0273-2289/96/5758—0923$7.50

Enhancement of Fe(III), Co(III), and Cr(VI) Reduction at Elevated Temperatures and by a Thermophilic Bacterium†

CHUANLUN ZHANG, SHI LIU, JOHN LOGAN, RAJA MAZUMDER, AND TOMMY J. PHELPS*

Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

ABSTRACT

An unusual thermophilic bacterium has been isolated from deep subsurface sediments and tested for its ability to enhance Fe(III), Co(III), and Cr(VI) reduction. Without the bacterium, abiotic metal reduction was insignificant at temperatures below 45°C, but became a major process at 75°C. Addition of the bacterium enhanced the reduction of these metals up to fourfold, probably by nonspecific mechanisms. This study demonstrates abiotic and biotic metal reduction under organic-rich thermic conditions, and suggests that thermally and/or biologically enhanced metal reduction may provide an alternative for remediating metal contamination.

Index Entries: Thermophilic bacterium; metal reduction; ferric citrate; Co(III)-EDTA; chromate.

INTRODUCTION

Bacterial reduction of Fe(III), Mn(III, IV), and other metals under mesophilic conditions (growth temperature <45°C) is well documented *(1–4)*. For example, Lovley and Phillips *(5)* isolated an obligately anaerobic bacterium, *Geobacter metallireducens*, from fresh water sediments of the Potomac River, MD, which reduces amorphic Fe(III) oxide to magnetite in a reaction coupled to acetate oxidation. Myers and Nealson *(6)* isolated a facultative bacterium, *Shewanella putrefaciens*, from the anoxic sediments of Lake Oneida, NY, which uses diverse electron acceptors (O_2, NO_3^-, NO_2^-, $S_2O_3^=$, and S^0) and reduces Fe(III) or Mn(IV) anaerobically when growing on formate or lactate. Recent summaries of the knowledge of metal reduction by bacteria can be found in Lovley *(3)* and in Nealson and Saffarini *(4)*.

† The submitted manuscript has been coauthored by a contractor of the US government under contract No. DE-AC05-960R22464. Accordingly, the US government reserves a nonexclusive royalty-free license to publish or reproduce the published form of this contribution or allow others to do so, for US government purposes.

*Author to whom all correspondence and reprint requests should be addressed.

Geological evidence suggests that bacteria may play an important role in metal reduction under thermic conditions. For example, Machel and Burton *(7)* found that authigenic magnetite, which can be formed by bacterial reduction of ferric iron *(8)*, co-occurred with thermally generated hydrocarbons. Information on metal reduction by thermophilic anaerobic bacteria is scarce. However, Boone et al. *(9)* reported anaerobic Fe(III) and Mn(IV) reduction by a thermophilic bacterium that grows on formate or lactate as an energy source. Examination of metal reduction at higher temperatures and by thermophilic bacteria is needed to elucidate the roles of biotic and abiotic processes in natural geothermal environments. Furthermore, information from such studies may lead to alternative techniques for abiotic and/or biotic remediation of metal contamination in thermal environments.

During a 1992 drilling operation in the Taylorsville Triassic Basin in Virginia, thermophilic bacteria were recovered from low-porosity sandstones and shales at depths of about 2.7 km below land surface. Most of these bacteria are gram-negative, rod-shaped cells capable of fermentation, denitrification, sulfate reduction, and Fe(III) or Mn(IV) reduction *(10)*. Enrichments for these types of microorganisms from drilling fluids or surface sediments were not successful, indicating that these microorganisms likely originated in the deep subsurface. Geological and hydrological evidence suggests that these microorganisms may have survived *in situ* for a long time. In this study, we examined anaerobic reduction of Fe(III), Co(III), and Cr(VI) by an anaerobic fermentative thermophilic bacterium isolated from the Taylorsville samples, and compared the biotic (bacterial) and abiotic metal reduction under similar conditions.

