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[Cobalt(III)–EDTA]$^-$ reduction by thermophilic methanogen *Methanothermobacter thermautotrophicus*

Rajesh Singh [a], Hailiang Dong [a,b,*], Deng Liu [c], Amy R. Marts [d], David L. Tierney [d], Catherine B. Almquist [e]

[a] Department of Geology and Environmental Earth Science, Miami University, Oxford, OH 45056, United States
[b] State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Beijing, 100083, China
[c] State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan, 430074, China
[d] Department of Chemistry and Biochemistry, Miami University, Oxford, OH 45056, United States
[e] Department of Paper and Chemical Engineering, Miami University, Oxford, OH 45056, United States

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ABSTRACT

Cobalt is a metal contaminant at high temperature radioactive waste disposal sites. Past studies have largely focused on mesophilic microorganisms to remediate cobalt, despite the presence of thermophilic microorganisms at such sites. In this study, *Methanothermobacter thermautotrophicus*, a thermophilic methanogen, was used to reduce Co(III) in the form of [Co(III)–EDTA]$^-$. Bioreduction experiments were conducted in a growth medium with H_2/CO_2 as a growth substrate at initial Co(III) concentrations of 1, 2, 4, 7, and 10 mM. At low Co(III) concentrations (<4 mM), a complete reduction was observed within a week. Wet chemistry, X-ray absorption near-edge structure (XANES) and electron paramagnetic resonance (EPR) analyses were all consistent in revealing the reduction kinetics. However, at higher concentrations (7 and 10 mM) the reduction extents only reached 69.8% and 48.5%, respectively, likely due to the toxic effect of Co(III) to the methanogen cells as evidenced by a decrease in total cellular protein at these Co(III) concentrations. Methanogenesis was inhibited by Co(III) bioreduction, possibly due to impaired cell growth and electron diversion from CO_2 to Co(III). Overall, our results demonstrated the ability of *M. thermautotrophicus* to reduce Co(III) to Co(II) and its potential application for remediating ^{60}Co contaminant at high temperature subsurface radioactive waste disposal sites.

1. Introduction

A number of taxonomically diverse microorganisms, including archaea and bacteria that are capable of dissimilatory metal reduction have been isolated from a variety of environments (Lloyd, 2003; Gadd, 2010; Lovely, 2013; Satyanarayana et al., 2013). Their ability to interact with various metals and radionuclides is not only important to the ecology of these microorganisms but also offers a promising method to remediate metals at contaminated sites (Liu et al., 2002; Lloyd, 2003; Gadd, 2010; Cheng et al., 2012; Satyanarayana et al., 2013; Lovely, 2013). Although various physical and chemical methods have been developed for metal remediation purpose, their high cost, low efficiency, and generation of large sludge volumes make these methods less attractive (Kazy et al., 2006). In contrast, microbial interactions are widely known to effectively affect environmental metal mobility, bioavailability, and toxicity as a result of the small size, high surface area to volume ratio, and metabolic versatility of microorganisms (Lovley, 2000; Barkay and Schaefer, 2001; Lloyd, 2003; Gadd, 2004, 2010; Dong, 2012; Lovely, 2013).

Metal reduction by thermophiles is less studied. Since the mid-1990s, thermophilic bacteria and archaea have been reported to grow either organotrophically with fermentable substrates or chemolithotrophically with molecular hydrogen when coupled to the reduction of solid state Fe(III) to Fe(II) (Slobodkin et al., 1997; Vargas et al., 1998; Kashefi et al., 2002; Gavrilov et al., 2003; Zavarzina et al., 2007). More recently, various thermophilic and hyperthermophilic bacteria and archaea have been shown to grow in the presence of iron-bearing clay minerals such as smectite, suggesting a possible microbial role in iron cycling in high temperature environments (Zhang et al., 2007, 2013; Kashefi et al., 2008; Dong et al., 2009; Dong, 2012). In particular, *Methanothermobacter thermautotrophicus*, a thermophilic methanogen, has been reported to reduce structural Fe(III) in smectite minerals at 65 °C (Zhang et al., 2013). Cooperative and competitive microbial reduction of structural Fe(III) in clay minerals and methanogenesis could have important implications for understanding the biogeochemical cycles of methane and iron.

