Title
Bioelectrical Perchlorate Reduction and Characterization of Novel Dissimilatory Perchlorate Reducing Bacteria

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Publication Date
2009

Peer reviewed|Thesis/dissertation

Bioelectrical Perchlorate Reduction and Characterization of Novel Dissimilatory Perchlorate Reducing Bacteria

by

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor John D. Coates, Chair
Professor Lisa Alvarez-Cohen
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Fall 2009

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Abstract
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Perchlorate (ClO_4^-) is a soluble anion that occurs naturally in small concentrations, however, as a widely used oxidant in solid munitions, it has become a significant contaminant in ground water throughout the United States due to unregulated disposal of this compound prior to 1997. As a competitive inhibitor of iodine uptake in the thyroid gland, perchlorate ingestion can lead to lower thyroid hormone production, which is of particular concern for proper pre- and neonatal development. Recent reports have documented perchlorate in dairy and human breast milk, indicative of its movement to the top of the food chain. Current remediation of this compound usually involves ion exchange technologies, which although effective, simply concentrate the perchlorate out of the treated water into brine solutions. In contrast, many microorganisms are capable of respiring perchlorate, transforming it into harmless chloride. As a result, bioremediation has been identified as the most effective means of contaminant removal and degradation, and many strategies have been developed to take advantage of these dissimilatory perchlorate reducing bacteria (DPRB).

Traditional bioremediation strategies have been based on stimulating DPRB with cheap and easily available organic electron donors such as ethanol and acetate. While effective at stimulating perchlorate reduction, these compounds also stimulate considerable microbial growth, both of DPRB and non-target organisms. The excessive growth of organisms leads to biofouling, which can cause treatment failure and the stimulation of unwanted metabolisms such as iron and sulfate reduction resulting in the production of toxic and malodrous compounds. Further, addition of labile organics gives poor feedback control over bioremediation schemes and in the case of drinking water treatment, can contribute to downstream disinfection byproducts (DBPs).

To address these issues, an electrochemical system was investigated for stimulation of DPRB. A variety of electrochemical systems have been developed to stimulate microbial metabolism (Chapter 1), but none had been applied to perchlorate reduction. The system was attractive due to the ability to supply reducing equivalents for microorganisms to utilize in reducing perchlorate without adding carbon that would stimulate growth. In addition, the ability to alter both the available potential and current offered the possibility of tighter feedback control and thermodynamic targeting of perchlorate but not more electronegative electron acceptors.

Experiments to make use of cathodic electrodes as electron donors for perchlorate reduction were investigated (Chapter 2). Pure cultures of previously isolated DPRB were tested in the cathodic chamber of the bioelectrical reactor (BER) in cell suspensions utilizing anthraquinone-2,6-disulfonate (AQDS) as an electron shuttle. These experiments served as proof of concept, and demonstrated that organisms could successfully reduce perchorate in this manner. However, since these pure cultures could not survive in the BER under growth conditions, an enrichment was performed in the cathodic chamber to isolate organisms that would function for extended periods. Two novel DPRB were isolated from this enrichment, and

one, strain VDY, was tested further. Strain VDY was capable of reducing perchlorate in the cathodic chamber both with and without AQDS as a shuttle. As a result, the organism was used to inoculate up-flow BERs that were designed for continous treatment of perchlorate. These reactors functioned effectively under a variety of perchlorate concentrations, both with and without the shuttle AQDS, and did not suffer from biofouling.

The other organism, strain MP, isolated from the BER enrichment was of unique phylogenetic affiliation for DPRB (Chapter 3). Most DPRB have been isolated from two genera within the *Betaproteobacteria*- the *Dechloromonas* and the *Azospira*. Strain MP, in contrast, was most closely related to another undescribed DPRB, strain CR, and during phylogenetic characterization of these and one other strain, LT-1, all were identified as members of novel clades with no previously known DPRB. Strains MP and CR were members of the genus *Propionivibrio*, which previously consisted of obligate fermentative organisms. Strain LT-1 constituted a new genus in the *Rhodocyclaceae*, named *Dechlorobacter*, which was most closely related to the *Azonexus*, a genus also with no known DPRB.

Strain VDY was physiologically unique compared to other DPRB because it could survive and function in the cathodic chamber of the BER under growth conditions. As a result, the organism was fully characterized physiologically and phylogenetically (Chapter 4) in an attempt to better understand the basis for this success. VDY was most closely related to another DPRB in the *Alphaproteobacteria*, strain WD. Together these organisms make up a perchlorate-reducing, non-magnetosome forming clade within the genus *Magnetospirillum*. VDY did not contain copies of the *mamI* or *mamL* genes, necessary for magnetosome formation, and was unable to form magnetosomes under the physiological conditions tested. The ability of strain VDY to exist in the BER without an added carbon source was most likely due to autotrophic carbon fixation. This was supported by the demonstrated presence of the RuBisCO *cbbM* gene, which was expressed under autotrophic growth on hydrogen, but not during heterotrophic growth on acetate.

Since VDY was capable of reducing perchlorate in the BER without an added mediator, it was hypothesized that the organism might be able to utilize other inorganic electron donors, including reduced iron coupled to perchlorate reduction. VDY was tested for its ability to oxidize iron(II) in the form of $FeCl_2$ (Chapter 5). Washed cell suspensions were capable of oxidizing iron coupled to both perchorate and nitrate. However, Fe(II) oxidation was not stoichiometrically balanced when the cells were reducing perchlorate, and indicated the presence of stored reducing equivalents, even when the culture was pre-grown in electron donor-limited conditions. The organism was capable of oxidizing iron(II) under growth conditions, but could not couple this metabolism to growth. Cells without any added electron donor reduced an equivalent amount of perchlorate as those cultured with iron(II), further demonstrating that the observed Fe(II) oxidation was not coupled to perchlorate reduction. Iron toxicity tests showed that in the presence of either ferrous or ferric iron, growth of VDY on acetate and perchlorate was inhibited, although the effect of ferrous iron was more deleterious. As a result, it was determined that while strain VDY is capable of concomitant iron(II) oxidation during perchlorate reduction, this process is not metabolically coupled and growth is in fact prevented by the presence of iron(II).

The work within this dissertation describes the successful development of a new reactor system for the treatment of perchlorate-contaminated influents and the complete characterization of two novel organisms isolated from original enrichments with that system. Further investigation of perchlorate-dependent iron(II) oxidation was also carried out in strain VDY. As

a result of these studies, a new bioelectrical option now exists for perchlorate remediation strategies, two new DPRB have been fully characterized and described, and the known phylogenetic diversity of DPRB within the *Betaproteobacteria* has been significantly expanded.

For my grandfathers

James O. Thrash and Wallace S. Cordray

Who showed me what a man could be

And their children

Virginia S. Thrash and James E. Thrash

Who have taught me how to become one

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Acknowledgements

An attempt to acknowledge everyone who has contributed to my completion of this dissertation would necessarily be impossible, as quite literally, I would not be here without the participation of everyone I have ever known, regardless of how small their input, and I can't hope to remember such a crowd, owing to the incredible volumes of other stuff I've had to memorize to get through this Ph.D. Nevertheless, I will attempt to highlight those bright stars who have guided my way through the darkness, and I suppose I should start at the beginning of the path.

None of what is in the following pages would have happened without the mentorship and guidance of Dr. Monica J. Carson, who was kind enough to hire an unemployed, inexperienced UCSD biology grad off the street and show him what a life in research had to offer. Monica, you steered me into what has been the most profound process of my life, and almost single-handedly ushered me into my calling. A thousand thank yous. Also, thank you Darren Burton, who was there at the beginning, and continues to offer his magnificent sense of humor, perspective, geekiness, mention charm, dramatic impersonations, love of all things Eddie Izzard (thanks to E.I. for all the laughs btw) and Star Wars, and aquatic companionship in assistance of my pursuits. You are an incredible friend. Thank you to Vanessa Tipon, who provided the love and support I needed during those days when I was first finding my way in the lab, and thanks to Brian Russell, Craig Woelfel, JJ, and Eric Epperson for the good times during those early steps. You all challenged me to become great. Thanks to Rick Cowley, who taught me how to live every day fully, and to Ted Reckas, who, in the multitude of other ways he supported me, taught me one of the most valuable things I've ever learned- how to surf! Thank you to Cary and Judy Bickler, who were a second family to me during my transition. Your unconditional love was inspiring and taught me to be open to every possibility. And to Gia Brazil, who marched with me from San Diego to the Bay, thank you for your love and companionship, your voice and your care. You were there when it was darkest, and I will always treasure that.

To my fellow Monkeys at Cal: you were my first friends, comrades, colleagues, and I'm so thankful for all of you. You all made my first year, and I am so proud to have watched where we have landed. At least it's been on our feet! Regardless, you are the best class in PMB history, and I'm thankful to have been one of you. To the shala crew, whom I've not seen in some time, the Ashtanga sangha was what got me through countless rough patches, and even though most of you I never knew well, you influenced me. To Vance Selover, thank you for the constant care during my early years here. You showed me the true nature of yoga, the true nature of asana, and the true nature of practice. I carry those principles with me always. Doug Bushey and Sikina Jinnah, you helped me bridge my practice and my graduate lifestyle, and your influence on me has been lasting and continues. I'm grateful to have met you both. Laura Blau, you showed me both sides of the coin, and you will always have a place in my heart. Thank you for all of your care, for breaking barriers with me, and for having the courage to go for it twice in a row.

To the Co-opted Mafia, Paul Garton and Mikey Stevens, man have we torn it up in the last few years. I am so thankful for your music and your friendship. John Howland, who continues to dazzle- I'm glad I have your voice in my head frequently. Nicole Hayden, who always amazes me with her work, I can't wait till I can afford your paintings! All of you artists have kept my process in context, and kept me inundated with wonderfully different perspectives.

Madelaine Bartlett, thanks for always being able to laugh with me. You've made life so light! I really appreciate it. Yoshihiro Katsuura, you've been the voice of my conscience so many times. Thanks for your amazing loyalty and for always calling it like you see it. I admire your strength and determination with, well, everything. Bryan Miller, thanks for keeping me in the wind and on the water on a regular basis- you've helped keep me sane, and it's been fantastic being able to geek out with you regarding everything nautical. Jason Staijch and Amy Steelman, thanks for the parties, for unexpected excitement, and your wonderful lighthearted presence. Jason, thank you for all your help with my burgeoning computational desires. Tom Sharpton and Lauren O.U. Sharpton, I treasure you both, for your friendship, for your love, for showing me how smooth a relationship could be. Tommy, thank you for the years of conversation, and for the unquestionable steadfast integrity. You are a role model to me, as well as a friend. I'm so glad we get to take some steps forward together professionally as well as personally. Erik Jacobsen and Melissa Chevalier, you two together have been my rock. I cannot possibly express how much your unending support and enthusiasm for me has powered me forward. I cherish you both as my own blood. I'm thankful that we have been able to explore so many landscapes of this life together already, and I look forward to so many more.

