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[Cobalt(III)-EDTA]⁻ reduction by thermophilic methanogen
Methanothermobacter thermautotrophicus

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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 H₂/CO₂ 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 CO₂ 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 ⁶⁰Co contaminant at high temperature subsurface radioactive waste disposal sites.

Keywords: cobalt, methanogen, bioreduction, methanogenesis, XANES, EPR

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 et al., 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-1990's, 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; Kashefi et al., 2008; Dong et al., 2009; Dong

2012; Zhang et al., 2013). 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 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, ⁶⁰Co has received considerable attention, largely due to its potential to migrate in the subsurface. Radioactive ⁶⁰Co 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 ($\log K_{\text{Co(II)-EDTA}} = 18.3$; $\log K_{\text{Co(III)-EDTA}} = 43.9$) (Brooks et al., 1996; Brooks et al., 1999). $[\text{Co(III)-EDTA}]^-$ is kinetically inert, resistant to ligand exchange reactions (Brooks et al., 1996), and thus promotes Co transport over a long distance. In contrast, $[\text{Co(II)-EDTA}]^{2-}$ 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 et al., 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 H₂/CO₂ as substrate in a concentration-dependent manner. These results have important implications for our understanding of the fate and transport of ⁶⁰Co 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 KH₂PO₄, 1.6 g Na₂HPO₄, 0.29 g NH₄Cl, 0.29 g NaCl, 0.0096 g

CaCl₂·2H₂O, 0.096 g MgCl₂·H₂O, 4 g NaHCO₃, 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 pre-washed with 10% nitric acid and later with DI water followed by degassing with O₂-free H₂/CO₂ gas mix (80:20) by passing through a hot copper column. After autoclaving at 121°C for one hour, mixed H₂/CO₂ (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% N₂ and 5% H₂, 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 N₂, 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 H₂/CO₂ (80:20). The serum bottles were sealed with butyl rubber stoppers and aluminum crimps. After autoclaving for one hour at 121°C, mixed H₂/CO₂ (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 x 10⁸ 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, 1.54 x 10⁸ cells mL⁻¹) were injected into the serum bottles before the addition of [Co(III)-EDTA]⁻. All solutions and cultures were transferred using sterile needles and syringes inside an anaerobic glove box and incubated at 65°C. The bioreduction experiments were run for a total of 370 h.

2.4 Analytical methods

2.4.1 Co(III) measurement

Time course loss of Co(III) in aqueous solution during bioreduction was determined spectrophotometrically at 535 nm wavelength (Caccavo et al., 1994). For the 1 mM [Co(III)-EDTA]⁻ bioreduction experiment, the kinetics of [Co(III)-EDTA]⁻ reduction was continuously monitored by observing gradual disappearance of the characteristic peak of Co(III) at 535 nm. To test if the presence of cells in the suspension would interfere Co(III) measurement, a separate experiment was designed, where cells were removed by centrifugation. The data showed that no difference was observed in Co(III) concentration with and without cells, suggesting that Co(III) measurement was not affected by the presence of cells in solution.

2.4.2 Protein assay measurement

This experiment was carried out to evaluate the change of biomass upon cell exposure to various [Co(III)-EDTA]⁻ concentrations (2, 4, 7 and 10 mM). Two separate experiments were carried out to test the effects of other compounds in the experimental treatment on cell growth. In the first experiment to test whether the organic compounds contained in the growth medium (yeast extract, tryptone, and peptone) can be a source of reducing power, a cell growth experiment without H₂ was conducted with a final cell concentration of $\sim 7 \times 10^7$ cells mL⁻¹. In the second experiment to test if potassium acetate had any inhibitory effect on cell growth, an experiment was conducted with 2 and 10 mM potassium acetate and a final cell concentration of $\sim 7 \times 10^7$ cells mL⁻¹.

Aliquots of cell suspension (1 mL in volume) were collected from the experimental serum bottles with a syringe and placed into 1.5 mL centrifuge tubes, followed by centrifugation at 5000 g for 10 min. The resulting cell pellet was re-suspended in water and washed three times with pre-filtered (pore size, 0.2 µm) quartz distilled water to remove residual materials from the

growth medium (peptone, tryptone and yeast extract). To lyse the cells and release protein, 0.9 mL of the re-suspended culture was added to 0.1 mL of 0.2 M NaOH and heated for 10 min at 100°C. After centrifugation of the suspension at 5000 g for 10 min, 0.8 mL supernatant was mixed with 0.2 mL Bradford reagent (Sigma Co. St. Louis, Mo) and the mixture was allowed to react for about 5 min. Protein concentration was measured spectrophotometrically at 595 nm with bovine serum albumen (BSA) as a standard (Bradford, 1976). All measurements were carried out in duplicate.

