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Mechanism and Consequences of Anaerobic Respiration of Cobalt by *Shewanella oneidensis* Strain MR-1▽

Heidi H. Hau,[1,3] Alan Gilbert,[2,3] Dan Coursolle,[1,3] and Jeffrey A. Gralnick[1,3]*

Departments of Microbiology[1] and Chemical Engineering[2] and BioTechnology Institute,[3] University of Minnesota—Twin Cities, St. Paul, Minnesota 55108

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Bacteria from the genus *Shewanella* are the most diverse respiratory organisms studied to date and can utilize a variety of metals and metal(loid)s as terminal electron acceptors. These bacteria can potentially be used in bioremediation applications since the redox state of metals often influences both solubility and toxicity. Understanding molecular mechanisms by which metal transformations occur and the consequences of by-products that may be toxic to the organism and thus inhibitory to the overall process is significant to future applications for bioremediation. Here, we examine the ability of *Shewanella oneidensis* to catalyze the reduction of chelated cobalt. We describe an unexpected ramification of [Co(III)-EDTA]$^-$ reduction by *S. oneidensis*: the formation of a toxic by-product. We found that [Co(II)-EDTA]$^{2-}$, the product of [Co(III)-EDTA]$^-$ respiration, inhibited the growth of *S. oneidensis* strain MR-1 and that this toxicity was partially abolished by the addition of $MgSO_4$. We demonstrate that [Co(III)-EDTA]$^-$ reduction by *S. oneidensis* requires the Mtr extracellular respiratory pathway and associated pathways required to develop functional Mtr enzymes (the *c*-type cytochrome maturation pathway) and ensure proper localization (type II secretion). The Mtr pathway is known to be required for a variety of substrates, including some chelated and insoluble metals and organic compounds. Understanding the full substrate range for the Mtr pathway is crucial for developing *S. oneidensis* strains as a tool for bioremediation.

Radioactive cobalt-60 (^{60}Co) is used for radiotherapy in hospitals and for the "cold pasteurization" of spices and other foods and is a by-product of nuclear and defense-related industries. The most mobile form of ^{60}Co contamination stemming from leaks, spills, or decontamination operations is [^{60}Co(III)-EDTA]$^-$ (26). In general, chelated forms of ^{60}Co are more mobile than nonchelated forms; however, stability and mobility are directly related to the oxidation state of ^{60}Co, and both the oxidized {[^{60}Co(III)-EDTA]$^-$} and reduced {[^{60}Co(II)-EDTA]$^{2-}$} forms are present in contaminated environments. The half-life of ^{60}Co is 5.27 years; therefore, waiting for decay is one method for treating contamination, provided that the contamination is contained. Unfortunately, [Co(III)-EDTA]$^-$ is both stable and highly mobile in groundwater systems (26), increasing the difficulty of containing leaks or spills. [Co(II)-EDTA]$^{2-}$ can dissociate more readily than the oxidized form, allowing free cobalt ions to be sorbed onto iron oxide surfaces, thus preventing further migration and potentially facilitating removal (14). Cobalt can exist in the +3 oxidation state, but Co(III) is not thermodynamically stable and requires a ligand, such as EDTA, for persistence in aqueous environments (19); otherwise, cobalt oxide minerals will form.

Microbial redox transformations of toxic metals represent one strategy for in situ remediation of contaminated areas (47). Members of the genus *Shewanella* can respire a diverse repertoire of compounds in the absence of oxygen, including metals and radionuclides, and for this reason are considered to be outstanding candidates for potential applications in the bioremediation of pollutants (34, 47). Several different *Shewanella* species have demonstrated the ability to reduce [Co(III)-EDTA]$^-$ anaerobically (9, 14, 23, 45). Importantly, *Shewanella alga* BrY can reduce [^{60}Co(III)-EDTA]$^-$ at or above concentrations found at contaminated sites without any noted deleterious effects of radiation on the bacterium (14).

One proposed strategy to facilitate remediation at [^{60}Co(III)-EDTA]$^-$-contaminated sites involves bacterium-mediated reduction to [^{60}Co(II)-EDTA]$^{2-}$; however, the mechanism of cobalt reduction remains undefined (14, 24). The elucidation of the main pathway for cobalt reduction is important for engineering microorganisms for specific bioremediation applications or increasing the efficiency of indigenous microbial populations. The findings from *S. oneidensis* strain MR-1 whole-genome DNA microarray studies suggest that similar pathways are utilized to reduce a diverse array of metal electron acceptors ranging from soluble metal complexes to insoluble metal hydr(oxy)oxides (3). The primary mechanism in *S. oneidensis* for the reduction of iron (Fe)- and manganese (Mn)-containing compounds is the Mtr respiratory pathway (11, 16, 43). Interestingly, the mechanisms of reduction of vanadium(V), uranium(VI), technetium(VII), and 9,10-anthraquinone-2,6-disulfonic acid appear to require the same cluster of genes that includes *omcA*, *mtrC* (*omcB*), *mtrA*, and *mtrB* (4, 10, 22, 25, 33, 42), although not all substrates have been tested with all mutant strains. Additional pathways required for this process are *c*-type cytochrome maturation (8), menaquinone biosynthesis (31), and type II secretion (13).