MATERIALS AND METHODS

Medium Preparation for Abiotic and Biotic Metal Reduction

Experiments for abiotic and biotic metal reduction were performed using 25-mL pressure tubes (from Bellco Glass Inc., Vineland, NJ) that contained 9 mL of a medium having the following ingredients (g/L): 5–70 NaCl, 0.2 $MgCl_2 \cdot 6H_2O$, 0.1 $CaCl_2 \cdot 2H_2O$, 1.0 NH_4Cl, 0.1 3-(*N*-morpholino)-propanesulfonic acid, 2.5 $NaHCO_3$, 1.0–1.5 yeast extract, 1.0 peptone, and trace mineral and vitamin solutions *(11)*. The medium was prepared anaerobically under an atmosphere of N_2/CO_2 gas mixture.

Before each experiment, pressure tubes containing the medium prepared above were incubated at 65°C for 5–10 d. During this period, yeast extract and peptone in the medium consumed trace oxygen and decreased the redox potential. No other reductant was added. The tubes were cooled to room temperature before other chemical components or the bacterial inoculum was added. Sterile phosphate was added to give a final concentration of 2 m*M* and glucose was added to give final concentrations of 5 or 10 m*M*. Sterile solutions of ferric citrate, Co(III)-EDTA, and potassium chromate were added to give final concentrations of 14, 0.5–10, and 0.5–2.5 m*M*, respectively. The final pH was 7.5–8.0. Tubes used for bacterial metal reduction experiments were inoculated with 0.5 mL of freshly grown culture, and tubes for abiotic metal reduction experiments were not. Metal reduction by the selected thermophilic bacterium was examined at 55 and 65°C. Abiotic metal reduction without the presence of the bacterium was examined at temperatures ranging from 25 to 75°C.

Bacterial Strain Selection and Growth

The bacterial strain (TOR 39) used for this study is an anaerobic, gram-negative, rod-shaped fermentative eubacterium that can ferment glucose and other carbohydrates (data not shown). Phospholipid fatty acid analysis performed on TOR 39 (data not shown) corroborated with the above phenotypic description in that phospholipids were ester-linked and contained oxygenated polar functional groups typical of thermophilic eubacterial species *(9)*. TOR 39 grows at temperatures from 50 to 70°C and at NaCl concentrations from 0.1 to 5% (w/v).

Cell growth during the reduction of Fe(III), Co(III), and Cr(VI) was monitored by acridine-orange direct count method using an epifluorescence microscope. Subsamples (0.5–1 mL) were diluted with sterile phosphate buffer (pH 7.0), filtered onto a black Nuclepore filter (0.2-μm pore diameter), stained with 1 mL of a particle-free acridine-orange solution for 2 min, and observed microscopically.

Spectrophotometric Analysis of Fe(III), Co(III), and Cr(VI) Reduction

Reduction of Fe(III) to Fe(II) was measured as the increase in Fe(II) concentration by the ferrozine method *(12)*. Subsamples (0.1 mL) were withdrawn with a syringe and needle and diluted 10- to 40-fold with anaerobic water before reacting with ferrozine. After 20 s of mixing, samples were filtered (using a 0.2-μm pore diameter PVDF filter from Whatman, Clifton, NJ) and the concentration of Fe(II) in the filtrate was measured at A_{562}. The anaerobic water/ferrozine extraction method used here underestimated the amount of Fe(II) produced, because it did not extract solid Fe(II) compounds, such as $FeCO_3$; consequently, the mass balances of iron were typically <50%.

