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# Dual-Bioaugmentation Strategy To Enhance Remediation of Cocontaminated Soil

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**Although metals are thought to inhibit the ability of microorganisms to degrade organic pollutants, several microbial mechanisms of resistance to metal are known to exist. This study examined the potential of cadmium-resistant microorganisms to reduce soluble cadmium levels to enhance degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) under conditions of cocontamination. Four cadmium-resistant soil microorganisms were examined in this study. Resistant up to a cadmium concentration of 275 μg ml$^{-1}$, these isolates represented the common soil genera *Arthrobacter*, *Bacillus,* and *Pseudomonas*. Isolates *Pseudomonas* sp. strain H1 and *Bacillus* sp. strain H9 had a plasmid-dependent intracellular mechanism of cadmium detoxification, reducing soluble cadmium levels by 36%. Isolates *Arthrobacter* strain D9 and *Pseudomonas* strain I1a both produced an extracellular polymer layer that bound and reduced soluble cadmium levels by 22 and 11%, respectively. Although none of the cadmium-resistant isolates could degrade 2,4-D, results of dual-bioaugmentation studies conducted with both pure culture and laboratory soil microcosms showed that each of four cadmium-resistant isolates supported the degradation of 500-μg ml$^{-1}$ 2,4-D by the cadmium-sensitive 2,4-D degrader *Ralstonia eutropha* JMP134. Degradation occurred in the presence of up to 24 μg of cadmium ml$^{-1}$ in pure culture and up to 60 μg of cadmium g$^{-1}$ in amended soil microcosms. In a pilot field study conducted with 5-gallon soil bioreactors, the dual-bioaugmentation strategy was again evaluated. Here, the cadmium-resistant isolate *Pseudomonas* strain H1 enhanced degradation of 2,4-D in reactors inoculated with *R. eutropha* JMP134 in the presence of 60 μg of cadmium g$^{-1}$. Overall, dual bioaugmentation appears to be a viable approach in the remediation of cocontaminated soils.**

Cocontaminated soils, soils contaminated with both metals and organics, are considered difficult to remediate because of the mixed nature of the contaminants. A treatment alternative to expensive excavation and incineration (9) of metal-contaminated soils is bioaugmentation with metal-detoxifying and/or organic-degrading microorganisms (1, 3, 4, 6, 18). Many microorganisms are known to degrade a variety of organics, and likewise, a number of metal-resistant microorganisms are known to detoxify metals, such as selenium, mercury, and cadmium (23, 27). In cocontaminated sites, metal toxicity inhibits the activity of organic-degrading microorganisms (24). Consequently, bioremediation efforts focus on reducing metal toxicity in sites with mixed contaminants. Until recently, bioaugmentation studies focused on the introduction of a microorganism that was both metal resistant and capable of organic degradation. Under field conditions, such an approach is often unsuccessful. One reason may be that the energy requirements to maintain concurrent metal resistance and organic degradation are too high, and the introduced organism cannot perform both activities under environmental conditions. The issue of cocontamination is a serious one, since approximately 37% of all contaminated sites in the United States alone contain both metal and organic contaminants (20; W. W. Kovalich, Jr., keynote lecture, 4th World Congr. Chem. Eng., p. 281–295, 1991).

The approach used in this study was to coinoculate with a metal-detoxifying population and an organic-degrading population that cooperatively functioned to remediate both metal and organic pollutants in a cocontaminated system. We hypothesized that the metal-resistant population could protect the metal-sensitive organic-degrading population from metal toxicity. Stephen et al. (27) used metal-resistant bacteria to protect indigenous soil β-subgroup proteobacterium ammonia oxidizers.

Metals, including cadmium, lead, and mercury, are, in most cases, microcidal; however, some bacteria have developed the ability to resist and detoxify these metals. Metal detoxification strategies, including those for cadmium, may include metal sequestration and precipitation (2, 10, 14, 26), which reduce soluble metal concentrations. Unlike organics, metals cannot be degraded, and thus most biological metal remediation approaches rely on the detoxification and immobilization of the metal both to reduce the biological toxicity and to retard metal transport.

The objective of this study was to determine the efficacy of dual bioaugmentation with metal-detoxifying and organic-degrading bacteria to facilitate organic degradation within cocontaminated systems. This objective was examined in coamended solution studies, in cocontaminated soils in the laboratory, and in a pilot field experiment. Four different cadmium-resistant bacterial isolates that did not degrade 2,4-dichlorophenoxyacetic acid (2,4-D) were tested for the ability to allow 2,4-D degradation to occur in the presence of toxic levels of cadmium, using *Ralstonia eutropha* JMP134 as the 2,4-D degrader.

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TABLE 1. Cadmium-resistant isolates and examined mechanisms of cadmium resistance

| Isolate | Cd MRL[a] ($\mu$g ml$^{-1}$) | Size of plasmid (bp) | Presence of gene | | EPS production | Intracellular accumulation | Extracellular accumulation |
|---|---|---|---|---|---|---|---|
| | | | *cad*A | *cad*C | | | |
| *Pseudomonas* sp. strain I1a | 20 | ND[b] | – | – | Yes | No | Yes |
| *Arthrobacter* sp. strain D9 | 50 | ND | – | – | Yes | No | NE[c] |
| *Pseudomonas* sp. strain H1 | 225 | 18,504 | – | – | No | Yes | No |
| *Bacillus* sp. strain H9 | 275 | 10,408 | – | – | No | Yes | No |

[a] Cadmium maximum resistance level.
[b] ND, not detected.
[c] NE, not examined.

## MATERIALS AND METHODS

**Bacterial strains.** Four highly cadmium-resistant soil bacteria were chosen for this study: *Arthrobacter* sp. strain D9, *Bacillus* sp. strain H9, *Pseudomonas* sp. strain H1, and *Pseudomonas* sp. strain I1a (Table 1). The isolation and characterization of these isolates have been described by Roane and Pepper (22). Cadmium-resistant bacteria were cultured on a defined mineral salts medium (MSM) amended with soluble cadmium as $CdCl_2$ in concentrations from 0 to 45 $\mu$g ml$^{-1}$ to represent concentrations observed at contaminated sites. The MSM contained the following: 0.5 g of sodium citrate ($C_6H_5Na_3O_7$), 0.1 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1.0 g of ammonium sulfate [$(NH_4)_2SO_4$], 1.0 g of glucose ($C_6H_{12}O_6$), and 0.1 g of sodium pyrophosphate [$Na_4P_2O_7(H_2O)_{10}$], buffered to pH 6.0 with potassium phthalate ($KHC_8H_4O_4$). In the 2,4-D biodegradation studies, a modified MSM was used wherein the glucose was replaced with 500 $\mu$g of 2,4-D ml$^{-1}$, and 2-[*N*-morpholino]ethanesulfonic acid ($C_6H_{13}NO_4S$) replaced potassium phthalate, which interfered with the 2,4-D absorbance readings.