In this work, we demonstrate that *S. oneidensis* strain MR-1, one of the best-studied organisms for bioremediation applications, can couple growth to the anaerobic respiration of

* Corresponding author. Mailing address: BioTechnology Institute, University of Minnesota, 140 Gortner Lab, 1479 Gortner Ave., St. Paul, MN 55108. Phone: (612) 626-6496. Fax: (612) 625-5780. E-mail: gralnick@umn.edu.

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$[Co(III)\text{-}EDTA]^-$. Interestingly, the toxicity of the respiration by-product $[Co(II)\text{-}EDTA]^{2-}$ initially masked this finding. The toxicity of $[Co(II)\text{-}EDTA]^{2-}$ could be overcome through the addition of $MgSO_4$, likely serving to form a complex with free EDTA that dissociated from Co(II). Furthermore, we demonstrate that the Mtr respiratory pathway is essential for $[Co(III)\text{-}EDTA]^-$ reduction by *S. oneidensis*, providing a molecular mechanism for anaerobic $[Co(III)\text{-}EDTA]^-$ respiration in this bacterium.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and growth media. *S. oneidensis* strain MR-1 was originally isolated from sediments of Lake Oneida in New York (27). Strains lacking *cymA*, *menC*, *dsbD*, *mtrB*, and *mtrC* (*omcB*) were generated by random transposon mutagenesis as described previously (44) and isolated in a screen for mutants defective in amorphous iron oxy(hydr)oxide reduction (J. A. Gralnick and D. K. Newman, unpublished data).

Strains were cultured aerobically and anaerobically as described previously (15) in Luria broth (LB) or minimal medium consisting of (per liter) 0.225 g of K_2HPO_4, 0.225 g of KH_2PO_4, 0.46 g of NaCl, 0.225 g of $(NH_4)_2SO_4$, 0.117 g of $MgSO_4$-$7H_2O$, and 100 ml of 1 M HEPES buffer adjusted to a final pH of 7.2. In addition, 5 ml/liter each of vitamins and modified trace minerals (2) from sterile filtered stock solutions was added to the minimal medium. The mineral solution was modified to include (per liter) 0.13 g of $ZnCl_2$ (instead of $ZnSO_4$), 0.025 g of Na_2MoO_4-$2H_2O$, 0.024 g of $NiCl_2$, and 0.025 g of Na_2WO_4-$2H_2O$. Anaerobic manipulations and 96-well-plate assays were performed in an anaerobic chamber (5% H_2, 20% CO_2, and 75% N_2 atmosphere; Coy).

$[Co(III)\text{-}EDTA]^-$ preparation. $[Co(III)\text{-}EDTA]^-$ was prepared according to the method of Taylor and Jardine (46) by adding (per 150 ml) 20 g of $Co(II)Cl_2$-$6H_2O$, 2.5 g of EDTA, and 5 g of potassium acetate and heating almost to a boil. Thirty-two milliliters of 7% H_2O_2 was added with continuous stirring. The solution was cooled to room temperature, and 100 ml of 100% ethyl alcohol (EtOH) was added slowly with stirring. Precipitated crystals were washed twice with 100% EtOH and allowed to dry. Crystals were then dissolved in a small volume of double-distilled water (ddH_2O), recrystalized in EtOH, and subjected to two additional EtOH washes. Crystals were allowed to dry on low heat until all EtOH was evaporated and were stored at 4°C. Products were examined spectrophotometrically at 535 nm for $[Co(III)\text{-}EDTA]^-$ and 490 nm for $[Co(II)\text{-}EDTA]^{2-}$. A 520 mM stock solution of $[Co(III)\text{-}EDTA]^-$ in ddH_2O was prepared, and stock solutions were sterilized by using a 0.22-μm-pore-size filter.

Viability staining and flow cytometry. A FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) was used for the determination of culture viability and cell concentrations. A 15-mW laser (Spectra-Physics, Mountain View, CA) with a wavelength of 488 nm was used for excitation. Cell concentrations were determined through the calibration of the sample volume by using standard beads of known concentrations from a kit, while the viability was assayed using the stains SYTO9 and propidium iodide from the LIVE/DEAD *Bac*Light bacterial viability and counting kit (Invitrogen, Carlsbad, California). The green fluorescence exhibited after SYTO9 staining was measured at 530 ± 30 nm. Propidium iodide fluorescence was measured at 585 ± 42 nm, and the electronic signals were compensated to adjust for the spectral overlap (data not shown). The fluorescence properties of live cells were determined by the staining of mid-exponential-phase cells cultivated aerobically in LB. The fluorescence properties of dead cells were determined by the staining of cells treated with isopropyl alcohol and confirmed by the staining of cells that were heat killed. Samples of approximately 7×10^5 cells per ml were inoculated in triplicate under anaerobic conditions, and the cells were assayed every 24 h over 96 h for growth in lactate and $[Co(III)\text{-}EDTA]^-$.

