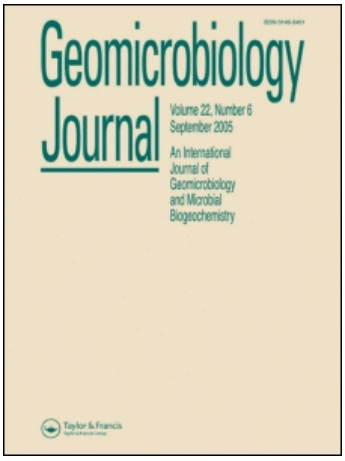

Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713722957

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Online publication date: 15 January 2010

To cite this Article Gao, Weimin, Gentry, Terry J., Mehlhorn, Tonia L., Carroll, Susan L., Jardine, Philip M. and Zhou, Jizhong(2010) 'Characterization of Co(III) EDTA-Reducing Bacteria in Metal- and Radionuclide-Contaminated Groundwater', Geomicrobiology Journal, 27: 1, 93 — 100
To link to this Article: DOI: 10.1080/01490450903408112
URL: http://dx.doi.org/10.1080/01490450903408112

Geomicrobiology Journal, 27:93–100, 2010

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490450903408112

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Characterization of Co(III) EDTA-Reducing Bacteria in Metal- and Radionuclide-Contaminated Groundwater

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The Waste Area Grouping 5 (WAG5) site at Oak Ridge National Laboratory has a potential to be a field site for evaluating the effectiveness of various bioremediation approaches and strategies. The site has been well studied in terms of its geological and geochemical properties over the past decade. However, despite the importance of microorganisms in bioremediation processes, the microbiological populations at the WAG5 site and their potential in bioremediation have not been similarly evaluated. In this study, we initiated research to characterize the microbial populations in WAG5 groundwater. Approximately 100 isolates from WAG5 groundwater were isolated and selected based on colony morphology. Fifty-five unique isolates were identified by BOX-PCR and subjected to further characterization. 16S rRNA sequences indicated that these isolates belong to seventeen bacterial genera including *Alcaligenes* (1 isolate), *Aquamonas* (1), *Aquaspirillum* (1), *Bacillus* (10), *Brevundimonas* (5), *Caulobacter* (7), *Dechloromonas* (2), *Janibacter* (1), *Janthinobacterium* (2), *Lactobacillus* (1), *Paenibacillus* (4), *Pseudomonas* (9), *Rhodoferax* (1), *Sphingomonas* (1), *Stenotrophomonas* (6), *Variovorax* (2), and *Zoogloea* (1). Metal respiration assays identified several isolates, which phylogenically belong or are close to *Caulobacter, Stenotrophomonas, Bacillus, Paenibacillus* and *Pseudomonas,* capable of reducing Co(III)EDTA$^-$ to Co(II)EDTA^{2-} using the defined M1 medium under anaerobic conditions. In addition, using WAG5 groundwater directly as the inoculants, we found that organisms associated with WAG5 groundwater can reduce both Fe(III) and Co(III) under anaerobic conditions. Further assays were then performed to determine the optimal conditions for Co(III) reduction. These assays indicated that addition of various electron donors including ethanol, lactate, methanol, pyruvate, and acetate resulted in metal reduction. These experiments will provide useful background information for future bioremediation field experiments at the WAG5 site.

Keywords bioremediation, dissimilatory metal reduction, groundwater, metal reduction, molecular ecology

INTRODUCTION

Many U.S. Department of Energy (DOE) sites are contaminated with radioactive materials as a result of World War II and Cold War era nuclear weapons research and production (http://www.em.doe.gov/pages/History.aspx). Offsite migration of these radionuclides poses a threat to public groundwater and surface water supplies. Many of these sites also contain a plethora of mixed wastes, including various organic compounds and metals, which further complicate containment and remediation efforts. One such location, the Waste Area Grouping 5 (WAG5) site at the Oak Ridge Reservation in Oak Ridge, TN, contains hundreds of unconfined shallow land burial trenches which received mixed wastes. The hydrological and transport processes at WAG5 have been well characterized (Jardine et al. 1999), and the area has been used as a model site to investigate the impact of hydrological and chemical processes on the fate and transport of various contaminants including Cd, Co, Cr, and trichloroethylene (TCE) (Table 1) (Jardine et al. 2002; Lenczewski et al. 2003). However, to date, the microbial populations which may be integral to these processes at the WAG5 site have not been similarly characterized.