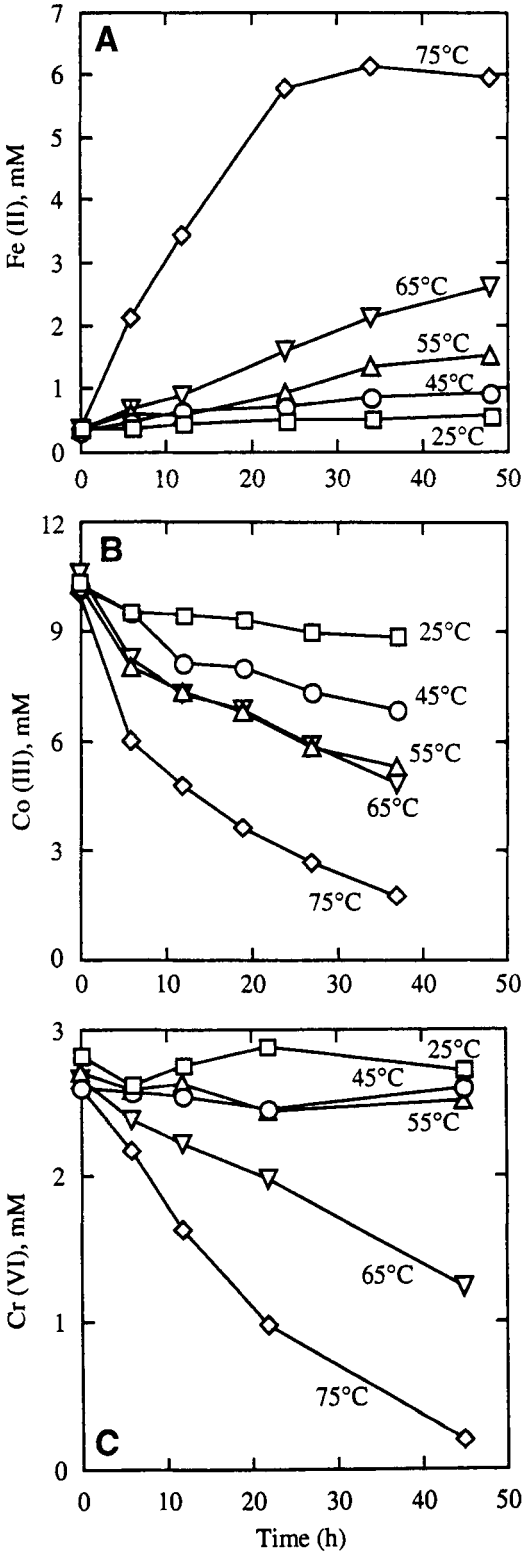
Reduction of Cr(VI) to Cr(III) was measured as the decrease in Cr(VI) concentration by the diphenylcarbazide method *(13)* according to Lovley and Phillips *(14)*. Subsamples (0.1 mL) were withdrawn with a syringe and needle, and diluted 10- to 600-fold before reacting with diphenlycarbazide reagent (0.025 g of sym-diphenylcarbazide in 10 mL of acetone). Samples were filtered as before, and the concentration of Cr(VI) in the filtrate was measured at A_{540}. Reduction of Co(III) to Co(II) was measured as the decrease in Co(III) concentration. Subsamples (0.5 to 1 mL) were diluted with 2 mL of distilled water and filtered as before. The concentration of Co(III) in the filtrate was measured at A_{548}.

RESULTS

Temperature Effect on Fe(III), Co(III), and Cr(VI) Reduction

The abiotic reduction of Fe(III), Co(III), and Cr(VI) increased as a function of temperature. As shown in Fig. 1A, no significant increase in Fe(II) was detected at 25 and 45°C (<1% of initial Fe[III] concentration), and only a slight increase was observed at 55 and 65°C (<3% of initial Fe[III] concentration), after 6 h of incubation; however, there was a dramatic increase in Fe(II) (about 13% of initial Fe[III] concentration) at 75°C during this period. At the end of the experiment, the amount of Fe(II) produced was <5% of initial Fe(III) concentration at 25 and 45°C, 9% at 55°C, 16% at 65°C, and ~50% at 75°C.

Figure 1B shows decreased Co(III) concentrations for all temperatures after 6 h of incubation. Again, similar to Fe(II) production profiles, the fastest reduction was at 75°C (0.67 vs 0.13 m*M*/h at 25°C). The amount of Co(III) reduced increased from 14% at 25°C to 82% at 75°C.