Co(II) ion, EDTA, and $[Co(II)\text{-}EDTA]^{2-}$ toxicity assays. A 0.5 M Co(II) stock solution was prepared by adding 5.94 g of $Co(II)Cl_2$-$6H_2O$ to 50 ml of ddH_2O and filter sterilizing. A stock solution of $[Co(II)\text{-}EDTA]^{2-}$ was prepared by adding equal molar amounts of Co(II) and EDTA stock solutions.

Toxicity to cells on agar plates, in sealed tubes, or in the wells of 96-well plates was evaluated. MR-1 was streaked directly onto LB or minimal medium agar plates that contained lactate, fumarate, and $[Co(II)\text{-}EDTA]^{2-}$. Plates were incubated at 30°C under aerobic or anaerobic conditions. The toxicity of $[Co(II)\text{-}EDTA]^{2-}$ was scored based on the appearance of colonies compared to that of a positive control grown without $[Co(II)\text{-}EDTA]^{2-}$. The toxicity of $[Co(II)\text{-}EDTA]^{2-}$ to cells grown anaerobically in a mixture of lactate, fumarate, and $[Co(II)\text{-}EDTA]^{2-}$ was monitored. Samples were withdrawn anaerobically and then plated onto LB agar plates, which were incubated aerobically at 30°C. Anaerobic-to-aerobic transition appeared to not be a concern in these experiments, as the CFU quantified by plate counts were equivalent to population estimates by flow cytometry (data not shown). The toxicity of Co(II), EDTA, and $[Co(II)\text{-}EDTA]^{2-}$ to cultures grown anaerobically in 96-well plates with continuous shaking at 30°C inside an anaerobic chamber was monitored spectrophotometrically at 600 nm. Aerobically grown overnight cultures were diluted to 10^6 cells per ml in anoxic minimal medium and then inoculated into 96-well plates containing lactate and fumarate, with or without Co(II) ions, EDTA, $[Co(II)\text{-}EDTA]^{2-}$, and 25 mM $MgSO_4$.

Growth in the presence of $[Co(III)\text{-}EDTA]^-$. Anaerobic growth on agar plates and in sealed tubes with $[Co(III)\text{-}EDTA]^-$ as the sole final electron acceptor was examined. MR-1 was streaked directly onto LB or minimal medium agar plates equilibrated under anaerobic chamber conditions for at least 48 h and containing 20 mM lactate and 5 mM [Co(III)-EDTA]. Growth at 30°C was scored based on the appearance of colonies compared to that of colonies on a positive control plate. Additionally, anaerobic growth in sealed tubes of minimal medium with lactate and $[Co(III)\text{-}EDTA]^-$ was monitored. Samples were withdrawn with a sterile syringe from sealed tubes in an anaerobic chamber at experimental time points and analyzed aerobically by using flow cytometry or spread plate counts. Sample dilutions were performed with minimal medium, and dilutions were spread plated onto LB agar plates, which were incubated aerobically at 30°C. The toxicity of $[Co(III)\text{-}EDTA]^-$ was determined by streaking samples onto LB or minimal medium agar plates containing 20 mM lactate and 5 mM $[Co(III)\text{-}EDTA]^-$ under aerobic or anaerobic conditions as described above. The medium was supplemented with $MgSO_4$ where indicated.

$[Co(III)\text{-}EDTA]^-$ reduction. $[Co(III)\text{-}EDTA]^-$ reduction was examined under anaerobic conditions at 30°C with polystyrene 96-well plates. $[Co(III)\text{-}EDTA]^-$ reduction was evaluated by the inoculation of approximately 2×10^7 cells per ml of an aerobically grown overnight culture diluted in anoxic minimal medium into wells containing 20 mM lactate and 5 mM $[Co(III)\text{-}EDTA]^-$ in minimal medium supplemented with vitamins and minerals. $[Co(III)\text{-}EDTA]^-$ reduction under these conditions was dependent on lactate addition (data not shown). Plates were shaken continuously, and $[Co(III)\text{-}EDTA]^-$ concentrations were monitored spectrophotometrically at 535 nm (7, 46) with a SpectraMax M2 microplate reader (Molecular Devices) in an anaerobic chamber. Total cellular protein levels were determined by the bicinchoninic acid method with bovine serum albumin as a standard.

