Engineering TCE-Degrading Rhizobacteria for Heavy Metal Accumulation and Enhanced TCE Degradation

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Abstract: Many superfund sites are currently co-contaminated with organic pollutants such as trichloroethene (TCE) and heavy metals. A promising strategy to address these mixed-waste situations is the use of TCE-degrading rhizobacteria that will survive and thrive in soil heavily polluted with heavy metals. In this work, a gene coding for the metal-binding peptide, EC20, was introduced into rhizobacteria engineered for TCE degradation, resulting in strains with both metal accumulation and TCE degradation capabilities. EC20 was displayed onto the cell surface of *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D using an ice-nucleation protein (INP) anchor. Expression of EC20 was confirmed by Western blot analysis and cells with EC20 expression showed sixfold higher cadmium accumulation than non-engineered strains in the presence of 16 μM $CdCl_2$. As expected, the TCE degradation rate was reduced in the presence of cadmium for cells without EC20 expression. However, expression of EC20 (higher cadmium accumulation) completely restored the level of TCE degradation. These results demonstrated that EC20 expression enhanced not only cadmium accumulation but also reduced the toxic effect of cadmium on TCE degradation. We expect that similar improvements will be observed when these engineered rhizobacteria are inoculated onto plant roots.

Keywords: heavy metal removal; bioremediation; rhizoremediation; TCE; phytochelatins

INTRODUCTION

Since World War II, large quantities of trichloroethene (TCE) have been used extensively as a solvent for the metal and textile industries (Environmental health center, 1997; Ochsner et al., 1979). Unfortunately, TCE has been found in recent years to be a suspected carcinogen, and its widespread usage has made TCE one of the most common groundwater pollutants at hazardous waste sites (McCarty, 1997). In the US, 54% of the US EPA Superfund sites contain TCE (Adamson and Parkin, 2000). Although natural attenuation of TCE can occur via anaerobic dechlorination, the reaction is slow and often incomplete resulting in accumulation of lesser-chlorinated ethenes, including the more toxic vinyl chloride (Sharma and McCarty, 1996; Yeager et al., 2001).

Aerobic degradation of TCE has been demonstrated by many different oxygenases. Toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4, for example, has been shown to oxidize TCE to primarily CO_2 and Cl_2 (Nelson et al., 1986). In addition, TOM can degrade other reductive-dehalogenation products of TCE such as *cis*-DCE and *trans*-DCE (Canada et al., 2002; Shim et al., 2000), making TOM a promising candidate for bioremediation of TCE. Recombinant strains expressing TOM have been shown to effectively degrade a mixture of these chlorinated ethenes (Canada et al., 2002; Luu et al., 1995; Shields et al., 1995).

In situ remediation of TCE-contaminated soil has recently been demonstrated by exploiting the symbiotic plant-microbe relationship in a rhizosphere (rhizoremediation) (Yee et al., 1998). Rhizoremediation is an attractive process since plant roots provide a large surface area for a large population of bacteria and transport the colonizing bacteria to a depth of 10–15 m in the soil (Anderson et al., 1993). In addition to improving the survival of the colonizing bacteria, the plant roots help to increase the availability of the pollutant by breaking and aerating soil particles as well as by pumping contaminated water to the root-colonizing bacteria. The TOM gene was stably integrated into several naturally occurring root-colonizing bacteria (rhizobacteria) such as *Pseudomonas* Pb2-1 and *Rhizobium* strain 10320D that colonize the roots of poplar tree, enabling the establishment of a bacterium-plant-soil microcosm (Shim et al., 2000).

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Treatment of TCE-contaminated surface and near-surface soil was demonstrated, with complete removal of TCE occurred within 13 days (Shim et al., 2000). More importantly, the use of indigenous rhizobacteria enables the engineered population to remain competitive in the rhizosphere for over 8 months (Wood et al., 2000).