All subsequent culturing took place in 25 ml of MSM amended with cadmium and incubated at 28°C on a rotary shaker at 180 rpm.

The maximum resistance level (MRL) was defined as the highest concentration of cadmium at which at least $10^4$ cells ml$^{-1}$ remained culturable after 48 h from an initial inoculation of $10^6$ cells ml$^{-1}$. The MRL of cadmium reflected the degree of resistance to cadmium. Cadmium concentrations were determined using a flame atomic absorption spectrophotometer following centrifugation at $10{,}000 \times g$ for 20 min and filtration of the sample with a 0.2-$\mu$m-pore-size filter.

*Ralstonia eutropha* JMP134 (previously *Alcaligenes eutrophus* JMP134) contains the 80-kb pJP4 plasmid that codes for the degradation of 2,4-D to 3-oxoadipate (13). The degradation to succinic acid is in part mediated by chromosomally encoded enzymes (19, 25, 28).

**Cadmium fate experiment.** Following individual inoculation with each of the isolates, any reduction in the amount of soluble cadmium in the broth was measured using atomic absorption. Thus, any reduction in bioavailable cadmium due to specific microbial interactions could be evaluated. In replicate flasks, each isolate was grown in 25 ml of MSM broth for 48 h at 28°C at various cadmium levels up to the MRL. Precipitated and cell-associated cadmium was collected following centrifugation at $10{,}000 \times g$ for 20 min and acidified with 1 N HCl to solubilize the cadmium. An initial microscopic assessment was performed to confirm cell lysis upon acidification. Both the supernatant and the acidified cell suspension were examined for cadmium.

**Mechanism of resistance to cadmium. (i) Detection of the Cad operon.** Primers developed by Endo and Silver (7) were used to detect the Cad operon, coding for a cadmium efflux pump. Chromosomal DNA was extracted using direct cell lysis at 98°C. The alkaline lysis procedure of Kado and Liu (12) was used to isolate and purify plasmid DNA.

Touchdown PCR with step annealing temperatures ranging from 54 to 67°C was used to amplify target sequences in lysed cell extracts and from plasmid DNA. The primer concentration used was 7.7 pmol per reaction. PCR products were run on a Tris-borate-EDTA–1.2% agarose gel at 100 V cm$^{-1}$, stained with ethidium bromide (1 $\mu$g ml$^{-1}$), and viewed under UV light.

**(ii) Production of extracellular polymers.** Many microorganisms have extracellular polymeric layers that confer metal resistance. These polymeric layers are anionic in nature and thus attract and sequester cationic metals. The rapid screening method developed by Liu et al. (17) was used to screen the cadmium-resistant isolates for the production of two bacterial exopolysaccharides (EPSs), succinoglycan or galactoglucon (EPS II). The method relies on the differential staining of polymer-producing versus nonpolymer-producing organisms.

**(iii) TEM.** Transmission electron microscopy (TEM) was used to assess morphologic changes in response to cadmium exposure. Bacteria (1.5 ml) grown in MSM (pH 6.0) containing 20 $\mu$g of cadmium ml$^{-1}$ for *Pseudomonas* strain I1a and *Arthrobacter* strain D9 and 125 $\mu$g of cadmium ml$^{-1}$ for *Pseudomonas* strain H1 and *Bacillus* strain H9 were harvested during logarithmic growth by pelleting at $14{,}000 \times g$ for 2 min. The cells were rinsed in sterile deionized water and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.0, saturated with oxine (Sigma Inc.), using microwave fixation (8, 16) to minimize the cadmium leaching associated with traditional fixation. Oxine reacts specifically with heavy metals, increasing contrast under the TEM (30) for visual affirmation of metal deposits. Cells were postfixed in 2% osmium in 0.1 M cacodylate buffer, pH 6.0. Following fixation, cells were dehydrated using a reagent-grade ethanol-$H_2O$ gradient at each concentration: 30, 50, 70, 95, 100, 100, and 100% (11). Cells were infiltrated with Spurr resin at concentrations of 50% and then 100% (Ted Pella Inc., Redding, Calif.). Samples were polymerized overnight at 70°C, thin sectioned with an RMC MT-7000 Microtome (Research Manufacturing Corp., Tucson, Ariz.), and viewed at 60 kV with a Philips 420 TEM (Philips Electron Optics Inc., Mahwah, N.J.). Cadmium accumulation was confirmed using Noran Series Voyager II X3 elemental dispersive spectroscopy (Noran Instruments, Inc., Middleton, Wisc.).

**(iv) Plasmid profiles and curing.** Since metal resistance can be plasmid encoded, the four isolates were examined for the presence of plasmids ranging in size from 2.6 to 350 MDa following incubation in the presence of 3 or 12 $\mu$g of cadmium ml$^{-1}$ depending on the resistance level of the isolate. Plasmid extractions used the alkaline lysis procedure of Kado and Liu (12).

Cadmium-resistant isolates containing plasmids were cured of their plasmids using increased temperature and a nonselective medium. Isolates underwent four successive transfers (0.1 ml of isolates into 25 ml of broth) into nutrient broth (Difco, Baltimore, Md.) that did not contain cadmium, thereby removing any possible selection pressure. Isolates were incubated at 37°C on a rotary shaker at 180 rpm. Plasmid extractions were performed at the end of the four transfers. "Cured" isolates were then reinoculated into MSM with and without cadmium stress to reevaluate the MRL of each isolate to cadmium.

**Degradation studies.** *R. eutropha* JMP134 was cadmium sensitive in this study in that at levels greater than 3 $\mu$g of cadmium ml$^{-1}$, no viable *R. eutropha* cells were detected ($<10^2$ cells ml$^{-1}$ from an initial inoculum of $10^4$ cells ml$^{-1}$). Note, however, that cadmium sensitivity is inoculant concentration dependent, and very high cell concentrations of *R. eutropha* JMP134 are more cadmium tolerant (K. L. Josephson, personal communication). Since degradation is often inhibited in the presence of metal(s), the ability of cadmium-resistant isolates to support 2,4-D degradation by *R. eutropha* JMP134 was examined. The degradation of 2,4-D by *R. eutropha* JMP134 was monitored in the presence of various cadmium levels upon inoculation with one of the cadmium-resistant isolates. Concentrations of 2,4-D in culture extracts were measured every 24 h at 230 nm following centrifugation at $14{,}000 \times g$ for 2 min to remove cell debris. The relationship between the concentration of 2,4-D and its absorbance at 230 nm was linear, with a $y$ value of $0.03x - 0.07$ ($r^2 = 0.991$).