Deletion and complementation strain construction. Deletion mutation and complementation analyses used methods described previously (17, 37). The ligation of upstream fragments (amplified with P1 and P2) and downstream fragments (amplified with P3 and P4) was facilitated by overlapping ends generated using the restriction enzyme BsaI. Primers for deletion constructs were as follows: *omcA*, P1SacI (NNNGAGCTCAGATAGTGTCTTAGTGGA), P2BsaI (NNNGGTCTCCATTAGTCGTATTTAATG), P3BsaI (NNNGGTCTCATAATTATCTGAATCGAG), and P4BamHI (NNNGGATCCCTTCGTGGCTAACGATAT); *mtrC*, P1SpeI (NNNACGAGTCAGCTGATCTAACTGGTA), P2BsaI (NNNGGTCTCTTTTTGTGCGTTCATCAT), P3BsaI (NNNGGTCTCGAAAATGTAATTTGCCCA), and P4SacI (NNNGAGCTCCATTCACACTACCGTGAG); *mtrA*, P1SacI (NNNGAGCTCCACAATTAGTGTTCAAGT), P2BsaI (NNNGGTCTCATGGTAAGTGCCGGCAC), P3BsaI (NNNGGTCTCAACCATCCATCTGGCAA), and P4SpeI (NNNACTAGTCATGGGTATTGAGCTGA); and *mtrB*, P1SacI (NNNGAGCTCATTGGGAAGCCTATTATG), P2BsaI (NNNGGTCTCATGGTTAGAACCATGAACv), P3BsaI (NNNGGTCTCAACCATTGAGTCTGAACA), and P4BamHI (NNNGGATCCTATGCATATTATCCATTA). Complementation primers used to amplify *mtrA* from wild-type genomic DNA were SalImtrAFor (CTCGACTTTCTTGAATTTTGTTGGGA) and SacIImtrARev (CCGCGGTGTTGGCTAATAAACGCTA). The amplification product was digested with SalI and SacII and then cloned into a similarly digested vector, pBBR1-MCS2 (18), to create pMTRA1. The orientation of the insert ensured expression under the control of the vector-carried *lac* promoter, and the insert was verified by sequence analysis. Strains used in the complementation analysis of the *mtrA* deletion strain (JG476) were JG553 (*mtrA* pBBR1-MCS2), JG554 (*mtrA* pMTRA1), and JG168 (MR-1 pBBR1-MCS2), and kanamycin was provided at 50 μg/ml.

RESULTS AND DISCUSSION

Anaerobic respiration of $[Co(III)\text{-}EDTA]^-$ by MR-1 leads to cell death. The anaerobic growth of other species of *Shewanella* coupled to $[Co(III)\text{-}EDTA]^-$ reduction has been reported previously; however, it has not been experimentally

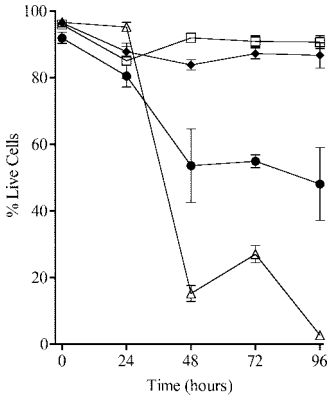

FIG. 1. Live/dead flow cytometric analysis of *S. oneidensis* MR-1 anaerobic growth using [Co(III)-EDTA]$^-$. Shown are percentages of live cells in samples during anaerobic growth in minimal medium containing 20 mM lactate and fumarate (●), 20 mM lactate and 5 mM [Co(III)-EDTA]$^-$ (△), lactate only (□), and 5 mM [Co(III)-EDTA]$^-$ only (◆). Error bars represent the standard errors of the means (SEM) from experiments performed in triplicate.

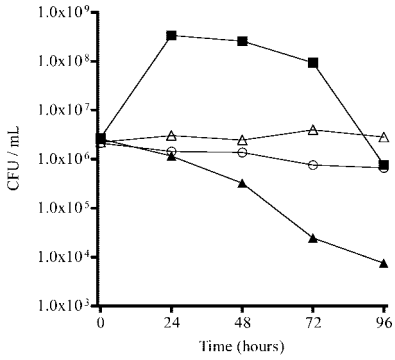

FIG. 2. Toxicity of [Co(II)-EDTA]$^{2-}$ during the growth of *S. oneidensis* MR-1. Plate counts were carried out over 96 h to monitor the anaerobic growth of cultures in tubes of minimal liquid medium containing 20 mM lactate, 20 mM fumarate, and various concentrations of [Co(II)-EDTA]$^{2-}$ as follows: 0 mM (control; ■), 1 mM (△), 3 mM (○), or 5 mM (▲). Data presented are the averages of results from experiments performed in duplicate (SEM, <10% deviation).

verified for *S. oneidensis* strain MR-1. No growth was visible when MR-1 was incubated on plates containing lactate and [Co(III)-EDTA]$^-$ (provided at concentrations ranging from 0.5 to 20 mM) as the sole anaerobic electron acceptor in rich or defined minimal medium (data not shown). These results suggested that MR-1 was unable to couple [Co(III)-EDTA]$^-$ reduction with anaerobic growth, that the substrate was limiting, or that a component of the reduction reaction inhibited growth.

We tested the growth of MR-1 in liquid culture, where [Co(III)-EDTA]$^-$ could be more available to growing cells than on solid medium. To obtain cell-specific measurements for each cell population, flow cytometric analysis was performed to examine both the viability and growth of MR-1 using [Co(III)-EDTA]$^-$ as the sole electron acceptor. At each time point, total cell numbers and the percentage of live cells were analyzed. Viable and nonviable cells have similar forward light scattering characteristics, which correlate with cell size, but exhibit different green fluorescence patterns. Cells with damaged membranes, indicative of death, retain less green fluorescence. Due to the accuracy of flow cytometry and the ease of determining pertinent cell concentrations by this method, the growth of MR-1 from concentrations as low as 10^4 cells per ml can be assayed directly.