One specific radionuclide, which is of concern of DOE due to its strong potential for offsite migration, is ^{60}Co. Organic chelating agents, such as EDTA which is present at WAG5 , can produce stable complexes with ^{60}Co thus increasing the aqueous solubility of ^{60}Co, reducing the interactions of ^{60}Co with subsurface minerals, and increasing the potential for transport of ^{60}Co offsite (Means et al. 1978). One possible means to reduce this

Received 3 February 2009; accepted 24 August 2009.
We appreciate insightful comments and suggestions of anonymous reviewers on the improvement of this manuscript. This research was supported by The United States Department of Energy under the Laboratory Directed and Research Program at ORNL. Oak Ridge National Laboratory is managed by the University of Tennessee-Battelle LLC for the Department of Energy under contract DE-AC05-00OR22725.
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TABLE 1
Selected chemical properties of WAG5 groundwater

Component	mg/L	Component	mg/L
Al	0−0.1	Mn	1−10
Br	0.1−0.2	Na	15−35
Ca	100−180	NO_2	0−0.03
Cd	0−0.2	NO_3	0−0.4
CH_4	1−4	PO_4	0−0.02
Cl	8−20	S_2^-	0.01−0.5
Co	0.01−0.5	Si	7−14
Fe(II)	0.2−5	SO_4	1−5
Fe(III)	0	DO	0.02−1
HCO_3	670−900	TOC	1−4
K	2−15	^3H	0.06*
Mg	9−23	pH	6.7−7.0

*μCi/ml.

migration is via microbial reduction of Co(III)-EDTA$^-$ to the less stable Co(II)-EDTA^{2-}. Co(II)-EDTA^{2-} can dissociate in the presence of cationic metals and is also sorbed more strongly to subsurface solids than is Co(III)EDTA$^-$ (Girvin et al. 1993; Brooks et al. 1999; Jardine et al. 2002; Krumholz et al. 2003). Several different bacteria have been identified that either directly or indirectly reduce Co(III)EDTA$^-$ to Co(II)EDTA^{2-} (Caccavo et al. 1994; Gorby et al. 1998; Blessing et al. 2001; Roh et al. 2002). It is possible for Fe- and Mn-oxides to reverse this process by catalyzing the oxidation of Co(II)EDTA^{2-}to Co(III)EDTA$^-$ (Gorby et al. 1998), but previous research indicated that this reverse reaction was limited under anaerobic conditions at the WAG5 site (Jardine et al. 2002).

The primary goals for this project were to identify the Co(III)EDTA$^-$-reducing bacteria present in WAG5 groundwater and to determine what electron donor and nutrient amendments best stimulated microbial Co(III)EDTA$^-$ reduction. Our results have showed that several isolates are capable of reducing Co(III) to Co(II) using the defined M1 medium under anaerobic conditions. As well, this study indicated that addition of various electron donors including ethanol, lactate, methanol, pyruvate, and acetate resulted in metal reduction using WAG5 groundwater as a basal medium.

Bacterial Enumeration and Isolation of Potential Co(III)EDTA$^-$-Reducing Bacteria

The WAG5 site and sampling wells has been described in detail previously (Jardine et al. 1999; Lenczewski et al. 2003). Groundwater from WAG5 wells 2–4, 10 and 16 was collected for isolation of culturable bacteria in this study and the collection procedure was described previously (Lenczewski et al. 2003). These wells are about between 4 to 6 meters deep and within 12 meters of each other. The above mean sea level (amsl) for these sampling wells are about 232.2, 234.5 and 233.5 meter for well 2–4, 16 and 10, respectively. Groundwater was collected in purged anaerobic bottles using a peristaltic pump. Bacteria in all sampled wells of WAG5 were enumerated with spread plating and direct counts. Groundwater was serially diluted in 0.1% $Na_4P_2O_7$ then plated onto R2A, trypticase soy agar (TSA), and nutrient agar (Difco). Triplicate plates were incubated in the dark at room temperature for 5 days prior to counting. Direct counts were performed on groundwater with acridine orange staining and epifluorescence microscopy (Lawrence et al. 2002). Log_{10}-transformed bacterial numbers from each medium were compared using ANOVA ($P = 0.05$) with Microsoft Excel 2003.