**(i) Pure culture.** In replicate pure culture experiments, 25 ml of MSM buffered to pH 6.0 with 2-[morpholino]ethanesulfonic acid (Sigma Inc.) was amended with 500 $\mu$g of 2,4-D ml$^{-1}$ and either 12 or 24 $\mu$g of cadmium ml$^{-1}$ depending on the MRL of each individual isolate. Each culture flask was inoculated with $10^4$ CFU of either cadmium-resistant isolate *Arthrobacter* strain D9, *Pseudomonas* strain H1, *Bacillus* strain H9, or *Pseudomonas* strain I1a ml$^{-1}$ and incubated at 28°C for 48 h at 180 rpm.

**(ii) Soil microcosms.** Once established in pure culture, the abilities of successful isolates to protect *R. eutropha* JMP134 from cadmium toxicity were examined in artificially metal-contaminated soil. To determine if 2,4-D degradation could be facilitated in cadmium-contaminated soil, 100 g of an uncontaminated Brazito sandy loam soil was amended with 1% (wt/wt) glucose, 500 $\mu$g of 2,4-D ml$^{-1}$, and 60 $\mu$g of cadmium ml$^{-1}$ (final concentrations). Glucose was used as a readily metabolizable carbon source to support the cadmium-resistant populations. Soil

microcosms (500-ml wide-mouth polypropylene jars) were incubated at 28°C and kept at 14% (wt/wt) soil moisture (75% of field capacity). Similar to the pure culture experiments, each soil microcosm was inoculated with one of the cadmium-resistant isolates ($10^4$ CFU $g^{-1}$) by including the isolate with the initial moisture amendment (to 75% field capacity) followed by vigorous soil mixing. Following a 48-h incubation, appropriate microcosms were inoculated with $10^4$ CFU of *R. eutropha* JMP134 $g^{-1}$, again in conjunction with moisture amendment. Control microcosms consisted of soil amended with glucose, 2,4-D, and cadmium without inocula and soil amended with glucose, 2,4-D, and cadmium inoculated with only a cadmium-resistant isolate or *R. eutropha* JMP134.

Concentrations of 2,4-D in soil extracts were measured daily for a total of 50 days. One-to-ten soil slurries were made using 0.1% (wt/vol) sodium pyrophosphate to neutralize soil particle charge, centrifuged at $14,000 \times g$ for 10 min, and read spectrophotometrically at 230 nm. Samples were analyzed in duplicate, and the soil microcosm experiment was performed three times. Soil samples without 2,4-D were used as blanks to subtract background absorbance, which was <1% of the total absorbance.

**(iii) Field bioreactors.** Laboratory soil microcosm studies with the isolate *Pseudomonas* strain H1 were repeated at an intermediate field scale level (other isolates were not examined at the field scale). *Pseudomonas* strain H1 was chosen because of its cadmium resistance (to a concentration of 225 μg $ml^{-1}$) and culturability. The *Bacillus* strain H9 isolate was not examined even though it was also highly resistant (to a concentration of 275 μg $ml^{-1}$), so as to avoid complications resulting from spore formation. Bioreactors were set up under field conditions to confirm laboratory microcosm results. The field study was initiated in June 1998 and concluded in September 1998.

Five-gallon polypropylene bioreactors (45.7 by 76.2 cm), located at the University of Arizona Campbell Avenue Agricultural Station, Tucson, were placed under a constructed shaded area so as to preclude direct sunlight, since daytime temperatures were routinely in excess of 37.8°C (100°F). Each reactor contained approximately 27 kg of Brazito sandy loam at 14% (wt/wt) moisture content amended with 500 μg of 2,4-D $g^{-1}$ and/or 60 μg of cadmium $g^{-1}$. The *Pseudomonas* sp. strain H1 isolate and the 2,4-D degrader *R. eutropha* JMP134 were inoculated at $10^4$ CFU g (dry weight) of $soil^{-1}$.

Inoculants and soil amendments were thoroughly mixed into the soil using a cement mixer (rinsed with 70% ethanol between treatments) prior to the start of the experiment while providing a 48-h incubation period between inoculation with *Pseudomonas* strain H1 and addition of *R. eutropha* JMP134. Treatments were set up so as to minimize cross-contamination between the amendments, e.g., 2,4-D-only treatments followed by 2,4-D-plus-cadmium treatments followed by 2,4-D-plus-*R. eutropha* followed by *Pseudomonas* strain H1 and so forth. There were 7 treatments (see Table 4), each replicated twice for a total of 14 bioreactors. Soil moisture was maintained at 14% (wt/wt) throughout the experiment. Ambient air temperature ranged from 16.7°C (62°F) to 48.9°C (120°F). Soil cores (45 by 2.5 cm) were collected weekly and analyzed for 2,4-D concentrations. Background absorbance at 230 nm was monitored in reactors without 2,4-D amendment and was subtracted from each sample 2,4-D reading. To eliminate cross-contamination, the soil corer was disinfected with 10% bleach after each sample collection.

Culturable 2,4-D-degrading microorganisms were enumerated on an eosin methylene blue (EMB) medium developed by DiGiovanni et al. (5). The acidity produced during 2,4-D degradation caused the eosin blue to stain the colony dark purple. Previous studies in our laboratory have confirmed that the purple colony appearance is indicative of 2,4-D degradation (5). Some of the isolates from the EMB medium were further screened to identify possible transconjugants. The screening process included performing enterobacterial intragenic consensus PCR for genomic fingerprints (29) and PCR to amplify the *tfdB* gene found on pJP4 (5), a plasmid profile to detect the 80-kb pJP4 (12), and analysis of 2,4-D degradation using either high-pressure liquid chromatography or spectroscopy at 230 nm. Comparison of results from the screening process to those with *R. eutropha* JMP134 allowed transconjugant enumeration.