A
75°C
65°C
55°C
45°C
25°C
Fe (II), mM
B
25°C
45°C
55°C
65°C
75°C
Co (III), mM
C
25°C
45°C
55°C
65°C
75°C
Cr (VI), mM
Time (h)

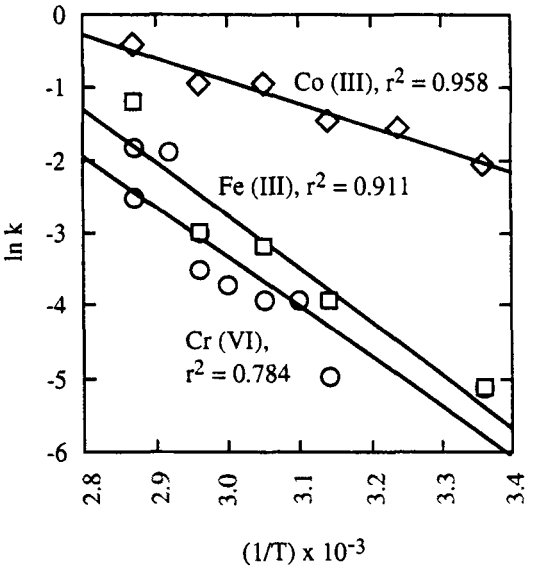


Fig. 2. Temperature (in kelvin) dependency of the abiotic reaction rate (k) for Fe(III) (measured as increase in Fe[II] production), Co(III), and Cr(VI). Fe (III) and Co(III) data were from Fig. 1A and B, respectively. Cr(VI) data were from Fig. 1C and other experiments. The slope of the curve fit equals $-E/R$ in Arrhenius equation, $k = k_o e^{-E/RT}$, where k_o is the frequency factor, E the activation energy of the reaction, and R the gas constant *(15)*.

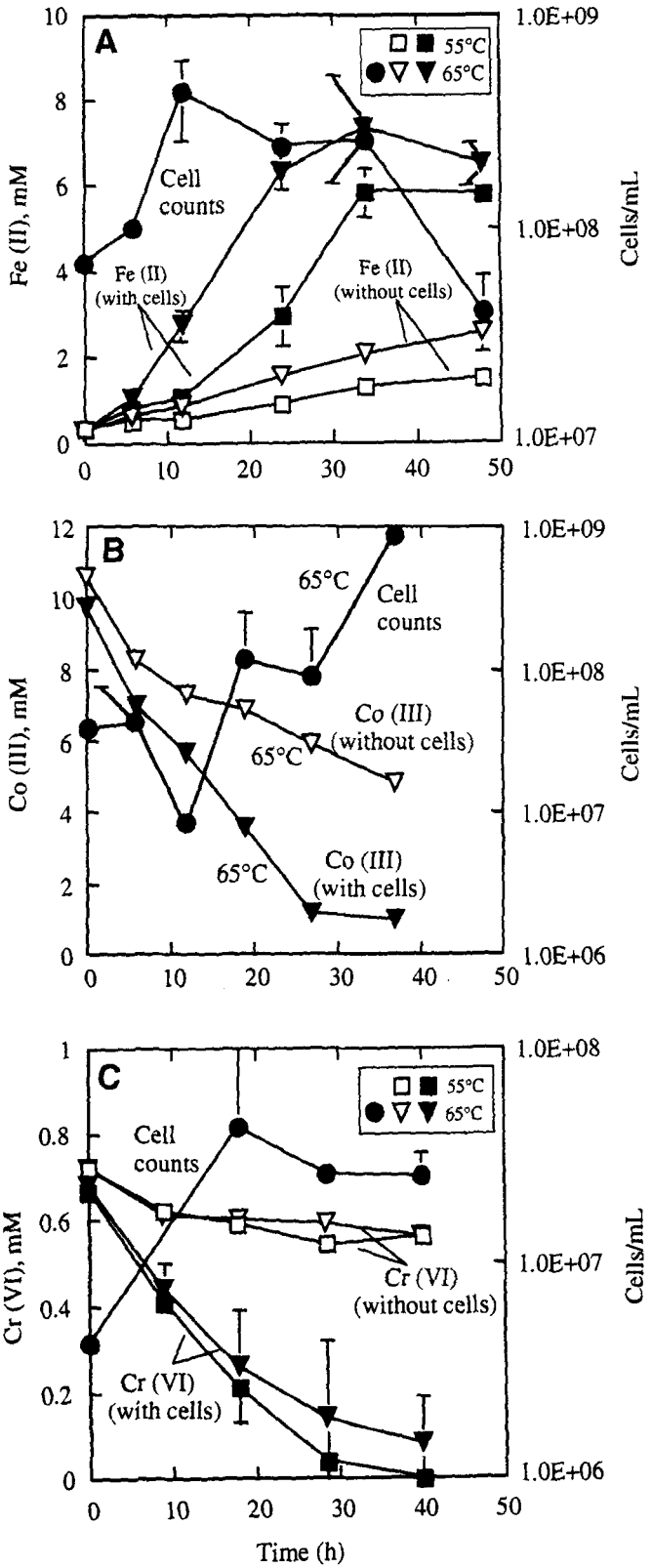
Abiotic reduction of Cr(VI) at 65 and 75°C (Fig. 1C) was rapid and linear, in contrast to the variable results obtained at lower temperatures. At 45 and 55°C, Cr(VI) reduction showed the same trend with a general decrease in Cr(VI) concentration between 0 and 22 h. Reduction of Cr(VI) at 25°C showed a less regular trend. Final reduction for Cr(VI) was 92% at 75°C, 52% at 65°C, and <10% between 25 and 55°C.