Culturable bacteria were isolated from WAG5 wells 2–4, 10, and 16 by spread-plating groundwater onto agar either directly or following enrichment. Media included: 1) aerobic mR2A agar; 2) aerobic R2A; 3) aerobic nutrient agar; 4) aerobic TSA; 5) anaerobic mR2A broth followed by isolation on aerobic or anaerobic mR2A agar (Fries et al. 1994); 6) NO_3^- broth [1 g KNO_3/L and 20 mM lactate; in NATE mineral medium: 1 g $MgSO_4$; 1 g K_2SO_4; 1 g Na_3PO_4; 0.2 g $CaCl_2$; 0.1 g NH_4Cl; 2.7 mg $FeCl_3{\cdot}6H_2O$; 50 μg $CuSO_4$; 10 μg $MnSO_4$; 70 μg $Zn(NO_3)_2$; 10 μg $CoCl_2$; 10 μg MoO_3/L] enrichment followed by isolation onto R2A, TSA, or nutrient agar; or 7) H_2+ Co(III) broth [1 mM ^{59}Co(III)EDTA$^-$ and 20 mM lactate in NATE mineral medium with a headspace containing 7.9% H_2, 5.2% CO_2, and 86.9% N_2] enrichment followed by isolation onto R2A, TSA, or nutrient agar. A solution of ^{59}Co(III)EDTA$^-$ was prepared according to the method of Taylor and Jardine (1995).

The results showed that culturable aerobic, heterotrophic bacterial counts from well 16 on R2A, TSA, and NA were not significantly different ($P = 0.24$) with 9.00 ($\pm$ 0.78) $\times$ 10^4, 6.77 ($\pm$ 1.86) $\times$ 10^4, and 7.33 ($\pm$ 1.27) $\times$ 10^4 CFU/mL, respectively. Direct counts were approximately 40 times higher than culturable counts at 3.38 $\times$ 10^6 cells/mL. The colony forming unit (CFU) counts on R2A agar with groundwater from well 2–4 and well 10 were also performed, and they were 5.5 ($\pm$1.52) $\times$ 10^5 and 1.45 ($\pm$1.24) $\times$ 10^6, respectively. The direct counts from these two wells were 1.35 $\times$ 10^7 and 3.6 $\times$ 10^7, respectively. Although within 12 meters of each other at the similar water levels, our results from bacterial enumeration showed that there were differences of bacterial density between wells and this suggested that the geochemical heterogeneity may exist among these closely located wells. Finally, totally 95 isolates were obtained from the various media.

Characterization of Isolated Bacteria

Isolated bacteria were grouped with BOX-PCR fingerprinting. Each isolate was transferred to 0.04 M NaOH (50 μl) with a small inoculation loop and then frozen at −80°C. The samples were then quickly thawed at 65°C, and subsequently heated to 96°C for 10 min. A 1-μL aliquot of lysed cells was used for BOX-PCR (25 μL total reaction volume) according to the protocol (Rademaker et al. 1998). The PCR parameters were: 95°C for 7 min; 35 cycles of 94°C for 1 min, 53°C for 1 min, and

65°C for 8 min; and 65°C for 16 min. Samples were observed on 1.5% TAE agarose gels with ethidium bromide staining. Gels were analyzed and compared with Molecular Analyst Software 1.6 (BioRad, Inc.).

Our results showed that 95 isolates obtained from the various media represented 55 unique BOX-PCR fingerprints (Table 2). Unique isolates were further characterized by partial sequencing of their 16S rRNA genes. Bacterial total DNA was isolated with Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The 16S rRNA genes were amplified from isolates with the PCR primers FD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′). PCR conditions are described as in references (Ye et al. 2004), and PCR products were purified with the ArrayIt PCR Purification Kit (TeleChem International, Inc.) or treated with ExoSAP-IT® (US Biochemical Corporation) according to manufacturer's instructions. DNA sequences were determined with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using a 3700 DNA Analyzer (Perkin-Elmer) according to the manufacturer's instructions using the primer 529R (5′-CGC GGC TGC TGG CAC-3′). Sequences were compared against those in GenBank using BLAST 2.2.10 (Altschul et al. 1997). Phylogenetically, the unique isolates were evenly distributed among the α-, β-, and γ-*Proteobacteria* and *Firmicutes* (Table 2). Sequences were deposited in GenBank under accession numbers DQ268765 to DQ268824.