2.4.3 Methane and hydrogen measurements

Time course methane and hydrogen concentrations were measured to determine the stoichiometry of methanogenesis at various concentrations of [Co(III)-EDTA]⁻. One mL of headspace gas was withdrawn and analyzed for CH₄ and H₂ concentrations with an Agilent Technologies gas chromatographic system (6890 N series). CH₄ concentration was analyzed with a flame ionization detector (FID) separated on a capillary column (HP-MOLSIV, 30 m x 0.53 mm; J&W Scientific) and H₂ concentration was analyzed with a thermal conductivity detector (TCD) with a packed column (Shin Carbon 100/120, 2 m x 1 mm; Restek, Bellefonte, PA). Based on measured partial pressures of CH₄ and H₂ in the headspace, the aqueous CH₄ and H₂ concentrations at equilibrium were calculated by the Gas law and Henry's law using their respective Henry's constants (where K_H for CH₄ at 65°C = 1.51 x 10⁻³ mol L⁻¹ atm⁻¹, Schwarzenbach et al., 1995; K_H for H₂ at 65°C = 9.5 x 10⁻⁴ mol L⁻¹ atm⁻¹, Canfield et al., 2005).

2.4.4 X-ray absorption near edge structure (XANES)

XANES spectroscopy was used to monitor the oxidation state of cobalt in the supernatant, before and after bioreduction of 1 mM [Co(III)-EDTA]⁻. Samples were drawn at various time points (0 to 49 h), loaded into lucite cuvettes with 6 µm polypropylene windows and frozen rapidly in liquid nitrogen. X-ray absorption spectra were measured at the National Synchrotron Light Source (NSLS), beamline X3A, with a Si(111) double crystal monochromator; harmonic rejection was accomplished using a Ni focusing mirror. Fluorescence excitation spectra for all samples were measured with a 13-element solid-state Ge detector array. Samples were held at ~ 15 K in a Displex cryostat during XAS measurements. X-ray energies were calibrated by reference to the absorption spectrum of a cobalt metal foil, measured concurrently. All of the data shown represent the average of 3 scans, measured using 10 eV steps in the pre-edge region (1 s integration time), 0.3 eV steps in the edge region (7690 – 7750 eV; 2s), and 5 eV steps in the EXAFS region (7750 – 8200 eV; 1s). Data reduction and normalization were accomplished following published procedures (Tierney and Schenk, 2014).

2.4.5 *Electron paramagnetic resonance (EPR)*

The bioreduction of Co(III) was also monitored by EPR, using an initial concentration of 2 mM [Co(III)-EDTA]⁻. Spectra were collected from samples taken at different time points (0 to 108 h) using a Bruker EMX EPR spectrometer equipped with an ER4116DM dual-mode resonator. Temperature was maintained with an Oxford Instruments ESR900 cryostat and temperature controller ($\pm$ 0.05 K). All measurements were carried out at 4.5 K, with the following instrument settings: ν_{MW} = 9.62 GHz (2 mW); receiver gain = 10^6 ; 10 G field-modulation (100 kHz); time constant/conversion time = 41 ms.

3. RESULTS

3.1 Bioreduction of [Co(III)-EDTA]⁻ by *M. thermautotrophicus*

Negligible amounts of Co(III) reduction were observed in either no-cell or heat-killed controls (Fig. 1). However, in the presence of *M. thermautotrophicus*, a complete reduction was observed within 200 h at Co(III) concentrations of 1, 2 and 4 mM with reduction rates of 90, 20 and 18 $\mu\text{M h}^{-1}$, respectively (Fig. 1 and Table 1). At higher Co(III) concentrations, bioreduction was incomplete, and rates and extents were lower. Bioreduction of 1 mM Co(III) was further supported by the gradual disappearance of the characteristic UV-vis absorption peak at 535 nm (Fig. 2).

