

The Role of Cell Bioaugmentation and Gene Bioaugmentation in the Remediation of Co-contaminated Soils

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Soils co-contaminated with metals and organics present special problems for remediation. Metal contamination can delay or inhibit microbial degradation of organic pollutants such that for effective *in situ* biodegradation, bioaugmentation is necessary. We monitored the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) or 3-chlorobenzoate (3-CB) in two different soils with and without cadmium (Cd) contamination. Additionally, we evaluated the ability of bioaugmentation to enhance organic degradation in these co-contaminated soils. Finally, we determined whether enhanced degradation was due to survival of the introduced organism (cell bioaugmentation) or plasmid transfer to indigenous microbial populations (gene bioaugmentation). In Brazito soil, dual inoculation with a Cd-resistant bacterium plus a known 2,4-D-degrading bacterium, *Ralstonia eutropha* JMP134, enhanced 2,4-D degradation. *Escherichia coli* D11, which lacks chromosomal genes necessary for complete 2,4-D mineralization, was used for gene bioaugmentation in Madera soil. Significant gene transfer of the plasmid to the indigenous populations was observed, and the rate of 2,4-D degradation was enhanced relative to that of controls. Cell bioaugmentation was further demonstrated when *Comamonas testosteroni* was used to enhance biodegradation of 3-CB in Madera soil. In this case no transfer of plasmid pBRC60 to indigenous soil recipients was observed. For the Madera soil, nonbioaugmented samples ultimately showed complete 2,4-D degradation. In contrast, nonbioaugmented Brazito soils showed incomplete 2,4-D degradation. These studies are unique in showing that both cell bioaugmentation and gene bioaugmentation can be effective in enhancing organic degradation in co-contaminated soils. Ultimately, the bioaugmentation strategy may depend on the degree of contamination and the time frame available for remediation. **Key words:** 2,4-dichlorophenoxyacetic acid, bioaugmentation, bioremediation, co-contaminated soil, gene transfer, heavy metals. *Environ Health Perspect* 110(suppl 6):943–946 (2002).

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Terrestrial environments such as soils or the vadose zone are typically complex microbial environments that contain large, diverse microbial populations. Of the many types of microflora found within soils, bacteria are particularly critical for *in situ* bioremediation. Soil bacteria are simple prokaryotic organisms with diverse characteristics, including variable terminal electron acceptors that allow for aerobic or anaerobic modes of respiration, as well as heterotrophic and autotrophic modes of nutrition. Coupled with this is their capability of remaining dormant for long periods of time within soil, and yet they are biologically engineered for rapid growth and fluid genetic changes. Thus, they are perfectly designed for and adapted to soils, which consist of an inorganic and organic matrix with fluctuating abiotic conditions. Normally, soil bioavailable microbial substrate becomes self-limiting, and soil bacteria for the most part exist under starvation conditions. Overall, then, soils are a harsh environment for bacteria, and yet they normally support diverse culturable bacterial populations of 10⁸ to 10⁹ organisms per gram of soil (1).

The addition of metal or organic contaminants to soils can impose additional stress on microbial communities, resulting in decreased

viable bacterial populations and/or activities (2–7). This situation can be exacerbated when pollution results in soils co-contaminated with both metals and organics. In this case the double stress imposed on soil bacterial communities means that for effective *in situ* bioremediation of the organic contaminant, there must be metal-resistant microbes with appropriate degradative genes, or a consortia of metal-resistant microbes with the appropriate catabolic capabilities. High soil metal concentrations can inhibit the microbial degradation of organics that are normally easily degraded within soils (4,8). In such cases bioaugmentation may enhance degradation and may even be a prerequisite for effective bioremediation.

