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Impact of Zinc on Biological Fe(III) and Nitrate Reduction by *Shewanella putrefaciens* CN32

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ABSTRACT

The impact of zinc on the reductive dissolution of hematite by the dissimilatory metal-reducing bacterium *Shewanella putrefaciens* CN32 was studied in batch culture mode. Experiments were conducted with hematite (2.0 g L^{-1}) in 10 mM PIPES (pH 6.8) and H_2 as the electron donor under nongrowth conditions (10^8 cell mL^{-1}), spiked with zinc (0 to 0.23 mM), and incubated for 5 days. Experiments were also conducted with ferric citrate (2.0 mM) and nitrate (1.43 mM NO_3-N L^{-1}) to evaluate the effect of zinc with soluble electron acceptors. A 50% inhibitory concentration (IC_{50}) was defined as the zinc concentration that decreased the extent of electron acceptor consumption by 50% compared to a no-zinc biotic control. The total zinc IC_{50} values for hematite, ferric citrate, and nitrate reduction were 0.21, 0.28, and 0.049 mM, respectively. The free zinc (Zn^{2+}) IC_{50} values for hematite, ferric citrate, and nitrate reduction were 0.13, 0.00016, and 0.049 mM, respectively. The low Zn^{2+} IC_{50} for ferric citrate was attributed to citrate complexation of zinc. The free Zn^{2+} IC_{50} for aerobic cell growth on lactate in zinc-containing defined growth medium was 0.050 mM. Differences between free Zn^{2+} IC_{50} values for soluble and solid phase electron acceptors indicate that zinc adsorption to hematite and cell surfaces may control inhibition during solid-phase iron reduction. Greater zinc tolerance observed during solid phase iron reduction compared to soluble electron acceptors may be attributed to the formation of biofilms on hematite surfaces, resulting in higher zinc tolerance compared to freely dispersed cells.

Key words: bioreduction; *Shewanella*; hematite; zinc; toxicity; inhibition

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INTRODUCTION

The microbial reduction of Fe(III) has been recognized as a promising process for the bioremediation of heavy metals and radionuclides within contaminated aquifers (Lovley *et al*., 1996; Fredrickson *et al*., 1998; Roden and Urrutia, 1999; Urrutia *et al*., 1999). Recent research has focused on the use of dissimilatory metal-reducing bacteria (DMRB) to promote the mobilization or immobilization of selected contaminants through the reductive dissolution or precipitation of these metals and associated iron oxides (Zachara *et al*., 1998, 2002; Burgos *et al*., 2002). Many studies have documented metal–microbe interactions (Baath, 1989; Kushner, 1993; Gadd, 1996; Warren and Haack, 2001), but few recent studies (Cooper *et al*., 2000; Parmar *et al*., 2001) have specifically focused on the impact of metals (zinc and nickel) on iron reduction by the DMRB *Shewanella putrefaciens*. This Gram-negative facultative anaerobe can reduce crystalline (Cooper *et al*., 2000; Zachara *et al*., 2001; Royer *et al*., 2002a, 2002b) and poorly crystalline iron oxides (Fredrickson *et al*., 2001), soluble Fe(III) complexes (Liu *et al*., 2001), nitrate, and a variety of toxic metals and radionuclides such as Cr(VI) (Fendorf *et al*., 2000; Wielinga *et al*., 2001), U(VI) (Brooks *et al*., 2003), and Tc(VII) (Wildung *et al*., 2000; Liu *et al*., 2002a).

Heavy metals are known to affect bacteria in a number of ways including altering key enzymes regulating intracellular transport (Welp and Brummer, 1997a) or exchanging essential cations on carboxyl and phosphoryl reactive sites on the cell wall (Fein *et al*., 2001). Previous studies related to heavy metal toxicity have focused on the influence of metals on microbial growth (Shuttleworth and Unz, 1991; Sani *et al*., 2001) or respiration (Knight *et al*., 1997) and on metal accumulation or precipitation on microbial surfaces (Poulson *et al*., 1997). Heavy metals such as zinc, copper, and cobalt are essential micronutrients for micro-organisms at low concentrations (μM range) (Raven, 1984), but when these beneficial concentrations are exceeded, microbial enzyme activity can be permanently altered. Zinc is a common ground water pollutant, and has been found in ground waters at U.S. Department of Energy (DOE) facilities at concentrations exceeding 10.7 mM total zinc, easily the highest heavy metal concentration reported (Riley and Zachara, 1992). Other studies have reported zinc concentrations of 5.0 mM (Lighthart *et al*., 1983) and 7.0 mM (Dueck *et al*., 1986) in polluted soils.

The purpose of this research was to investigate the effects of zinc on the biological reduction of hematite by the DMRB *S. putrefaciens* CN32. The initial hypothesis was that zinc could inhibit solid-phase biological iron reduction by at least two mechanisms: via direct toxicity or lethality to the DMRB, or via modification of the oxide and/or DMRB surfaces by sorbed zinc, which may affect DMRB–oxide contact or DMRB–oxide electron transfer (Roden and Urrutia, 2002). To differentiate between the proposed mechanisms of inhibition, similar experiments were conducted using soluble electron acceptors, specifically ferric citrate and nitrate, for comparative purposes.

EXPERIMENTAL PROTOCOLS

Microorganism and culture conditions

Shewanella putrefaciens CN32 was provided courtesy of Dr. David Balkwill (Subsurface Microbial Culture Collection, Florida State University). *S. putrefaciens* CN32 was isolated from an anaerobic subsurface core sample (250 m below ground surface) from the Morrison Formation in northwestern New Mexico (Fredrickson *et al*., 1998). The cultures were grown aerobically on tryptic soy broth without dextrose (TSB-D) at 20°C. Cells were harvested by centrifugation (4,900 $\times$ *g*, 10 min, 20°C) from a 16-h-old culture (late log phase) under nongrowth conditions (no added inorganic nutrients or vitamins during experiments). The cells were washed three times in 10 mM 1,4-piperazinediethanosulfonic acid (PIPES; pH = 6.8), with the final wash undergoing deoxygenation to remove residual oxygen. Cell pellets were resuspended in 5–15 mL of deoxygenated 10 mM PIPES buffer in an anaerobic chamber (Coy; Grass Lakes, MI) under a N_2:H_2 (ca. 97.5:2.5%) atmosphere and the cell density was determined by absorbance at 420 nm. Cells were assumed to be cylindrical shape (0.5 μm diameter by 2 μm length) with a mass of 6.4×10^{-10} mg cell^{-1} (Liu *et al*., 2002b).