Isolated bacteria were tested for the ability to reduce Co(III)EDTA$^-$, Fe(III), and fumarate in M1 medium containing Co(III)EDTA$^-$ (0.2 mM), Fe(III) citric acid (10 mM), or fumarate (10 mM), respectively. M1 medium [15 g $N(CH_2COOH)_3$;10 g NaCl; 10 g $CaCl_2{\cdot}2H_2O$; 1.19 g $(NH_4)_2SO_4$; 0.56 g KH_2PO_4; 2.17 mg Na_2SeO_4; 2.0 g $FeCl_2{\cdot}4H_20$; 1.0 g $MgCl_2{\cdot}6H_2O$; 0.2 g Na_2WO_4; 1.0 g $MnCl_2{\cdot}4H_2O$; 1.0 g $CoCl_2{\cdot}6H_2O$; 0.5 g ZnCl2; 20 mg $CuCl_2{\cdot}2H_2O$; 50 mg H_3BO_3; 0.1 g $Na_2MoO_4{\cdot}2H_2O$; 0.17 g Na_2SeO_3; 0.24 g $NiCl_2{\cdot}6H_2O$/L] was prepared on a gas station to introduce anaerobic conditions before filling in glass tubes (10 ml per tube) anaerobically, followed by stoppering and autoclaving. Inoculation was carried out in an anaerobic glove box using 0.2 ml culture of each isolate grown in mR2A liquid medium without shaking under dark for 2 days at room temperature. Assays were performed in triplicate for each isolate. During 1 month of incubation at room temperature in the dark, tubes were checked for reduction of Co(III) and Fe(III) according to colorimetric changes of medium, whereas fumarate reduction was indicated by turbidity of medium.

It was found that many of the isolates could reduce fumarate, but none could reduce iron under the tested conditions (Table 2). Seven distinct isolates could reduce Co(III)EDTA$^-$ including three *Firmicutes* (isolates 3B4, 5F11, and 9F3; *Bacillus* and *Paenibacillus* spp.), two α-*Protebacteria* (isolates 1E4 and 3G9; *Caulobacter* spp.), and two γ-*Proteobacteria* (isolates 2B9 and 6H10; *Pseudomonas* and *Stenotrophomonas* spp.).

Seven distinct, relatively diverse bacteria isolated from WAG5 could reduce Co(III)EDTA$^-$. This is not surprising given the wide range of Co(III)EDTA$^-$-reducing bacteria that have been reported in the literature including *Deferribacteres* [*Geovibrio* sp. (Caccavo et al. 1996)], *Firmicutes* [*Thermanaerobacter* spp. (Roh et al. 2002)], *Deinococcus-Thermus* [*Thermus* sp. (Kieft et al. 1999)], γ-*Proteobacteria* [*Shewanella* spp. (Gorby et al. 1998; Liu et al. 2002)] and δ-*proteobacteria* [*Geobacter* and *Desulfovibrio* spp. (Caccavo et al. 1994; Blessing et al. 2001)]. A hyperthermophilic Archaea (*Pyrobaculum islandicum*) has also been reported to reduce Co(III)EDTA$^-$ (Kashefi and Lovley 2000).

It was recently shown that Co(III) reduction can be facilitated by a cytochrome-mediated process in Shewanella (Hau et al. 2008). However, to our knowledge, the *Caulobacter* spp. (isolates 1E4 and 3G9) isolated from WAG5 are the first α-*Proteobacteria* reported to reduce Co(III)EDTA$^-$. *Caulobacter* spp. are commonly found in aquatic, oligotrophic environments and are known to degrade hydrocarbons and aromatic compounds (Poindexter 1999). A previous research also revealed that members of Caulobacter could be highly resistant to heavy metals including uranium (Hu et al. 2005). There is also evidence for Hg^{2+} reduction by *Caulobacter* spp., but this is likely part of a detoxification mechanism (Ji et al. 1989). In this study, we observed that only two (1E4 and 3G9) out of six tested isolates of *Caulobacter* spp. performed cobalt reduction (Table 2). This may reflect that the conditions for cobalt reduction of other isolates were not met in our research. Due to the toxicity of Co(II)EDTA^{2-} to bacteria (Hau et al 2008), it is also likely that those isolates incapable of cobalt reduction in our experiments lack the capability to detoxify such a byproduct derived from metal transformation.