Bioaugmentation has been defined as the introduction of specific microbes into a contaminated site for the purpose of enhancing the biological activity of the existing populations (9). The major problems associated with bioaugmentation are *a*) the rapid decline in numbers or death of the introduced microbe that can occur because of biotic or abiotic stress and *b*) the difficulty in getting the introduced microbes dispersed throughout the contaminated site. These problems can occur when the expected enhanced degradation is

caused by activity from the introduced whole cells (cell bioaugmentation). Because of this, we now define gene bioaugmentation as the process of obtaining enhanced activity after gene transfer from an introduced donor organism into a member of the indigenous soil population. In this case, death of the more “fit” indigenous members is less likely, and the resultant transconjugants can “grow” through the contaminated site. In this article we examine different scenarios that use cell and/or gene bioaugmentation to enhance bioremediation of an organic within a soil co-contaminated with metal.

Materials and Methods

Soils

Soils were collected from two areas near Tucson, Arizona: Madera Canyon (Madera Canyon soil), and the University of Arizona Campus Agricultural Center (Brazito sandy loam). Soils have had no known previous exposure to cadmium (Cd), 2,4-dichlorophenoxyacetic acid (2,4-D), or 3-chlorobenzoate (3-CB). Surface soils were sieved (pore size, 2 mm) and if not used within 1 week of collection, stored at 4°C. Properties of each soil type are shown in Table 1.

Bacteria Used in Bioaugmentation Studies

Ralstonia eutropha JMP134 (pJP4). This bacterium contains plasmid pJP4, an 80-kb catabolic plasmid that codes for the initial breakdown of 2,4-D to 2-chloromaleylacetic acid. Further degradation from 2-chloromaleylacetic acid to succinic acid is dependent on chromosomal genes located within JMP134. Plasmid pJP4 belongs to the IncP1 group and also encodes resistance to mercuric ions.

Escherichia coli D11. This bacterium contains plasmid pJP4 but does not have the chromosomal genes necessary for the transformation of 2-chloromaleylacetate to

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succinic acid. Therefore, it will be selected against, in media in which 2,4-D is the sole carbon source. In contrast, transconjugants that acquire pJP4 and that do contain the appropriate chromosomal genes can easily be detected on selective media with 2,4-D as the sole carbon source.

Comamonas testosteroni BR60. This bacterium was isolated by Wyndham et al. (10) from surface runoff waters near an industrial landfill. *C. testosteroni* BR60 was found to degrade 3-CB via the protocatechuate pathway contained on the 85-kb plasmid pBRC60 (11). *C. testosteroni* BR60 has also been shown to degrade various other chlorine and methyl-substituted benzoates and to be capable of transferring its degradative genes to indigenous bacteria.

Pseudomonas sp. H1. This bacterium was isolated and characterized by our laboratory. It is resistant to 225 µg/mL Cd in solution. It is also able to sequester Cd from solution, thus reducing bioavailable Cd levels (5).

Soil Microcosms and Bioreactors

Bioaugmentation studies in soil were conducted either in 0.5-L polypropylene wide-mouth screw-cap jars (microcosms) or in 20-L polypropylene containers (bioreactors). All studies were replicated 3 times unless indicated otherwise. Appropriate chemical (Sigma Chemical Company, St. Louis, MO, USA; 2,4-D, minimum 95% purity; Cd, 99.3% purity) and biological amendments were made to the soils. At designated time intervals, a subsample of soil was extracted for microbial and chemical assays.

Microbial assay: isolation and characterization of presumptive transconjugants containing plasmid pJP4. Indigenous soil bacterial recipients of plasmid pJP4 are referred to as transconjugants. These were isolated on 2,4-D-selective media (12) and distinguished via ERIC (enterobacterial repetitive intergenic consensus) polymerase chain reaction (PCR) fingerprinting techniques (13). A modified miniscreening procedure for large plasmids was used to assess the presence of an 80-kb plasmid (14). Finally, the presence of pJP4 plasmid was confirmed via PCR detection of the *tfdB* gene (15).

Chemical assay: quantitation of organic biodegradation. The concentration of 2,4-D or 3-CB within soil was monitored using a Waters Associates (Milford, MA, USA) high-performance liquid chromatograph

(HPLC) system with a wavelength of 235 nm and a Waters C₁₈ column (14).