3.2 Cell growth measurement

An adverse effect of Co(III) on bacterial cell biomass was observed, and the extent of growth inhibition was proportional to initial Co(III) concentration (Fig. 3). At 2 and 4 mM [Co(III)-EDTA]⁻ concentrations, total protein concentration initially decreased but increased following the reduction of Co(III) (Compare Fig. 1 and Fig. 3 inset). At higher Co(III) concentrations (7 and 10 mM), cell growth recovery was slower, and protein concentrations did not return to their onset levels (Fig. 3), apparently because large amounts of toxic Co(III) remained at the end of the experiment. An additional experiment conducted without hydrogen showed a five-fold cell growth, suggesting that organic compounds in the growth medium served as a source of energy for cells (data not shown). Another experiment conducted with potassium acetate but without Co(III) showed that a 2 mM potassium acetate solution did not have any inhibitory effect, but a 10 mM solution inhibited cell growth by a factor of 2. Because the

concentrations of potassium acetate used in our experiments were in the range of 0.2 to 1 mM, this inhibitory effect could be ignored.

3.3 Stoichiometry of methanogenesis at various concentrations of [Co(III)-EDTA]⁻

This experiment was conducted to determine the stoichiometric ratio of H₂ consumption and CH₄ production in the methanogenic system with and without the addition of [Co(III)-EDTA]⁻. In Co-free control bottle, a significant amount of methane was produced over time (Fig. 4a & b). The amount of H₂ consumed (0.15 mmol) was stoichiometric with the amount of CH₄ produced (0.037 mmol) according to Equation 1.

However, exposure to various concentrations of [Co(III)-EDTA]⁻ inhibited methanogenesis to different extents. At 2 mM Co(III), the amount of H₂ consumption was still the same (~ 0.155 mmol) but the amount of methane generation was less (~ 0.025 mmol). Approximately 0.088 mmol of Co(III) was reduced (Fig 4a, b, Table 2). According to the 4:1 stoichiometric ratio of H₂ and CH₄, the amount of H₂ required to produce 0.025 mmol CH₄ should be ~ 0.1 mmol, leaving ~ 0.055 mmol for Co(III) bioreduction. Hence, the observed ratio of H₂ oxidation and Co(III) reduction was 0.055:0.088, reasonably close to the theoretical ratio of 1:2 according to the following equation,

The observed ratios of H₂ oxidation to Co(III) reduction were fairly close to the expected ratio of 1:2 for other concentrations of Co(III) as well (Table 2), suggesting that reduction of two moles of [Co(III)-EDTA]⁻ required 1 mole of H₂ to produce 2 moles of [Co(II)-EDTA]²⁻

according to Eq. 2. Co(III) reduction continuously consumed H_2 , even after methane accumulation stopped (Figs 1, 4a, b). Furthermore, a comparison between the cell growth curve (Fig. 3) and methanogenesis (Fig. 4) revealed that even after methane production ceased in both the control and the Co(III)-EDTA containing treatments, cell growth continued, consistent with our observation above (Section 3.2) that organic compounds in the growth medium could serve as an energy source to support cell growth.

3.4 Determination of cobalt oxidation state by XANES

The XANES spectrum is sensitive to both the oxidation state of the metal, through modulation of the edge energy, and its local geometry, through the relative breadth of common features. As expected, at time zero, the absorption edge suggests the presence of both Co(III) and Co(II), consistent with our independent test showing that ~10% Co(II) remained when hydrogen peroxide was used to prepare the [Co(III)-EDTA]⁻. Interestingly, the Co(II) component virtually disappeared during the first 1.5 hours of incubation (Fig. 5), based on a shift to higher energy of the white line absorption at ~ 7720 eV, and a sharpening of the first post-edge absorption feature at 7740 eV. At some point between 3 and 11 hours of exposure, a nearly complete reduction of Co(III) to Co(II) occurred for 1 mM Co(III) concentration experiment, as evidenced by a shift to lower energy and sharpening of the white line absorption at ~ 7720 eV as well as disappearance of the first post-edge absorption feature at 7740 eV. These changes are more easily seen in the XANES derivatives shown in the inset, where peak corresponds to the mid-point of the rising absorption edge. Based on the derivatives, the edge shift to lower energy by ~ 2 eV from the 3

hour time point to 11 hours is consistent with one electron reduction of the starting Co(III), establishing that [Co(III)-EDTA]⁻ is reduced to Co(II) (Tierney and Schenk, 2014).