Iron oxide

An iron oxide powder was obtained from J.T. Baker, heated to 550°C in air overnight before use to remove residual carbon, and identified by X-ray diffraction and Mössbauer spectroscopy to be hematite (α-Fe_2O_3) of greater than 99% purity. The hematite had an average particle diameter of 1.0 μm measured by laser diffraction and a specific surface area of 9.04 m^2 g^{-1} measured by five-point N_2-BET (Jeon *et al*., 2001). The zero point of charge was pH 8.5, as determined by electrophoretic mobility and proton titrations (Jeon *et al*., 2001). The hematite surface site density for Fe(II) was 5.1 sites per nm^2 based upon adsorption of Fe(II) (Jeon *et al*., 2001). Hematite was added to anaerobic PIPES buffer at least 24 h prior to any experiment to allow for hydration.

AU1

Bioreduction experiment preparation

A "master reactor" approach was used to prepare all experiments (Royer *et al*., 2002a) to ensure consistent chemical and biological conditions. All preparations were performed within an anaerobic chamber. A master reactor was prepared by combining the electron acceptor and the inoculum in a 120-mL serum bottle. All solutions (except the zinc stock) were prepared in 10 mM PIPES (pH 6.8). Before zinc addition, three 10-mL aliquots were removed, which served as biotic no-zinc controls. Zinc was incrementally added to the master reactor from an acidified and deoxygenated $ZnCl_2$ solution (1,000 mg L^{-1} AAS certified standard) along with an equal volume of 0.1 N NaOH (for pH maintenance). For each zinc concentration tested, 10 mL of the suspension was transferred to 20 mL amber serum bottles (in triplicate), sealed within the anaerobic chamber (97.5:2.5 N_2H_2), and incubated in the dark at 20°C on orbital shakers (150 rpm) outside of the anaerobic chamber for up to 5 days. Hydrogen from the anaerobic chamber atmosphere was used as the electron donor in all experiments except for the DMRB growth experiments where lactate (30 mM) was used. The pH of the experiments never varied outside the range of 6.6–7.0.

Hematite bioreduction

Experiments were performed with 2.0 g L^{-1} hematite (25 mM Fe(III) L^{-1}), final cell density (target) of 10^8 cells mL^{-1}, and zinc concentrations ranging from 0 to 0.23 mM. After incubation times of 1, 2, 3, 4, and 5 days, reactors were sacrificed for the measurement of dissolved and 0.5 N HCl extractable ("adsorbed plus dissolved" as defined in Jeon *et al*., 2003) Fe(II), dissolved and 0.5 N HCl extractable zinc, pH, and freely dispersed cell viability. Cell viability was operationally defined using the LIVE/DEAD© *Bac*light™ bacterial viability kit (Molecular Probes; Eugene, OR).

Ferric citrate bioreduction

Ferric citrate reduction was tested in solutions containing 2 mM ferric citrate (dissolved in 10 mM PIPES), 4 mM citric acid, a final cell density of 10^8 cells mL^{-1}, and variable concentrations of zinc (0 to 1.38 mM), all in 10 mM PIPES. Solution pH of 6.8 was maintained with 3 N NaOH or 3 N HCl. A 3:1 molar ratio of citrate:Fe(III) was selected to prevent the formation of amorphous ferric hydroxide based on speciation calculations made with MINTEQA2 (Allison *et al*., 1991) using published thermodynamic data (Liu *et al*., 2001; Smith and Martell, 1997). The program was run at fixed hydrogen ion concentrations assuming solution equilibrium was established. The calculation of free metal activity using MINTEQA2 has been used in several studies (Shuttleworth and Unz, 1991, 1993) for metal–microbe systems containing high cell concentrations. Reactors were prepared in clear glass serum bottles (250 mL) containing between 100 to 200 mL of solution media, crimp-sealed with thick butyl rubber stoppers and aluminum caps, and sealed serum bottles were incubated in the dark at 20°C on orbital shakers outside of the anaerobic chamber. Larger reactor volumes (compared to 10 mL used during hematite bioreduction) ensured that adequate sample volume existed throughout duration of experiment. After incubation times of ca. 0, 1, 2.5, 3.75, and 6 h, reactors were sampled within the anaerobic chamber by piercing the septa with a sterile needle and syringe. Samples were analyzed for dissolved Fe(II), dissolved zinc, viability of freely dispersed cells, and pH.

Nitrate bioreduction

Experiments were performed in 10 mM PIPES (pH 6.8) containing 1.43 mM NO_3-N L^{-1} (added as KNO_3), a final cell density of 10^8 cells mL^{-1}, and variable concentrations of zinc (0 to 0.46 mM). Reactor preparation and reactor sampling were performed using the same techniques as the ferric citrate experiments. Samples were collected after incubation times of ca. 0, 5, 11, 21, and 31 h, and were used to measure nitrate–nitrogen, dissolved zinc, viability of freely dispersed cells, and pH.

DMRB growth

Cells were prepared as above; however, the M1 defined growth medium (Myers and Nealson, 1988) was used for the final two aerobic rinses to remove residual TSB-D. Rinsed cells were inoculated at 5.0×10^7 cells mL^{-1} into reactors containing the M1 medium, 30 mM lactate, and variable concentrations of zinc (0 to 3.06 mM total zinc) and were grown aerobically on a rotary shaker (150 rpm). Cell concentrations were measured by absorbance at 420 nm after incubation times of 0 and 21 h. Absorbance values were compared to abiotic, no-zinc controls.

Zinc sorption

Experiments were performed to determine the sorption of zinc to 10^8 pasteurized cells mL^{-1} (140 m^2 g^{-1} surface area; Fein *et al*., 1997; Sokolov *et al*., 2001), 2.0 g L^{-1} hematite (9.04 m^2 g^{-1} surface area; Jeon *et al*., 2001), and both viable and sterilized suspensions containing 2.0 g L^{-1} hematite plus 10^8 cells mL^{-1}. Zinc con-

centrations ranged from 0.02 to 0.69 mM total zinc. Sterilized cells were pasteurized at 63°C for 30 min prior to inoculation. The effectiveness of this sterilization procedure was confirmed using *Bac*light. The viable cells plus hematite suspensions contained between 0.088 and 0.30 mM Fe(II). All sorption experiments were performed at pH 6.8 within the anaerobic chamber in triplicate using experimental procedures described above including an equilibration time of 5 days. Duplicate measurements of extractable and dissolved metal concentrations for each sample from each reactor were taken (for a total of $n = 6$) using methods described below.