Regardless of the trauma, I am thankful for my qualifying exam committee: Dr. Steve Lindow, Dr. Lisa Alvarez-Cohen, Dr. Doug Clark, and Dr. Kara Nelson. You forced me to do during my third year that which I should have done during my second, but regardless, I grew tremendously through your crucible, and I'm grateful. Matt Traylor and Jasqueline Pena, who got me through my third year classes, thanks a million for the extra hours. Thank you Lisa again, and Dr. Garrison Sposito, for taking the time to serve on my dissertation committee. You have shown me the way to the bottom of the hill, and this work is framed by that guidance.

I also need to express my deep gratitude to several establishments that have helped me through this process: India Palace, Biryani House, Jupiter, Triple Rock, Brick, Jose's Coutroom, 500 Club, Puerto Allegre, Casanova, Bigfoot Lounge, Lanesplitter Pizza, The Avenue, The Albatross, Priya, Cesar, Sconehenge, Yali's, and Bobby G's. Without your ample supply of food and alcohol, I most certainly would not have succeeded in finishing this dissertation!

To the Coates Lab, my second family, of rotating membership, for the last five years- nothing that I have accomplished has been separate from you. You all have made me feel at home, accepted, and have helped in thousands of little ways to make this dissertation happen. Most special thanks to Dr. Karrie Weber, who trained me in almost all of my techniques and was never too busy to help me further my abilities. Thank you for taking the time to teach me so much about how to become an independent scientist, for demonstrating how to hold oneself to a high standard, and above all, for showing me and all of us that persistence and dedication pay off! You have been and continue to be an inspiration. Dr. Ian Van Trump, thank you for all of your help, your insightful commentary, your additional testosterone (we sometimes needed more of it around here!), your existentialism, your music, and for being that person who seemed to intuitively always know how to make people felt cared for. I'm grateful to have been able to work with you all these years. Dr. Yvonne Sun, thanks for your wonderful sense of humor and your team attitude. You always made the lab feel connected. Dr. Kathy Bryne-Bailey, our new lab mom, thanks for your steadiness, Dr. Mark Heinnickle, thank you for your unending enthusiasm, and Dr. Saumyadita Bose, thank you for your unique perspective on...everything! Forest Kaser, thanks for showing us how to charge, to find a way to accomplish whatever is needed, no matter how technical. A million thanks to Sarir Ahmadi, who even in her comparatively short time here, has done amazing things to help me finish this dissertation. You

have been great, and I'm glad you joined our team. Joey Pakes, thank you for the love and care, the music, thoughtful conversation, for dreaming big with me, and for saving me from dying in an underwater cave! I'm so glad we've been able to work together as colleagues and as friends. Kelly Wrighton, my most constant companion, my partner in crime- I could never have achieved this without you. You have been my boxing manager, always in my corner, always backing me up, always on my side. Your steadfast willingness to listen, to share, to brainstorm, and to care has been more meaningful to me than perhaps you can imagine, and I am so incredibly grateful for your presence in my life. We've given this Ph.D. thing a run for its money, and I'm so excited about the work we will be able to accomplish together in the future. You're the best friend/colleague/superhero/drinking-buddy/workout-partner/badass-scientist anyone could ask for!

John Coates. I know you don't like it when your students call you "Dr." I know other things, like what "great gobshite" means, what cats are truly for, what the "mushroom treatment" is, and most importantly, to remember the "suck-it-and-see" experiment- all because of your wonderfully colored perspective. This has been no small part of what has made the journey through your lab the most rewarding process of my life. I am truly appreciative of your mentorship, your guidance, and your advice over these years. Thank you for taking me under your wing, and especially for showing me how to think. Nowhere else would I have learned the qualities, techniques, and approaches that I have learned in your lab. You've taught me more than you will ever know, and my ability to move forward now is on the strength of the foundation you've helped me build. I am truly grateful.

Finally, I would like to thank the most important people in my life- my family. Mom, Dad, Corinne, Adriane, Pop, Velma, Papa Wally, Louise, Chris, Lia, Joji- from you I have learned the meaning, and perhaps more inconceivably, the feeling of unconditional love. Your unending support, encouragement, involvement, and interest has fueled my various quests over the years, including this Ph.D. and the steps I now take beyond it. The knowledge that you have been behind me, no matter what, has given me the courage to take big risks, to follow my dreams, and to fight through any and all adversity. This dissertation is yours as much as it is mine, and I am forever grateful to all of you. I have been and will always be so very honored to be your son, brother, grandson, brother in-law, uncle. I love you all.

Chapter 1
Direct and Indirect Electrical Stimulation of Microbial Metabolism

Abstract

All organisms require an electron donor and acceptor, frequently in chemical form, but an elegant alternative is to supply these via direct electrochemical means. Electricity has been used to stimulate microbial metabolism for over 50 years. Since the first report of oxygenating media using anodic oxygen generation from electrolysis in 1956, researchers have made use of applied power systems to supply energy for microbial respiratory processes from fermentations to anaerobic reduction of toxic pollutants. Bioelectrical reactors (BERs) have been utilized for culturing organisms, influencing metabolite production, and biotransformation of a wide array of compounds. Both enrichment and pure cultures have been cultivated in the presence of applied current, showcasing the applicative diversity of these systems. As the need for more environmentally conscious solutions to waste-treatment, remediation, and cultivation efforts increases, systems that supply energy to microorganisms without chemical amendment are becoming more attractive. Additionally, the essential flexibility of BERs offers an almost unlimited range of solutions for metabolic stimulation and downstream application.

Introduction

Usually, culture of non-phototrophic microorganisms has involved providing the energy source- the flow of electrons- through solely chemical means. However, much research has gone into providing needed electron flow through direct and indirect electrical stimulation of microbial metabolism. Direct stimulation involves the interaction of electron transport chain components with a working electrode surface. In contrast, indirect stimulation involves the transfer of electrons from a working electrode to a microorganism either through a soluble mediator or a gas (usually hydrogen or oxygen produced by electrolysis of water). In both cases the applied current completes one side of the microbial metabolism, providing either the electron acceptor or donor.

Applying power to microorganisms is accomplished with electrochemical cells whose structure and composition can vary greatly, although they generally come in two main configurations, single- or dual-chamber. These systems, reviewed below, are defined here as bioelectrical reactors (BERs) in order to make a distinction between them and the closely related microbial fuel cell (MFC), which has garnered much attention recently (1). In a MFC, a potential difference is created and resistance placed across an electrochemical cell to *extract* useful current from the microbial metabolism analogous to chemical batteries (hence the commonly-used term to describe MFCs: biobatteries). In contrast, BERs *apply* current to the microorganisms for the purpose of stimulating microbial metabolism. Since MFC systems and BERs share similar characteristics from the standpoint of circuitry, chamber construction, and basic electrochemical parameters, that have been reviewed previously by Lovley (1) and Logan et al. (2), this review will not focus on these common features but rather focus on those unique to applied power BERs.

In a BER where bacteria are present and power is applied, two routes of stimulation are available (Fig 1). In one, cathodic reduction of either a mediator or part of the bacterial electron transport chain serves as the energy source for the bacteria. The bacteria pass these electrons through their electron transport chain to terminal reductases which then reduce an oxidized substrate (e.g. a contaminant like nitrate or uranium). Alternatively, the BER system provides a continuous supply of a suitable electron acceptor either through direct anodic oxidation of a terminal reductase or through indirect anodic oxidation of a soluble mediator which is used by the microorganism as a suitable electron acceptor to oxidize various reduced substrates such as ammonia or ferrous iron.

Important Electrochemical Factors Influencing System Effectiveness

Since the eventual goal of the BER is to provide essential electron flow for a microorganism the efficacy of the stimulation is determined by the interaction of that microorganism with the electrochemical system. The exact genetic and biochemical mechanisms of interaction between an organism and a working electrode remain unknown. However, potential at the working electrode and the abiotic reactions occurring at the electrode surface are two electrochemical factors that can affect system performance.

Potential

The amount of energy (as ATP) a microbe can obtain from a given metabolic process is directly proportional to the potential energy difference, ΔE^o (in volts), between the electron

donor and the electron acceptor. Potential in this review is reported versus a normal hydrogen electrode (NHE). In a BER, the applied potential at the electrode takes the place of the potential of a chemical electron donor or acceptor. Thus the electrochemical ("redox") couple that a microorganism is exposed to between a working electrode and a corresponding electron donor or acceptor determines the maximum available energy that can be used for growth and/or metabolism.

BER potential is commonly set in two different ways. Two-electrode systems set the working electrode vs. a counter electrode and are frequently used in electrolysis-based systems (e.g. (3)). Three-electrode systems poise the working electrode potential vs. a constant potential reference electrode. Figure 1 includes a reference electrode in the cathodic chamber. In three-electrode systems, the counter electrode potential can be varied to maintain the potential difference between the reference and working electrodes. Three-electrode systems are beneficial for precise maintenance of potential at the working electrode and are frequently used with electron shuttles (4) or during direct electrode oxidation (5)(see below) to ensure precise redox potential in a cathodic or anodic chamber.

Abiotic reactions at the electrode surface

In addition to direct and indirect electron transfer to a microbe, other reactions may take place at the electrode surface that can directly impact microorganisms. Table 1 describes the variety of biologically pertinent abiotic reactions that are found in applied power systems. If the potential difference between the anode and cathode is set beyond 1.2V (vs. NHE), oxidative electrolysis of water and reduction of protons will occur (6) (Table 1). Assuming the electrode material is non-reactive, these oxidation and reduction reactions produce anodic O_2 and H^+ and cathodic H_2 (6). At ~2V (vs. NHE), cathodic reductive electrolysis of water is possible, adding OH^- ions in addition to H_2 (Table 1). H_2 and O_2 are widely utilized by microorganisms as an electron donor and acceptor, respectively. Additionally, the presence of H^+ and OH^- ions will have a direct and dramatic effect on pH in the localized vicinity of the electrodes in a non-buffered system, which if unchecked can cause deterioration of microbial metabolism. Oxygen in the BER can also react cathodically to form H_2O_2 at low pH (6, 7), which is toxic to microorganisms (8); a concern for BERs used to culture acidophilic iron-oxidizing organisms (see below). Alternatively, Cl^- ions, in high enough concentration, can be oxidized at the anode to form Cl_2 (6, 9) that readily reacts with water to form hypochlorous acid (HOCl) (6), a common disinfection agent (10). The production of both H_2O_2 and Cl_2 therefore can be detrimental to BER performance.