## RESULTS

**Resistance to cadmium.** As summarized in Table 1, the four isolates were resistant to a wide range of cadmium concentrations, from 20 to 275 μg $ml^{-1}$. Only *Pseudomonas* strain H1 and *Bacillus* strain H9 had plasmids, of 18.5 and 10.4 kb, respectively. Upon plasmid curing, neither H1 nor H9 remained cadmium resistant, and they were unable to grow in the presence of 125 μg of cadmium $ml^{-1}$ (levels approximately half of the MRLs), respectively. The Cad operon was not identified in any of the isolates.

Observed mechanisms of cadmium resistance included extracellular and intracellular sequestration. Extracellular binding of cadmium was observed with *Arthrobacter* strain D9 and *Pseudomonas* strain I1a (data for I1a shown; Fig. 1a and b). After EPS production in isolate *Pseudomonas* strain I1a was confirmed with staining, the response of the isolate to 20 μg of cadmium $ml^{-1}$ was observed under the TEM. Cadmium sequestration by the EPS layer was evident as a dark precipitate surrounding the cells (Fig. 1b).

In contrast, intracellular accumulation of cadmium in *Pseudomonas* strain H1 and *Bacillus* strain H9 cultures was observed (data for H1 shown; Fig. 1c and d). For *Pseudomonas* strain H1, under TEM and in the presence of 125 μg of cadmium $ml^{-1}$, large intracellular accumulations of cadmium were confirmed with EDS (Fig. 1d). There was also an increase in cellular density indicative of nonspecific cadmium binding to the cells. Negative controls included cells grown in the absence of cadmium.

The effect of bacterial growth and metal resistance on cadmium solubility was also examined in a cadmium fate experiment (Table 2). The amount of cadmium present in solution decreased with isolates I1a, D9, H1, and H9 with growth from $10^4$ to $10^8$ CFU $ml^{-1}$. The most dramatic decreases in levels of soluble cadmium were seen with *Pseudomonas* strain H1 and *Bacillus* strain H9, such that an average 36% was lost with growth. Growth of *Arthrobacter* strain D9 and *Pseudomonas* strain I1a resulted in 22 and 11% decreases in soluble cadmium. Based on controls with metal and no inocula, >99% of the total cadmium remained soluble.

**Degradation studies.** Several experiments were conducted to determine the method of coinoculation. It was found that if a cadmium-resistant population and *R. eutropha* JMP134 were coinoculated at the same time, no degradation occurred and *R. eutropha* JMP134 was not recoverable. The same result was found if *R. eutropha* JMP134 was inoculated 24 h after the cadmium-resistant population was added to the cadmium–2,4-D culture medium. However, following a 48-h postinoculation with a cadmium-resistant population, the culture flasks inoculated with $10^4$ CFU of *R. eutropha* JMP134 $ml^{-1}$ did show degradation. A small inoculating biomass was used in both the pure culture and the soil experiments to assess the abilities of the inoculated populations to grow and perform under contaminated and, in the soil, nonsterile conditions. Augmentation with a smaller biomass in field scenarios can be desirable. It was also found that $>10^5$ cells $ml^{-1}$ "artificially" decreased levels of soluble metal due to increased cell binding.

In order for the cadmium-sensitive 2,4-D degradation to occur in the presence of cadmium, bioavailable cadmium concentrations had to be detoxified. The abilities of the four cadmium-resistant soil isolates, *Arthrobacter* strain D9, *Pseudomonas* strain H1, *Bacillus* strain H9, and *Pseudomonas* strain I1a (Table 1), to detoxify cadmium such that *R. eutropha* JMP134 could degrade 500-μg $ml^{-1}$ 2,4-D was determined first in broth (Fig. 2). Experiments showed that $10^4$ CFU of *R. eutropha* JMP134 $ml^{-1}$ alone in the presence of >3 μg of cadmium $ml^{-1}$ did not degrade 2,4-D, presumably because of cadmium toxicity. Additionally, none of the cadmium-resistant isolates could degrade 2,4-D (data not shown). Consequently, the dual-bio-