Analytical techniques

Fe(II) was reported as dissolved and extractable. For dissolved Fe(II), samples were filtered (0.2 μm cellulose acetate) and Fe(II) was measured by ferrozine (1.96 mM ferrozine in 50 mM HEPES, pH 8.0; Stookey, 1970). Solution pH of the filtrate was determined in the anaerobic chamber using a combination pH electrode. For extractable Fe(II), an unfiltered sample was acidified with HCl to achieve a final solution normality of 0.5 N. The solution was mixed for ca. 24 h, filtered (0.2 μm), and Fe(II) in the filtrate was measured by ferrozine. Dissolved and total zinc was measured from the corresponding dissolved and extractable Fe(II) filtrate samples by flame atomic absorption spectrometry (AAS) after preservation with conc. HNO_3. Nitrate was measured by a Hach 2010 spectrometer (Hach Company, Loveland, CO) using the high range nitrate (0–30 mg L^{-1}) Test 'n Tube method 322. Abiotic reduction of hematite by 0.25 M hydroxylamine hydrochloride in 0.25 M HCl (HA-HCl) (Roden and Zachara, 1996; Hacherl *et al*., 2001) with variable zinc (0 to 0.23 mM) showed zinc did not significantly ($p > 0.05$) affect Fe(II) production.

The *Bac*light bacterial viability kit includes mixtures of green fluorescent nucleic acid stain SYTO 9 and red fluorescent nucleic acid stain propidium iodide (PI). The SYTO 9 stain generally labels all bacteria while the PI stain penetrates only bacteria with damaged membranes causing a displacement of the SYTO 9 stain (Boulos *et al*., 1999). Thus, viability is defined and determined solely on cell membrane integrity. Nonviable cells were counted by their fluorescent red color, while viable cells were counted by their fluorescent apple green color (Sani *et al*., 2001; Hu *et al*., 2003; Teitzel and Parsek, 2003). A 30-μL aliquot of a stain mixture containing 20 μL PI and 10 μL SYTO 9 stock solutions dissolved in 1 mL MilliQ water was added to 1 mL of unfiltered bacteria-containing suspension. A 10-μL sample was removed from the treated 1-mL sample and placed on a glass slide beneath a 4.84 cm^2 cover slip. Cell viability was calculated based upon the average from five field counts (each field being 2.64×10^{-4} cm^2) for each sample (one per reactor) using 64× magnification with the aid of a Zeiss epifluoresence microscope (Zeiss; Jena, Germany). Cells not visibly attached to hematite particles were counted as freely dispersed cells (Langley and Beveridge, 1999). Cell viability counts for cells physically adhered to hematite (i.e., localized biofilms) were not counted due to uncertainties of consistent stain uptake by cells within biofilms. Previous studies have found *Bac*light staining to correlate positively with plate counting techniques (Virta *et al*., 1998; Auty *et al*., 2001) and our plate counts compared favorably ($p > 0.05$) with *Bac*light freely dispersed cell counts under experimental conditions tested (hematite bioreduction containing variable zinc).

Data analysis

The quantification of zinc inhibition during aerobic cell growth and the anaerobic respiration of hematite, ferric citrate and nitrate by *S. putrefaciens* CN32 was assessed using microbial toxicity definitions developed by Sani *et al*. (2001). Specifically, we defined inhibition as the percent change of bioreduction extent due to zinc addition relative to its biotic no-zinc control. This definition may be expressed as:

$$\%\text{Inhibition} = \left(\frac{\text{Bioreduction Extent with Zinc}}{\text{Bioreduction Extent without Zinc}}\right) \times 100 \quad (1)$$

where the bioreduction extent was measured after 5 days for hematite, 6 h for ferric citrate, and 9 h for nitrate. These end points were selected because they occurred at times when the pseudofirst-order rates were still operative [i.e., plots of $\ln(C/C_0)$ highly linear; initial rates yet to decrease]. In addition, data analyses comparing bioreduction extent values to bioreduction rate values yielded essentially identical IC_{50} estimates. For aerobic growth experiments, the extent of growth was measured after 21 h. The inhibitory concentration of zinc required for 50% reduction in microbial activity (IC_{50}) was calculated according to the equation:

$$IC_{50} = 10\left(\frac{50\% - \beta_0}{\beta_1}\right) \quad (2)$$

where β_0 is the y-axis intercept of the log zinc vs. linear percent inhibition regression line and β_1 is the slope of the log zinc vs. linear percent inhibition regression line.

"Free" zinc (Zn^{2+}) concentrations reported were estimated using MINTEQA2 (Allison *et al*., 1991) speciation analysis based upon published thermodynamic data (Smith and Martell, 1997; Liu *et al*., 2001). Zinc speciation analyses, determined using zinc cyclic voltammetry (CV-50W Voltammetric Analyzer by Bioanalytical Systems, West Lafayette, IN), indicated Zn^{2+} was the

dominant zinc species for the experimental conditions. Measured soluble and total zinc concentrations and pH were used as input for MINTEQA2 calculations of free Zn^{2+} to account for sorption to cells and hematite.

Zinc sorption isotherms were presented using the Freundlich adsorption isotherm equation:

$$\text{LOG } q = \text{LOG } K_f + \frac{1}{n}\text{LOG } C \qquad (3)$$

where q is the equilibrium adsorbent phase concentration, K_f is the Freundlich adsorption capacity factor, $1/n$ is the Freundlich intensity factor, and C is the dissolved phase equilibrium concentration.

RESULTS

Hematite bioreduction

For the purpose of this study, zinc toxicity was quantified as the inhibition of the biological reduction of the provided electron acceptor (hematite, ferric citrate, or nitrate) by *S. putrefacians* CN32. For hematite, this effect was based upon a decrease in 0.5 N HCl extractable (adsorbed plus dissolved) Fe(II) produced after a 5-day incubation period. While others have shown the inability of 0.5 N HCl to extract all Fe(II) associated with hematite (Jeon *et al*., 2001; Hansel *et al*., 2004), "total" Fe(II) and zinc using 3.0 or 6.0 N HCl extraction was not measured due potential interferences caused by hematite dissolution (Jeon *et al*., 2003). Extractable Fe(II) production as a function of added zinc (Fig. 1A) revealed that the rate and 5-day extent of biogenic Fe(II) production was dependent on the presence of total zinc. Abiotic controls (results not shown) resulted in no Fe(II) production. Total zinc concentrations less than 0.13 mM resulted in either no change or increased Fe(II) production relative to the biotic no-zinc control. Only the highest zinc concentration tested (0.23 mM) resulted in 36% decreased Fe(II) production relative to the biotic no-zinc control.

Increasing zinc concentrations generally decreased freely dispersed cell viability over time (Fig. 1B). For the biotic no-zinc control (Fig. 1B), 82% of the freely dispersed cells were viable after 5 days. No viable cells remained in the presence of 0.23 mM zinc after 5 days. The average total number of dispersed (viable and nonviable) cells counted was $1.32 \times 10^8 \pm 4.63 \times 10^7$ colony-forming units (CFU) mL^{-1}, respectively.