3.5 EPR analysis

In its standard configuration, with $B_0 \perp B_1$, an EPR experiment is sensitive to species with an odd number of unpaired electrons, and insensitive to those with an even number of unpaired electrons (Kramers' rule). [Co(III)-EDTA]⁻ is an octahedral complex of a d^6 ion found exclusively in its low-spin, diamagnetic form (EPR-silent). Consistent with this, the EPR spectrum at time zero was flat, aside from a small signal near $g \sim 2$ that likely arose from trace paramagnetic species in the medium. In contrast, after 40 hours of exposure, a clear signal spanning *ca.* 800 to 3500 G was observed, and this signal became stronger in intensity up to a complete reduction of Co(III) to Co(II) at 108 h (Fig. 6). The appearance of this signal requires formation of the one electron reduced Co(II) ion (d^7), and its shape is consistent with high-spin Co(II). These data are consistent with the UV-vis and XANES spectra.

4. DISCUSSION

4.1 Bioreduction of [Co(III)-EDTA]⁻ to [Co(II)-EDTA]²⁻ by *M. thermautotrophicus*

Our data suggest that the thermophilic methanogen *M. thermautotrophicus* was capable of reducing Co(III) to Co(II), with different bioreduction rates and extents depending on initial Co(III) concentration (Table 1). A very limited number of bioreduction kinetics and reduction efficiency of Co(III) by other thermophilic microorganisms have been reported previously. *Thermus scodectus*, SA-01, isolated from groundwater sampled at 3.2 km depth in a South African Gold mine completely reduced 0.15 mM [Co(III)-EDTA]⁻ at 65° C with a rate of 7.5 μ M

h⁻¹ when coupled with oxidation of lactate (Kieft et al., 1999). Similarly, an anaerobic hyperthermophilic archaeon *Pyrobaculum islandicum* was reported to reduce [Co(III)-EDTA]⁻ completely at 100° C with H₂ as electron donor (Kashefi and Lovely, 2000). Another thermophilic, anaerobic fermenter, *Thermoanaerobacter* sp. TOR 39, was capable of reducing 10 mM [Co(III)-EDTA]⁻ at 65° C with a very high reduction rate of 237 μM h⁻¹. Our measured Co(III) bioreduction rates (13-90 μM h⁻¹) and extents (48-100%) by *M. thermautotrophicus* fall within these previously reported values.

Compared to thermophiles, a considerable body of work has demonstrated the ability of mesophilic microorganisms to gain energy through dissimilatory reduction of chelated Co(III) when coupled with oxidation of H₂ or other organic compounds. Among them, *Shewanella alga* BrY was able to reduce 50 μM [Co(III)-EDTA]⁻ at a rate of 258 μM h⁻¹ with lactate as electron donor, accompanied by a concomitant increase in cell numbers (Gorby et al., 1998). The sulfate-reducing bacterium *Desulfovibrio vulgaris* also promoted complete reduction of 0.5 mM [Co(III)-EDTA]⁻ at a rate of 3.8 μM h⁻¹ when coupled with oxidation of ethanol, and the reduction was enhanced by sulfate addition to the system (Blessing et al., 2001). Similarly, an alkaliphilic metal reducing bacterium, *Alkaliphilus metalliredigens*, efficiently reduced 4.5 mM Co(III)-EDTA⁻ at a rate of 37 μM h⁻¹ with yeast extract as electron donor, in the presence of 20 g/L NaCl. Relative to the rates and extents of Co(III) reduction by various mesophilic microorganisms, the bioreduction rates and extents by *M. thermautotrophicus* reported here are higher, which could be partly due to the temperature effect. The positive effect of high temperature on bioreduction kinetics has been observed previously, where the rates of structural Fe(III) reduction in clay minerals by thermophilic methanogens are typically higher than those by mesophilic methanogens (Zhang et al., 2012, 2013).

XANES spectroscopy of Co(III) before and after bioreduction showed clear evidence of reduction of cobalt from Co(III) to Co(II). The Co(III) spectra (at 1.5 and 3 h) showed characteristic absorption edge peaks consistent with a Co(III) 1s to 4p transition (Sano et al., 1988), comparable to previous studies where XANES was used to monitor the reduction of cobalt in biological systems (Bonnitcha et al., 2006). After complete reduction ($t > 11$ h, Fig. 1), the spectra were representative of biologically reduced Co(II) (Bonnitcha et al., 2006).

In addition to the XANES signature of reduction, a strong EPR signal (*ca.* 800 to 3500 G), whose intensity grew with increasing exposure time, was observed. This must be taken as additional evidence for the formation of Co(II) as the reduction product in our bioreduction experiment (Fig. 6). The vast majority of Co(II) species are high-spin, with three unpaired electrons, while Co(III) species are almost exclusively low-spin and diamagnetic. While it is impossible to attribute this signal to a single species, its shape is inconsistent with that of [Co(II)-EDTA]²⁻ (Coronado et al., 1988), but similar to a typical protein-bound high-spin Co(II), suggesting that the Co(II) formed may be protein-bound (Bennett, 2010), where the proteins may have been possibly derived from cells. Production of extracellular polymeric substances (EPS) by various microorganisms has been reported previously in response to environmental stresses (Aquino et al., 2004; Sheng et al., 2005; Kilic et al., 2008).