It was shown that other α-*Proteobacteria* have been shown to reduce different metals including Fe(III) (Kusel et al. 1999). Interestingly, several researchers have found large populations of α-*Proteobacteria* at the DOE Environmental Remediation Sciences Program Oak Ridge Field Research Center (FRC) site which is also in Oak Ridge, TN (Yan et al. 2003; North et al. 2004; Peacock et al. 2004; Fields et al. 2005). The FRC site is contaminated with several compounds including metals, nitrate, and organic solvents along with uranium, plutonium, and technetium radionuclides (http://www.esd.ornl.gov/orifrc/). It is unclear what ecological function that the α-*Proteobacteria* are performing at the FRC site, but they appear to be enriched in the contaminated environment with one study reporting over 50% of 16S rRNA clones in contaminated sediment to be from α-*Proteobacteria* as compared to about 10% in uncontaminated sediment (North et al. 2004).

We did not explicitly test whether the *Caulobacter* spp. in this study directly reduced Co(III)EDTA$^-$ as an electron acceptor or indirectly via the production of reducing compounds such as sulfide (Blessing et al. 2001), but the enrichment medium only contained small amounts of alternative electron acceptors such as sulfate and no production of sulfides was observed thus suggesting that the Co(III)EDTA$^-$ was directly reduced. Our isolation methods undoubtedly underestimated

TABLE 2
Characteristics of bacteria isolated from WAG5 groundwater

Isolate	Well	Source[1]	Agar[2]	Closest Match in GenBank (%)[3]	GenBank Accession No.	Reduced[4]		
						Co(III)	Fe(III)	Fumarate
1D1	2–4	GW	mR2A	*Stenotrophomonas maltophilia C20 (99%)*	DQ268783	no	no	no
1D2	2–4	GW	mR2A	*Brevundimonas vesicularis DW-1 (86%)*	DQ268784	no	no	yes
1D5	2–4	GW	mR2A	*Caulobacter sp. FWC17 (93%)*	DQ268817	–	–	–
1D8	2–4	GW	mR2A	*Brevundimonas sp. pfB9 (99%)*	DQ268785	no	no	no
1D9	2–4	GW	mR2A	*Zoogloea ramigera (96%)*	DQ268786	–	–	–
1D10	2–4	GW	mR2A	*Variovorax paradoxus dS (99%)*	DQ268787	–	–	–
1D11	2–4	GW	mR2A	*Variovorax paradoxus P1 (100%)*	DQ268788	–	–	–
1D12	2–4	GW	mR2A	*Stenotrophomonas maltophilia C20 (99%)*	DQ268789	no	no	no
1E1	2–4	GW	mR2A	*Caulobacter crescentus CB15 (98%)*	DQ268790	no	no	yes
1E4	2–4	GW	mR2A	*Caulobacter sp. (100%)*	DQ268818	yes	no	yes
1E5	2–4	GW	mR2A	*Caulobacter crescentus CB15 (99%)*	DQ268791	no	no	yes
1E7	2–4	GW	mR2A	*Caulobacter sp. FWC17 (94%)*	DQ268792	no	no	yes
1E9	2–4	mR2A	mR2A	*Stenotrophomonas maltophilia c20 (100%)*	DQ268793	no	no	no
1E11	2–4	mR2A	mR2A	*Alcaligenes xylosoxidans H (100%)*	DQ268794	–	–	–
1E12	2–4	mR2A	mR2A	*Lactobacillus plantarum LP3 (99%)*	DQ268795	no	no	yes
2B9	10	GW	mR2A	*Stenotrophomonas maltophilia SM14U (94%)*	DQ268775	yes	no	yes
2B11	10	GW	mR2A	*Aquaspirillum delicatum (98%)*	DQ268776	–	–	–
2C1	10	GW	mR2A	*Pseudomonas lanceolata (99%)*	DQ268777	–	–	–
2C4	10	GW	mR2A	*Dechloromonas sp. ED1 (95%)*	DQ268778	–	–	–
2C5	10	GW	mR2A	*Aquamonas fontana (99%)*	DQ268779	–	–	–
3A1	16	GW	mR2A	*Janthinobacterium sp. HHS7 (99%)*	DQ268765	–	–	–
3A8	16	GW	mR2A	*Janibacter sp. Ho-13 (98%)*	DQ268766	no	no	no
3A10	16	GW	mR2A	*Sphingomonas suberifaciens (93%)*	DQ268768	no	no	yes
3A11	16	GW	mR2A	*Stenotrophomonas sp. MFC-C (94%)*	DQ268815	–	–	–
3A12	16	GW	mR2A	*Brevundimonas vesicularis (99%)*	DQ268769	–	–	–