Ferric citrate bioreduction

Experiments were performed with ferric citrate to evaluate the inhibitory effect of zinc using a soluble instead of solid-phase electron acceptor. MINTEQA2 calculations indicated that ferrihydrite formation would be thermodynamically favorable in 10 mM PIPES at pH 6.8 with a 1:1 molar ratio of citrate:Fe(III) (2 mM ferric citrate), therefore, a 3:1 molar ratio was obtained by adding 4 mM citric acid. Under these conditions, MINTEQA2 calculations indicated the formation of zinc–citrate was most favorable (90.8 to 93.3% of total zinc), while free Zn^{2+} accounted for only 0.053 to 0.076% of the total zinc for the

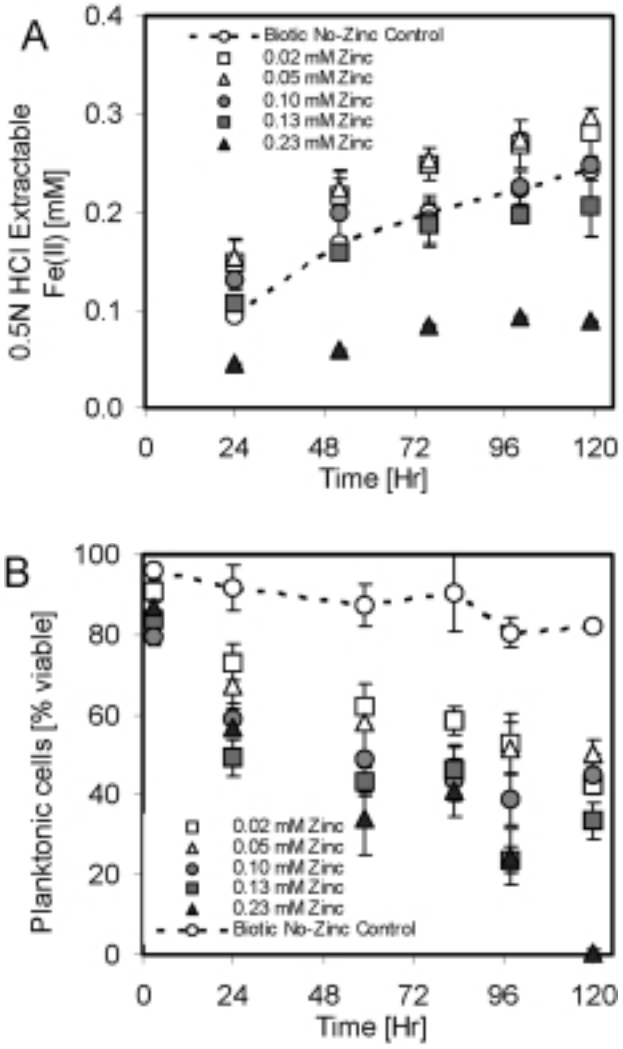

Figure 1. (**A**) 0.5 N HCl extractable Fe(II) production, (**B**) freely dispersed cell viability, for *Bac*light LIVE/DEAD cell counts as a function of time (0 to 5 days) for the biological reduction of 2 g L^{-1} hematite at pH 6.8 in 10 mM PIPES. Total zinc concentration ranged from 0 mM for the biotic and abiotic controls to 0.23 mM. *Shewanella putrefaciens* CN32 was used (10^8 cells mL^{-1}) under nongrowth conditions. Abiotic controls with hematite produced 0.0 mM Fe(II). Biotic controls without hematite produced 0.003 mM Fe(II). Values are means of three replicates (±standard deviation).

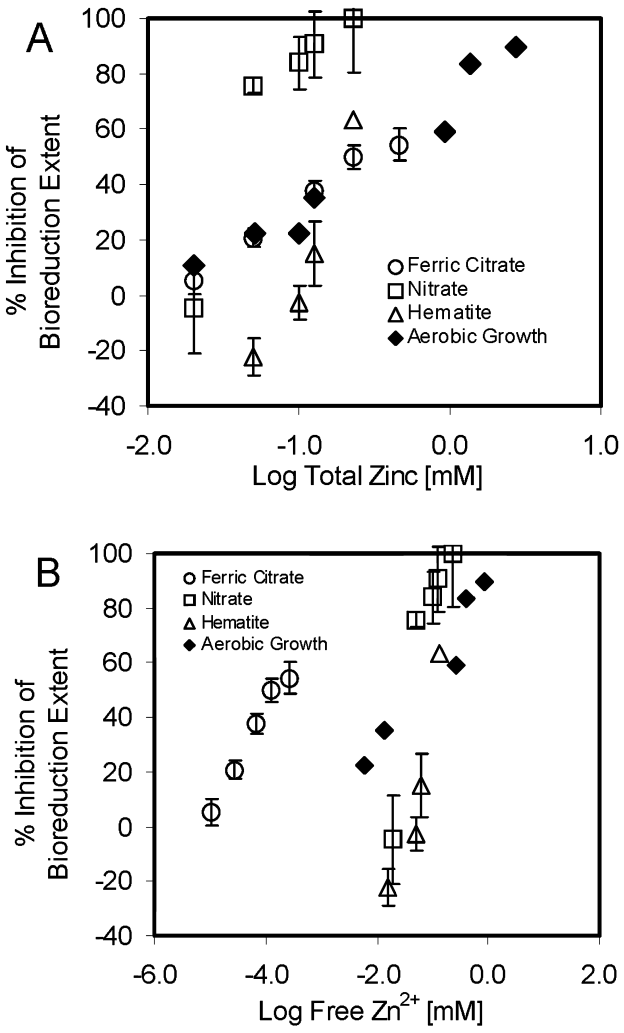

Figure 2. Inhibition of the extent of bioreduction using *S. putrefaciens* for 2 g L^{-1} hematite, 2 mM ferric citrate, 1.43 mM NO_3-N, and *S. putrefaciens* aerobic growth in M1 media as a function of (**A**) log total zinc, (**B**) log free Zn^{2+}. Free Zn^{2+} determined from MINTEQA2 calculations based on measured dissolved zinc concentrations and pH. Values are means of three replicates (±standard deviation).

lowest and highest total zinc concentrations tested (0.02 and 1.38 mM, respectively). The dominant Fe(III) species in this system was Fe(III)–citrate (99.9%), similar to the ASM-I model results found by Liu *et al*. (2001).