The use of carbon electrodes introduces another reaction, as carbon is electrochemically active. If carbon is used at the anode, it can be oxidized. The oxidation of carbon to CO_2 occurs at ~ -0.21V (vs. NHE) (11) (Table 1) and in an aqueous system adds protons and bicarbonate (HCO_3^-) through equilibration with water, lowering the pH, but also providing buffering capacity. This can be useful for systems that have no intrinsic buffering capacity (12), but because the reaction involves the transfer of a solid to a dissolved gas, eventually the carbon anode will dissolve completely and need replacement.

Multiple materials and configurations of electrodes have been utilized in BERs, including titanium (13), platinum (14), stainless steel (15), and carbon in the form of graphite (5), activated carbon (16), amorphous carbon (12), glassy carbon (17), and carbon felt (18) (Table 2). Although good for conductivity, titanium (19) and platinum (9) electrodes are more rarely used due to cost. Stainless steel can be used effectively (20) but most research has been done with

carbon due to its irregular surface, which is good for bacterial adhesion, low cost, and variety of forms for enhancing surface area. Since the active interface of a BER is the electrode surface, the larger the surface area, the more effective the reactor. Examples of electrode structures include cylinders/rods (3), blocks (5), and higher geometric surface area configurations such as metal mesh (21), powdered graphite (4) and graphite felt (18).

Electron Transfer Mechanisms and Applications

Electron transfer between a microorganism and a working electrode is the primary operational objective of a BER. In BERs tested thus far, the transfer of electrons between a working electrode and a bacterial cell can occur either directly at the electrode surface or indirectly mediated by a soluble electron shuttling agent, or the electrolysis of water (Fig 2).

Electrolysis of Water

Figure 2 shows the cathodic electrolysis of water to produce hydrogen, which can be oxidized by microorganisms coupled to the reduction of many substrates, including nitrate (pictured). Hydrogen is utilized as an electron donor by a wide variety of microorganisms and electrolytic production of hydrogen is therefore an effective strategy for stimulating metabolism and growth of those organisms (e.g. (22, 23), refs in Table 4). The reverse process, anodic generation of oxygen for microbial reduction as an electron acceptor, has been studied in fewer cases but is nonetheless effective for stimulating bacterial growth and metabolism (e.g., (9, 24, 25)).

Aerobic rich media oxidation

As oxygen is the most energetically favorable electron acceptor a wide variety of bacteria make use of it, but liquid culturing therefore requires adequate oxygenation of media, usually via shaking or sparging of air. The first report of a BER for stimulation of bacterial culture came in 1956 when Sadoff et al. used an applied current to supply oxygen via electrolysis of water for *Pseudomonas fluorescens* (9). This method sparged the media with fine bubbles of O_2, supplying equivalent oxygen to that of traditional shake flasks. However, the authors did report the presence of hypochlorite (OCl^-) under some conditions and abiotic scrubbing of O_2 via displacement by cathodically generated H_2 in uninoculated cultures, illuminating for the first time some additional concerns in electrolysis of microbial media for culturing.

Aerobic hydrogen oxidation

The aerobic chemolithotrophs that make use of hydrogen for their electron donor take advantage of the largest possible electrochemical potential energy difference for their metabolism and are often called the “knallgas” bacteria, a German word meaning literally “bang-gas” referring to the combination of oxygen and hydrogen. Schlegel and Lafferty electrolytically generated both cathodic hydrogen and anodic oxygen for the cultivation of the knallgas bacteria, specifically *Ralstonia eutropha* H16 in 1965 (22). This was of particular relevance since alternative methods of providing both hydrogen and oxygen to the culture medium presented significant dangers due to the explosive combination of these gases.

Nitrification/Denitrification

Microorganisms are responsible for all the major reactions in the nitrogen cycle. The reduction of N_2 to ammonia (nitrogen fixation) is carried out solely by microbes and is a process on which all complex life depends as it is the sole entry point of nitrogen into the food web. Aerobic microorganisms such as *Nitrosomonas* and *Nitrobacter*, in consecutive steps, catalyze the nitrification of NH_3 to NO_3^- (8) while many anaerobes such as *Azoarcus* can mediate denitrification of NO_3^- back to N_2 (26). Nitrification and denitrification are significant processes as they can effectively remove fixed nitrogen from the environment since the endproduct is nitrogen gas, a fact exploited during wastewater treatment where there is an over-abundance of nitrogen, frequently in the form of ammonia or nitrate. Nitrate represents a significant human health threat because it is broken down in the liver to nitrite, which inhibits proper oxygen transfer by hemoglobin (27). Since nitrate is frequently present in wastewater and reduced forms of nitrogen can be oxidized to nitrate, all forms of nitrogen must be removed to properly treat wastewater.

BER systems that rely on electrolysis of water have been studied extensively for use in wastewater treatment. Table 3 describes the configurations and operational parameters of reactors described in the literature examined for total nitrogen removal from wastewater. Common to all of these systems is the electrolysis of water and the presence of an enriched biofilm community on the working electrodes. Most make use of electrolytically generated hydrogen gas as an electron donor for denitrification (3, 12, 15, 16, 19, 20, 24, 28-41) but several studies also take advantage of electrolytic oxygen generation for aerobic nitrification (16, 24, 25, 42, 43).

The first use of a BER hydrolysis-mediated denitrification system was reported by Sakakibara and Kuroda in 1993(*3*). The same group followed this publication with a detailed mathematical model of the process (28) and another similar study utilizing carbon anodes for the neutralization of cathodically formed hydroxide ions (29). The occurrence of denitrification inhibition in the electrolysis BER prompted the development of a more sophisticated model by Flora et al. (33). This study found that cathodic overproduction of hydrogen could have an inhibitory effect on the denitrifying ability of the biofilm community, and the improved model agreed well with experimental results. Sakakibara et al. followed up their original reactor model with another study incorporating dissolved oxygen (DO) and sulfate to more accurately simulate contaminated groundwater (30).

Following this early work, multiple reports evaluating the viability of the electrolysis-based denitrification BER system were published concerning a variety of operational parameters, including electrode material composition and copper sensitivity (15), long-term operation (12, 34), selective ion removal (12), and alternative electrode configurations (16, 35, 36, 39, 40), including a sand column BER system which, while effective, suffered from local pH changes around the electrodes which proved deleterious to microbial activity (20). Simultaneous nitrification and denitrification in a BER was first reported by Kuroda et al. (24). Reactors were successful in supplying both oxygen for ammonium oxidation and hydrogen for nitrate reduction and this study was followed up by another using specific enrichments for anodic nitrification and cathodic denitrification (25). Subsequently Goel and Flora demonstrated nitrification in a single-chamber BER (42). However, a later attempt to combine nitrification and denitrification in a sequential two-chamber reactor system was only partially successful due to ammonium diffusion through the cation-specific membrane and detrimental pH changes (43).

Electron shuttles

Many electro-active substrates, such as quinines (4), phenazines (44), and humic substances (45, 46), can be used in a non-degradative manner by bacteria as electron donors and/or acceptors. Microorganisms can selectively oxidize or reduce these species without consuming them, leaving them free for recycling at an electrode. In this manner, electrons are "shuttled" between microbes and electrodes. Figure 2 shows the example of cathodic reduction of anthroquinone-2,6-disulfonate (AQDS, E^o = -0.184 V) for subsequent oxidation by an organism coupled to perchlorate reduction as reported by Thrash et al. (4). Investigations using BERs have made use of many types of shuttles, described in detail below, that are listed in Table 4 along with their function as either an electron acceptor or donor and corresponding electrochemical potential.

Acidophilic aerobic iron-oxidation

Aerobic iron-oxidizing bacteria are of particular interest for their role in bioleaching of metals (47). Organisms such as *Acidithiobacillus ferrooxidans* (formerly *Ferrobacillus sulfooxidans* and *Thiobacillus ferrooxidans* (48)) make use of reduced iron (II) as an electron donor coupled to aerobic respiration. Both abiotic, and biotic oxidation of iron (II) sulfides, such as pyrite (FeS), leads to the production of sulfuric acid, and thus *A. ferrooxidans* is acidophilic, thriving in low pH environments (49)(and references therein). This leaching process requires two steps (50). The first is abiotic oxidation of the iron sulfide by atmospheric oxygen yielding ferric iron:

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O$$

and is followed by reduction of the ferric iron by sulfide from the iron sulfide yielding sulfuric acid:

$$FeS_2 + 14\ Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

However, the first reaction at low pH is slow abiotically. Organisms like *A. ferrooxidans*, which couple the oxidation of ferrous iron to aerobic respiration, can dramatically enhance the rate of ferric iron formation with subsequent acidification and metal dissolution (51).

It is just this dissociation of metal sulfides in application to leaching of valuable ores that makes studies of *A. ferrooxidans* so attractive. However, growth of aerobic, iron-oxidizing cultures is limited because of the bioenergetics of the metabolism. Due to the relatively small electrochemical difference between oxygen and iron at acidic pH (ΔE^o ~ 0.06V for O_2 and iron (II) at pH 2 compared to ΔE^o = 1.2V for oxygen and hydrogen, for example (8)), and the fact that oxidation of iron (II) only yields one electron while reduction of $\frac{1}{2}O_2$ to H_2O requires two electrons, the available energy for growth is small. It is impractical to simply add more iron (II) due to solubility issues at high concentration, product inhibition, and precipitation of the oxidized iron (III) (52, 53).

This conundrum motivated the first study, by Kinsel and Umbreit, of a bioelectrical system based on iron (III) regeneration for oxidation by *A. ferrooxidans* in 1964 (54). By doing so they reported up to 20-fold improvement in cell yield. Supporting BER studies on the same organism were subsequently released by Kovrov et al. and Denisov et al. (55, 56). Since then many researchers have investigated electrical stimulation of the organism owing to its prevalence in bioleaching and desulfurization of coal (18, 52, 57-69). Commonly, these authors use cathodic reductive regeneration of iron (II) to stimulate higher cell numbers and better growth rates while the organism grew aerobically. The basic system has been explored in a multitude of ways to improve the growth yield further by making use of single chamber systems (60), separate growth and iron-reduction chambers (52, 61), combined cathodic iron-reduction and

anodic oxygen production (18), potentiostatic control (62), and bubbled air in a separate growth chamber (59). Further, this BER design has been utilized for the growth of another species, *Leptospirillum ferrooxidans* (63), and mathematically modeled to determine that cathodic reduction of iron was the rate-limiting step (64).