(Continued on next page)

TABLE 2
Characteristics of bacteria isolated from WAG5 groundwater *(Continued)*

Isolate	Well	Source[1]	Agar[2]	Closest Match in GenBank (%)[3]	GenBank Accession No.	Reduced[4]		
						Co(III)	Fe(III)	Fumarate
3B2	16	GW	mR2A	*Brevundimonas sp. pfB9 (98%)*	DQ268770	no	no	no
3B3	16	GW	mR2A	*Rhodoferax antarcticus (96%)*	DQ268771	–	–	–
3B4	16	GW	mR2A	*Bacillus sp. AH533 (99%)*	DQ268772	yes	no	yes
3G1	16	GW	NA	*Bacillus pumilus (98%)*	DQ268821	–	–	–
3G4	16	GW	R2A	*Bacillus pumilus (98%)*	DQ268823	no	no	yes
3G7	16	GW	R2A	*Brevundimonas sp. 44/11 (99%)*	DQ268803	–	–	–
3G9	16	GW	R2A	*Caulobacter sp. (100%)*	DQ268804	yes	no	yes
3G12	16	GW	R2A	*Caulobacter sp. (100%)*	DQ268807	no	no	yes
4G3	16	GW	TSA	*Bacillus cereus T1 (97%)*	DQ268822	–	–	–
4G5	16	GW	TSA	*Pseudomonas sp. MSB2046 (100%)*	DQ268802	–	–	–
4G10	16	GW	TSA	*Paenibacillus sp. (98%)*	DQ268805	–	–	–
4G11	16	GW	TSA	*Bacillus sp. BacB2 (98%)*	DQ268806	–	–	–
4H7	16	GW	TSA	*Bacillus sp.Tf253 (98%)*	DQ268808	no	no	yes
4H4	16	H_2+ Co	NA	*Pseudomonas sp. (97%)*	DQ268825	no	no	yes
4H11	16	H_2+ Co	NA	*Bacillus chitinolyticus (94%)*	DQ268811	–	–	–
5F7	16	H_2+ Co	R2A	*Stenotrophomonas maltophilia alfa-2 (99%)*	DQ268799	no	no	yes
5F8	16	H_2+ Co	R2A	*Paenibacillus phyllosphaerae (92%)*	DQ268800	no	no	yes
5F4	16	H_2+ Co	R2A	*Paenibacillus sp. (98%)*	DQ268797	yes	no	yes
6F6	16	H_2+ Co	TSA	*Pseudomonas fluorescens (99%)*	DQ268798	no	no	yes
6H10	16	H_2+ Co	TSA	*Pseudomonas sp. 12M9 (89%)*	DQ268810	yes	no	yes
7B5	16	mR2A	mR2A	*Bacillus cereus KNUC55 (93%)*	DQ268773	–	–	–
7B6	16	mR2A	mR2A	*Bacillus sp. P3 (89%)*	DQ268774	–	–	–
7C6	16	mR2A	mR2A	*Dechloromonas sp. SIUL (100%)*	DQ268780	–	–	–
7C8	16	mR2A	mR2A	*Janthinobacterium sp. HHS32 (94%)*	DQ268781	–	–	–
7C12	16	mR2A	mR2A	*Pseudomonas lanceolata (99%)*	DQ268782	–	–	–
7H12	16	mR2A	mR2A	*Pseudomonas sp. pfB13 (100%)*	DQ268812	no	no	yes
8G2	16	NO_3	NA	*Bacillus pumilus (98%)*	DQ268801	no	no	yes
8H5	16	NO_3	R2A	*Pseudomonas sp. CH8 (94%)*	DQ268826	no	no	no
9F3	16	NO_3	TSA	*Paenibacillus polymyxa KCTC3717 (95%)*	DQ268820	yes	no	yes
9G8	16	NO_3	TSA	*Pseudomonas sp. CH8 (90%)*	DQ268824	no	no	no

[1]Direct from groundwater (GW) or enrichment with mR2A, NO_3^-, or H_2+ Co(III) broth.
[2]Final agar used to isolate culture – nutrient agar (NA), mR2A, R2A, or trypticase soy agar (TSA).
[3]Based on partial 16S rDNA sequences. Percent similarity is indicated in parentheses.
[4]Isolates not tested are indicated by –.

the diversity of Co(III)EDTA$^-$-reducing bacteria at the site since we first isolated the bacteria primarily on aerobic, heterotrophic media before testing for Co(III)EDTA$^-$ reduction, and this would have precluded the growth of many potential Co(III)EDTA$^-$- reducers including the many obligate anaerobes in the *δ-Proteobacteria*.