In addition to organic pollutants, over 40% of the Superfund sites in the US are co-contaminated with heavy metals (Sandrin et al., 2000). Remediation of sites co-contaminated with organic and metal pollutants is a complex problem, as the two components often must be treated differently. It is well known that the presence of heavy metals can inhibit a broad range of microbial processes including metabolism, growth, and the aerobic biodegradation of a variety of organic pollutants (Said and Lewis, 1991; Sandrin and Maier, 2003). A promising strategy to address the mixed-waste situation requires the use of rhizobacteria that will survive and thrive in soil heavily polluted with heavy metals.

Immobilization of heavy metals by cysteine-rich peptides is a major mechanism employed by nature (animals and plants) for counteracting heavy metal toxicity. Synthetic phytochelatins (ECs) with the general structure $(Glu\text{-}Cys)_n$ Gly, which are protein analogs of the naturally occurring metal-binding peptide, phytochelatin (PC), have been shown to bind heavy metals with high affinity (Bae et al., 2000). Several bacteria with surface-expressed EC20 (20 cysteines) have been shown to accumulate up to tenfold more cadmium than the unmodified strains (Bae et al., 2000). Introduction of EC20 into rhizobacteria that are engineered for TCE degradation would endow them with both TCE degradation and metal remediation capabilities.

In this work, EC20 was displayed onto cell surface of two TCE-degrading rhizobacteria, *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D, using with the ice-nucleation protein (INP) anchor. Our results demonstrate that EC20 expression enhances not only cadmium accumulation but also reduces the toxic effect of cadmium on TCE degradation. We expect that similar improvements on TCE degradation could be observed when these engineered rhizobacteria are inoculated into the plant roots.

MATERIALS AND METHODS

Strains, Plasmid, and Media

Pseudomonas strain Pb2-1 (a natural isolate from the poplar root) and *Rhizobium* strain 10320D (ATCC 10320) were used for Cd^{2+} binding and TCE degradation experiments (Shim et al., 2000). The TOM gene was stably integrated into the chromosome of these strains using the mini-Tn5 transposon system containing a kanamycin selection marker. *Escherichia coli* strain XL1-Blue (*recA1 endA1 gyrA96 thi*-1 hsdR17 supE44 relA1 lac [F′*proAB lacI*^{q}*ZΔ*M15 Tn10 (Tet^r)]) (Stratagene, La Jolla, CA) was used for plasmid cloning and propagation. Plasmid pVINPEC20-t containing a tetracycline marker was used to express the INP-EC20 fusion protein.

Luria-Bertani (LB) medium (Sambrook and Russell, 2001) containing 50 μg/mL of tetracycline (Sigma, St. Louis, MO) was used for *E. coli.* Low phosphate medium (MJS; 12.5 mM Tris at pH 7.2, 50 mM NaCl, 20 mM NH_4Cl, 1 mM KCl, 1 mM $MgCl_3$, 0.1 mM $CaCl_3$, 0.05 mM $MnCl_2$, 0.4% [vol/vol] glycerol, 0.8% [wt/vol] casamino acid, 0.005% thiamine hydrochloride) (Sambrook and Russell, 2001) containing either 50 μg/mL of kanamycin (plasmid-free cells) or 50 μg/mL of both kanamycin and tetracycline (cells containing pVINPEC20-t) was used for *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D.

Construction of pVINPEC20-t for the Expression of INP-EC20

Since both *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D are kanamycin resistant, the *inp*-ec20 fragment was transferred from pVINP20 (Bae et al., 2002) into pVLT31 containing a tetracycline resistance marker to create pVINPEC20-t. The *inp*-ec20 fragment was PCR amplified from plasmid pVINP20 using a forward primer (P1-HindIIIINP): 5′-ACTCAGAAGCTTAGGAAACGATGAA-TATCG-3′ and a reverse primer (P2-EcoRIINP): 5′-ACT-CAGGAATTCTTAACCACATTCACATTC-3′. The amplified fragments were digested with *Eco*RI and *Hind*III, and ligated with similarly digested pVLT31 (Lorenzo et al., 1993).