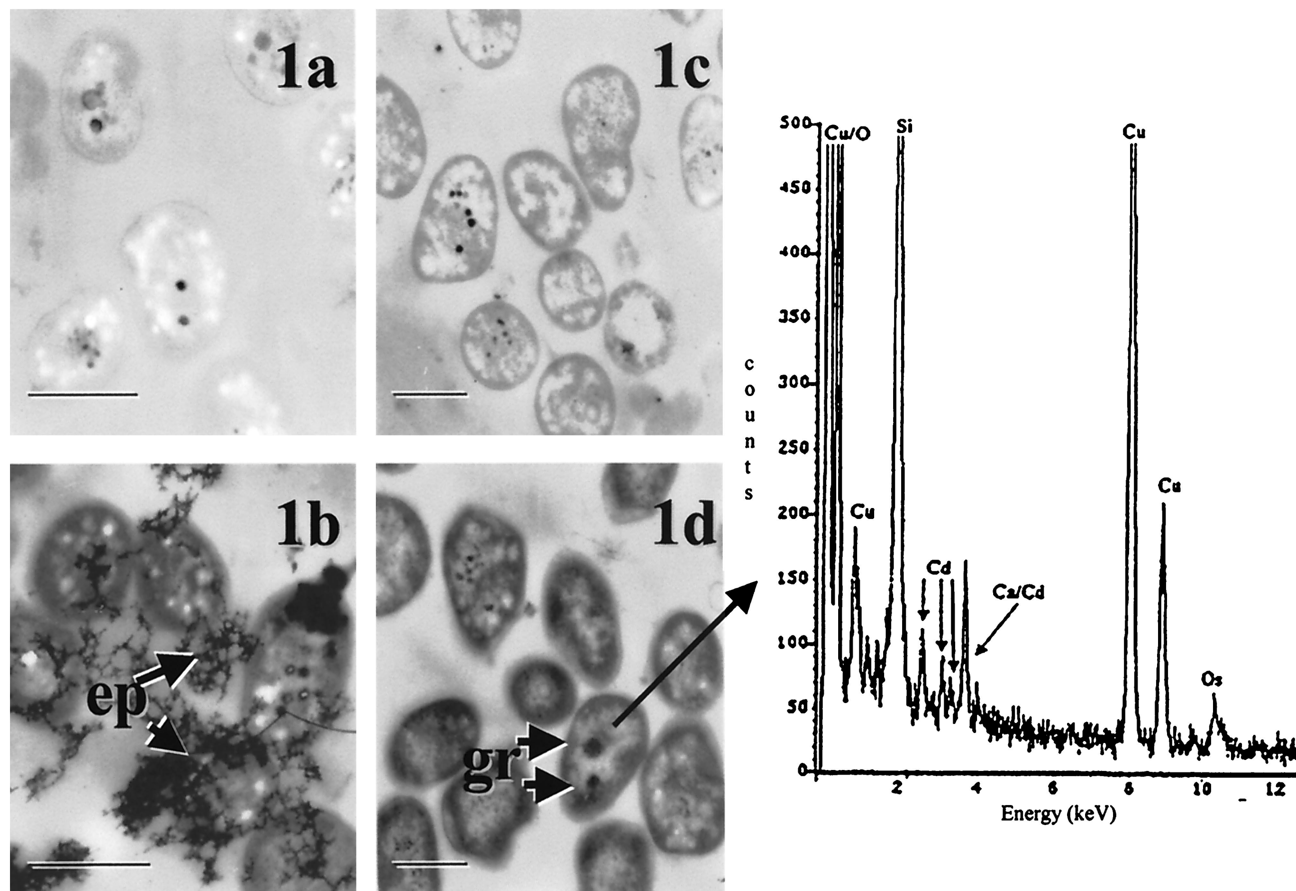


FIG. 1. TEM micrographs of *Pseudomonas* strain I1a in the absence of cadmium (a) and when exposed to 20 μg of cadmium $ml^{-1}$ (b). Note the dark precipitate (ep) associated with the surrounding EPS layer (b), confirmed as cadmium by elemental X-ray analysis. TEM micrographs of *Pseudomonas* strain H1 in the absence of cadmium (c) and when exposed to 125 μg of cadmium $ml^{-1}$ (d) are shown. Note the dense accumulations (gr), confirmed to be cadmium with elemental analysis. In both panels b and d, overall cellular density increased due to nonspecific metal binding. Bars equal 0.5 μm. In the spectrum provided, the copper (Cu) peaks were from the copper grid, the silicon (Si) peak was from the embedding medium Spurrs, and the osmium (Os) peak was from staining with $OsO_4$. The cadmium (Cd) peaks confirmed the presence of cadmium.

augmentation approach was initially examined with MSM broth amended with 500 μg of 2,4-D and cadmium $ml^{-1}$, wherein the cocontaminated broth was inoculated with $10^4$ CFU of a cadmium-resistant isolate $ml^{-1}$, incubated for 48 h at 28°C, and then reinoculated with $10^4$ CFU of *R. eutropha* JMP134 $ml^{-1}$. Complete 2,4-D degradation by *R. eutropha* JMP134 occurred in the presence of 12 μg of cadmium $ml^{-1}$ with the cadmium-resistant isolate *Pseudomonas* strain I1a and 24 μg of cadmium $ml^{-1}$ with the cadmium-resistant isolates *Arthrobacter* strain D9, *Bacillus* strain H9, and *Pseudomonas* strain H1. Interestingly, when the levels of soluble cadmium following growth of each of the cadmium-resistant isolates

TABLE 2. The influence of microbial growth from $10^4$ to $10^8$ cells $ml^{-1}$ on the solubility of cadmium in MSM broth, pH 6.0

| Total cadmium (μg/25 ml) | % Total cadmium remaining soluble (48 h postinoculation) with: | | | | |
|---|---|---|---|---|---|
| | No inoculum | *Pseudomonas* strain H1 | *Bacillus* strain H9 | *Arthrobacter* strain D9 | *Pseudomonas* strain IIa |
| 300 | 99.9 | 79 ± 0.4 | 85 ± 4.6 | | |
| 625 | 99.9 | 76 ± 2.5 | 58 ± 10.7 | | |
| 1,250 | 98.8 | 67 ± 4.0 | 75 ± 29.3 | | |
| 1,575 | 98.2 | 40 ± 0.0 | 35 ± 5.5 | | |
| 3,125 | 98.5 | 56 ± 5.0 | 68 ± 0.1 | | |
| 75 | 99.9 | | | ND[a] | 86 ± 1.9 |
| 150 | 99.9 | | | 82 ± 6.6 | 89 ± 4.2 |
| 300 | 99.9 | | | 86 ± 2.3 | 95 ± 2.0 |
| 625 | 99.9 | | | 84 ± 1.6 | 85 ± 6.5 |
| 950 | 99.9 | | | 62 ± 1.4 | ND |

[a] ND, not determined.

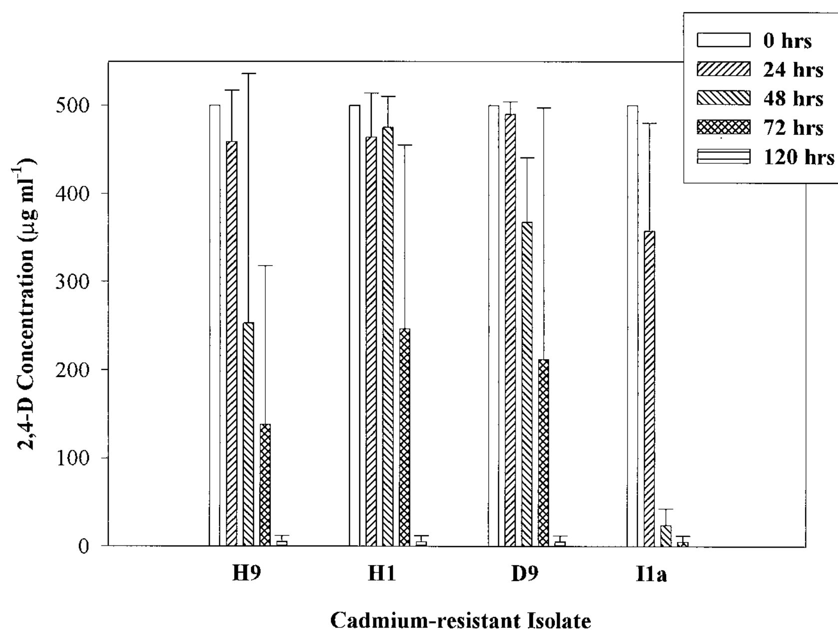


FIG. 2. In broth, cadmium detoxification by isolates *Arthrobacter* strain D9, *Bacillus* strain H9, *Pseudomonas* strain H1, and *Pseudomonas* strain I1a allowed 2,4-D degradation by cadmium-sensitive *R. eutropha* JMP134 in the presence of 12 μg of cadmium $ml^{-1}$ for isolate I1a and 24 μg of cadmium $ml^{-1}$ for isolates D9, H1, and H9. Within 120 h, all isolates allowed the degradation of 500-μg $ml^{-1}$ 2,4-D to undetectable levels. Times as indicated were 48 h following inoculation with the cadmium-resistant isolate.

analyzed in Table 2 were examined, it seemed that the levels of cadmium solubility remained too high to support degradation of 2,4-D by *R. eutropha* JMP134. There may have been additional unidentified mechanisms of cadmium detoxification that did not reduce soluble cadmium concentrations but did render the cadmium nontoxic, as seen with chelation.