F2 The results shown in Fig. 2 (○ symbols) demonstrate that low concentrations of zinc did not stimulate ferric citrate bioreduction (i.e., no negative values for inhibition), as observed with hematite bioreduction [Figs. 1A and 2 (△ symbols)]. Because of the high citrate concentration (6 mM), even the highest dissolved zinc concentration (0.46 mM) resulted in a very low free Zn^{2+} concentration (0.00027 mM) as calculated using MINTEQA2 (Fig. 2B). *Bac*light counts (results not shown) revealed that freely dispersed cell viability was not significantly affected (all counts greater than 99% viable after 6 h) for dissolved zinc concentrations up to 1.38 mM. However, an ancillary experiment using 6.88 mM total zinc yielded no viable cells. Higher zinc concentrations decreased the 6-h extent of ferric citrate reduction compared to its corresponding biotic no-zinc control (Fig. 2).

Nitrate bioreduction

Experiments were performed with nitrate to evaluate the effect of zinc using a soluble electron acceptor that does not contain a high ligand concentration (i.e., citrate). *S. putrefaciens* can reduce nitrate in the absence of oxygen with hydrogen as the electron donor (Kim and Picardal, 1999; Cooper *et al*., 2000). The effect of zinc inhibition on the 9-h extent of nitrate reduction (Fig. 2; □ symbols) indicates low concentrations of zinc did not stimulate nitrate reduction, consistent with ferric citrate results. Zinc concentrations greater than 0.02 mM decreased the extent of nitrate reduction compared to its corresponding biotic no-zinc control. Viability of freely dispersed cells ($1.43 \times 10^8 \pm 3.43 \times 10^7$ CFU mL^{-1}) was variable under nitrate-reducing conditions with increasing zinc concentrations (Fig. 3); however, the highest zinc concentration (0.46 mM zinc) resulted in <5% viable cells remaining. F3

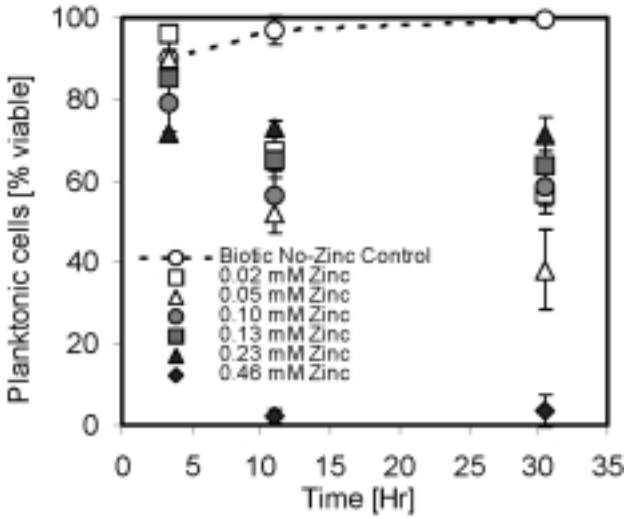

Figure 3. Freely dispersed viable cells as a function of time (0 to 31 h) for the biological reduction of nitrate with variable zinc. Solution consisted of 10 mM PIPES, 1.43 mM NO_3-N (added as KNO_3), 10^8 cells mL^{-1} at pH 6.8. Values are means of three replicates (±standard deviation).

DMRB growth

Aerobic growth experiments were conducted to determine whether zinc would inhibit the growth of *S. putrefaciens* CN32, and to establish whether the inhibition concentration was similar to concentrations observed for other respiration conditions (i.e., hematite, nitrate, and ferric citrate bioreduction). Growth experiments were conducted for 21 h, corresponding to the early stage stationary growth. The defined growth medium (M1) (Myers and Nealson, 1988) allowed the determination of free Zn^{2+} using MINTEQA2. Calculated free Zn^{2+} concentrations ranged between 5.8 and 30.8% of the measured dissolved zinc concentrations under conditions tested. For total zinc concentrations between 0 to 2.75 mM (corresponding free Zn^{2+} concentrations displayed in Fig. 4), inhibition of aerobic growth of *S. putrefaciens* CN32 increased with increased free Zn^{2+} concentrations. The highest level of inhibition (90%) occurred at the highest free Zn^{2+} concentration (0.85 mM Zn^{2+} or 2.75 mM dissolved zinc). The zinc concentration resulting in 50% inhibition of aerobic cell growth ($IC_{50,\ growth}$) occurred at 0.050 mM free Zn^{2+} based on the regression equation established (Fig. 4).

IC_{50} values

The IC_{50} values presented in Table 1 help to quantify and compare the effects of zinc addition under the experimental conditions tested. Results show total zinc inhibition was greater for the two soluble electron acceptors, ferric citrate and nitrate, compared to solid-phase hematite (Fig. 2A). Decreased biological activity (i.e., consumption of electron acceptors) occurring at lower zinc concentrations indicated a greater inhibitory effect. Nitrate bioreduction was most sensitive to zinc, with an IC_{50} value of 0.049 mM total zinc. The IC_{50} values were 0.21, 0.28, and 0.30 mM total zinc for hematite, ferric citrate, and cell growth experiments, respectively.

The IC_{50} values were also calculated using free Zn^{2+} concentrations (Fig. 2B). For nitrate bioreduction, MINTEQA2 calculations indicated total zinc concentrations were equal to the dissolved zinc concentrations, while free Zn^{2+} concentrations were equal to ca. 99.4% of the dissolved zinc concentrations. Thus, the IC_{50} for free Zn^{2+} was 0.049 mM. For hematite bioreduction, the lower dissolved zinc concentrations were due to increased sorption onto hematite and cells, and MINTEQA2 calculations showed free Zn^{2+} concentrations were equal to ca. 99.7% of the dissolved zinc concentrations. The free Zn^{2+} IC_{50} for hematite bioreduction was 0.13 mM. For the ferric citrate experiments, the free Zn^{2+} concentrations were significantly less than the total zinc concentrations due to high citrate concentrations. The free Zn^{2+} IC_{50} for ferric citrate as determined by MINTEQA2 was 0.00016 mM. For the cell growth experiments, MINTEQA2 calculations found zinc preferentially associated with lactate and SeO_4^{2-} present in the M1-defined growth media, yielding an IC_{50} value of 0.050 mM free Zn^{2+}.