Transformation of pVINPEC20-t into *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D was performed using the electroporation method of Grag et al. (1999). After the cultures were grown to an optical density (OD_{600}) of 0.4–0.6, cells were harvested and placed on ice for 30 min. Cells were then washed four times with ice-cold sterile deionized water and recovered by centrifugation at 9,000*g* for 10 min at 4°C. After an additional wash with 10% sterilized ice-cold glycerol, cells were resuspended in 10% glycerol at 10^{10}–10^{11} cell/mL and 90 μL of cell suspension was mixed with 2 μg of plasmid DNA. After vortexing for 10 s, the sample was kept on ice for 30 min before electroporation (2.5 kV, 25 μF, 200 Ω). Transformed cells were incubated in LB medium for 24 h and cells were selected on LB agar plates containing 50 μg/mL of kanamycin and tetracycline.

Western Blot Analysis

Western blot analysis was performed as described (Sambrook and Russell, 2001). Cells were inoculated in a selective medium from overnight grown seed culture at an initial $OD_{600} = 0.1$ and induced with 0.8 mM of isopropy-β-D-thiogalactopyranoside (IPTG) at $OD_{600} = 0.5$. After overnight cultivation, cells were harvested and concentrated to $OD_{600} = 10$ with a SDS gel-loading buffer and boiled for 10 min. The cell lysate was loaded onto 12% (w/v) polyacrylamide gel and the proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a nitrocellulose membrane and incubated with a polyclonal rabbit anti-INPNC serum overnight (Kwak et al., 1999). Western blot analysis

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was performed with a Bio-Rad Immun-Blot GAR-AP kit (Bio-Rad, Hercules, CA). A prestained protein standard marker (Kaleidoscope Standards (Bio-Rad)) was used to determine protein molecular weights.

Cadmium Binding Experiments

Cells were inoculated into 20 mL of MJS medium containing the appropriate antibiotics in a 125-mL flask at an initial $OD_{600} = 0.1$. When the OD reached 0.5, INP-EC20 production was induced with 0.8 mM IPTG and $CdCl_2$ was added to a final concentration of 16 μM. Samples were obtained at various culture times to determine the cell density and whole-cell accumulation of cadmium. For whole-cell Cd^{2+} content, cells were washed with 5 mM HEPES buffer containing 0.8% NaCl for three times before drying at 65°C for 24 h. The dried cell pellet was digested with 100 μL of concentrated nitric acid for 2 days. The total Cd^{2+} content was measured using atomic adsorption spectroscopy (Perkin Elmer, Inc., Wellesley, MA) in the flame mode.

TCE Degradation Experiments

Cells were cultured and induced as described above. After measuring the OD_{600} of overnight grown cells, cells were centrifuged at 10,000*g* for 1 min using a type JA-17 rotor (Beckman Instruments, Fullerton, CA). Cells were washed with 0.1 M potassium phosphate buffer (pH 7.0) and diluted to an $OD_{600} = 1$ with the same buffer. Each sample (50 mL of cell culture) was prepared in 200 mL bottles (Wheaton, Millville, NJ) that are capped with a Teflon-coated silicone septum and an aluminum crimp seal (Fisher Scientific, Hampton, NH). Fifty microliters of a TCE stock solution (10 mM TCE in *N,N*-dimethylformamide) (Fisher Scientific) was injected directly into the cell suspension to a final TCE concentration of 10 μM. Each bottle was incubated in a 30°C incubator-shaker (I2400 incubator shaker; New Brunswick Scientific, Edison, NJ) at 300 rpm. Gas phase samples (5 mL) were taken using a 10-mL gas-tight syringe (Hamilton, Reno, NV) every 10 min to measure the amount of TCE remained by a gas chromatograph (Hewlett Packard 5890 series II, Palo Alto, CA) using a flame ionization detector and a Supelcowax 10 capillary column (30 m × 0.53 mm × 1 μm: Supelco, Billefonte, PA).