In comparison with extensive studies of metal reduction by mesophiles, only a few studies have focused on the reduction of toxic metals and radionuclides by thermophiles. Among them, an anaerobic fermenter, *Thermoanaerobacter ethanolicus* isolated from the Triassic Taylorsville Basin in Virginia, was able to reduce Fe(III), Mn(IV), Cr(VI), U(VI) and [Co(III)–EDTA]$^-$ at 65 °C (Zhang et al., 1996; Roh et al., 2002). *Thermus scotoductus* SA01 isolated from a South African

* Corresponding author at: Department of Geology and Environmental Earth Science, Miami University, Oxford, OH 45056, United States. Tel.: +1 513 529 2517; fax: +1 513 529 1542.
E-mail address: dongh@miamioh.edu (H. Dong).

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gold mine and *Pyrobaculum islandicum*, a hyperthermophilic archaeon, were able to reduce various metals and radionuclides including Fe(III), Mn(IV), U(VI), Tc(VII), Cr(VI) and $[Co(III)–EDTA]^-$ at 65 °C and 100 °C, respectively (Kieft et al., 1999; Kashefi and Lovely, 2000). Similarly, both thermophilic methanogen *M. thermautotrophicus* (optimal temp. 65 °C) and mesophilic methanogen *Methanosarcina mazei* (optimal temp. 37 °C) have been demonstrated to possess the ability to reduce V(V) (Zhang et al., 2014). More recently, the ability of *M. thermautotrophicus* to reduce toxic Cr(VI) to non-toxic Cr(III) has been demonstrated, highlighting a potential application for the reduction and immobilization of Cr(VI) at high temperature radioactive disposal sites (Singh et al., 2015).

Among these metals and radionuclides, ^{60}Co has received considerable attention, largely due to its potential to migrate in the subsurface. Radioactive ^{60}Co typically moves in ground water along with ethylenediaminetetraacetic acid (EDTA) (Riley et al., 1992; Brooks et al., 1996; Gao et al., 2010), a persistent organic compound used in cleaning and decontamination operations (Xue and Traina, 1996; Brooks et al., 1999). While EDTA generally enhances the solubility of ^{60}Co, the geochemical stability of Co–EDTA complexes, and thus the fate of ^{60}Co moving through the subsurface is strongly related to the oxidation state of cobalt ($\log K_{Co(II)–EDTA} = 18.3$; $\log K_{Co(III)–EDTA} = 43.9$) (Brooks et al., 1996, 1999). $[Co(III)–EDTA]^-$ is kinetically inert, resistant to ligand exchange reactions (Brooks et al., 1996), and thus promotes Co transport over a long distance. In contrast, $[Co(II)–EDTA]^{2-}$ can dissociate more readily, allowing free cobalt ions to be sorbed onto oxide minerals, preventing further spreading in natural environment (Szecsody et al., 1994; Gorby et al., 1998; Brooks et al., 1999; Jardine et al., 2002).

The co-occurrence of cobalt and EDTA has been reported at the U.S. Department of Energy (DOE) sites, such as the Waste Area Grouping 5 (WAG5) site at the Oak Ridge Reservation in Oak Ridge, TN (Gao et al., 2010), Hanford Site, WA (Fruchter et al., 1984, 1985; Jones et al., 1988), and Chalk River Nuclear Laboratories in Canada (Killey et al., 1984). ^{60}Co has been detected in groundwater in the 200 East Area of Hanford with concentrations above 100 picocuries (pCi) per liter (ANL, 2001). Thermal insulation in these areas usually leads to elevated temperatures, due to the decay of long-lived radionuclides (e.g., ^{137}Cs and ^{90}Sr) (Agnew and Corbin, 1998). Methanogens are known to exist at similar DOE waste disposal sites, such as the Waste Isolation Pilot Plant (WIPP) in southern New Mexico (Wang and Francis, 2005), as well as other subsurface sites such as the Äspö Hard Rock Laboratory in Sweden (Pedersen, 1999; Kotelnikova, 2002). While methanogens have been shown capable of reducing structural Fe(III) in clay minerals (Zhang et al., 2013), and heavy metals V(V) (Zhang et al., 2014) and Cr(VI) (Singh et al., 2015), their ability to reduce and detoxify other heavy metals such as Co, especially at elevated temperatures, has not been explored. Indeed, thermophilic methanogens are among the least studied of any microorganisms that have the ability to carry out important redox reactions in nature.