Results

Experiment 1: Evaluation of 2,4-D degradation within Cd-contaminated Madera Canyon soil by the indigenous soil microflora.

This experiment was a laboratory microcosm study performed in triplicate. Madera sandy loam soil was amended with 1,000 µg 2,4-D/g soil and 0, 60, 120, 180, or 240 µg Cd/g soil added as CdCl₂. A subsample of moist soil from each microcosm jar was extracted and analyzed for 2,4-D concentration via HPLC analysis. Sampling was performed immediately after chemical additions and weekly thereafter until 2,4-D degradation was complete. Figure 1 shows the effects of Cd on the ability of the indigenous soil population to degrade 2,4-D, with a distinct lag phase followed by active degradation. With no Cd amendment of the soil, complete degradation of 2,4-D by the indigenous population was accomplished in 21 days. The addition of Cd to soil at levels of 60, 120, 180, and 240 µg Cd/g soil resulted in a progressive increase in the adaptation period before the onset of degradation. Total disappearance of the 2,4-D at the highest level of 240 µg Cd/g soil required 35 days, 2 weeks longer than the control soil receiving no Cd addition.

The mineralization of the organic compound 2,4-D was preceded by an acclimation or adaptation period, the length of time between the addition of the organic compound and the onset of its degradation. In this study of the indigenous population response, there was a progressive increase in the time necessary for the disappearance of added 2,4-D as increased Cd levels were added to soil. This appeared to be due to an increase in the adaptation period rather than decreased degradation rates. Once this lag phase ended, degradation proceeded rapidly regardless of Cd level. It is important to note that the bioavailable Cd concentrations in the soil were measured and remained constant throughout the experiment (data not shown).

Experiment 2: Cell bioaugmentation using *Ralstonia eutropha* JMP134 and *Pseudomonas* H1. Here *Pseudomonas* sp. H1 and *R. eutropha* JMP134 were inoculated individually into bioreactors containing Brazito sandy loam co-contaminated with Cd (60 µg/g) and 2,4-D (500 µg/g). Each treatment was replicated twice. Table 2 shows

the results of these intermediate field-scale trials. In the absence of inoculum, some 2,4-D loss (from 500 µg/g to 400 µg/g) was observed within the 70-day field trial. Upon the introduction of JMP134 (with 2,4-D only), degradation from 500 µg/g to 200 µg/g was apparent by day 70. Bioaugmentation with the metal-resistant H1 alone did not improve biodegradation. In the presence of Cd, degradation by JMP134 was completely inhibited and only occurred upon the co-augmentation with JMP134 and H1. Ninety percent of the recovered 2,4-D-degrading isolates were not JMP134, indicating the potential for gene transfer via gene bioaugmentation. In addition, however, cell bioaugmentation via H1 was also necessary.

Experiment 3: Cell bioaugmentation and 3-CB degradation within Madera Canyon sandy loam. Inoculation of Madera Canyon soil with *C. testosteroni* BR60 increased the rate of 3-CB degradation in both the 500 and 1,000 µg 3-CB/g soil microcosms (Figure 2). Each treatment was performed in triplicate. In the 500 µg 3-CB/g soil microcosms, 3-CB was reduced to undetectable levels within 7 days in the BR60-inoculated soil but persisted until 21 days in the uninoculated soil. The inoculation effect was more pronounced in the 1,000 µg 3-CB/g soil microcosms, with 3-CB being undetectable within 14–21 days, whereas 562.9 ± 5.7 µg 3-CB/g soil remained in the uninoculated microcosms after 28 days.

In the inoculated treatments the total number of culturable degraders increased from the initial inoculum level of 10⁶ colony-forming

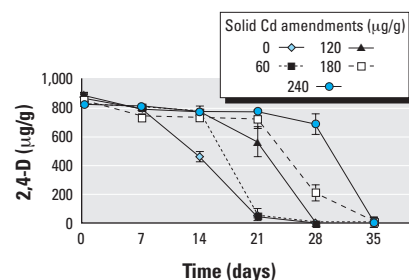


Figure 1. Influence of Cd on 2,4-D degradation by the indigenous microbial population within Madera Canyon soil. Error bars represent standard deviation of three replicate microcosms.