Investigating this system for more applied purposes, Lopez-Lopez et al. utilized an *A. ferrooxidans* culture as a catholyte to lower cathodic potential and improve cost efficiency of electrochemical treatment of wastewater (57). Harvey and Crundwell developed a feeback circuit based on redox potential to keep concentrations of iron (II) constant during experiments with *A. ferrooxidans* to test the growth inhibition of the organism by arsenite, common in the bioleaching process (65, 66). Further bioleaching experiments of iron, zinc, and copper from pyrite, sphalerite, and chalcopyrite were conducted by Natarajan and collegues in a series of studies (67-69) where it was found that positive potentials at the working electrode were more effective for bioleaching because abiotic mineral oxidation created starting products (S^0 and Fe^{2+}) for further bacterial oxidation. The bacterial oxidation of iron (II) to iron (III) enhanced dissolution of the minerals and thereby accelerated the leaching process.

Anaerobic lithotrophic iron reduction

Microorganisms can make use of ferric iron as a terminal electron acceptor, in effect "breathing" the metal. Microbial iron reduction is a major metabolism in all anoxic ecosystems and plays an important role in global geochemical cycles (70)(and references therein). The first unequivocal demonstration of dissimilatory iron-reduction by a pure culture was by Balashova and Zavarzin in 1979 (71). Since then, a multitude of studies have shown the ubiquity and diversity of the organisms that carry out this metabolism (70), and the particular use of iron (III) as an electron acceptor and hydrogen as an electron donor has been proposed as an early form of microbial metabolism on ancient earth (72).

An innovative method for culturing such chemolithoautotropic iron-reducers using a BER was conceived by Ohmura et al. in which cathodic production of hydrogen from electrolysis was combined with anodic iron (III) regeneration (23). They used this system to culture *A. ferrooxidans*, which although it has been studied extensively for its aerobic iron-oxidation metabolism, is also capable of anaerobic growth on iron coupled to hydrogen oxidation. This system utilized the BER to provide both the electron donor and acceptor for *A. ferrooxidans* through electrochemical means, similarly to Schlegel and Lafferty's knallgas culture system.

Sulfate reduction

Microbial sulfate reduction is of major importance in the global sulfur cycle and specifically in the carbon cycling of marine sediments (51). Since microorganisms can utilize sulfur in the form of sulfate (as well as sulfite, thiosulfate, and elemental sulfur) as a terminal electron acceptor, this metabolism has been applied to remove the sulfur content from petroleum (73)(and references therein) and coal (58), a process that is necessary to remove this ubiquitous fuel contaminant. A BER system was investigated by Kim et al. to stimulate sulfate reduction for sulfur removal from petroleum. They showed that electrically-reduced methyl viologen could stimulate bacterial sulfate reduction in *Desulfovibrio desulfuricans* and that sulfur could be biologically removed from Kuwait crude oil using this process (74).

Glucose fermentation

The fermentation of glucose is one of the most fundamental metabolic pathways in microbiology. Heterotrophic breakdown of glucose and other complex organic molecules yields important fermentation products which are utilized by many anaerobic microbes as electron donors for anaerobic respiration and methane production. Importantly, the fermentation of glucose has many possible pathways both within and between microbial species, and as such has been studied extensively to understand the metabolic flux of many organisms. In particular, alteration of the conditions of glucose fermentation can lead to different breakdown endproducts, for example with *Clostridium acetobutylicum*, where increasing the partial pressure of hydrogen in the media improved the relative yield of butanol (75, 76).

Given the importance of studying the pathways of glucose fermentation, it is not surprising that many researchers have investigated the use of electrical stimulation for better understanding this metabolism. Emde and Schink demonstrated the ability to direct glucose fermentation endproducts by providing an electrochemically reduced electron shuttle to *Propionibacterium freudenreichii* (21). The presence of either AQDS or cobalt sepulchrate increased propionate formation over acetate from 73% to 90% or 97%, respectively although the shuttle had no effect on the endproducts of lactate fermentation. In a contrasting study using a similar BER with cobalt sepulchrate again as an electron donor shuttle, Schuppert et al. were able to shift propionate formation from 68% to 100% during lactate fermentation with *P. acidi-propionici* (77). In both studies growth yields of the organisms were decreased, indicating some shunting of electrons for purposes other than growth as a result of the electron shuttle, the applied redox potential, or both.

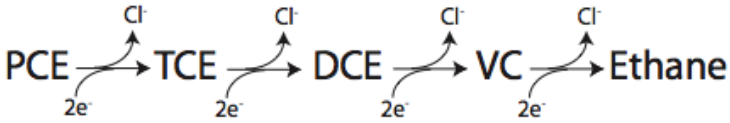
Studies carried out on *C. acetobutylicum* in a BER suggested a possible mechanism for redox-mediated metabolism changes. The idea of utilizing a BER system to control the redox potential of a culture was first tested by Thompson and Gerson, but they did not address mechanisms for redox effect on cultures (78). Kim and Kim showed that electrically-reduced methyl viologen could act as a direct electron donor to NAD^+ for alteration of fermentation endproducts (79). Building on this system, Peguin et al. reported a direct correlation between decreasing redox potential and increasing rate of $NAD(P)^+$ reduction (13), leading to an increase in the $NADH/NAD^+$ ratio. Based on this and two previous studies (80, 81), one of which showed that glyceraldehyde-3-phosphate dehydrogenase for *C. acetobutylicum* was inhibited by a high NADH/NAD+ ratio (81), they hypothesized that continuous increase of NADH concentrations due to decreased redox potential would inhibit glucose consumption and therefore NAD^+ regeneration leading to growth defects. Supporting this study, She et al. reported cathodic inhibition of *Enterobacter dissolvens* through electrolysis of water (82) and demonstrated a depression in NAD^+ levels compared to NADH in this system.

Park and Zeikus used neutral red as an electron shuttle (first reported in a BER by Hongo and Iwahara (14, 83)) for the purpose of improving glucose utilization by *Actinobacillus succinogenes* and increasing cell yield, ethanol, and succinate end concentrations (84). They showed that neutral red could also be used to directly reduce NAD^+ and developed a model for the role it could play in influencing cellular metabolism. The model agreed with their data and described neutral red interacting not only with NAD^+ but also with the fumarate reductase complex resulting in increased proton translocation (and energy generation) compared to those cells grown without the electrically reduced shuttle. A more recent study has confirmed the influence of BER-applied potential on the fermentation end products of another *Clostridia* species, *C. thermocellum*, and a yeast, *Saccharomyces cerevisiae* (an idea first reported by Krizaj and Lestan (85)) (86). Both organisms showed increased ethanol production compared to acetate

at lower applied potentials. However, since the applied potentials were more electronegative than that expected for electrolysis of water, it may be that the effect reported was due to increased hydrogen partial pressures in the media, as opposed to simple applied potential.

Dechlorination

Chlorinated solvents are almost entirely anthropogenic and many are highly toxic or carcinogenic. Fortunately, microorganisms can reduce some chlorinated solvents by using them as terminal electron acceptors (87). Both chlorinated phenols and chlorinated ethenes can be degraded by bacteria under highly reducing conditions coupled to oxidation of elemental hydrogen. Chlorinated phenols can be reduced sequentially to phenol and then further mineralized to CO_2 (88). Perchloroethene (PCE) is reduced stepwise to trichloroethene (TCE), dichloroethene (DCE), and then vinyl chloride (VC), with some organisms capable of transforming VC completely to harmless ethane (87):



The final conversion to ethene is important to achieve, because although PCE and TCE are toxic, DCE and VC are even more so, and are known carcinogens (89, 90)(and references therein). Therefore, partial dechlorination represents an even greater health threat than the parent compounds.

Two groups have looked at the use of BERs to stimulate microbial dechlorination processes. Skadberg et al. utilized an electrolysis-based system for degradation of 2,6-dichlorophenol to 2-chlorophenol and in addition showed a significant deleterious impact of copper on the system (91). Aulenta and colleagues used electrically-reduced methyl viologen to stimulate trichloroethene (TCE) reduction (17). Their system was able to reduce TCE to vinyl chloride and even ethane/ethene in small concentrations. However, recent studies have indicated the abiotic electrochemical reduction and subsequent dechlorination of TCE and its breakdown products completely to ethene at low potential differences with non-metallic electrodes (92, 93). These studies indicate that the electrochemical reduction of chlorinated ethenes works effectively enough in the absence of microbes that further study on BER application to these compounds may be irrelevant.

Perchlorate reduction

Perchlorate is widely used as an oxidant in solid munitions. Its chemical properties make it highly soluble, poorly adsorbed, and non-reactive in the natural environment. Although toxic to humans because of competitive inhibition of iodine uptake in the thyroid gland, many microorganisms can readily utilize perchlorate as a terminal electron acceptor, reducing it to harmless chloride (94). This, and the fact that dissimilatory perchlorate-reducing bacteria (DPRB) are ubiquitous in the environment (95) makes microbial perchlorate reduction the most cost-effective and readily available means of remediating this contaminant. As is the case for many of the other contaminants listed above, ex-situ treatment by chemical stimulation can be plagued by overgrowth of the organisms leading to increased cost, biofouling, and treatment failure. In addition, chemical addition to treatment streams can lead to the abiotic production of carcinogenic downstream disinfection byproducts (96). To overcome these issues, a BER system was recently explored for the treatment of perchlorate (4).

Thrash et al. demonstrated a system that was capable of stimulating a native perchlorate-reducing community from local groundwater in the presence of the electron shuttle AQDS. The enrichment reactor operated successfully for over 70 days and a novel perchlorate-reducer, *Dechlorospirillum* strain VDY was isolated from the system. Strain VDY was shown to be capable of continuously removing 100mg/L influent perchlorate at 100% efficiency both with and without AQDS as a shuttle. Phenotypic studies indicated that strain VDY was capable of utilizing hydrogen for perchlorate reduction, and headspace measurements from abiotic reactors indicated the presence of enough hydrogen to account for the removed perchlorate. These studies demonstrated the BER as an effective alternative to chemical addition for the continuous treatment of perchlorate.