Among the three metals tested, Co(III) had the highest reduction rates for all temperatures, ranging from 0.13 mM/h at 25°C to 0.67 mM/h at 75°C. Fe(III) and Cr(VI) reduction rates were <0.05 mM/h at temperatures below 65°C and increased to 0.3 and 0.08 mM/h at 75°C, respectively. All three metals showed a temperature dependency of the reduction rate, which can be described by the Arrhenius law (Fig. 2). The correlation between ln k and 1/T was significant with p <0.01 for Co(III) and Cr(VI) and with p <0.05 for Fe(III).

Microbial Enhancement of Fe(III), Co(III), and Cr(VI) Reduction by TOR 39

The potential enhancement of Fe(III), Co(III), and Cr(VI) reduction by bacterial strain TOR 39 was examined after freshly grown inocula were transferred into the medium containing the oxidized metals. Figure 3A shows the production of Fe(II)

Fig. 1. *(opposite page)* Abiotic reduction of Fe(III), Co(III), and Cr(VI) at different temperatures. **(A)** Reduction of initial 14 mM Fe(III) measured as the production of Fe(II) with time. **(B)** Reduction of initial 10 mM Co(III) with time. **(C)** Reduction of initial 2.5 mM Cr(VI) with time. Glucose concentration was 5 mM in A and 10 mM in B and C. Yeast extract and peptone concentrations were 0.1–0.15% in A, B, and C.


A
55°C
65°C
Cell counts
Fe (II) (with cells)
Fe (II) (without cells)
Fe (II), mM
Cells/mL
1.0E+09
1.0E+08
1.0E+07
B
65°C
Cell counts
Co (III) (without cells)
65°C
Co (III) (with cells)
Co (III), mM
Cells/mL
1.0E+09
1.0E+08
1.0E+07
1.0E+06
C
55°C
65°C
Cell counts
Cr (VI) (without cells)
Cr (VI) (with cells)
Cr (VI), mM
Cells/mL
1.0E+08
1.0E+07
1.0E+06
Time (h)

reduced from 14 m*M* ferric citrate in the presence or absence of the bacterium. Also shown are cell counts during biotic Fe(II) production at 65°C. After a lag phase of 6 h, production of Fe(II) at 65°C increased dramatically in the presence of TOR 39 with a rate of 0.29 m*M*/h between 6 and 24 h, whereas the abiotic production rate was only 0.05 m*M*/h during this period. The amount of Fe(II) produced in the presence of TOR 39 reached the maximum value at 34 h, which was about 3.5 times higher than in the absence of the bacterium.

Bacterial production of Fe(II) at 55°C followed the trend observed at 65°C (Fig. 3A); however, a dramatic increase in Fe(II) at 55°C was observed after 24 h of incubation, and the maximum amount of Fe(II) produced at 34 h was less (5.8 vs 7.4 m*M*) than at 65°C. Similarly, abiotic Fe(II) production at 55°C was slower (0.02 m*M*/h vs 0.05 m*M*/h) and less (1.5 vs 2.6 m*M*) than at 65°C.