Total cell numbers increased approximately 100-fold within 48 h in the presence of lactate and fumarate (data not shown). The percentage of live cells among all cells in the sample decreased within the first 48 h (Fig. 1). Once stationary phase was reached, live cell numbers remained consistent for the duration of the experiment. We hypothesize that cell death observed in the positive control was due to the culture's reaching stationary phase. Two negative controls were prepared: one lacking an electron donor but containing [Co(III)-EDTA]$^-$ and another containing lactate but no electron acceptor. In each case, the total number of viable cells remained constant (Fig. 1). The high percentages of live cells present in both negative controls suggest that [Co(III)-EDTA]$^-$ itself is not toxic to cells, an observation confirmed by monitoring aerobic growth on agar plates containing [Co(III)-EDTA]$^-$ (data not shown). No difference in total cell numbers in samples containing an electron donor alone or a donor and [Co(III)-EDTA]$^-$ as the electron acceptor was observed (data not shown). Therefore, the results of this experiment do not indicate if *S. oneidensis* strain MR-1 can grow anaerobically with [Co(III)-EDTA]$^-$ as an electron acceptor. However, unlike that in the lactate-only control, the population of viable cells in the samples containing lactate and [Co(III)-EDTA]$^-$ declined significantly over time, with less than 3% of the population remaining viable at the conclusion of the experiment (Fig. 1). Cell death occurred rapidly and correlated with the visual observation that [Co(III)-EDTA]$^-$ had been reduced, suggesting that [Co(II)-EDTA]$^{2-}$ was responsible for the observed decline in cell viability in these cultures.

[Co(II)-EDTA]$^{2-}$ is toxic to MR-1. Based on flow cytometry results, MR-1 was plated onto LB agar containing lactate, fumarate, and 0.5, 5, or 20 mM [Co(II)-EDTA]$^{2-}$. Under aerobic conditions, concentrations of up to 5 mM [Co(II)-EDTA]$^{2-}$ did not inhibit growth; however, concentrations above 0.5 mM [Co(II)-EDTA]$^{2-}$ prevented growth anaerobically (data not shown). A similar trend was noted when the experiment was performed with minimal medium, except that concentrations higher than 0.5 mM [Co(II)-EDTA]$^{2-}$ inhibited growth under both aerobic and anaerobic conditions (data not shown). The difference in the toxicity of [Co(II)-EDTA]$^{2-}$ to MR-1 in rich and defined media is likely to be due to the availability of high concentrations of various nutrients found in LB, allowing cells to devote more energy to respond to the [Co(II)-EDTA]$^{2-}$ stress. To better define the concentrations of [Co(II)-EDTA]$^{2-}$ tolerated by MR-1 during growth, we inoculated cultures into minimal medium containing lactate, fumarate, and [Co(II)-EDTA]$^{2-}$ and monitored anaerobic growth by determining numbers of CFU (Fig. 2). No overall increase in CFU in cultures grown in the presence of 1 or 3 mM [Co(II)-EDTA]$^{2-}$ was noted, but a constant number of cells remained viable for the duration of the experiment. At [Co(II)-EDTA]$^{2-}$ concentrations of 4 mM (data not shown) or

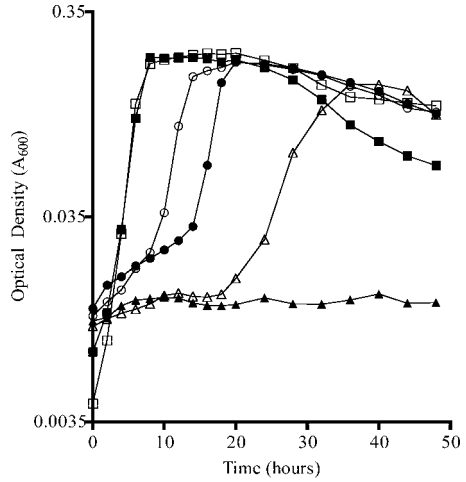

FIG. 3. $MgSO_4$ suppresses $[Co(II)\text{-}EDTA]^{2-}$ toxicity during growth. Anaerobic growth was monitored in minimal medium containing 20 mM lactate and 20 mM fumarate (■) in the presence or absence of $[Co(II)\text{-}EDTA]^{2-}$ and 25 mM $MgSO_4$, as follows: $MgSO_4$ (□), 0.5 mM $[Co(II)\text{-}EDTA]^{2-}$ (●), 0.5 mM $[Co(II)\text{-}EDTA]^{2-}$ and $MgSO_4$ (○), 1 mM $[Co(II)\text{-}EDTA]^{2-}$ (▲), or 1 mM $[Co(II)\text{-}EDTA]^{2-}$ and $MgSO_4$ (△). Data presented are the averages for duplicate experiments; error bars were removed for clarity (SEM, <10% deviation).