4.2 Resistance of *M. thermautotrophicus* to [Co(III)-EDTA]⁻

Although, small amounts of cobalt are beneficial for microbial metabolism (Ankel-Fuchs and Thauer, 1988; Nies, 1992, 1999), millimolar concentrations of [Co(III)-EDTA]⁻ apparently were toxic to *M. thermautotrophicus* and inhibited its growth (Fig. 3). Measurement of time

course protein concentration showed initial inhibition of cell growth at all levels of Co(III), indicating the toxic effects of Co(III). However, at lower Co(III) concentrations, there was a small amount of resumption of cell growth after significant Co(III) reduction occurred, similar to previous studies on microbial reduction of Cr(VI) (Cheung and Gu, 2003; Viamajala et al., 2004; Singh et al., 2015). This inhibition-recovery cycle of *M. thermautotrophicus* cells during [Co(III)-EDTA]⁻ bioreduction (Fig. 3 inset) could be attributed to the enzymes present in its genome such as superoxide dismutase which is known to possess the ability to repair damaged cellular components. A similar mechanism has been proposed to explain the Cr(VI) toxicity to *M. thermautotrophicus* (Singh et al., 2015). However, incomplete reduction at higher Co(III) concentrations (7 and 10 mM) was likely caused by a greater toxicity of cobalt to *M. thermautotrophicus*, as evidenced by inhibition of cell growth at these concentrations (Fig. 3). This toxicity was also supported by the observed inhibition in methanogenesis during this experiment (Fig. 4). An inhibitory effect of Co(III) has been observed previously on *Alkaliphilus metalliredigens* during the reduction of 4.5 mM Co(III), where the growth rate was more than 2-fold slower with Co(III) than with 15 mM Fe(III) (Ye et al., 2004).

4.3 Inhibition of methanogenesis by [Co(III)-EDTA]⁻ bioreduction

The observed inhibition of methanogenic activity by [Co(III)-EDTA]⁻ bioreduction could be ascribed to two possible reasons (Fig. 4a & b). First, the observed growth impairment of *M. thermautotrophicus* cells by [Co(III)-EDTA]⁻ would inhibit methanogenesis. Alternatively, diversion of electron flow from CO₂ to [Co(III)-EDTA]⁻ reduction could be responsible. In the normal pathway of methanogenesis, hydrogenases in hydrogenotrophic methanogens can oxidize H₂ to protons while releasing two electrons, and these two electrons are utilized to reduce

410 methyl-coenzyme M ($\text{CH}_3\text{-S-CoM}$) to CH_4 (Deppenmeier et al., 1999). However, it has been
411 documented that, in the presence of bioavailable Fe(III), these electrons could divert to reduce
412 Fe(III) to Fe(II) (Bond and Lovley, 2002, Zhang et al., 2013). Therefore, in our experiment,
413 these electrons could have been diverted to reduce $[\text{Co(III)-EDTA}]^-$ to $[\text{Co(II)-EDTA}]^{2-}$. This
414 electron diversion hypothesis was supported by the stoichiometric oxidation of H_2 with reduction
415 of $[\text{Co(III)-EDTA}]^-$ (Fig. 1, Fig. 4b, Table 2, and Eq. 2), implying that electrons used to reduce
416 $[\text{Co(III)-EDTA}]^-$ were derived from H_2 . This electron diversion hypothesis was further supported
417 by a continued decline of H_2 even after methane accumulation stopped in treatments with
418 $[\text{Co(III)-EDTA}]^-$ (compare Figs 1 and 4a, b).