Drug synthesis

BERs have also been used in pharmaceutical production research. Many drugs are produced using microorganisms as catalysts for individual synthesis steps or in some cases, multiple steps (97). Frequently these organisms are engineered, but wild-type strains are also used. Shin et al. showed effective BER stimulation of the yeast *Trichosporon capitatum* for an important drug synthesis reaction (98). *T. capitatum* reduces Br-ß-tetralone to Br-ß-tetralol, an intermediate in potassium channel blocker synthesis. The authors utilized a BER for reduction of neutral red to effectively improve Br-ß-tetralone reduction rates and final yields of Br-ß-tetralol.

Direct electron transfer

There are many examples in the MFC literature of direct anodic electron transfer for the purpose of power generation (1) (and references therein). However, in the field of applied power systems, only Gregory et al. have confirmed direct cathodic transfer of electrons to a bacterial cell (5). In addition to an enrichment culture, pure cultures of *Geobacter metallireducens* and *G. sulfurreducens* were shown to utilize electrons directly from the surface of graphite electrodes for the purpose of reducing nitrate and fumarate, respectively. Scanning electron microscopy of the surface of the electrodes revealed a monolayer of bacterial cells, and replacement of media to remove soluble mediators and planktonic cells did not affect current utilization (5). Current flow was directly correlated with electron acceptor utilization over multiple cycles. This finding is significant because in contrast to the many reports of organisms capable of reduction of insoluble electron acceptors like electrodes and iron oxides, reports of direct oxidation of solid surfaces are much more limited. The mechanism for direct electrode oxidation is unknown although *Geobacter* species have been shown to have many redox-active components on their outer membranes including cytochromes and conductive pili, so-called “nanowires” (99, 100). Whether these could be used for accessing electrons in an oxidative manner remains to be seen. Another possibility that still exists is that the organisms were not conserving energy via cathodic oxidation, but rather that cathodic electrons were passed directly to the nitrate reductase for reducton of nitrate.

However, several lines of evidence support the possibility of direct cathodic oxidation for the purpose of energy conservation by a microorganism. Observations by Weber et al. (70, 101-103), Chaudhuri et al. (104), and Shelobolina et al. (105), described nitrate-dependent microbial oxidation of solid-phase ferrous iron. *Azospira suillum* strain PS oxidized several forms of solid-phase iron (II), including arsenopyrite, chromite, siderite, and importantly, the silicacious iron minerals almandine and staurolite, the biological oxidation of which was previously unknown

(104). Shelobolina et al. demonstrated oxidation of nontronite, another ferrous iron-containing silicate, by *Desulfitobacterium frappieri* (105). These studies confirm that a variety of microbes can access electrons from solid minerals and combined with the presence of organisms other than Geobacteraceae in the BER enrichment culture leave open the possibility that other species with additional reductive metabolisms are capable of direct electrode oxidation.

Uranium reduction

Although normally found in nature as only a trace metal, human enrichment of uranium for munitions has transformed it into a significant health risk. Uranium in the hexavalent oxidation state U(VI) is soluble at circumneutral pH and can therefore move into groundwater. However, a variety of microorganisms can reduce soluble U(VI) to the insoluble tetravalent form [U(IV)], precipitating it out of solution, and this metabolism has been proposed as a remediation strategy for precipitation, and therefore immobilization, of uranium (106).

Gregory and Lovley have applied their BER system to uranium bioremediation (107). In studies using defined cultures of *G. sulfurreducens*, U(VI) was removed from solution at a poised potential of -500mV vs. Ag/AgCl with concomitant current flow. When power was removed, uranium remained out of solution in biotic systems but returned to solution in abiotic controls, indicating the biological influence on uranium removal. Similar results were obtained with sediment batch reactors and continuous flow-through sediment columns. The authors suggested that such a system may be able to selectively precipitate uranium on electrode surfaces that could then be extracted to remove the metal from a system entirely.

Future Directions- Understanding the bugs

While significant progress has been made in understanding the physics and chemistry in BERs, the transition of such systems from bench-top experimental settings to widespread use in treatment and synthesis applications will require a better understanding of their biology. Key problems exist with the majority of reactor designs tested thus far. Most of the reactors described here have no reported data for efficiency of electron transfer, i.e., what percentage of applied electrons go to the desired reaction. As such it is impossible to know accurate power requirements for any metabolic process. Hydrolysis-based BERs, while effective, may be restricted from large-scale application due to inefficiencies and the high cost of running power at the necessary potentials as compared to the cost of chemical electron donor addition. Mediator-based systems can be run with lower power requirements, but the addition of a chemical electron shuttle can be expensive, toxic for other applications, or inappropriate in the case of drinking water treatment or in-situ bioelectrical stimulation. In any case, future studies must take into account the overall efficiency of electron transfer for their economic viability to be assessed and for designs to be compared effectively.

With such problems taken into consideration, BERs show the most promise in direct electron transfer to and from microorganisms- ironically, the area of research that is most neglected up to now. The ability of a microbe to directly utilize a working electrode as an electron donor or acceptor in a BER could allow significant reduction in power requirements as well as remove the necessity of an electron shuttle. Also, such systems are inherently more efficient as there is no loss of electrons to unused mediators that either do not come in contact with the organism or diffuse out of the media before being utilized (e.g. hydrogen gas). Additionally, a recent innovative study has demonstrated that cathodic power supply for

biological denitrifcation can be supplied directly from microorganisms transferring electrons to an electrode in the anodic chamber (108). This represents the first example of MFC technology driving a BER process and shows promise for hybridizing the two processes. Direct interactions of microorganisms with electrodes has been heavily utilized in MFC research, and some promising studies have demonstrated potential routes of electron transfer through and out of outer membranes (1, 100, 109-111), but as yet the mechanisms of electron transfer to electrodes are still poorly understood. In any case there may be distinct differences between the process by which a cell receives electrons from a solid surface and by which it donates electrons to such a surface.

Regardless of these differences, the future of BER research will depend on a better understanding of the means by which we can directly stimulate microbial metabolism using electric current. Such understanding can only come from getting to know the microorganisms that can interact directly with electrodes. The variety of pure cultures used in BERs (Table 5) demonstrates the viability of detailed metabolic investigation. Direct electrode oxidation has been demonstrated and studies focused on microbial oxidation of solid-phase iron minerals add credence to the attempt to cultivate more organisms with these capabilities. Enrichments of microorganisms from low-power BERs should yield interesting candidates for further examination. In addition, genetically modified organisms, already a staple of bioreactors in the pharmaceutical industry, would offer even broader opportunities for the interface of biology and electrochemistry. The ability to offer electrons at virtually any metabolically relevant potential can open many new doors for our understanding and application of microbial metabolism. Electrical stimulation of microbial metabolism will yield even more impressive results when we move beyond the limitations of current systems.

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Tables and Figures

Table 1. Biologically important abiotic reactions at the electrode surface. Many abiotic reactions at the electrode surface can influence microbial behavior independent of the stimulated metabolism. Electrolysis reactions can have pH effects due to creation of protons and hydroxide ions. Hydrogen peroxide and chlorine gas, which are both potent inhibitors of some microbial metabolisms, can also be generated. With carbon anodes, oxidation of the electrode to carbon dioxide can influence system pH, alkalinity, and eventually dissolve the electrode itself. Values reported vs. normal hydrogen electrode.

Reaction	E^o (V) vs. NHE	Ref
$O_{2(g)} + 4H^+ + 4e^- \leftrightarrows 2H_2O$	1.230	(6)
$2H_2O + 2e^- \leftrightarrows H_{2(g)} + 2OH^-$	-0.828	(6)
$2H^+ + 2e^- \leftrightarrows H_{2(g)}$	0.0	(6)
$O_{2(g)} + 2H^+ + 2e^- \leftrightarrows H_2O_2$	0.695	(6)
$Cl_{2(g)} + 2e^- \leftrightarrows 2Cl^-$	1.358	(6)
$CO_{2(g)} + 4H^+ + 4e^- \leftrightarrows C + 2H_2O$	-0.213	(11), w/ adjustment from SHE

Table 2. Electrode material diversity. A variety of materials have been employed in BERs for stimulation of microbial metabolisms. Shown are the materials and their used form along with an example reference.

Material	Type	Example Ref
Platinum	Wire	(14)
Platinum	Foil	(9)
Platinum	Mesh	(21)
Platinum	Plate	(61)
Titanium	Wire	(13)
Titanium	Plate	(19)
Iron	Rod	(9)
Lead	Plate	(65)
Stainless steel	Plate	(15)
Stainless steel	Rod	(20)
Stainless steel	Mesh	(91)
Carbon	Amorphous	(12)
Carbon	Activated	(16)
Carbon	Glassy	(17)
Carbon	Graphite block	(5)
Carbon	Graphite powder	(4)
Carbon	Graphite felt	(18)

Table 3. Electrolysis-based BERs for wastewater treatment. Much work has been completed on the use of electrolysis-based BERs for the purpose of nitrogen removal, particularly for the application to wastewater treatment. Although all the reactors listed aim to stimulate nitrification and/or denitrification by electrolytic generation of oxygen and/or hydrogen, there are many unique elements in the design parameters of the various systems.