The uncontaminated soil used in the laboratory soil microcosms was a Brazito sandy loam with 12% clay, 0.21% organic matter, pH 8.2, and no known previous metal exposure. Indigenous microbial numbers in the soil were $3.2 \times 10^7 \pm 9.1 \times 10^6$ culturable CFU $g^{-1}$ of dry weight on R2A medium (Difco, Baltimore, Md.) and $7.2 \times 10^7 \pm 3.2 \times 10^6$ total cells $g^{-1}$ of dry weight as determined by acridine orange direct microscopic counts.

In laboratory soil microcosms, the cadmium-resistant isolates *Pseudomonas* strain H1, *Bacillus* strain H9, *Arthrobacter* strain D9, and *Pseudomonas* strain I1a appeared to detoxify cadmium, thereby protecting *R. eutropha* JMP134 from cadmium toxicity as 2,4-D degradation occurred in the presence of 60 μg of cadmium $g^{-1}$. Soluble cadmium was not detectable in the amended soils; however, cadmium toxicity effects were observed in the contaminated soils as *R. eutropha* JMP134 2,4-D degradation was inhibited. Table 3 summarizes the specific rates of degradation for each isolate. Within 50 days, the cadmium-resistant *Pseudomonas* strains H1 and I1a allowed the complete degradation of 500-μg of 2,4-D $ml^{-1}$. Upon addition of cadmium-resistant *Bacillus* strain H9 and *Arthrobacter* strain D9, degradation occurred within 35 days. Interestingly, neither the indigenous microbial flora nor the cadmium-resistant isolates could degrade 2,4-D in the cadmium-contaminated soil system within the 50-day time frame. Under the conditions of this experiment, *R. eutropha* JMP134 also did not degrade 2,4-D when cadmium was present. In Brazito soil amended only with 2,4-D, complete 2,4-D degradation by *R. eutropha* JMP134 occurred within 5 days. It should be noted that the Brazito soil used in this study was not sterile and consequently presented competitive challenges for the introduced organisms, and yet degradation still occurred in the cocontaminated soils upon inoculation with the cadmium-resistant isolates.

We also tested the dual-bioaugmentation strategy in an intermediate field scale experiment. At the intermediate field scale, more variability was evident than in the bench-scale studies (Fig. 3). However, several conclusions can still be drawn. When 2,4-D was added to the Brazito soil without cadmium, slow rates of degradation ultimately occurred without bioaugmentation with *R. eutropha* JMP134 (Fig. 3a), as

TABLE 3. Degradation of 500-μg $g^{-1}$ 2,4-D by *R. eutropha* JMP134[a] in laboratory soil microcosms to undetectable levels with 60-μg $g^{-1}$ cadmium and a cadmium-detoxifying isolate present

| Day | 2,4-D concn (μg $g^{-1}$) with: | | | |
|---|---|---|---|---|
| | *Pseudomonas* strain I1a | *Pseudomonas* strain H1 | *Bacillus* strain H9 | *Arthrobacter* strain D9 |
| 7 | 412 ± 123 | 400 ± 143 | 550 ± 19 | 500 ± 118 |
| 14 | 505 ± 7 | 550 ± 50 | 555 ± 59 | 538 ± 48 |
| 21 | 550 ± 0 | 500 ± 0 | 472 ± 39 | 158 ± 0 |
| 28 | 433 ± 10 | 495 ± 10 | 113 ± 0 | 146 ± 30 |
| 35 | 300 ± 50 | 289 ± 28 | 10 ± 0 | 10 ± 2 |
| 42 | 300 ± 35 | 238 ± 14 | ND[b] | ND |
| 50 | 10 ± 5 | 10 ± 12 | ND | ND |

[a] *R. eutropha* JMP134 alone degraded the 500 μg of 2,4-D $g^{-1}$ in the absence of cadmium to an undetectable level in 5 days.

[b] ND, not detected.