RESULTS

Expression of EC20 Onto the Surface of *Pseudomonas* Strain Pb2-1 and *Rhizobium* Strain 10320D

Our initial goal in this study was to demonstrate the functional expression of EC20 onto the surface of two root-colonizing TCE degraders, *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D, and to investigate the effects on cadmium accumulation and TCE degradation. The truncated ice-nucleation protein (INPNC) anchor, which has been used

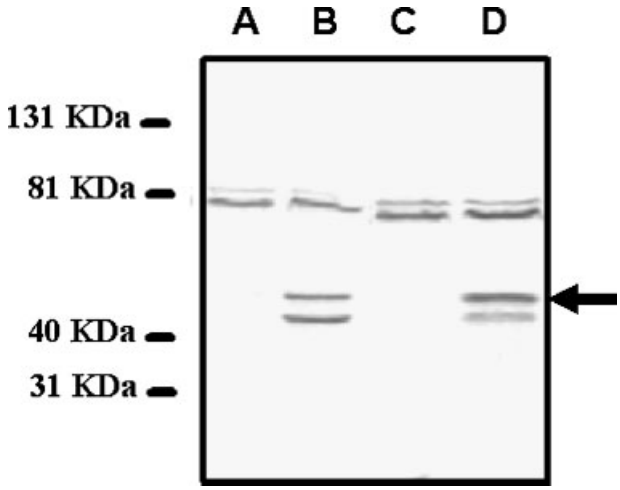

Figure 1. Expression of the INP-EC20 fusion protein in *Pseudomonas* strain Pb2-1 (**lane B**) and *Rhizobium* strain 10320D (**lane D**) harboring pVINPEC20-t. Cells carrying pVLT31 (**lanes A** and **C**) were used as controls. The INP-EC20 fusion protein is indicated by an arrow. Total cell proteins were separated on SDS–PAGE (12.5% (w/v) polyacrylamide) and transferred to a nitrocellulose membrane. Western blot analysis with an anti-INP serum was performed at 1:3,000 dilution.

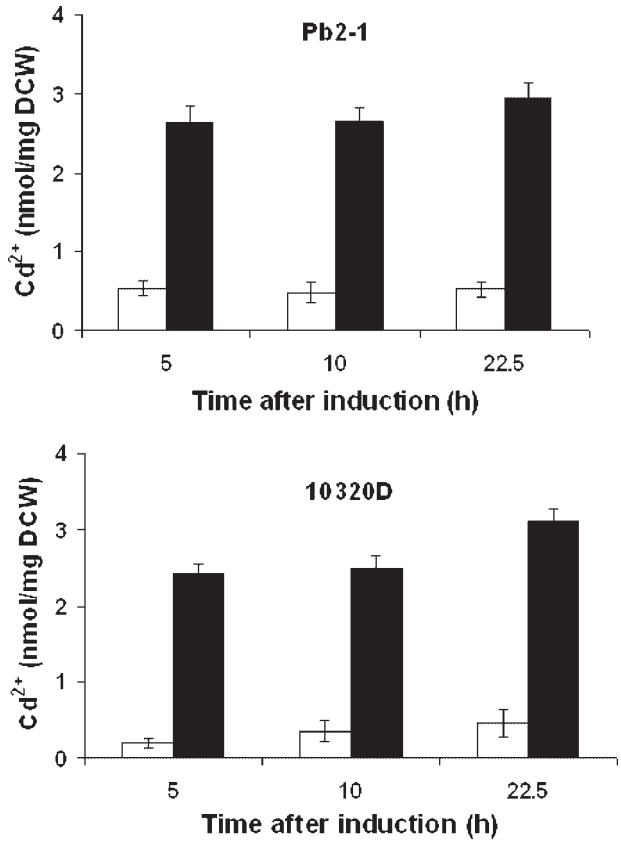

Figure 2. Cd^{2+} binding by *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D harboring pVLT31 (empty bar) or pVINPEC20-t (filled bar). Cells were grown in the presence of 16 μM of $CdCl_2$. Results obtained from three independent experiments were presented with standard deviations.