Treatment	Reactor configuration	Volume	Cathode	Anode	Current range	Voltage	Cathodic current density	Temp	Treated concentration	Year	ref
Denitrification	Two-chamber/no membrane	2.4L × 2	n/a	n/a	10-40mA	0-37V	0.02-0.08mA/cm^2	25-30°C	44mg/L NO_3^-	1993	(3, 29)
Denitrification/ Neutralization/ Pesticide removal	Single chamber	0.205L	Stainless steel	Carbon rod	2.5mA	2.2V	0.01mA/cm^2	25°C	62mg/L NO_3^-	1994	(28, 30), (12, 31, 32)
Denitrification	Single chamber	1.48 L	Carbon	Carbon	0-100mA	n/a	0.0-0.16mA/cm^2	25°C	89mg/L NO_3^-	1994	(33)
Nitrification/ Denitrification	Single chamber	7.9L	Carbon rods	Carbon rod	100mA	n/a	0.08 mA/cm^2	38°C	155mg/L NO_3^- 64mg/L NH_4^+	1996	(24)
Denitrification	Single chamber	2.8L	Carbon cylinder	Carbon rod	100mA	n/a	0.11mA/cm^2	n/a	42.5mg/L NO_3^-	1996	(24)
Denitrification	Single chamber	~1.5 L	Carbon cylinder	Carbon rod	0-100mA	n/a	0.0-0.16mA/cm^2	n/a	20mg/L NO_3^-	1998	(34)
Denitrification	Single chamber sand column	4.5 L no sand	Stainless steel	Graphite	0-20mA	n/a	0.0-1.42mA/cm^2	n/a	89mg/L NO_3^-	1998	(20)
Denitrification	Single chamber	3.5L	Steel & graphite	Graphite	12mA	n/a	0.28mA/cm^2*	n/a	722mg/L NO_3^-	1998	(15)
Denitrification	Two-chamber	0.750L	Steel, graphite, copper	Steel	1mA	n/a	0.03mA/cm^2*	n/a	89mg/L NO_3^-	1998	(15)
Nitrification/ Denitrification	Single chamber	100L	Activated carbon packed bed	Activated carbon packed bed	0-450mA	0-50V	0.0-7.5mA/cm^2	10-25°C	613mg/L NO_3^- 3mg/L NH_4^+	2000	(16)
Denitrification	Two-chamber	0.017L	Graphite	Platinum	20, 40mA	n/a	0.2, 0.41 mA/cm^2	26°C	111mg/L NO_3^-	2000	(35), (19)
Denitrification	Two-chamber	36L	Titanium × 8	Pt-coated metal × 2	80-960mA	n/a	n/a	25°C	89mg/L NO_3^-	2001	(36)
Denitrification/ Neutralization/ Metal removal	Single chamber	~5L	Carbon rod	Carbon rod	n/a	n/a	0.0-0.09mA/cm^2	35°C	886mg/L NO_3^-	2001	(37, 38)
COD removal	Single-chamber	27L	Stainless steel	Titanium	0-500mA	n/a	0.0-25mA/cm^2	n/a	~400mg/L COD	2002	(112)
Denitrification	Two-chamber	0.6L	Granular activated carbon × 5	Pt-coated titanium	40-300mA	n/a	0.05-0.4mA/cm^2	n/a	177mg/L NO_3^-	2002	(39, 40)
Nitrification/ Denitrification	Single chamber	3.8L	Expanded metal	Expanded metal	0-51mA	n/a	0.0-0.3mA/cm^2	30°C	221mg/L NO_3^- 64mg/L NH_4^+	2002	(25)
Nitrification	Single chamber	0.175L	Stainless steel	Titanium	25-50mA	n/a	1.25-2.5mA/cm^2	20°C	~45mg/L NH_4^+	2005	(42)

Nitrification/ Denitrification	Two-chamber	3.2L	Stainless steel	Titanium	10-20mA	n/a	0.5-1.0 mA/cm^2	23°C	63mg/L NH_4^+	2005	(43)
Denitrification	Two-chamber	1.0L	Graphite felt	Dimension-ally stable	0-500mA	n/a	0.0-4.8mA/cm^2	30°C	2179mg/L NO_3^-	2005	(41)

*stainless steel electrodes had steel mesh connected, thereby decreasing reported current density.

Table 4. Electron shuttles used in BERs. Shuttles used by the studies reviewed here, along with their corresponding electrochemical potential.

Shuttle	Use (e-donor/acceptor)	Reduction potential E^o (V)	Ref
Methyl viologen	Donor	-0.450	(17), (79), (13, 80)
Cobalt sepulchrate	Donor	-0.350	(21)
Neutral red	Donor	-0.325	(14, 83), (84, 113)
AQDS	Donor	-0.184	(21), (4)
Iron	Donor	0.760	Examples in Refs (18), (60), (54).
Iron	Acceptor	0.760	(23)

Table 5. Pure cultures used in BERs. A wide variety of organisms have been used in BER studies. The functional flexibility and ultimate applicative diversity of BERs depends on the microbes within them. As pure culture studies have demonstrated, microbes from all over the phylogenetic tree have the ability to not only survive the BER environment, but also make very good use of electrical stimulation.

Domain	Organism	Stimulated Metabolism	Ref
Bacteria	*Acidithiobacillus ferrooxidans*	Aerobic iron oxidation/ Anaerobic iron reduction	(54), etc./ (23)
	Actinobacillus succinogenes	Glucose fermentation	(84, 113)
	Brevibacterium flavum	Glucose fermentation	(14, 83)
	Clostridium acetobutylicum	Glucose fermentation	(79), (13, 80)
	Clostridium thermocellum	Complex media fermentation	(86)
	Dechlorospirillum strain VDY	Perchlorate reduction	(4)
	Desulfovibrio desulfuricans	Sulfate reduction	(74)
	Enterobacter dissolvens	Glucose fermentation	(82)
	Escherichia coli	Aerobic complex media oxidation	(78)
	Geobacter metallireducens	Nitrate reduction	(5)
	Geobacter sulfurreducens	Fumarate reducton Uranium reduction	(5, 107)
	Leptospirillum ferrooxidans	Aerobic iron oxidation	(64)
	Propionibacterium acidi-propionici	Complex media fermentation	(77)
	Propionibacterium freudenreichii	Glucose fermentation	(21)
	Pseudomonas fluorescens	Aerobic glucose oxidation	(9)
	Ralstonia eutropha H16	Aerobic hydrogen oxidation	(22)
Eukarya	*Saccharomyces cerivisiae*	Complex media fermentation	(85), (86)
	Trichosporon capitatum	Br-ß-tetralone reduction	(98)

Figure 1. Electrical stimulation of microorganisms
BERs can stimulate microbial metabolism by acting as cathodic electron sources or anodic electron sinks. In each case, reduction or oxidation, respectively, of a substrate is coupled to the electrical stimulus by the microorganism.

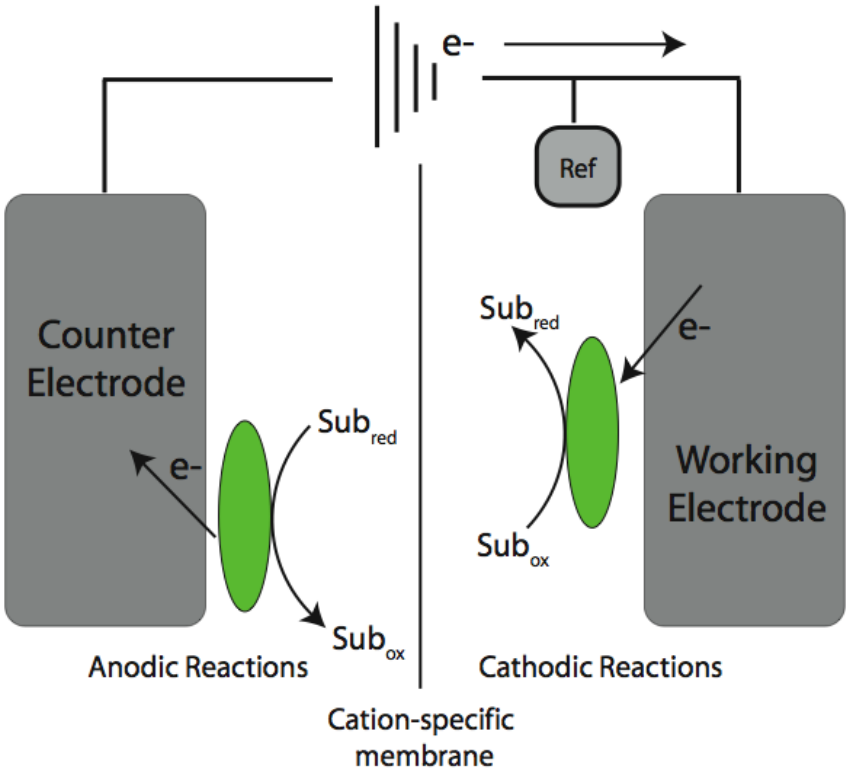
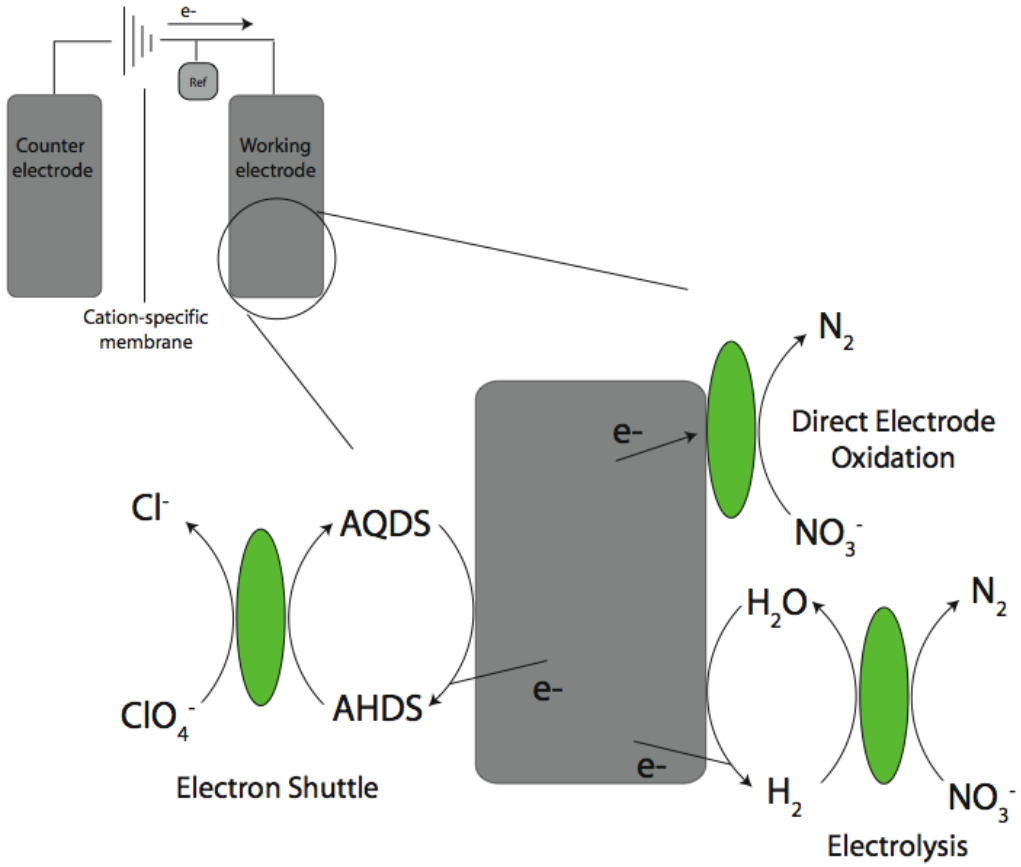


Figure 2. Mechanisms of electron transfer
The three means of transferring electrons between electrodes and microorganisms are pictured here with representative cathodic reaction examples. Indirect methods: electrolysis of water to hydrogen can provide electron-donating capacity for organisms reducing nitrate; electron shuttling by AQDS can stimulate microbial perchlorate reduction. Direct electrode oxidation has been used for reduction of nitrate.