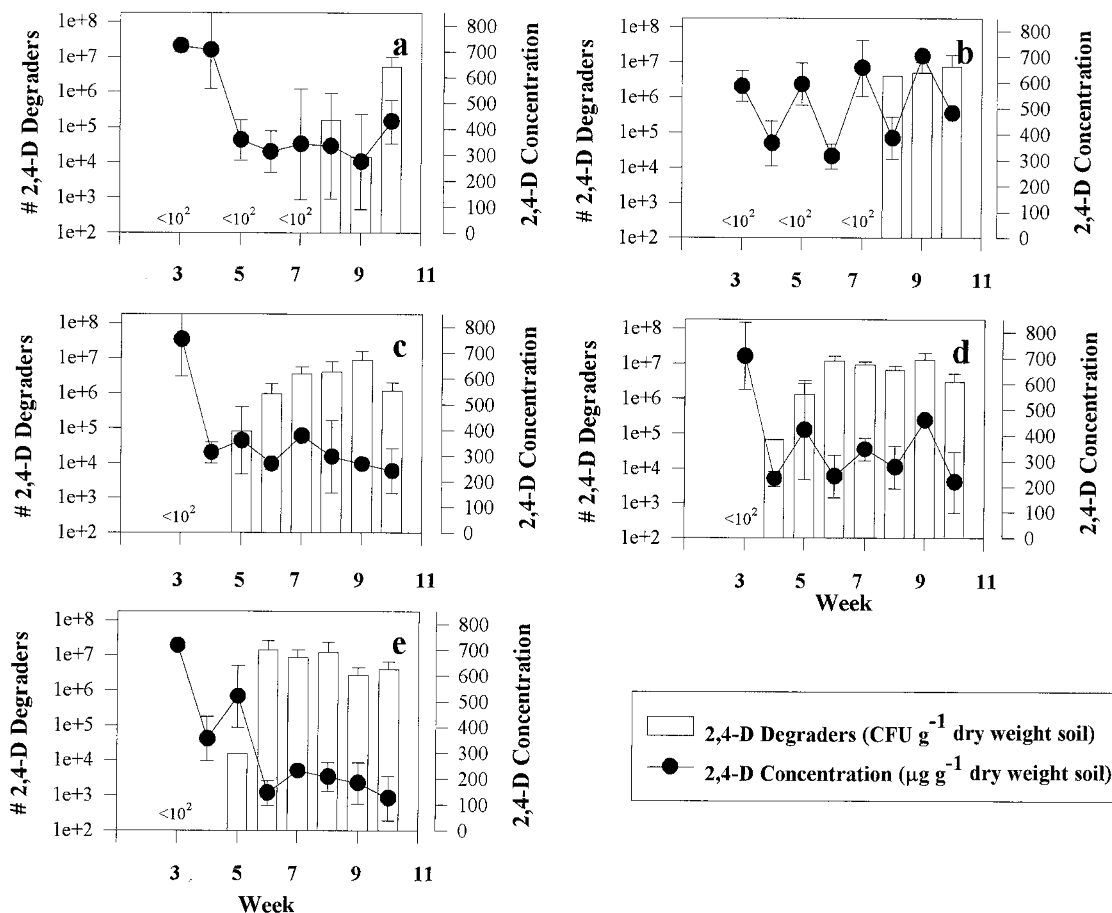


FIG. 3. The degradation of 500-μg $g^{-1}$ 2,4-D and the appearance of 2,4-D-degrading microbial populations with time in weeks, as detected in pilot field scale bioreactors containing Brazito soil amended with 2,4-D only (Treatment 1) (a); 2,4-D and 60 μg of cadmium $g^{-1}$ (Treatment 2) (b); 2,4-D and $10^4$ CFU of *R. eutropha* JMP134 $g^{-1}$ (Treatment 3) (c); 2,4-D, 60 μg of cadmium $g^{-1}$, and $10^4$ CFU of *R. eutropha* JMP134 $g^{-1}$ (Treatment 4) (d); 2,4-D, 60 μg of cadmium $g^{-1}$, $10^4$ CFU of *R. eutropha* JMP134 $g^{-1}$, and $10^4$ CFU of cadmium-resistant *Pseudomonas* strain H1 $g^{-1}$ (Treatment 7) (e). The original number of 2,4-D degraders was fewer than $10^2$ CFU $g^{-1}$ of soil prior to bioaugmentation. Treatment 5 (2,4-D and *Pseudomonas* strain H1) and Treatment 6 (2,4-D, 60 μg of cadmium $g^{-1}$, and *Pseudomonas* strain H1) are not shown.

indigenous microbial populations acclimated to the 2,4-D. However, even after 10 weeks, degradation was incomplete, and 2,4-D levels did not decrease between weeks 5 and 10. The apparent degradation observed in the absence of inoculation in the field indicates the presence of a native population of 2,4-D-degrading microorganisms that were not detected in the soil microcosm experiments. In the presence of 60 μg of cadmium $g^{-1}$, indigenous degradation appeared to be inhibited in the absence of bioaugmentation (Fig. 3b) even though 2,4-D degraders were culturable within the soil after 8 weeks. Under all treatment conditions, in the absence of *R. eutropha* JMP134 inoculation, 2,4-D-degrading organisms did not appear until week 8. The occurrence of these degraders may have been due to microsite variation in cadmium concentrations in soil or the use of alternate carbon sources available in the soil. In the laboratory, we have observed bacterial populations that were cadmium resistant and could degrade 2,4-D that were unable to resist cadmium and degrade 2,4-D concurrently, possibly due to the energy demand placed on the organism. Consequently, the indigenous 2,4-D degraders may not have been able to degrade 2,4-D in the presence of the cadmium, since the EMB medium used to select for 2,4-D-degrading organisms did not contain cadmium. In the reactors inoculated with *R. eutropha* JMP134, the number of 2,4-D-degrading organisms fell from the inoculated $10^4$ CFU of *R. eutropha* JMP134 $g^{-1}$ to $<10^2$ CFU of 2,4-D-degrading organisms $g^{-1}$ during weeks 1 through 3. By week 4 or 5, however, the number of 2,4-D degraders increased dramatically, to $10^7$ CFU $g^{-1}$ (Fig. 3c to e).

In Treatment 3 (Fig. 3c), with 2,4-D and *R. eutropha* JMP134, as observed in all the reactors inoculated with *R. eutropha* JMP134, a 2,4-D-degrading population was evident by week 5 ($10^5$ CFU $g^{-1}$). The concentration of 2,4-D in Treatment 3 was reduced to 300 mg $kg^{-1}$ in the first 4 weeks and then remained stable, indicative of incomplete degradation or slow rates of degradation. Interestingly, degradation occurred prior to the appearance of 2,4-D degraders, probably due in part to some nonculturable degraders on the EMB medium. In Treatment 4 reactors (cadmium and *R. eutropha* JMP134; Fig. 3d), degradation was less apparent, and even though by week 6, $10^7$ 2,4-D degraders $g^{-1}$ could be recovered, cadmium appeared to have an effect on 2,4-D degradation.

TABLE 4. Comparison of 2,4-D degradation levels in field study treatments based on analysis of variation followed by Fisher's protected least-significant-difference pairwise comparison

| Treatment comparison | Significance ($P$ value)[f] |
|---|---|
| 2,4-D[a] vs 2,4-D + Cd[b] (1 vs 2)[c] | 0.0226 |
| 2,4-D vs 2,4-D + JMP[d] (1 vs 3) | 0.2258 |
| 2,4-D + Cd vs 2,4-D + Cd + JMP (2 vs 4) | 0.0183 |
| 2,4-D + JMP vs 2,4-D + Cd + JMP (3 vs 4) | 0.0520 |
| 2,4-D + Cd + JMP vs 2,4-D + Cd + JMP + H1[e] (4 vs 7) | 0.0150 |
| 2,4-D vs 2,4-D + H1 (1 vs 5) | 0.1405 |
| 2,4-D + Cd vs 2,4-D + Cd + H1 (2 vs 6) | 0.2933 |
| 2,4-D + Cd + H1 vs 2,4-D + Cd + JMP + H1 (6 vs 7) | 0.0061 |

[a] 500 μg g of soil$^{-1}$ (dry weight).
[b] 60 μg g of soil$^{-1}$ (dry weight).
[c] Treatment numbers are in parentheses.
[d] JMP, *R. eutropha* JMP134 (2,4-D degrader).
[e] H1, *Pseudomonas* strain (cadmium-resistant).
[f] $P$ values of ≤0.05 indicate 95% significance.