421 **4.4 Implication for bioremediation**

422 Thermophilic methanogens are among the least studied microorganisms that have the
423 ability to carry out important redox reactions in nature. Their ability to respire toxic metals at
424 high-temperature, contaminated sites could offer an alternative opportunity for immobilizing
425 toxic metals and radionuclides in such environments. Therefore, an improved understanding of
426 the kinetics of $[\text{Co(III)-EDTA}]^-$ reduction by thermophilic *M. thermautotrophicus* is important
427 for predicting the fate of ^{60}Co in such environments. In deep subsurface environments,
428 thermophilic methanogens could use hydrogen as a sole energy source and inorganic carbon as
429 their carbon source (Pederson, 1996; Kotelnikova, 2002), and in doing so, they could play a vital
430 role in minimizing migration of ^{60}Co (Szecsody et al., 1994; Gorby et al., 1998; Brooks et al.,
431 1999; Jardine et al., 2002). For that reason, this finding raises the possibility of bioremediation
432 by injecting methanogens into high-temperature subsurface radioactive waste disposal sites,

where heavy metals and radionuclides may be the predominant forms of contaminants (Riley et al., 1992; Thomas et al., 1998; Palmisano and Hazen, 2003; Cattant et al., 2008; Gao et al., 2010).

5. CONCLUSIONS

Thermophilic methanogen *M. thermautotrophicus* was capable of reducing Co(III) as a terminal electron acceptor at ~65° C when coupled with oxidation of H₂. This archaeon completely reduced Co(III) to Co(II) at concentrations as high as 4 mM. At higher Co(III) concentrations, reduction was incomplete, possibly due to the toxicity of Co(III). The observed inhibition of methanogenesis by Co(III) bioreduction could also be related to the toxicity of Co(III) and electron diversion from CO₂ to Co(III). Our results suggest that *M. thermautotrophicus* could be a desirable microorganism for remediating cobalt contamination in high-temperature radioactive waste environments.

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Table 1. Rates and extents of [Co(III)-EDTA]⁻ reduction by *M. thermautotrophicus* at various initial concentrations.

Initial [Co(III)-EDTA] ⁻ conc. (mM)	Rate of bioreduction (μM h ⁻¹)	Extent of bioreduction (%)
1	90	100
2	20	100
4	18	100
7	13.6	69.8
10	13.3	48.5

Table 2. H₂ consumption and the reduction of [Co(III)-EDTA]⁻ at various initial concentrations. The ratio between H₂ oxidation and Co(III) reduction is reasonably close to the expected ratio of 1:2.

Initial [Co(III)-EDTA] ⁻ conc. (mM)	[Co(III)-EDTA] ⁻ reduced (Initial-final) (mM)	[Co(III)-EDTA] ⁻ reduced (Initial-final) (mmol)	Total CH ₄ produced (mmol)	Stoichiometric consumption of H ₂ (mmol) to produce the observed amounts of CH ₄	Actual H ₂ consumption (mmol)	Amount of H ₂ used to reduce [Co(III)-EDTA] ⁻ (mmol)	Ratio of H ₂ oxidation to [Co(III)-EDTA] ⁻ reduction
2.2	2.2	0.088	0.025	0.100	0.155	.055	1:1.6
3.7	3.7	0.15	0.022	0.088	0.154	0.066	1:2.2
7.2	5.03	0.201	0.02	0.08	0.161	0.081	1:2.4
10.2	4.95	0.198	0.018	0.072	0.166	0.094	1:2.1

Figure captions

Fig. 1. Bioreduction of $[\text{Co(III)-EDTA}]^-$ by *M. thermautotrophicus* at various concentrations. A 100% reduction was achieved at Co(III) concentrations of 1, 2 and 4 mM, whereas reduction was incomplete at 7 and 10 mM Co(III) concentrations. Controls (without cells and heat-killed cells) showed negligible levels of reductions. All the measurements were performed in duplicates and an average was reported.

Fig. 2. Time-course UV-vis spectra showing the gradual disappearance of the characteristic absorption peak at 535 nm over the course of bioreduction of 1 mM Co(III).

Fig. 3. Time course change of total cellular protein of *M. thermautotrophicus* upon exposure to various $[\text{Co(III)-EDTA}]^-$ concentrations. Cells were able to recover their growth after nearly complete reduction of 2 and 4 mM Co(III) but no significant growth recovery was observed at higher Co(III) concentrations. The inset figure shows the lower protein concentration range at an expanded vertical scale.

Fig. 4. Time course methane production (a) and hydrogen consumption (b) by *M. thermautotrophicus* with or without $[\text{Co(III)-EDTA}]^-$ at various concentrations.

Fig. 5. Co *K*-edge XANES at different time points over the course of Co(III) bioreduction with an initial concentration of 1 mM. Inset: Representative XANES derivatives for time = 0, 3 and 49 hr.

Fig. 6. EPR spectra of cobalt at different time points over the course of Co(III) bioreduction with an initial Co(III) concentration of 2 mM.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6