successfully to display EC20 in a wide range of bacteria (Bae et al., 2002; Shimazu et al., 2001), was used to target EC20 onto the cell surface. Plasmid pVINPEC20-t, carrying the *inpnc*-ec20 fusion was constructed by inserting the *ec20* fragment into the vector pVLT31 (Lorenzo et al., 1993). Expression of INPNC-EC20 was confirmed by Western blotting with an INPNC antiserum. A band corresponding to the INP-EC20 fusion was detected from cells carrying pVINPEC20-t (Fig. 1), while no such protein was detected with control cells carrying pVLT31. A slightly smaller band was also detected with the INP antiserum, suggesting the INP-EC20 fusion was partially degraded. The surface localization of the INPNC-EC20 fusion proteins was confirmed by immunofluorescence microscopy using a rabbit anti-INPNC serum as the primary antibody followed by straining with a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (data not shown).

Enhanced Cadmium Bioaccumulation by Rhizobacteria With Surface-Expressed EC20

The functionality of surface-expressed EC20 to bind Cd^{2+} was investigated. Cells were grown in MJS medium in the presence of 16 μM $CdCl_2$, and whole-cell accumulation of Cd^{2+} was determined at different growth phases. Both rhizobacteria expressing EC20 on the cell surface accumulated a significantly higher amount of Cd^{2+} than cells carrying pVLT31 (Fig. 2). The improvement in Cd^{2+} accumulation was sustained during the entire growth period, demonstrating that the inherent background binding level by these rhizobacteria is low. This increase in Cd^{2+} binding occurred without any observable differences in cell growth, indicating that neither the increased Cd^{2+} binding nor the surface expression of EC20 had noticeable effects on the engineered rhizobacteria. This result is important as it suggests the engineered rhizobacteria might survive in the poplar roots with similar stability, a condition necessary for the successful rhizoremediation of mixed metal-TCE wastes.

Restoration of TCE Degradation by Surface-Expressed EC20

To investigate the effect of cadmium on TCE degradation, *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D were grown overnight either in the presence or absence of $CdCl_2$ before harvesting and resuspending to a final OD_{600} of one in PBS buffer. Resting cells were then incubated with 10 μM TCE and the rate of TCE was determined either in the presence or absence of $CdCl_2$. In the presence of 16 μM Cd^{2+}, the rate of TCE degradation was decreased by 30% and 60% for *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D, respectively (Fig. 3). This result confirms that the presence of cadmium is detrimental to TCE degradation. In comparison, the TCE degradation rates were completely restored for both strains expressing EC20 on the surface. A similar protective effect on TCE degradation was observed even when the Cd^{2+}