This chapter was published in Environmental Science and Technology under the following reference:

Thrash, J. C., and J. D. Coates. 2008. Review: Direct and Indirect Electrical Stimulation of Microbial Metabolism. Environ. Sci. Technol. 42:3921-3931.

Chapter 2
Electrochemical Stimulation of Microbial Perchlorate Reduction

Abstract

As part of our studies into the diversity of dissimilatory perchlorate reducing bacteria (DPRB) we investigated the reduction of perchlorate in the cathodic chamber of a bioelectrical reactor (BER). Our results demonstrated that washed cells of *Dechloromonas* and *Azospira* species readily reduced 90 $mg.L^{-1}$ perchlorate in the BER with 2,6-anthraquinone disulfonate (AQDS) as a mediator. No perchlorate was reduced in the absence of cells, AQDS, or in an open circuit control. Similar results were observed when a natural microbial community was inoculated into a fed-batch BER. After 70 days operation, a novel DPRB, strain VDY, was isolated which readily reduced perchlorate in a mediatorless BER. Continuous up-flow BERs (UFBERs) were seeded with active cultures of strain VDY and perchlorate at a volumetric loading of 60 $mg.L^{-1}.day^{-1}$ was successfully removed. Gas phase analysis indicated that low levels of H_2 produced at the cathode surface through electrolysis may mediate this metabolism.

The results of these studies demonstrate that biological perchlorate remediation can be facilitated through the use of a cathode as the primary electron donor, and that continuous treatment in such a system approaches current industry standards. This has important implications for the continuous treatment of this critical contaminant in industrial waste streams and drinking water.

Introduction

Perchlorate (ClO_4^-), a soluble anion, is known to affect mammalian thyroid hormone production potentially leading to neonatal neuropsychological development deficiencies (1-3). It is predominantly a synthetic compound with a broad assortment of industrial applications ranging from pyrotechnics to lubricating oils (4). Ammonium perchlorate represents 90% of all perchlorate salts manufactured and is used as an energetics booster or oxidant in solid rocket fuels and munitions (4). Its presence in the environment primarily results from legal historical discharge of unregulated manufacturing waste streams, disposal pond leachate, and the periodic servicing of military inventories (5-7). Although a powerful oxidant, under most environmental conditions perchlorate is quite stable owing to the high energy of activation associated with its reduction (5,6). Perchlorate salts readily dissociate in aqueous phases because of the large molecular volume and single anionic charge (5). Furthermore, perchlorate does not significantly sorb to soils or sediments and, in the absence of any biological interactions, its mobility and fate are largely influenced by the hydrology of the environment (8).

Remediation efforts for perchlorate contamination have focused primarily on microbial reduction (5,9). Many recent studies have demonstrated that specialized microorganisms have evolved that can grow by the anaerobic reductive dissimilation of perchlorate into innocuous chloride (9,10). More than forty dissimilatory perchlorate-reducing bacteria (DPRB) are now in pure culture (9) and organisms capable of this metabolism are known to be ubiquitous in soil and sedimentary environments (11) making in-situ treatments relatively straightforward. Furthermore, several bioreactor designs are available for the ex-situ biological attenuation of perchlorate-contaminated waters. Recently, some of these reactor designs were approved by the California Department of Health Services for application in the treatment of perchlorate contaminated drinking water (URL http://www.safedrinkingwater.com/archive/sdwn051502.htm). However, these systems are still dependent on the continual addition of a chemical electron donor to sustain microbial activity and are always subject to biofouling issues (12,13). In addition, residual labile electron donor in the reactor effluent can stimulate microbial growth in water distribution systems and contribute to the formation of potentially toxic trihalomethanes during disinfection by chlorination (14,15). To overcome these problems, chemolithotrophic perchlorate-reducing bioreactors utilizing H_2 as an electron donor have been proposed (16,17). However, H_2 in bulk quantities is difficult to handle and is publicly perceived as representing a significant disaster threat due to its inherent explosive nature. Alternative inorganic electron donors including Fe(II), S^o, or H_2S may offer a more practical approach, however, regular additions of these compounds to bioreactors are still required. Furthermore, H_2S is a malodorous toxic compound which can cause corrosion issues while the particulate ferric (hydr)oxides resulting from Fe(II) oxidation result in unpleasant taste and odor, clogged pump-and treatment systems, and anodic corrosion of steel pipes and distribution lines (18-20).

Here we investigated the use of a negatively charged electrode (cathode) in the working chamber of a bioelectrical reactor (BER) as an electron donor for microbial perchlorate reduction. In this instance the DPRB can use the electrons on the electrode surface and can include indirect transfer as a source of reducing equivalents for perchlorate reduction, while assimilating carbon from CO_2 or alternative available carbon sources. Such a process has the advantage of long-term, low-maintenance operation while limiting the injection of additional chemicals into the water treatment process. This would negate downstream issues associated

with corrosion and biofouling of distribution systems and the production of toxic disinfection byproducts.

Materials and Methods

BER construction. The BER was constructed of two 50 mm diameter glass chambers with three sample ports, connected with a 30 mm pinch clamp assembly (Laboratory Glass Apparatus, Berkeley, CA). Chambers were separated with a cation exchange membrane, (Nafion 117). Sample ports were sealed with thick butyl rubber stoppers and aluminum crimp seals. The electrodes were 2.5 x 1.25 x 7.5 cm unpolished graphite (G-10 Graphite Engineering and Sales, Greenville, MI) connected with watertight threaded fittings (Impulse, San Diego, CA) to wires and sealed with conductive silver epoxy (Epoxy Technology, Billerica, MA). Wires were fed through stoppers in the top of each chamber. Prior to initial use and before each use thereafter, the electrodes were washed repeatedly in 1N HCl to remove residual minerals and any biomass buildup and rinsed in sterile deionized water. The silver (Ag/AgCl) reference electrode (Warner Instruments, Hamden, CT) was threaded through a butyl stopper after sterilization with ethanol. Prior to inoculation, the chambers and all stoppers were autoclaved to ensure sterility and subsequently flushed with filter sterilized (0.22 μm pore size) N_2-CO_2 (80:20; vol:vol) gas. Throughout the study the anodic chamber was filled with pre-sterilized, anaerobic 30 mM (1.83 $g.L^{-1}$) bicarbonate buffer (pH 6.8) while the cathodic chamber was filled with either bicarbonate buffer (cell suspensions) or basal growth medium (enrichment/growth studies) outlined below and amended with perchlorate as the sole electron acceptor. Unless otherwise noted 500 μM AQDS (183 $mg.L^{-1}$) was added as an appropriate electron shuttle. The growth media was further amended with acetate (5.9 $mg.L^{-1}$) as a suitable carbon source. Both chambers were bubbled continuously with filtered (0.22 μm pore size) N_2:CO_2 (80:20; vol:vol) gas to ensure anaerobic operation and maintenance of pH throughout operation. A custom-built potentiostat (Berkeley BioScientific; Dublin, Ireland) was connected to a digital multimeter (Thurlby Thandar Instruments, Cambridgeshire, UK) for control of the electrochemical potential.

When run in continuous-flow experiments, 1/16" ID Norprene tubing (Saint Gobain Performance Plastics, Paris, France) was threaded through 1ml syringe housings embedded in butyl stoppers in the ports of the cathodic chamber. Flow was established using a peristaltic pump (Rainin, Oakland, CA). The cathodic chamber was filled with powdered graphite that was held in place by a thin (0.5 cm) layer of acid washed sand. A single unpolished graphite (G-10 Graphite Engineering and Sales, Greenville, MI) electrode connected with watertight threaded fittings (Impulse, San Diego, CA) to wires and sealed with conductive silver epoxy (Epoxy Technology, Billerica, MA) was plunged into the powdered graphite bed to ensure electrical conductivity. The influent was pumped in an up-flow mode through the graphite bed, which was maintained at a potential difference of 500 mV relative to the reference electrode. The reactor was inoculated (10% by volume) with an active perchlorate-reducing culture of strain VDY which was spun down and resuspended in an equal volume of anoxic sterile growth media prior to injection into the graphite bed through the influent port.

Medium and culturing conditions. All pure cultures were obtained from lab stocks except strain VDY which was isolated in this study. Standard anaerobic culturing techniques were used throughout (21-23). The medium was boiled and cooled under N_2-CO_2 (80:20) to remove dissolved O_2 and then dispensed into anaerobic pressure tubes or serum bottles under N_2-CO_2, capped with thick butyl rubber stoppers, and sterilized by autoclaving. The basal medium was the bicarbonate-buffered freshwater medium that had previously been used for culturing perchlorate-reducing bacteria (24). Unless otherwise noted, sodium salts of acetate and

perchlorate at 10 mM each (590 $mg.L^{-1}$ and 990 $mg.L^{-1}$, respectively) were added from sterile anoxic aqueous stocks as the electron donor and acceptor respectively.

Alternative electron donors and acceptors were added from sterile anoxic aqueous stocks prepared from the sodium salts to give final concentrations of 10 mM in the culture medium. All culture incubations were performed at room temperature without agitation in the dark.

Washed cell suspensions. Cells were pre-grown anaerobically in 1L volumes with acetate (590 $mg.L^{-1}$) as the electron donor and perchlorate (990 $mg.L^{-1}$) as the sole electron acceptor. After 18 hours incubation the cells were harvested by centrifugation (3,000 x g) and washed with anoxic bicarbonate buffer (1.83 $mg.L^{-1}$) under an N_2:CO_2 (80:20; vol:vol) headspace. Washed cells were resuspended in anoxic bicarbonate buffer (1 ml) and sealed in a 10ml serum vial with a butyl stopper under the same headspace.

DPRB isolation. Perchlorate-reducing enrichments were established by transferring 1 g of electrode surface scrapings into 9 ml of prepared anoxic medium under a gas stream of N_2:CO_2 (80:20; vol:vol). Acetate (590 $mg.L^{-1}$) was the electron donor and perchlorate (990 $mg.L^{-1}$) was the electron acceptor. Incubations were done at 30 ^{o}C in the dark. Positive enrichments were identified by visual increase in optical density and by microscopic examination. Once a positive enrichment was established the perchlorate-reducing culture was transferred (10% inoculum) into 9 ml of fresh anoxic medium. Isolated colonies were obtained from transfers of positive enrichments by the standard agar shake-tube technique outlined previously (24) with acetate as the sole electron donor and perchlorate (590 $mg.L^{-1}$ and 990 $mg.L^{-1}$, respectively) as the sole electron acceptor.