Zinc sorption

The distribution of zinc between solid (hematite and cell surfaces) and solution phases is important due to competitive sorption or passivation effects that may occur during hematite bioreduction. Individual experiments were performed to determine the zinc sorption capacity of *S. putrefaciens* CN32, hematite, and hematite plus cells on a surface area basis. Nonviable cells were pasteurized to ensure no Fe(II) production occurred. Freundlich isotherms for zinc sorption (Fig. 5) indicate that, for a maximum dissolved zinc concentration of 0.55 mM, more zinc sorbed to pasteurized *S. putrefaciens* (0.0079 mmol zinc m^{-2}) than to hematite (0.0053 mmol zinc m^{-2}), pasteurized cells plus hematite (0.00052 mmol zinc m^{-2}), or viable cells plus hematite (0.00051 mmol zinc m^{-2}). Hematite plus viable cells had a greater Freundlich sorption intensity factor ($1/n$; 0.421) than hematite plus pasteurized cells (0.243), suggesting a greater zinc sorption capacity at high dissolved zinc concentrations. The additive sum of the individual hematite and pasteurized

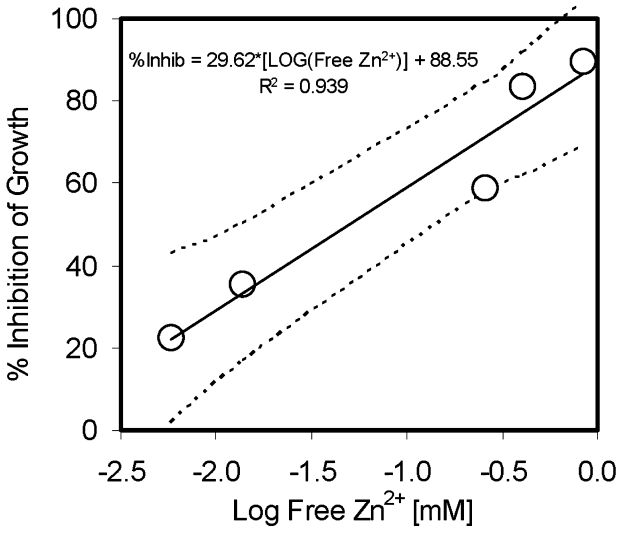

Figure 4. Inhibition of *S. putrefaciens* growth (21 h) as a function of free Zn^{2+} addition in M1 growth media at 20°C. Inhibition calculated as 420 nm absorbance difference between zinc containing samples and no-zinc controls. Free Zn^{2+} determined from MINTEQA2 speciation calculations based on measured dissolved zinc concentrations and pH. Solid line represents least square regression ($R^2 = 0.939$). Dashed lines represent 95% confidence interval.

Table 1. Summary of 50% inhibition concentrations (IC_{50}).

Process	*Zinc*	*Equation*	*IC_{50}*	R^2
Hematite reduction	Total Zinc	%Inhib = 129.5*LOG Zinc + 137.6	0.21 mM	0.925
	Free Zn^{2+}[b]	%Inhib = *LOG Zn^{2+} 127.2	0.13 mM	0.842
Nitrate reduction	Total Zinc	%Inhib = *LOG Zinc + 174.4	0.049 mM	0.840
	Free Zn^{2+}[b]	%Inhib = *LOG Zn^{2+} 174.6	0.049 mM	0.840
Ferric citrate reduction	Total Zinc	%Inhib = *LOG Zinc + 70.98	0.28 mM	0.984
	Free Zn^{2+}[b]	%Inhib = *LOG Zn^{2+} 189.6	0.00016 mM	0.981
Cell growth	Total Zinc	%Inhib = *LOG Zinc + 69.96	0.30 mM	0.951
	Free Zn^{2+}[b]	%Inhib = *LOG Zn^{2+} 88.55	0.050 mM	0.939

[a]Dose–response relationship where 50% reduction in microbial activity or response occurred relative to a no-zinc control.
[b]Free Zn^{2+} concentrations (Fig. 2B) determined from MINTEQA2 speciation model (Allison *et al*., 1991) using published thermodynamic data (Smith and Martell, 1997; Liu *et al*., 2001).

cell isotherms (dashed line within Fig. 5) was greater than the combined systems containing both hematite and cells (both pasteurized and viable), implying that surface site blockage for the combined system may occur. Sorbent surface areas were estimated as 9.04 $m^2\ g^{-1}$ hematite (Jeon *et al*., 2001) and 140 $m^2\ g^{-1}$ cells (Fein *et al*., 1997; Sokolov *et al*., 2001) using 0.064 g L^{-1} cells, with cell mass estimated using an average cell weight of 6.4×10^{-10} mg dry wt. $cell^{-1}$ for *S. putrefaciens* CN32 (Liu *et al*., 2002b). A summary of the Freundlich isotherm parameters (i.e., equation lines and residuals) is presented in Table 2. The "maximum" sorbed metal concentrations were calculated based on the maximum dissolved zinc concentrations measured using the equations in Table 2. Thus, maximum refers to the highest sorbed concentration attained under these experimental conditions.

DISCUSSION

The inhibitory effects of zinc are well documented in the literature, specifically with toxicological studies of filamentous bacteria (Shuttleworth and Unz, 1991) and sulfate reducing bacteria (Poulson *et al*., 1997; Sani *et al*., 2001; Utgikar *et al*., 2001, 2002) where zinc was shown to inhibit cell growth. A summary of zinc inhibition values from these and other studies is presented in Table 3. Our aerobic growth study indicates *S. putrefaciens* CN32 growth was also sensitive to the presence of free Zn^{2+}. Mechanistically, toxicity has previously been reported as closely associated with free metal activity (Knight and McGrath, 1995; Chaudri *et al*., 1999, 2000; Ritchie *et al*., 2001). B-type metal cations such as zinc (Stumm and Morgan, 1996), can also inhibit microbes via complexation with carboxyl and phosphoryl surface sites on cell walls (Fein *et al*., 2001). However, while microbial growth results clearly demonstrate the toxicity of zinc, the large discrepancies between the number of freely dispersed viable cells and the extent of Fe(II) production during hematite bioreduction (Fig. 1) for a given zinc concentration remains unclear. Our data suggest that viable cell counts were not necessarily a positive indicator of bioreduction performance. For example, extractable Fe(II) for the biotic no-zinc control and 0.13 mM zinc addition were similar (0.206 and 0.242 mM; Fig. 1A); however, Fe(II) production for 0.13 mM zinc were obtained with significantly fewer freely dispersed viable cells (33 compared to 82%; Fig 1B). These differences suggest that direct microbial toxicity by zinc may not be the primary mechanism responsible for the inhibition effects observed.