TOR 39 density increased from 6.9×10^7 cells/mL at 0 h to 4.4×10^8 cells/mL at 12 h during the early stage of biotic Fe(II) production at 65°C (Fig. 3A); however, the cell numbers decreased to 4.1×10^7 cells/mL by the end of the experiment. Bacterial cells were not counted during Fe(II) production at 55°C.

Figure 3B shows abiotic and biotic reduction of Co(III) (initial concentration of 10 m*M*) and bacterial cell counts at 65°C. Rates of abiotic and biotic reduction of Co(III) were similar (0.39 vs 0.46 m*M*/h) after 6 h of incubation, suggesting that the lag phase of bacterial growth was similar to that seen during Fe(II) production. After 6 h, the Co(III) reduction rate was about twice as fast in the presence of TOR 39 than in its absence. By the end of the experiment, the amount of Co(III) reduced was 1.5 times higher in the presence of TOR 39 than in its absence (Fig. 3B). Cell numbers also increased during bacterial reduction of Co(III).

Bacterial enhancement of Cr(VI) reduction was examined at Cr(VI) concentrations below 1.0 m*M* because Cr(VI) concentrations $\geq$ 1.0 m*M* inhibited the growth of TOR 39 (data not shown). Figure 3C shows the biotic and abiotic reduction of Cr(VI) at 55 and 65°C with an initial Cr(VI) concentration of about 0.7 m*M*. The rates of abiotic reduction of Cr(VI) at 55 and 65°C in the absence of TOR 39 were about 0.01 m*M*/h after 9 h of incubation. In contrast, rates of biotic Cr(VI) reduction were three times faster at both temperatures during the same period of incubation. Between 9 and 40 h, biotic reduction of Cr(VI) continued rapidly, whereas the abiotic reduction proceeded slowly at both temperatures. The increase in cell counts during the early stage of incubation was similar to the trend of cell counts during Fe(II) production. By the end of the experiment, the initial concentration of Cr(VI) was reduced biotically by 88% at 65°C and by 95% at 55°C. Abiotic reduction of Cr(VI) at 65 and 55°C was $\leq$ 20%. Figure 4 summarizes the biotic vs abiotic reduction of Fe(III), Co(III), and Cr(VI). This figure shows that TOR 39 significantly enhanced reduction of these metals at 55 and 65°C.

Fig. 3. *(opposite page)* Microbially enhanced reduction of Fe(III), Co(III), and Cr(VI) at 55 and/or 65,°C. **(A)** Time-course measurement of abiotic and biotic reduction of Fe(III) (measured as increase in Fe[II] production) and cell counts. **(B)** Time-course measurement of abiotic and biotic reduction of Co(III) and cell counts. **(C)** Time-course measurement of abiotic and biotic reduction of Cr(VI) and cell counts. *See* Fig. 1 for concentrations of glucose, yeast extract, and peptone in the medium. Results shown are the average of duplicate measurements ± 1 SD, except for cell numbers at 0 (A, B, and C) and 40 h (B), which represent a single measurement. For those points at which SDs are not visible, the error bars were smaller than the points as plotted.