It is interesting that none of the isolated bacteria could reduce Fe(III) since many of the Co(III)-reducing bacteria in

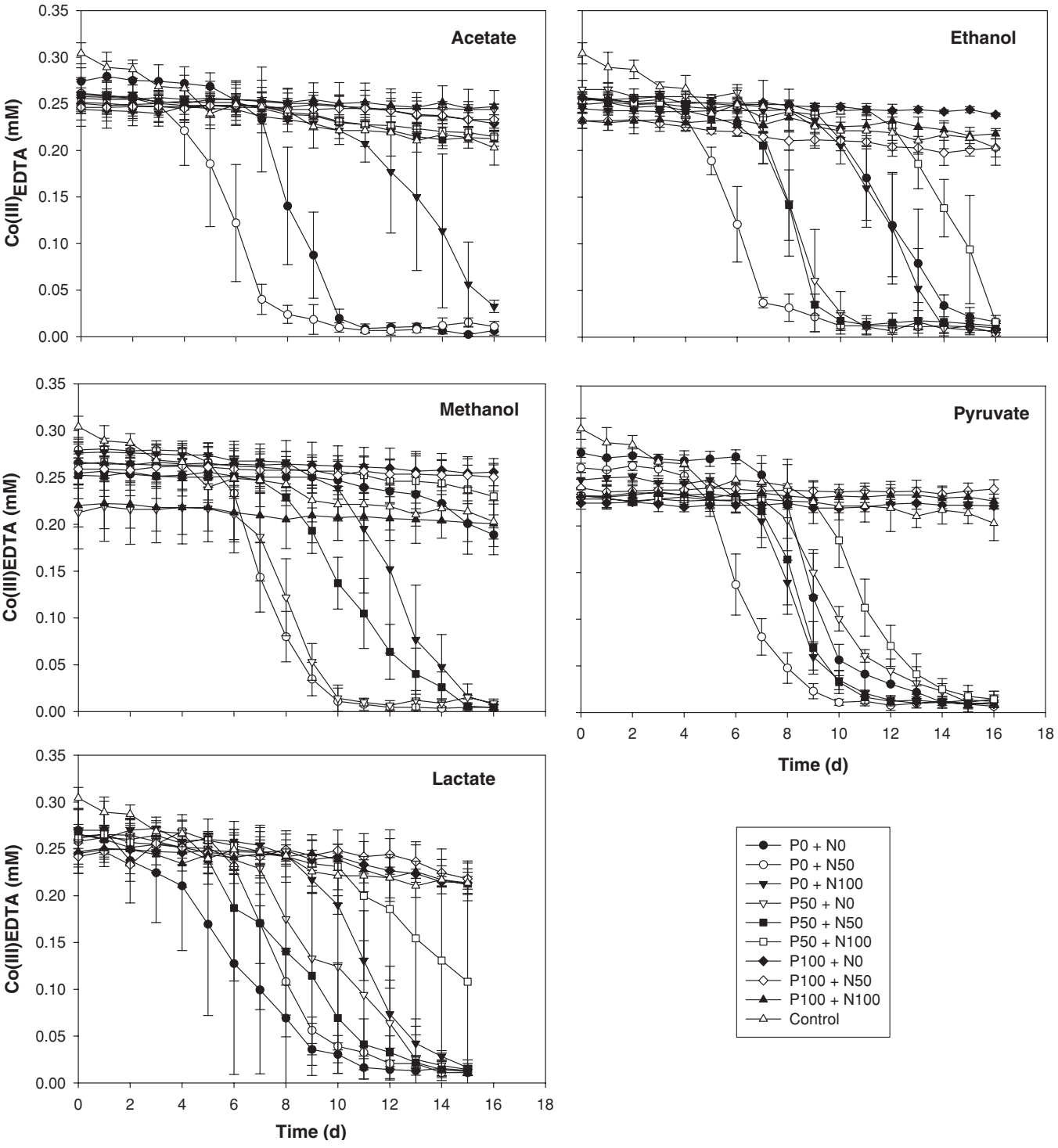

FIG. 1. Reduction of Co(III)EDTA$^-$ in WAG5 groundwater following the addition of different electron donors and varying levels of N and P. N as NH_4Cl (0, 50, or 100 mM) and P as KH_2PO_4/K_2HPO_4[pH = 7.0] (0, 50, or 100 mM) were added to each assay.