The objective of this research was to investigate the bioreduction kinetics of $[Co(III)–EDTA]^-$ by *M. thermautotrophicus*, a thermophilic methanogen. A laboratory based experiment was conducted at different $[Co(III)–EDTA]^-$ concentrations at an optimal growth temperature of 65 °C and pH 7, to address the following questions: (i) Is *M. thermautotrophicus* capable of reducing $[Co(III)–EDTA]^-$ in aqueous solution? (ii) If so, does the concentration of $[Co(III)–EDTA]^-$ affect the extent and rate of reduction and cell viability? What are the kinetics of $[Co(III)–EDTA]^-$ reduction? (iii) What is the oxidation state of the reduced Co(III)? Various wet chemistry and spectroscopic methods were employed to investigate the interactions of *M. thermautotrophicus* with $[Co(III)–EDTA]^-$ in aqueous solution. Our results demonstrate the ability of *M. thermautotrophicus* to utilize $[Co(III)–EDTA]^-$ as a terminal electron acceptor with H_2/CO_2 as substrate in a concentration-dependent manner. These results have important implications for our understanding of the fate and transport of ^{60}Co in a variety of high temperature subsurface environments.

2. Materials and methods

2.1. Cultivation of the methanogen

M. thermautotrophicus was kindly provided by Dr. Xiuzhu Dong (Institute of Microbiology, Chinese Academy of Sciences, Beijing, China). This strain was routinely cultured in a sulfate-free enrichment medium under strictly anaerobic conditions. The medium contained (per liter of DI water): 1.08 g KH_2PO_4, 1.6 g Na_2HPO_4, 0.29 g NH_4Cl, 0.29 g NaCl, 0.0096 g $CaCl_2 \cdot 2H_2O$, 0.096 g $MgCl_2 \cdot H_2O$, 4 g $NaHCO_3$, 1.6 g yeast extract, 0.5 g tryptone, 0.5 g peptone, 1 mL of vitamin solution (Kenealy and Zeikus, 1981), 1 mL trace metal solution (Zehnder and Wuhermann, 1977), and 1 mL of 0.1% resazurin (redox indicator). The pH of the medium was adjusted to 7.0 by adding 0.1 N HCl as needed. The medium was then added to 60 mL serum bottles that were prewashed with 10% nitric acid and later with DI water followed by degassing with O_2-free H_2/CO_2 gas mix (80:20) by passing through a hot copper column. After autoclaving at 121 °C for 1 h, mixed H_2/CO_2 (80:20) gas was injected into the headspace of the serum bottles until a pressure of 140 kPa was reached. The medium was then inoculated with *M. thermautotrophicus* inside a glove box (filled with 95% N_2 and 5% H_2, Coy Laboratory Products, Grass Lake, Michigan) and the serum bottles were incubated at 65 °C. Cells were transferred three times prior to bioreduction experiments.

2.2. $[Co(III)–EDTA]^-$ preparation

$[Co(III)–EDTA]^-$ was prepared following the method of Dwyer et al. (1955). A mixture of Co(II) chloride hexahydrate (8 g), potassium acetate (20 g) and ethylenediaminetetraacetic acid (EDTA) (10 g) was heated in DI water (60 mL) to near boiling, followed by gradual addition of 3% hydrogen peroxide (30 mL) to oxidize Co(II) to Co(III), while stirring continuously. The solution was cooled to room temperature and $[Co(III)–EDTA]^-$ was precipitated by slowly adding 100 mL of 100% ethanol. Precipitated crystals were washed twice with 100% ethanol and allowed to dry at room temperature, and stored at 4 °C for later use. Prior to their use in bioreduction experiments, the crystals of $[Co(III)–EDTA]^-$ were re-dissolved in DI water that had been degassed with N_2, and filter sterilized with a 0.2 μm filter. The resulting $[Co(III)–EDTA]^-$ solution was used as a stock for subsequent bioreduction experiments.

2.3. $[Co(III)–EDTA]^-$ bioreduction experiments

The sulfate-free enrichment medium (final volume 40 mL) was added to the serum bottles (total volume 120 mL) and purged with H_2/CO_2 (80:20). The serum bottles were sealed with butyl rubber stoppers and aluminum crimps. After autoclaving for 1 h at 121 °C, mixed H_2/CO_2 (80:20) gas was injected into the headspace of the serum bottles until a pressure of 140 kPa was reached. Cells in the exponential phase were injected into the serum bottles inside an anaerobic glove box with a final cell concentration of 1.54×10^8 cells mL^{-1} as determined by DAPI (4′,6-diamidino-2-phenylindole) fluorescent staining. Varying amounts of the $[Co(III)–EDTA]^-$ stock solution were then added to the bottles to achieve final concentrations of 1, 2, 4, 7 and 10 mM. Although these concentrations were much higher than naturally present Co levels at contaminated sites (<0.1 mM, such as the Hanford site in Washington State, United States), these experiments were designed to provide mechanistic insights (e.g., toxicity) within reasonable experimental timeframe. Two duplicate serum bottles were prepared for each concentration, along with abiotic controls (no cells) and heat-killed cells. For the heat-killed control experiment, autoclaved cells (the same final concentration as in live experiment,