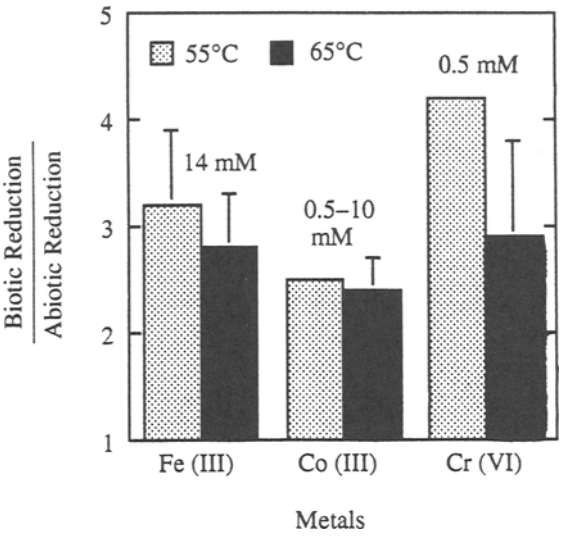


Fig. 4. Ratio of biotic vs abiotic metal reduction at 55 and 65°C with the indicated initial metal concentrations. Fe(III) reduction was measured as the increase of Fe(II). Results are the mean ± SE for two to four parallel experiments, except for Cr(VI) reduction at 55°C, which represents the average ± 1 SD of a single experiment (Fig. 3C). For those bars in which the error bars are not visible, the SE is <0.1.

Metabolic inhibition experiments (data not shown) indicated that TOR 39 was sensitive to the metal reductase inhibitor *p*-chloromercuriphenylsulfonate and to the dehydrogenase inhibitor quinacrine. Reduction of ferric citrate was not sensitive to the sulfate-reduction inhibitor molybdate, the nitrate-reductase inhibitor NaN_3, or the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, substantiating that the organism is a fermentative anaerobe.

DISCUSSION

Temperature profiles of abiotic reduction of Fe(III), Co(III), and Cr(VI) (Fig. 1A, B, and C) suggest that abiotic metal reduction was insignificant at mesophilic temperatures (<45°C), was significant above 45°C, and became a major process at 65 and 75°C. Although all three metals showed a temperature dependency of abiotic reduction, they have different activation energies, which are proportional to the slope in Fig. 2. Larger slopes (Fe[III] and Cr[VI]), thus have greater activation energies and indicate a higher temperature sensitivity than a smaller slope (Co[III]) *(15)*.

Results of Fe(III) reduction in this study support the current understanding that in many sedimentary environments of mesophilic temperatures, the potential for Fe(III) reduction coupled to organic matter oxidation by abiotic mechanisms is low *(2,16)*; however, abiotic reduction of Fe(III) may play an important role in higher temperature environments, such as geothermal vents and deep aquifers, where temperatures can be above 50°C.

The more than twofold increase in the amount of metals reduced at 55 and 65°C in the presence of TOR 39 relative to the abiotic reduction suggests that the bacte-

rium was responsible for the enhancement. Based on the susceptibility of TOR 39 to different metabolic inhibitors, it is likely that the bioenhancement of metal reduction reflects the potential for dumping reducing equivalents generated by TOR 39 metabolism rather than gaining more growth from respiratory metal reduction as other metal-reducing bacteria do *(3,4)*. Nevertheless, our data indicated that at thermal conditions, fermentative bacteria, such as TOR 39, may play a greater role in enhancing metal reduction than mesophilic fermentative bacteria, which typically transfer <5% of electrons to the oxidized metals *(2)*. The exact mechanisms operated by thermophilic fermentative bacteria in reducing metals, however, need further investigation.

In addition to enhancing the reduction of Fe(III), Co(III), and Cr(VI), TOR 39 also enhanced the reduction of other metals, such as Mn(IV) and U (VI) (data not shown). Similar thermophilic bacteria tolerated higher temperature (up to 75°C) and NaCl concentrations (up to 7%), while indicating the potential of reducing metals.

Fe(III) forms have a significant effect on Fe(III) reduction. Increasing crystallization results in less reduction of Fe(III), probably because of decreases in the solubility and surface area of the Fe(III) form *(1,17)*. The bacteria-enhanced Fe(III) reduction in this study was probably caused by the high solubility of ferric citrate. Other studies reported that Fe(III) reduction rates were stimulated by the addition of ferric citrate *(18,19)*.

Abiotic reduction of toxic Cr(VI) by reactive organic matter, such as phenol, at mesophilic temperatures has been well established *(20,21)*. Raising temperatures may increase the rate of Cr(VI) reduction, and adding thermophilic bacteria may enhance the reduction even further. Bioenhancement of Cr(VI) reduction has been demonstrated at mesophilic temperatures *(12)*, and has a potential application for removing Cr(VI) from contaminated water and waste streams.