5 mM, death was noted within 48 h and significant cellular death was evident at all subsequent time points (Fig. 2).

$[Co(II)\text{-}EDTA]^{2-}$ toxicity can be abolished by the addition of $MgSO_4$. Previous studies of other microorganisms determined that the toxicity of some divalent cations can be influenced by magnesium (Mg) concentrations (1). To suppress the detrimental effects of $[Co(II)\text{-}EDTA]^{2-}$, media were supplemented with 25 mM $MgSO_4$ and the anaerobic growth of MR-1 utilizing lactate and fumarate was analyzed (Fig. 3). The addition of $MgSO_4$ to cultures did not affect growth over 48 h as monitored by optical density (A_{600}). Cells grown in the presence of 0.5 mM $[Co(II)\text{-}EDTA]^{2-}$ with or without $MgSO_4$ achieved similar optical densities, but cells supplemented with $MgSO_4$ had a shorter lag time. The most significant effect was noted with cells grown in 1 mM $[Co(II)\text{-}EDTA]^{2-}$. Without the addition of $MgSO_4$, no increase in optical density was observed; however, the addition of $MgSO_4$ permitted growth after a considerable lag. The decrease in optical density in the cultures at later time points was due to cell death and the lysis of cells in this experiment.

Why is $[Co(II)\text{-}EDTA]^{2-}$ more toxic than $[Co(III)\text{-}EDTA]^{-}$? These complexes have substantially different dissociation constants, with $[Co(II)\text{-}EDTA]^{2-}$ being more likely to dissociate in solution (14). We sought to determine if the observed toxicity was due to free cobalt ions [Co(II)] or EDTA individually. The effects of Co(II) and EDTA during the anaerobic growth of MR-1 were evaluated, and both components were found to inhibit growth when present at levels as low as 0.25 mM (Fig. 4). At the Co(II) and EDTA concentrations tested, the addition of 25 mM $MgSO_4$ had no effect on Co(II) lethality (Fig. 4A) but partially abolished EDTA toxicity (Fig. 4B). This observation suggests that the toxicity of $[Co(II)\text{-}EDTA]^{2-}$ is due in part to free EDTA since adding $MgSO_4$ reversed the growth impairment. EDTA is able to chelate a wide range of metals and can destabilize or increase the permeability of the outer membrane in gram-negative bacteria (6, 20, 21). When we tested the toxicity of $[Co(II)\text{-}EDTA]^{2-}$ with excess Mg(II) (provided as $MgSO_4$), it is possible that dissociated EDTA chelated Mg(II), therefore preventing the deleterious effects of free EDTA (Fig. 3). Under these conditions, free Co(II) concentrations must remain low enough not to impair growth (Fig. 3).

$MgSO_4$-dependent growth of *S. oneidensis* MR-1 coupled to $[Co(III)\text{-}EDTA]^{-}$ respiration. Since the toxicity of $[Co(II)\text{-}EDTA]^{2-}$ was alleviated through the addition of $MgSO_4$, $[Co(III)\text{-}EDTA]^{-}$ utilization by MR-1 was reevaluated by adding 25 mM $MgSO_4$ to cultures and monitoring CFU over time (Fig. 5). A positive control respiring fumarate achieved approximately 2 logs of growth within 24 h, whereas samples lacking either an electron acceptor or a donor had an overall decrease in cell

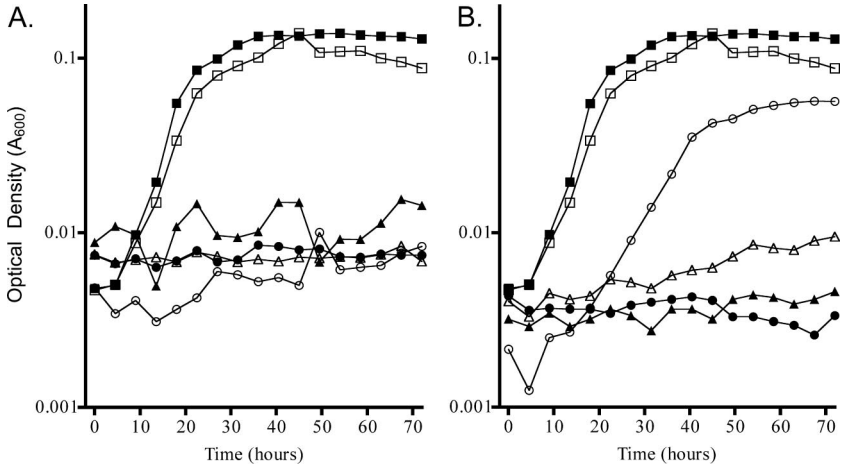

FIG. 4. Toxicity of Co(II) and EDTA during the growth of MR-1. Anaerobic growth was monitored for cells in minimal medium containing 20 mM lactate and 20 mM fumarate. (A) Toxicity of Co(II) with (empty symbols) and without (filled symbols) 25 mM $MgSO_4$. Symbols indicate no Co(II) addition (squares), 0.25 mM Co(II) (circles), or 2 mM Co(II) (triangles). (B) Toxicity of EDTA with (empty symbols) or without (filled symbols) 25 mM $MgSO_4$. Symbols indicate no EDTA addition (squares), 0.25 mM EDTA (circles), or 2 mM EDTA (triangles). Data presented are the averages for duplicate experiments; error bars were removed for clarity (SEM, <10% deviation).