the literature also reduce Fe(III) (Caccavo et al. 1996; Liu et al. 2002; Roh et al. 2002). This was likely due to the low number (<2 cells/ml) of Fe(III)-reducing bacteria at the site (Lenczewski et al. 2003). Also, it was possible that the suitable conditions including appropriate pH for successful Fe(III) reduction were not met in our experiments. Alternatively, since we used Fe(III)-citrate as the electron receptor instead of Fe(III)-EDTA, this may negatively effect the Fe reduction, as shown in a recent study that chelating status of Fe(III) could effect its reduction kinetics (Wang et al. 2008).

Effect of Electron Donor and Nutrient Level on Co(III)EDTA$^-$ Reduction

Groundwater was collected from WAG5 well 10 and filter sterilized through 0.2 μm filters. Nine ml of sterile groundwater was aliquoted into individual anaerobic pressure tubes under an N_2 atmosphere. The groundwater was amended with acetate, ethanol, lactate, methanol or pyruvate (12 mM) as an electron donor. N as NH_4Cl (0, 50, or 100 mM) and P as KH_2PO_4/K_2HPO_4[pH = 7.0] (0, 50, or 100 mM) were added to each tube. Co(III)EDTA$^-$ was added as the electron acceptor (0.2 mM). Tubes were inoculated with 1 ml of fresh WAG5 well 10 groundwater (except the control which received 1 ml of sterile groundwater) and incubated at room temperature in the dark. Co(III)EDTA$^-$ concentrations were determined every 24 h by measuring absorbance spectrophotometrically at 535 nm versus a standard curve (Spectronic 20, Milton Roy Company). Treatments were conducted in triplicate.

The results showed that each of the tested electron donors could stimulate Co(III)EDTA$^-$ reduction in WAG5 groundwater (Figure 1). Acetate and ethanol produced the most rapid reduction, with most of the Co(III)EDTA$^-$ being reduced within 7 d for the most effective nutrient amendment levels. The lactate results were highly variable as evidenced by the large error bars. Generally, the lower levels of N and P produced the most rapid Co(III)EDTA$^-$ reduction. For all electron donors, except lactate which produced highly variable results, addition of low levels of N (50 mM) and no P produced the highest rate of Co(III)EDTA$^-$ reduction. The other combinations were more variable in their effect.

Interestingly, the maximum observed rates of Co(III)EDTA$^-$ reduction were relatively similar for each of the tested electron donors. However, there were several differences when individual electron donor and N and P level combinations were compared. It is likely that these differences resulted from preferential stimulation of different Co(III)EDTA$^-$-reducing populations depending on what amendments were added to the WAG5 groundwater. Different bacteria have been shown to selectively or preferentially use specific electron donors during Co(III)EDTA$^-$ reduction (Liu et al. 2002).

Additionally, it was reported that use of different electron donors, including acetate, lactate, and glycerol, affected microbial growth and community composition in Fe(III)-reducing enrichments from uranium-contaminated sediments (Petrie et al. 2003). For all of the electron donors, except lactate which produced highly variable results, addition of low levels of nutrients produced the highest rate of Co(III)EDTA$^-$ reduction. Tests with groundwater from other sites produced similar results (data not shown). These results would be expected for microbial populations indigenous to WAG5, such as *Caulobacter* spp., which are adapted to an oligotrophic environment. This has implications for any *in situ* Co(III)EDTA$^-$ remediation efforts at WAG5 and similar sites. Our results here suggested that amendments of minimal rather than high levels of nutrients would be helpful of bioremediation in metal- and radionuclide-contaminated groundwater. This study provides a guideline for the future field experiments of bioremediation at WAG5 site of ORNL' DOE NABIR Field Research Center.

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