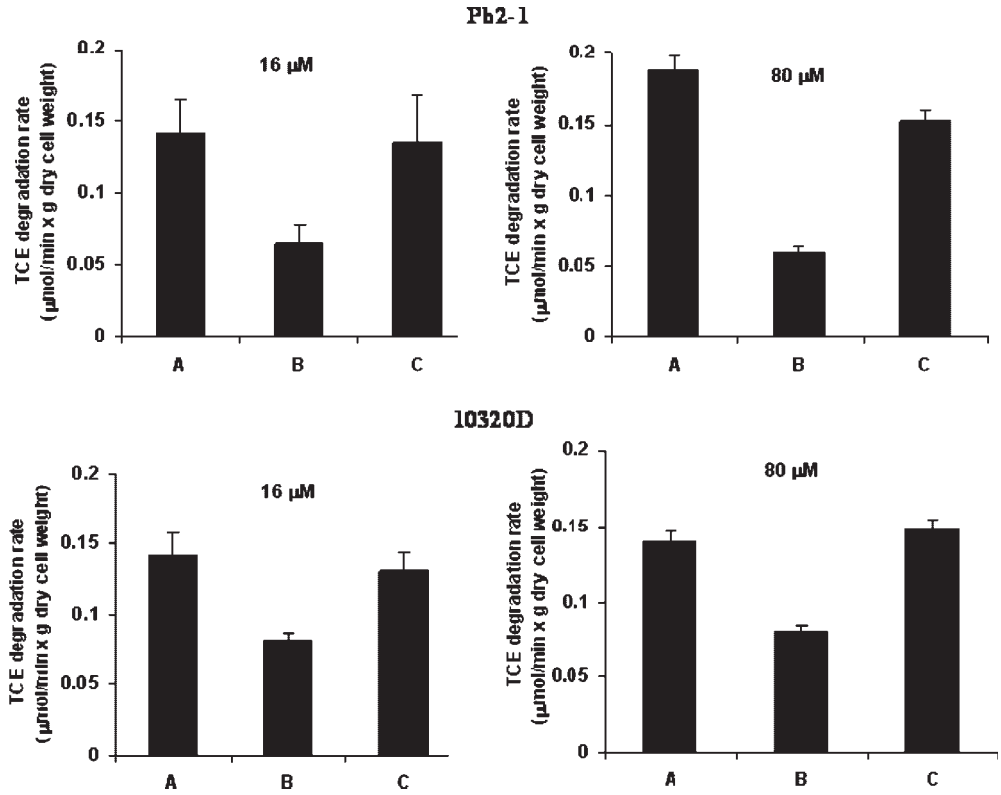

Figure 3. The TCE degradation rate for *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D harboring pVINPEC20-t incubated with either 16 or 80 μM $CdCl_2$. **A**: no induction and no Cd^{2+} added, (**B**) Cd^{2+} added but no induction, and (**C**) Cd^{2+} added and induction for EC20 expression. Results obtained from three independent experiments were presented with standard deviations.

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concentration was increased by fivefold to 80 μM. These results indicate that the surface-expressed EC20 moiety could effectively sequester Cd^{2+} ions, minimizing transport, and the inhibitory effect of intracellular Cd^{2+} on TCE degradation.

DISCUSSION

Bioremediation of soil co-contaminated with both heavy metals and organics is considered difficult since the presence of heavy metals is known to inhibit the activity of many organic-degrading microorganisms. Although co-inoculation of a metal-detoxifying population with an organic-degrading population has been shown to function cooperatively to remediate both metal and organic pollutants in a co-contaminated soil system, up to 48 h adaptation was required for metal detoxifying to occur before organic degradation was observed (Roane et al., 2001). For this reason, introduction of a single microorganism that is capable of maintaining their organic-degrading activity under mixed-waste conditions is an intriguing possibility. In this study, two TCE-degrading rhizobacteria, *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D, which have been shown to survive and degrade TCE effective in plant-soil studies were engineered with the capability to sequester heavy metals. The strategy is to display a metal-binding peptide EC20 that has high affinity for a wide range of heavy metals onto their cell surface in order to lower or eliminate the metal-induced inhibition on TCE degradation. As with most organic-degrading microorganisms, the TCE degradation rates were significantly impaired (up to 60%) in the presence of Cd^{2+}. Surface display of EC20 enabled not only enhanced cadmium binding but also protected the engineered strains against the toxic effects of cadmium on TCE degradation. These results suggest that the surface-displayed EC20 was effective in minimizing the inhibitory intracellular Cd^{2+} concentration by extracellular sequestration. Since the cadmium concentrations employed in this study are within the range of 4.5–348 μM found in contaminated soil, inoculation of these engineered rhizobacteria into plant roots should provide a technology useful for the remediation of TCE and cadmium co-contaminated soil. This feasibility is currently under investigation.

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