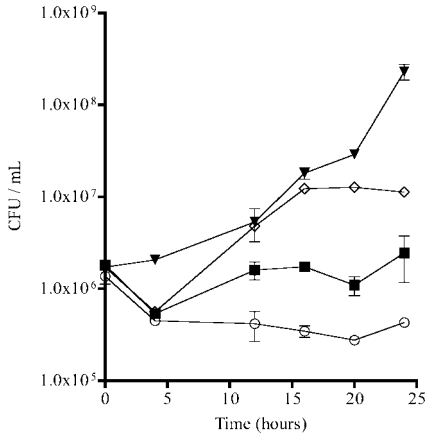

FIG. 5. Growth of *S. oneidensis* MR-1 coupled to the respiration of $[Co(III)\text{-}EDTA]^-$. Anaerobic growth in tubes of minimal liquid medium supplemented with 25 mM $MgSO_4$ was monitored. Additionally, tubes contained 20 mM lactate and 20 mM fumarate (▼), 20 mM lactate (○), 1 mM $[Co(III)\text{-}EDTA]^-$ (■), and 20 mM lactate and 1 mM $[Co(III)\text{-}EDTA]^-$ (◇). Growth was monitored by quantifying CFU aerobically using LB plates. Error bars represent SEM from experiments performed in triplicate.

numbers or no growth. Cell numbers increased by approximately 1 log when cells were cultured with lactate and $[Co(III)\text{-}EDTA]^-$, demonstrating $MgSO_4$-dependent growth of MR-1 coupled to $[Co(III)\text{-}EDTA]^-$ respiration.

Implications for remediation strategies. Considering that the potential application for metal-reducing bacteria is the bioremediation of contaminant metals or radionuclides in aquatic and subsurface environments (47), our finding that $[Co(II)\text{-}EDTA]^{2-}$ is toxic to MR-1 will have an impact on remediation strategies using this organism. For the successful application of *S. oneidensis* in promoting the reduction of $[Co(III)\text{-}EDTA]^-$, the nature of Co(II) ion and EDTA toxicity should be further studied to develop strains that can tolerate higher levels of $[Co(II)\text{-}EDTA]^{2-}$ than the present strains. An immediate strategy would be to supplement contaminated sites with $MgSO_4$ to mitigate EDTA stress on bioremediation bacteria.

The reduction of $[Co(III)\text{-}EDTA]^-$ requires the Mtr respiratory pathway. Though the reduction of $[Co(III)\text{-}EDTA]^-$ by other strains of *Shewanella* has been described previously, the genes required for this process have not been identified (3, 9, 14, 23, 45). Given the role of the Mtr respiratory pathway in reducing a variety of other substrates, we reasoned that it was likely to be involved in the reduction of $[Co(III)\text{-}EDTA]^-$. Transposon mutants lacking Mtr pathway genes (*mtrB* and *mtrC*) and lacking genes for processes known to be required for a functional Mtr pathway (*cymA*, *dsbD*, *menC*, and *gspD*) all had lower rates of $[Co(III)\text{-}EDTA]^-$ reduction than the wild type (Fig. 6A). These data demonstrate the requirement of the Mtr pathway (*mtrB* and *mtrC*), type II secretion (*gspD*) (13, 38), *c*-type cytochrome maturation (*dsbD*) (8, 12), menaquinone biosynthesis (*menC*) (31, 41), and the tetraheme *c*-type cytochrome CymA, thought to be the primary link between many respiratory pathways in *S. oneidensis* and the menaquinone pool in the cytoplasmic membrane (29, 39, 40). The magnitudes of $[Co(III)\text{-}EDTA]^-$ reduction deficiency in the transposon mutants examined here are similar to previously described defects of these mutant strains in the reduction of Fe and Mn (4, 5, 22, 30).