Table 2. Intermediate field scale trials: 70-day bioreactor results.

Treatment	Bioaugmentation	2,4-D degradation
2,4-D only	None	Yes
2,4-D only	JMP134	Yes
2,4-D + Cd	None	No
2,4-D + Cd	H1 (Cd detoxifier)	No
2,4-D + Cd	JMP134 (known 2,4-D degrader)	No
2,4-D + Cd	H1 + JMP134	Yes

Adapted from Roane et al. (5).

Table 1. Characteristics of Madera Canyon soil and Brazito sandy loam.^a

Soil	pH	Texture	Composition (%)			
			Sand	Silt	Clay	Organic matter
Brazito	8.5	Sandy loam	76	12	12	1.1
Madera	6.5	Sandy loam	78	16	6	3.3

^aAdapted from Newby et al. (14).

units (cfu)/g soil to approximately 10^8 cfu/g soil, an increase that was more rapid in the 500- than in the 1,000- μg 3-CB/g soil microcosms. In contrast, no indigenous 3-CB degraders were detected in the inoculated microcosms throughout the experiment because all degraders isolated from these inoculated microcosms were later confirmed to be the BR60 inoculant. In the uninoculated microcosms, culturable indigenous 3-CB degrader numbers increased in the uninoculated, 500 μg 3-CB/g soil microcosms from undetectable levels at 0 days to approximately 10^8 cfu/g soil by 14 days, and then decreased to less than 10^7 cfu/g soil by 28 days. In contrast, no 3-CB degraders were detected in the uninoculated, 1,000- μg 3-CB/g soil microcosms during the experiment. No transfer of plasmid pBRC60 from the BR60 inoculant to indigenous bacteria was detected, illustrating that the mechanism for enhanced degradation was apparently cell bioaugmentation.

Experiment 4: Comparison of bioaugmentation with two different pJP4 donors. An intermediate field-scale study was conducted to assess the degradation of 2,4-D in the presence and absence Cd in Madera Canyon sandy loam soil. Each treatment was performed in triplicate. When *E. coli* D11 was used as the introduced donor, no enhanced degradation was observed relative to that in noninoculated controls regardless of the presence of Cd. Degradation of 2,4-D was complete 49 days (no Cd) to 56 days (with Cd) after inoculation (Figure 3). When a portion of the soil was reamended with 500 $\mu\text{g}/\text{g}$ of 2,4-D, degradation in the inoculated soil was greater than that in the noninoculated controls regardless of the presence of the co-contaminant Cd (Figure 3). In these reamended soils, 2,4-D degradation was much more rapid than previously. Because *Escherichia coli* D11 lacked the chromosomal genes for complete mineralization of 2,4-D, degradation must have been enhanced by gene bioaugmentation. In fact, significant populations of transconjugants were observed in all

D11-inoculated treatments. The ubiquity and diversity of soil microbial populations capable of mineralizing 2,4-D suggest that many microbes contain the appropriate chromosomal genes to complement the pJP4-enhanced genes for 2,4-D degradation (16). Culturable transconjugant numbers reached approximately $10^7/\text{g}$ of soil in all inoculated treatments. All transconjugants were identified via sequencing of 16S rDNA as belonging to either the *Burkholderia* or *Ralstonia* genus. *Burkholderia graminis* was the dominant transconjugant.