While the data presented in Fig. 1B does suggest direct toxicity occurs in the presence of zinc, that data also

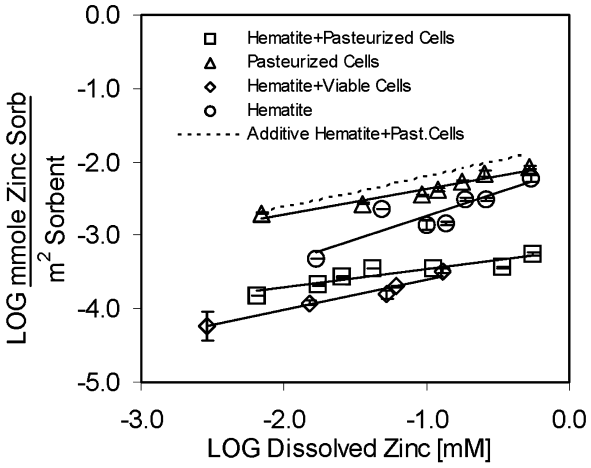

Figure 5. Distribution of zinc for (□) 2.0 g L^{-1} hematite, 10^8 pasteurized cells mL^{-1}, (△) 10^8 pasteurized cells mL^{-1}, (◇) 2.0 g L^{-1} hematite, 10^8 viable cells mL^{-1}, (○) 2.0 g L^{-1} hematite, and dashed line represents additive sum of hematite and pasteurized cell isotherms. Symbols represent mean values (n = 6) (±standard deviation).

Table 2. Summary of Freundlich isotherm parameters[a] for 5-day zinc sorption in 10 mM PIPES, pH 6.8 (Fig. 5).

Sorbent	*Equation*[b]	*Adsorp. Max*[c] *[mmol zinc m^{-2}]*	R^2
Hematite[d]	LOG q = 0.632*LOG C − 2.111	0.0053	0.788
Pasteurized *S. putrefaciens*[e,f]	LOG q = 0.349*LOG C − 2.013	0.0079	0.932
Hematite pasteurized *S. putrefaciens*[d,e,f]	LOG q = 0.243*LOG C − 3.219	0.00052	0.850
Hematite viable + *putrefaciens*[e,f] *S. putrefaciens*[d,f,g]	LOG q = 0.421*LOG C − 3.179	0.00051	0.957
Additive hematite + pasteurized *S. putrefaciens*	LOG q = 0.425*LOG C − 1.778	0.013	—

[a]Values derived using Freundlich isotherm equation: LOG q = LOG K_f + 1/n LOG C, with K_f the Freundlich adsorption capacity factor and 1/n the Freundlich adsorption intensity factor.

[b]Freundlich isotherm equation.

[c]Maximum values based on maximum dissolved zinc = 0.55 mM (Fig. 5).

[d]2.0 g L^{-1} hematite with estimated surface area of 9.04 m^2 g^{-1} (Jeon *et al*., 2001).

[e]Cells pasteurized to ensure nonviability and no biogenic Fe(II).

[f]Estimated from 10^8 cells mL^{-1} concentration of cells at 6.4×10^{-10} mg $cell^{-1}$ (Liu *et al*., 2002b). Surface area estimated as 140 m^2 g^{-1} (Fein *et al*., 1997; Sokolov *et al*., 2001).

[g]Results from no-amendment with-zinc hematite bioreduction experiments. Total biogenic Fe(II) ranged from 0.088–0.30 mM.

[h]Additive sum of individual hematite and pasteurized cell isotherms.

suggests other mechanisms such as sorption of Fe(II) or Zn^{2+} onto the cell or hematite surfaces or the formation of localized "biofilms" on the surface of the hematite [such as incomplete monolayer coverage; Hansel *et al*., 2004) may be responsible. For example, zinc was found to preferentially adsorb onto (nonviable) cells compared to suspensions containing either hematite or a mixture of (nonviable) cells and hematite (Fig. 5 and Table 2). While metal binding affinities have been reported as proportional to hydraulic radius, waters of hydration, and valance (Trivedi and Axe, 2000), zinc binding to *S. putrefaciens* CN32 (Fig. 5) was similar to the binding capacity reported for *B. subtilis* (0.70 mM g^{-1}; Beveridge and Murray, 1976). The high degree of zinc adsorption onto the hematite may have induced "surface passivation" (Urrutia *et al*., 1999; Roden and Urrutia, 2002) on the hematite, resulting in lowering the bioavailabity of "high" energy surface reductive sites found on hematite (Hacherl *et al*., 2001).

Interestingly, hematite bioreduction at low concentrations of zinc (below ca. 0.10 mM) resulted in a slight increase in 5-day biogenic Fe(II) production compared to its corresponding biotic no-zinc control (Fig. 1A and B). The ability of heavy metals to stimulate a biological process at low concentrations while becoming toxic at higher concentrations has previously been termed hormesis (Luckey, 1991). For example, zinc stimulation of microbial activity (at subtoxic concentrations) was reported to relieve nutrient deficiencies (Welp and Brummer, 1997b) and increase microbial growth rates (Shuttleworth and Unz, 1991). Our results were consistent with the "Type II" dose–response effects described by Welp *et al*. (Welp and Brummer, 1997b), where zinc or other micronutrients stimulated a biological response at low concentrations while subsequently inhibiting the response at higher concentrations. The microbial hormesis response observed may be due to the alleviation of a localized zinc deficiency impairing enzyme activity (Raven, 1984), disruption of potassium uptake and efflux resulting in increased metabolic respiration (Webster and Gadd, 1996), or the induction of a localized pH change allowing trace nutrients to become more bioavailable (Welp and Brummer, 1997b).

None of the above explanations, however, can account for the differences between the increase in hematite bioreduction occurring with a corresponding decrease in freely dispersed viable cells, especially at zinc concentrations where stimulation of Fe(II) production occurred. For example, 0.050 mM total zinc increased the 5-day Fe(II) production in the hematite system by 14% relative to the biotic no-zinc control (Fig. 1A), while the corresponding freely dispersed viable cell population decreased by 39% (Fig. 1B). The dispersed cells may respond to the presence of zinc by increasing their metabolic activity, so much that biological output (reduction of hematite) of the remaining live, viable cells is greater than the unaffected control cells (i.e., fewer cells operating at faster rate).

An alternative explanation may be the difference between metabolic activity and resistance to zinc for freely dispersed cells compared to cells adsorbed onto hematite.

Table 3. Summary of relevant zinc inhibition results.