To further quantify the role of the Mtr respiratory pathway in the reduction of $[Co(III)\text{-}EDTA]^-$, we generated deletions of the four genes that encode Mtr respiratory proteins: *mtrA*, *mtrB*, *mtrC*, and *omcA*. MtrA is a periplasmic decaheme *c*-type cytochrome thought to accept electrons from CymA (35) and donate electrons to the MtrB/MtrC/OmcA complex (36, 43). MtrB is an integral outer membrane protein (4), while MtrC and OmcA are both decaheme *c*-type cytochrome lipoproteins (32) localized on the outer leaflet of the outer membrane (28). Deletions in the individual genes did not alter the expression of other genes within this cluster, indicating that these genes do not affect polarity as measured by quantitative reverse tran-

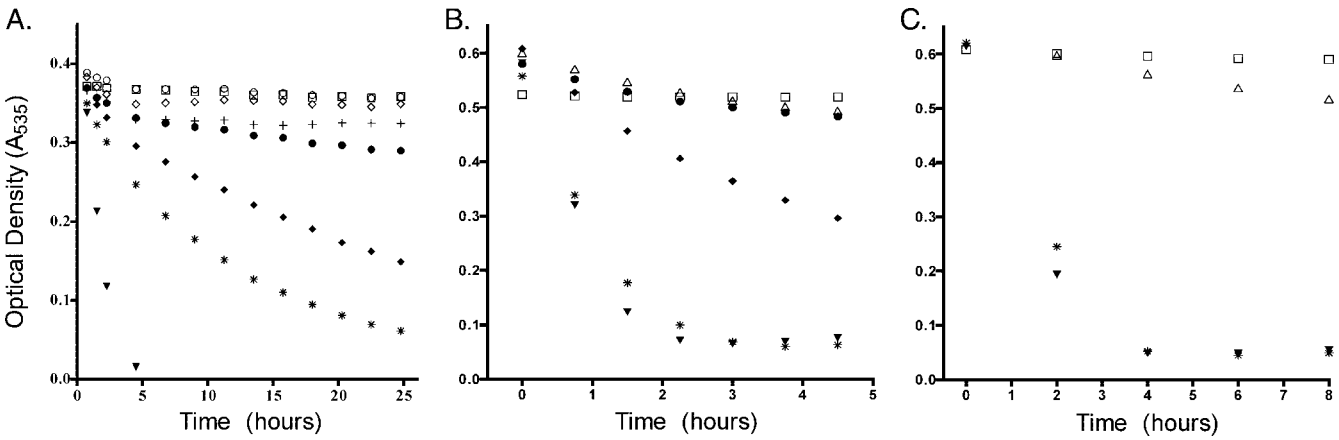

FIG. 6. Anaerobic reduction of $[Co(III)\text{-}EDTA]^-$ by *S. oneidensis* MR-1 and mutant strains. (A) Analysis of the *cymA* (JG107; ◇), *dsbD* (JG297; ○), *menC* (JG300; +), *mtrB* (JG299; ●), *mtrC* (JG298; ◆), and *gspD* (JG301; ∗) transposon mutants relative to the wild type (JG274; ▼) and the abiotic control (□). (B) Analysis of the *mtrA* (JG476; △), *mtrB* (JG467; ●), *mtrC* (JG420; ◆), and *omcA* (JG335; ∗) deletion mutants relative to the wild type (JG274; ▼) and the abiotic control (□). (C) Complementation of *mtrA* deletion strain JG476: *mtrA* pBBR1-MCS2 (JG553; △), *mtrA* pMTRA1 (JG554; ∗), wild type with pBBR1-MCS2 (JG168; ▼), and abiotic control (□). Data presented are the averages for triplicate experiments; error bars were removed for clarity (SEM, <10% deviation).

scription-PCR (data not shown). Strains lacking *mtrA* and *mtrB* were severely defective in [Co(III)-EDTA]$^-$ reduction, while *mtrC* mutants were partially defective (Fig. 6B). Interestingly, the *mtrC* transposon mutation caused a more striking defect (reduction to ~12% of the wild-type rate) than the *mtrC* deletion mutation (reduction to ~50% of the wild-type rate), suggesting some polarity of the transposon on *mtrA* and/or *mtrB*, the genes located immediately downstream of *mtrC*. Strains lacking *omcA* were only slightly defective (exhibiting 96% of the wild-type rate), indicating that OmcA is not required for [Co(III)-EDTA]$^-$ reduction under the conditions tested. These data suggest that MtrC is the primary reductase for [Co(III)-EDTA]$^-$ in *S. oneidensis* strain MR-1. The complementation of the *mtrA* deletion strain fully restored [Co(III)-EDTA]$^-$ reduction activity, as shown in Fig. 6C. The complementation of both *mtrB* and *mtrC* deletions also yielded significant increases in [Co(III)-EDTA]$^-$ reduction rates (data not shown). Taken together, the results of genetic analyses of [Co(III)-EDTA]$^-$ reduction demonstrate that the Mtr respiratory pathway is essential for this process. Given the ability of the Mtr respiratory pathway to facilitate electron transfer to the outside of the cell, we predict that *S. oneidensis* strain MR-1 can also reduce insoluble forms of cobalt {e.g., heterogenite [Co(III)OOH] or cobalt oxy(hydr)oxide (CoOOH)}. Studies are under way now to test this prediction.

This work has added [Co(III)-EDTA]$^-$ to the list of substrates reduced via the Mtr respiratory pathway of *S. oneidensis*. The promiscuity of this system is intriguing since it has a direct impact on the biochemical mechanism of electron transfer to these different substrates, the ecological utility of such a system to *Shewanella*, and the potential bioremediation applications of *Shewanella* bacteria.

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