When *R. eutropha* JMP134 was the introduced donor organism, different results were obtained. Note that enhanced degradation of 2,4-D via JMP134 could in fact be due to cell and/or gene bioaugmentation because JMP134 itself can mineralize 2,4-D or potentially transfer that capability to other indigenous soil recipients. In the initial soil bioreactors, inoculation with JMP134 resulted in significantly increased rates of 2,4-D degradation compared with those in noninoculated controls (Figure 3). All treatments showed slightly reduced rates of degradation in the presence of Cd. The mechanism of enhanced 2,4-D degradation was apparently cell bioaugmentation because very few transconjugant isolates were obtained. Upon reamendment with additional 2,4-D,

JMP134-inoculated treatments again showed enhanced degradation rates compared with those of noninoculated controls (Figure 3). In this latter case, all treatments again showed much faster rates of degradation than those in the original soil bioreactors. Transconjugants were detected in the reamended soils but at low levels. Therefore, in this case, enhanced degradation was due to JMP134 (cell bioaugmentation) and transconjugants (gene bioaugmentation).

It is interesting that in the original soil bioreactors, bioaugmentation with JMP134 resulted in the greatest rates of degradation relative to those in either D11-inoculated treatments or noninoculated control treatments. However, upon reamendment with 2,4-D, D11 treatments resulted in the highest degradation rates.

Discussion

Whereas 2,4-D was readily degraded within Madera soil by indigenous microorganisms, when the co-contaminant Cd was present, rates of degradation were decreased. These data show that co-contamination with a metal can delay or even inhibit indigenous microbial activity. Other studies have also shown adverse affects of metals on microbial activities and biomass (6,17).

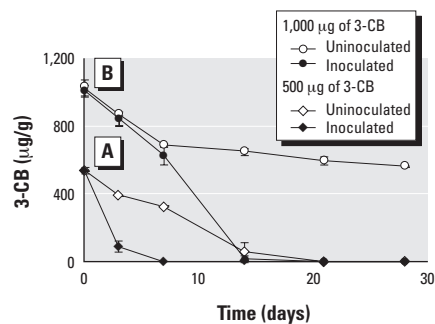


Figure 2. Concentrations of 3-CB in microcosms amended with (A) 500 μg 3-CB/g soil and (B) 1,000 μg 3-CB/g soil that were uninoculated or inoculated with *C. testosteroni* BR60. Error bars represent the standard deviation of three replicate microcosms. Figure adapted from Gentry et al. (18).

