underscores some of the difficulties in re-engineering metabolism, as observed previously. Recombinant strains carrying genes encoding a heterologous pathway may fail to express all the genes (Watts *et al.*, 2004), but the present study confirmed that most metabolic intermediates, and CO_2, were produced, indicating that the failure to grow must be due to other reasons. In other examples, bacteria engineered for 2-chlorotoluene (Haro & de Lorenzo, 2001) or cyclohexene metabolism (Swift *et al.*, 2001) expressed complete pathways, yet failed to grow, perhaps because of metabolic misrouting. Causes underlying the failure to grow can be subtle: a quinone oxidoreductase is required for growth of a *Pseudomonas* strain on terpenes, although the direct physiological role of this enzyme in the metabolism is unclear (Forster-Fromme & Jendrossek, 2005). Since *D. radiodurans* MD884 grew normally in the presence of fructose as the carbon source in the presence of toluene (Fig. 3 and 4), metabolite toxicity associated with toluene degradation appears to be unlikely (Lange *et al.*, 1998). Further, toluene was transformed into cellular macromolecules (Table 1) and CO_2 (Fig. 2 and 3), suggesting that metabolic misrouting within the recombinant pathway of MD884 was also not a problem. Instead, as indicated by the limited mineralization of toluene (Fig. 2a), the yield of pyruvate and acetate from the Tol/Xyl pathways might be insufficient to sustain levels of TCA-cycle-dependent energy production needed for growth.

While O_2 concentrations in sediments are variable (0–400 μM) (Smith, 1995), they are typically high enough to support Tod/Xyl functions (Costura & Alvarez, 2000). There are only two oxygen-dependent enzymes in the Tol/Xyl pathway: catechol-2,3-dioxygenase, which has a K_m for O_2 of 17 μM (Kukor & Olsen, 1996); and toluene dioxygenase, for which the K_m for O_2 is likely to be near 4·3 μM, as reported for the closely related benzoate-1,2-dioxygenase (Yamaguchi & Fujisawa, 1980). However, it should be noted that bacterial isolates from deeper radioactive unsaturated soil at Hanford [e.g. depth 84 ft (25·60 m); 21 μCi (777 kBq) ^{137}Cs [γ, β^-]^E (g soil)$^{-1}$] have been shown to comprise predominantly Gram-positive aerobic chemoheterotrophs (Balkwill *et al.*, 1997; Fredrickson *et al.*, 2004). Even in deep environments where O_2 concentrations could become limiting, bioventing and biosparging strategies could be applied as part of a biostimulation approach to help overcome O_2 limitations (Werner *et al.*, 1997).

The development of viable *in situ* bioremediation applications is a long-term goal of the DOE, including the use of engineered organisms (McCullough *et al.*, 2003), and a variety of DOE field-research efforts are currently under way (McCullough *et al.*, 2003). Genetically engineered micro-organisms have already been used successfully in non-DOE regulatory-agency-approved field-scale bioremediation (Ripp & Sayler, 2002; Strong *et al.*, 2000). Recombinant organisms have been considered as an option when naturally occurring organisms do not provide the complete suite of functions needed to deal with contaminant mixtures and sites. *D. radiodurans* is non-pathogenic, and indigenous to some contaminated DOE sites (Fredrickson *et al.*, 2004). In the present example of metabolically engineered *D. radiodurans* metabolizing radioactive, heavy metal and organic contaminant mixtures to reduce their toxicity and/or migration in the environment, strain MD884 is a possible candidate for future research with contaminated sediments *in situ* or *ex situ*.

ACKNOWLEDGEMENTS

This research was funded by the DOE (Office of Biological and Environmental Research) grant DE-FG02-04ER63918 from the Environmental Remediation Sciences Program. Pacific Northwest National Laboratory is operated for the DOE by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830.

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