The variation in 2,4-D degradation observed in Treatment 4 and Treatment 2 correlated with the cadmium amendment that resulted in sporadic degradation due to microscale toxicity effects. As expected from both the pure culture and soil microcosm experiments, cadmium-resistant *Pseudomonas* strain H1 (Treatment 5 and Treatment 6) did not degrade or facilitate the increased degradation (above background levels seen in Treatment 1) of 2,4-D within the 70-day time frame of the field study, regardless of whether cadmium was present or not.

In Treatment 7 reactors (2,4-D, cadmium; *R. eutropha* JMP134 and *Pseudomonas* strain H1), the extent of degradation was noticeably enhanced upon addition of the coinoculants (Fig. 3e). As observed in the soil microcosms, the reactors with 2,4-D and cadmium, coinoculated with cadmium-resistant *Pseudomonas* strain H1 and *R. eutropha* JMP134, exhibited substantial degradation in conjunction with the appearance of $>10^6$ CFU of 2,4-D-degrading organisms g$^{-1}$ of dry weight, suggesting that *Pseudomonas* strain H1 conferred a protective effect. Degradation to 100 mg of 2,4-D kg$^{-1}$ occurred by week 6 in conjunction with the appearance of $10^7$ 2,4-D degraders g$^{-1}$. Thus, it appears that initial inoculation with the cadmium-resistant isolate *Pseudomonas* strain H1 detoxified the cadmium. Interestingly, an estimated 90% of the recovered 2,4-D-degrading organisms were strains other than *R. eutropha* JMP134.

Mean values for duplicate 2,4-D readings at each time point for weeks 5 through 10 where 2,4-D degradation occurred were used for analysis of variation followed by Fisher's protected least-significant-difference pairwise comparison between treatments (Table 4). The finding of no significant difference between the 2,4-D (Treatment 1) and 2,4-D plus *R. eutropha* JMP134 (Treatment 3) treatments indicated that indigenous microbial populations were capable of some 2,4-D degradation. This was surprising, since no indigenous degradation was observed in the soil microcosms. However, in both soil microcosms and field bioreactors, cadmium inhibited both indigenous degradation and that by *R. eutropha* JMP134 (Treatment 2 and Treatment 4, respectively). With a $P$ value of ≤0.05 taken to be 95% confidence, there was not a significant difference in degradation rates between the *R. eutropha* JMP134 augmented reactors with and without cadmium ($P$ = 0.052). However, cadmium did affect the degradation by increasing the variability of the readings, and in Treatment 7 (Fig. 3d), the addition of *Pseudomonas* strain H1 significantly increased the rate of degradation of 2,4-D ($P$ = 0.015). Significant degradation was observed in treatments with both *Pseudomonas* strain H1 and *R. eutropha* JMP134 (Treatment 4 versus Treatment 7 and Treatment 6 versus Treatment 7). No degradation was observed in treatments with *Pseudomonas* strain H1 alone (Treatment 1 versus Treatment 5 and Treatment 2 versus Treatment 6).

## DISCUSSION

This study has demonstrated the use of a dual-bioaugmentation strategy in the remediation of cocontaminated systems. This strategy involved the coinoculation of a metal-resistant microbial population with an organic-degrading population, the primary mode of action being metal detoxification, such that organic degradation was no longer inhibited. Based on promising results in laboratory experiments with both pure culture and soil microcosms, we examined this strategy in a field trial. The rates of 2,4-D degradation by *R. eutropha* JMP134 in the presence of *Pseudomonas* strain H1 were surprisingly similar in both the laboratory soil microcosms and in the field study (approximately 50 days), though 2,4-D degradation was not complete in the field study. Also interesting was the observation that many of the 2,4-D-degrading isolates preliminarily examined for the pJP4 plasmid recovered in the field experiment were not *R. eutropha* JMP134. This and the observation that indigenous 2,4-D degradation was not significant with cadmium suggested that transfer of the pJP4 plasmid to indigenous populations occurred. The field study did demonstrate that bioaugmentation using coinoculants is a viable option for the remediation of metal- and organically contaminated soils.

While the Cad operon was not found in any of the isolates, this does not exclude the presence of a cadmium-efflux system; however, the isolates examined reduced soluble cadmium concentrations, indicating the use of an alternative mechanism of resistance. Since soluble metal is thought to be more toxic than bound or precipitated metal, each of the four isolates effectively reduced cadmium toxicity. Of the four isolates, *Pseudomonas* strain H1 and *Bacillus* strain H9 appeared to use an intracellular mechanism of cadmium sequestration. While intracellular microbial cadmium accumulation has not been well documented, the observed increase in outer membrane density upon exposure to cadmium indicated binding of metal to lipopolysaccharides, as found by Landley and Beveridge (15). Metallothionein production and polyphosphate precipitation represent two possible explanations wherein cadmium is sequestered intracellularly. However, the precise mechanism of intracellular accumulation of cadmium merits further investigation.

The two other cadmium-resistant isolates, *Pseudomonas* strain I1a and *Arthrobacter* strain D9, showed evidence of EPS production upon staining and under the TEM showed cadmium accumulation external to the cells. Similarly, a study by Roane (21) found that EPS production resulted in extracellular lead sequestration. Metal binding to exopolymers is known to reduce metal toxicity. While generally associated with ad-

hesion and protection against desiccation, exopolymers act as strong ionic attractants and, thus, readily bind metals.

It was interesting that the two most cadmium-resistant isolates were *Pseudomonas* strain H1 (resistant up to 225 μg $ml^{-1}$) and *Bacillus* strain H9 (resistant up to 275 μg $ml^{-1}$), which both exhibited intracellular cadmium accumulation. The resistance mechanisms of these two organisms were also plasmid encoded. Finally, the most dramatic decreases in soluble cadmium upon growth were seen with *Pseudomonas* strain H1 and *Bacillus* strain H9, in that there was a 36% loss in soluble cadmium with growth. *Arthrobacter* strain D9 and *Pseudomonas* strain I1a showed less detoxification, with a resulting decrease of 22 and 11% in soluble cadmium with growth.

This study found that while dual bioaugmentation with metal-detoxifying and organic-degrading microbial populations is effective at cocontaminant remediation, time must be allowed for metal detoxification to occur before organic degradation is observed. Evidence of this was observed in the 48 h needed between inoculation with the metal-detoxifying population and inoculation with the organic-degrading population. We found that the viability of the organic-degrading population decreased when it was added to the system prior to metal detoxification. Only using this staggered approach to bioaugmentation was remediation of a cocontaminated soil successful.

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