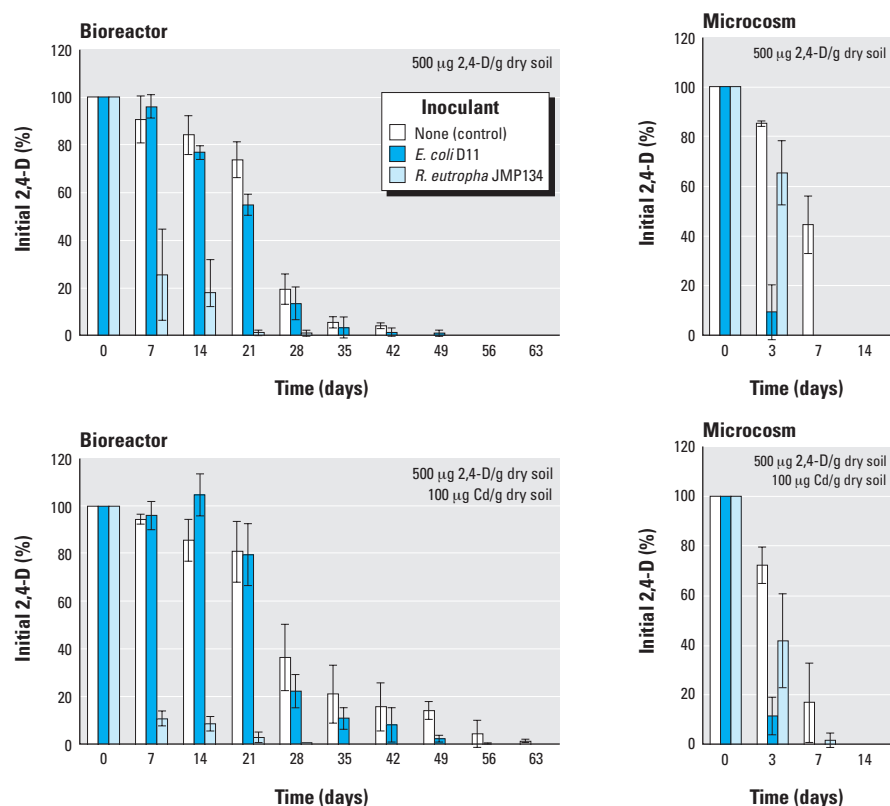


Figure 3. Degradation of 2,4-D in Madera Canyon soil bioreactors, presented as the percentage remaining of the initial 2,4-D added to each bioreactor or microcosm. Data points and error bars show means and standard deviations based on data from three replicate bioreactors or microcosms. Figure reproduced from Newby et al. (12) with permission from American Society for Microbiology.

The influence of Cd on 2,4-D degradation was clearly observed in experiment 2. Here, the low-nutrient desert soil, Brazito sandy loam, was unable to support degradation of 2,4-D in the presence of the co-contaminant Cd. This inhibition could only be overcome by inoculation with two bacterial isolates, JMP134 and H1. In this study, enhanced activity in the presence of Cd was likely due to dual cell bioaugmentation with JMP134 and *Pseudomonas* H1. The mechanism for enhanced degradation would appear to have been Cd detoxification by *Pseudomonas* H1 via cell bioaugmentation, which enhanced gene transfer from JMP134 to indigenous organisms.

Cell bioaugmentation was also successful in the studies with 3-CB. Inoculation of 3-CB-contaminated soil with *C. testosteroni* BR60 increased the rate of degradation at levels of 500 and 1,000 µg 3-CB/g soil. The increase in the rate of contaminant degradation compared with that of the indigenous soil microflora was most dramatic at the higher level of contamination. The higher concentration of 3-CB apparently inhibited the development of the indigenous 3-CB degrader population. Because no transfer of plasmid pBRC60 from the BR60 inoculant to indigenous bacteria was detected, cell bioaugmentation was assumed to be the mechanism for the increased degradation of 3-CB.

The comparison of bioaugmentation from two different pJP4 donors illustrated the complex dynamics that occur within soil microbial communities. Whereas initially, gene bioaugmentation with D11 did not enhance 2,4-D rates of degradation relative to that of noninoculated controls, cell bioaugmentation with JMP134 did. These results were consistent with or without the co-contaminant Cd. It is interesting to note that the influence of Cd was less detrimental in Madera soil than in Brazito soil, perhaps due to the higher organic matter content within the Madera soil. These results

can be explained by the fact that inoculation with a high concentration of JMP134 cells would allow instantaneous degradation of 2,4-D. In contrast, gene transfer events from D11 could have resulted in new 2,4-D-degrading individuals, but the cell density of these new transconjugants would have to increase via growth before significant degradation resulted from gene transfer. Upon reamendment with additional 2,4-D, significantly different results were observed. Now initially gene bioaugmented treatments (D11) resulted in the greatest rates of degradation. These observations suggest that by the time the soil had been re-exposed to the pollutant, transconjugant populations were sufficiently high to allow significant degradation—greater even than within JMP134-treated soils. This further suggests that the diversified transconjugant population generated from the *E. coli* D11 inoculation was better suited for subsequent 2,4-D degradation than the *R. eutropha* JMP134-inoculated soil, in which the presence of the 2,4-D-degrading inoculant repressed transconjugant growth.

Overall, these results support the premise that gene bioaugmentation with plasmid-bearing organisms may be particularly useful because of the possibility of gene transfer to indigenous populations. However, the utility of gene bioaugmentation depends on a relatively healthy potential recipient population. In severely co-contaminated systems, cell bioaugmentation that allows immediate degradation of the organic contaminant may be the viable alternative. Therefore, the ultimate choice of cell versus gene bioaugmentation depends on the degree of contamination and also the time frame available for remediation relative to the urgency of the situation.

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