Species	*Evaluation process*	*Zinc 50% inhibition*[a]	*Source*
E. coli	Bioluminescence in artificial soil solution	0.051 mM	(Ritchie *et al*., 2001)
E. coli	Bioluminescence in sewage sludge	0.086,[b] 0.038[c] mM	(Chaudri *et al*., 1999)
P. fluorescens	Bioluminescence in sewage sludge	0.34,[b] 0.15[c] mM	(Chaudri *et al*., 1999)
Rhizotox-c	Bioluminescence in sewage sludge	0.25,[b] 0.092[c] mM	(Chaudri *et al*., 2000)
Thiothrix	Cell growth	0.0019,[b] 0.050[c] mM	(Shuttleworth and Unz, 1991)
Nitrosomonas communis	Ammonium oxidation in soil	171 mg kg^{-1} soil	(Fruhling *et al*., 2001)
F. candida	Reproduction in soil	683 μg g^{-1} soil	(VanGestel and Hensbergen, 1997)
SRB[d]	Microbial community sulfate reduction in acid mine drainage	0.25 mM	(Utgikar *et al*., 2001)
Heterogeneous soil microbial population	Microbial community dehydrogenase activity in contaminated soil	115 mg $kg^{-1,b,e}$ 0.0029 mM[c]	(Welp, 1999)
D. desulfuricans	Microbial sulfidogenesis	0.20 mM	(Poulson *et al*., 1997)
S. putrefaciens CN32	Nitrate reduction	0.049 mM	Current study
	Ferric citrate reduction	0.28 mM	
	Cell growth	0.30 mM	

[a]Dose–response relationship where 50% reduction in microbial activity or response occurred relative to a no Zn(II) control.
[b]Total zinc within the soil.
[c]Soluble zinc measured within the soil solution.
[d]Unidentified culture of sulfate reducing bacteria found within acid mine drainage.
[e]Differences between metals soil and pore water concentrations occur due to variable affinity of metals to soil materials.
[f]Concentration where inhibition of sulfidogenic activity started.

The viability of adsorbed cell biofilms within our system were not estimated due to limitations associated with *Bac*light stain on cell aggregations; however, we frequently observed large hematite aggregates (>10-μm width, often much larger) surrounded by biofilm cell aggregations. Early work by Costerson *et al*. (1995) found biofilm cells more resistant to antibacterial agents than their freely dispersed counterparts. These differences were attributed to sigma factors repressing genes which resulted in a clear difference in cell phenotype. For example, bacterial biofilms have been shown to form exopolysaccharides (EPS) (Schmitt *et al*., 1995; Huang *et al*., 2000). EPS generated by the biofilms formed on the hematite surfaces could preferentially bind zinc (Teitzel and Parsek, 2003), preventing contact between zinc and planktonic cells, and thus becoming a preventative mechanism against zinc detoxification. This complexation could result in a zinc gradient within the biofilm, with highest zinc concentration found in the periphery and lowest in the substrata (Teitzel and Parsek, 2003). Thus, we believe zinc may be preferentially bound onto the cells–hematite matrix (i.e., cell biofilms formed on hematite surfaces) within our system, resulting in a mechanism of zinc detoxification for freely dispersed cells within our system. Additionally, it should be noted that with a solid-phase electron acceptor (hematite), available reduction sites could become rate limiting, while this electron acceptor limitation would likely not happen with a dissolved electron acceptor. In other words, the "net biological output" of the system may not be controlled by the total viable cell concentration but by the viable cell concentration directly attached to the oxide surface. While these explanations are speculative, observations supporting these arguments may be derived from the data.

The bioreduction of soluble electron acceptors (nitrate and ferric citrate) appeared more sensitive (i.e., inhibition occurring at lower concentrations of zinc) to free Zn^{2+} than solid-phase hematite (Fig. 2B). Bioreduction

of ferric citrate was found to be most sensitive to free Zn^{2+} (IC_{50} of 0.00016 mM); however, the estimates of free Zn^{2+} in the presence of 6 mM citrate demonstrate uncertainties regarding the formation and stability constants of the zinc–citrate complexes (Liu *et al*., 2001). Speciation calculations for the nitrate experiments, however, show that fewer solution chemicals may complex with zinc. For example, the formation of $ZnNO_3^+$ and $ZnOH^+$ amounted to only 0.24 and 0.32% of the total zinc concentrations, respectively, for all conditions tested. The similarity between IC_{50} values for between nitrate bioreduction (0.049 mM; Fig. 2B) and aerobic growth (0.050 mM) suggest a similar mechanism of zinc lethality occurs in these experiments. Interestingly, the IC_{50} value for hematite bioreduction was higher (i.e., zinc less toxic) than observed for other treatments. Zinc was found to have a high affinity to pasteurized cells in the absence of hematite (△ symbols, Fig. 5). These conditions are similar to nitrate reduction where the cells provided the only surface for zinc to partition onto. The difference between the additive sum of individual pasteurized cell and hematite isotherms (dashed line, Fig. 5) and the combined systems for both viable (◇ symbols) and pasteurized cells (□ symbols) plus hematite suggests that surface blockage by hematite–cell interactions may limit the number of zinc sorption sites. This blockage appears to be another mechanism of zinc resistance as indicative of the higher IC_{50} value for hematite bioreduction as compared to the other systems tested.

This greater degree of zinc resistance may also be due to competitive sorption between zinc and Fe(II) as observed in Fig. 5 where differences in zinc sorption between nonviable (pasteurized) cells (□ symbols) and viable cells (◇ symbols) occurred in the presence of hematite. At low zinc concentrations, the hematite plus viable cells system exhibits a lower quantity of zinc sorption (i.e., lower Freundlich capacity factor K_f) compared to the hematite plus pasteurized cells. However higher dissolved zinc concentrations result in greater zinc sorption due to the higher Freundlich intensity factor for the hematite plus viable cells system, likely attributed to the lower mass of Fe(II) produced at higher dissolved zinc concentrations. The sorption of biogenic Fe(II) to hematite has been thought to effect the rate and extent of bioreduction of solid-phase iron oxides, primarily through surface coverage or "passivation" of the oxide surface by sorbed Fe(II) (Urrutia *et al*., 1998; Roden and Urrutia, 1999). Sorbed Fe(II) may inhibit bioreduction by decreasing the number of bioavailable Fe(III)–oxide surface sites (Roden and Urrutia, 1999), preventing DMRB–oxide contact (Fredrickson *et al*., 2001), or increasing the passive resistance of electron transport (Fredrickson *et al*., 1998).

SUMMARY

The results of this study further the understanding of the inhibitory effects of zinc on the biological reduction of hematite, ferric citrate, and nitrate by the DMRB *S. putrefaciens* CN32. Zinc toxicity to *S. putrefaciens* CN32 was apparent during both aerobic cell growth and nitrate bioreduction experiments. The similarity between the free Zn^{2+} IC_{50} for both cell growth and nitrate reduction demonstrate the lethality of free Zn^{2+} to cellular respiration and growth. However, differences in free Zn^{2+} IC_{50} value between soluble (ferric citrate, nitrate, and oxygen) and solid-phase (hematite) electron acceptors may be attributed to a combination of surface sorption or attenuation of zinc onto hematite or cell surfaces, and ability of biofilms formed on the hematite to have a higher degree of zinc tolerance compared to freely dispersed cells.

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