In summary, this study demonstrated that abiotic reduction of Fe(III), Co(III), and Cr(VI) was insignificant in the presence of organic matter, such as glucose, yeast extract, and peptone, at mesophilic temperatures (<45°C). However, biotic reduction of these metals was significant above 45°C and became a major process at 75°C in the presence of organics. On the other hand, reduction of Fe(III), Co(III), and Cr(VI) was two to four times higher in the presence of the thermophilic bacterium than in its absence, and the number of bacterial cells increased concomitantly with the reduction of these metals, suggesting that the enhanced reduction of these metals is related to the fermentative activity of the thermophilic bacterium.

ACKNOWLEDGMENTS

We thank Philip M. Jardine and David L. Taylor for providing Co(III)-EDTA, David Ringelberg for performing lipid analysis, and Anthony V. Palumbo and Susan M. Pfiffner for technical assistance. This research was funded to T. J. Phelps by the US Department of Energy Subsurface Science Program (Deep Microbiology Subprogram), managed by Frank Wobber, under contract DE-AC05-960R22464 with Locheed Martin Energy Research Corp. C. Zhang, S. Liu, J. Logan, and R. Mazumder were supported through Oak Ridge Associated Universities, Oak Ridge, TN 37830. This is publication number 4520 of the Environmental Sciences Division, ORNL.

REFERENCES

1. Lovley, D. R. (1987), *Geomicrobiol. J.* **5**, 375–399.
2. Lovley, D. R. (1991), *Microbiol. Rev.* **55**, 259–287.
3. Lovley, D. R. (1993), *Annu. Rev. Microbiol.* **47**, 263–290.
4. Nealson, K. H. and Saffarini, D. (1994), *Annu. Rev. Microbiol.* **48**, 311–343.
5. Lovley, D. R. and Phillips, E. J. P. (1988), *Appl. Environ. Microbiol.* **54**, 1472–1480.
6. Myers, C. R. and Nealson, K. H. (1988), *Science* **240**, 1319–1321.
7. Machel, H. G. and Burton, E. A. (1991), *Geophys.* **56**, 598–605.
8. Lovley, D. R. (1990), *Iron Biominerals*, Frankel, R. B. and Blakemore, R. P., eds., Plenum, New York, pp. 151–166.
9. Boone, D. R., Liu, Y., Zhao, Z., Balkwill, D. L., Drake, G. R., Stevens, T. O., and Aldrich, H. C. (1995), *Intl. J. Syst. Bacteriol.* **45**, 441–448.
10. Balkwill, D. L., Boone, D. R., Colwell, F. S., Griffin, T., Kieft, T. L., Lehman, et al. (1994), *EOS* **75**, 385, 395–396.
11. Phelps, T. J., Raione, E. G., White, D. C., and Fliermans, C. B. (1989), *Geomicrobiol. J.* **7**, 79–91.
12. Stookey, L. L. (1970), *Anal. Chem.* **42**, 779–781.
13. Urone, P. F. (1955), *Anal. Chem.* **27**, 1354,1355.
14. Lovley, D. R. and Phillips, E. J. P. (1994), *Appl. Environ. Microbiol.* **60**, 726–728.
15. Levenspiel, O. (1972), *Chemical Reaction Engineering*, Wiley, New York.
16. Lovley, D. R., Phillips, E. J. P., and Lonergan, D. J. (1991), *Environ. Sci. Technol.* **25**, 1062–1067.
17. Kostka, J. E. and Nealson, K. H. (1995), *Environ. Sci. Technol.*, **29**, 2535–2540.
18. Arnold, R. G., Dichristina, T. J., and Hoffmann, M. R. (1986), *Appl. Environ. Microbiol.* **52**, 281–289.
19. Jones, J. G., Gardener, S., and Simon, B. M. (1983), *J. Gen. Microbiol.* **129**, 131–139.
20. Elovitz, M. S. and Fish, W. (1994), *Environ. Sci. Technol.* **28**, 2161–2169.
21. Palmer, C. D. and Wittbrodt, P. R. (1991), *Environ. Health Perspect.* **92**, 25–40.