16s rRNA gene sequencing and analysis. Cells from 10-ml cultures of the isolated perchlorate reducing bacteria were harvested by centrifugation, resuspended in 40 μl sterile water, and lysed by the addition of 5 μl chloroform with a 10 min incubation at 95 ˚C. Primers specific to bacterial 16S rDNA (8F: 5'- AGAGTTTGATCCTGGCTCAG-3'; 1525R: 5'-AAGGAGGTGATCCAGCC-3') were used in a polymerase chain reaction (PCR) that consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.2 mM $MgCl_2$, 0.2 mM each dNTP, 75 ng of each primer, 0.5 μl Taq polymerase (Gibco/BRL), and 1 μl of lysed cells in a 50 μl reaction. Amplifications were performed at these parameters: 94 ˚C for 3 min, followed by 30 cycles of 94 ˚C for 1 min, 55 ˚C for 1 min, and 72 ˚C for 2 min with a final incubation of 10 min at 72 ˚C. The amplification products were gel-purified (GeneClean II, BIO101) and cycle sequenced (ThermoSequenase, Amersham) using internal primers. Sequence entry and manipulation was performed with the MacVector 8.1 sequence analysis software program for the Macintosh (Oxford Molecular). Sequences of select 16S rRNAs were downloaded from the Ribosomal Database Project (25) and Genbank (26) into the computer program SeqApp (27). The 16S rDNA sequence from strain VD was manually added to the alignment using secondary structure information for proper alignment. Distance analysis of the aligned sequences was performed using PAUP* 4.0d65 (28).

Analytical methods. All experimental analyses were performed in triplicate to ensure reproducibility and the results are expressed as the mean of these determinations. Controls were complete in singlet unless otherwise stated. The concentration of perchlorate in cultures was determined using ion chromatography as previously described (29). Cell growth in active

cultures was monitored by optical density at 600nm (OD600), and by total direct cell count using phase-contrast microscopy as previously described (11).

Results

Bioelectrical reduction of perchlorate by pure cultures of DPRB. Active washed cell suspensions of *Dechloromonas agitata, D. aromatica, and Azospira suillum*, three representatives of the environmentally dominant perchlorate reducing bacterial genera, readily reduced 99 $mg.L^{-1}$ perchlorate over a twenty four hour trial when incubated in the cathodic chamber of the electrochemical cell at a poised potential of -450 mV (relative to the Ag reference electrode) containing 183 $mg.L^{-1}$ (500 μM) 2,6-anthraquinone disulfonate (AQDS) (Fig. 1, a representative example of the three separate incubations). In all cases, the rate and extent of perchlorate reduction was almost identical to that observed in the positive control to which acetate (59 $mg.L^{-1}$) was added as the sole electron donor (Fig. 1). In contrast, no significant perchlorate reduction was observed in identical incubations in which the electrical circuit was incomplete (open circuit control) (Fig. 1) suggesting that perchlorate reduction was an enzymatically-mediated reaction using electrons derived from the surface of the cathode. Although perchlorate was continuously removed none of the tested DPRB (*D. agitata, D. aromatica,* or *A. suillum*) grew in the BER, as demonstrated by unchanged OD 600, when similar experiments were performed in the BER using basal growth medium amended with 5.9 $mg.L^{-1}$ acetate as a suitable carbon source (data not shown).

In all incubations, no significant perchlorate reduction occurred relative to the open circuit control if the AQDS was omitted from the medium even after an extended incubation period of 72 hours (data not shown). In order to ensure that the observed perchlorate reduction was not due to the biological catabolism of the added AQDS, perchlorate concentrations were monitored in active anaerobic cultures of *D. agitata* incubated with AQDS as the sole electron donor in sealed serum vials. Again, no perchlorate reduction was observed unless acetate was added as an additional electron donor (data not shown). In contrast, if AH_2DS (the reduced hydroquinone form of AQDS) was provided *D. agitata* readily reduced the perchlorate with the concomitant oxidation of the AH_2DS to AQDS (Fig. 2). In the absence of either perchlorate or cells no AH_2DS oxidation was apparent and in the absence of AH_2DS no perchlorate reduction was observed (Fig. 2). Analysis of the concentration of AQDS and AH_2DS in the medium during incubation revealed that the total anthraquinone concentration remained constant during incubation and was not being biodegraded as a carbon source by the organisms (data not shown). Furthermore, the oxidation of 1.54 $g.L^{-1}$ (4.2 mM) AH_2DS resulted in the reduction of 98 $mg.L^{-1}$ (0.99 mM) of perchlorate giving a stoichiometry of 4.2, which is similar to the predicted theoretical ratio according to:

$$4C_{14}H_8O_8S_2^{2-}\ (AH_2DS) + ClO_4^- \rightarrow 4C_{14}H_6O_8S_2^{2-}\ (AQDS) + Cl^- + 4H_2O$$

These results indicate that perchlorate reduction in the BER was microbially mediated by electrons being shuttled from the cathode surface to the bacterial cells by the AQDS.

Bioelectrical reduction of perchlorate by natural DPRB populations. Significant perchlorate removal was observed under growth conditions when the cathodic chamber of the BER was filled with sterile anoxic basal media amended with perchlorate, 183 $mg.L^{-1}$ (500 μM) AQDS, poised at -500mV (vs. Ag/AgCl), and inoculated with 10% v/v water from Strawberry Creek on the UC Berkeley campus that had been filtered with 11μm filter paper. Over the first three weeks, almost 200 $mg.L^{-1}$ perchlorate was removed in the enrichment compared to an open-circuit control (Fig. 3). At this time the medium in both the open- and closed-circuit BER's was amended with 0.1 $mg.L^{-1}$ yeast extract for continued cell maintenance. As expected, this

resulted in a sharp increase in the amount of perchlorate consumed in both systems, but thereafter perchlorate levels stabilized in the open-circuit control while the closed-circuit BER continued to remove perchlorate. An amendment of perchlorate and an additional 0.1 $mg.L^{-1}$ yeast extract at day 30 showed similar results, and after 70 days of operation, the closed-circuit enrichment had removed 1000 $mg.L^{-1}$ more perchlorate than the open circuit control.

Isolation and characterization of strain VDY. To identify some of the DPRB present in the BER inoculated with Strawberry Creek groundwater, samples (1 g) were scraped from the surface of the cathode after the 70-day incubation and transferred into fresh basal medium with acetate as the electron donor and perchlorate as the sole electron acceptor. After two weeks incubation growth was visually apparent in the primary enrichments of these samples. These enrichments were transferred into fresh basal medium (10% inoculum) where good growth was again observed after 24 hours as determined by increase in optical density and microscopic examination. Highly-enriched, perchlorate-reducing cultures were obtained by sequential transfer over the following week prior to serial dilution into agar tubes. Small (1-2mm diameter) pink colonies of consistent morphology were apparent after two weeks of incubation, and a dissimilatory perchlorate-reducing isolate, strain VDY, was selected for further characterization.

Strain VDY was a gram-negative, facultative anaerobe. Cells, 0.2 μm diameter by 7 μm length showed a consistent spirillum morphology. Strain VDY completely oxidized organic electron donors to CO_2 in the presence of a suitable electron acceptor. Alternatively, strain VDY grew fermentatively in anoxic basal medium amended with glucose (1.80 $g.L^{-1}$), yeast extract (0.1 $g.L^{-1}$) and casamino acids (0.1 $g.L^{-1}$). Spores were not visible in wet-mounts by phase contrast microscopy and no growth was observed in fresh acetate-perchlorate medium after pasteurization at 80 ^{o}C for 3 minutes. In addition to acetate, strain VDY used lactate, AH_2DS, ethanol, and H_2 as electron donors and perchlorate, chlorate, nitrate, or O_2 as electron acceptors. Analyses of the 16S rDNA sequences indicated that strain VDY was closely related (>99% 16S rDNA sequence identity) to the known DPRB *Dechlorospirillum anomalous* strain WD in the alpha subclass of the proteobacteria (Fig. 4).

Pure culture BER studies with strain VDY. As with the other DPRB tested (*D. agitata, D. aromatica, and A. suillum*), perchlorate was rapidly removed in the cathodic chamber of a BER poised at -500 mV when inoculated with strain VDY (Fig. 5). Although no significant growth was observed the cell density in the BER remained constant throughout the incubation, while that of the open circuit control rapidly declined (data not shown). No perchlorate removal was observed in the open circuit control, however, in contrast to the results obtained with the other DPRB, strain VDY was capable of reducing perchlorate in the BER in the absence of the mediator AQDS (Fig. 5), although this removal was significantly slower than that in the BER amended with AQDS. Analysis of H_2 production in the BER indicated that under operational conditions 0.78 $\mu g.min^{-1}$ were produced through electrolysis of water at the surface of the cathode which is more than enough reducing equivalents to account for the observed reduction of the perchlorate (2 $\mu g.min^{-1}$) in the mediatorless BER throughout the incubation assuming a theoretical stoichiometry of

$$4H_2 + ClO_4^- \rightarrow Cl^- + 4H_2O$$

Continuous treatment of perchlorate in an UFBER. In order to determine the applicability of the BER system combined with strain VDY to the continuous treatment of perchlorate, bench-

scale up-flow bioelectrical reactors were constructed in triplicate in the same BER architecture used for the batch experiments. The active chamber contained a packed-bed of graphite powder charged by a graphite electrode inserted into the bed to maximize the surface area available for electron donation by the electrode. The reactors were inoculated with anaerobic active washed cells of strain VDY previously grown on acetate and perchlorate. To promote establishment of the DPRB culture in the bed and to provide a starting baseline for the closed- and open-circuit reactors, acetate (82 $mg.L^{-1}$) was added with strain VDY. AQDS was added at 183 $mg.L^{-1}$ (500 μM) to these initial experiments and the hydraulic loading rate of perchlorate in the influent was initially set at 60 $mg.L^{-1}.day^{-1}$ for the first twelve days. As expected in response to the acetate injections, perchlorate levels in the effluent of all reactors was negligible for the first three days operation (Fig. 6). However, the perchlorate levels in the open-circuit control soon began to rebound to the influent concentration and stabilized at that level for the remainder of the incubation (Fig. 6). In contrast, perchlorate was consistently removed throughout the incubation in the closed-circuit reactors and at least 80% removal efficiency was continuously achieved in each of the duplicate reactors for the three weeks of operation (Fig. 6). Variations in effluent perchlorate concentration throughout the incubation were due to adjustments of the reactor residence time, which was optimized to 3 days at day 16, corresponding to a volumetric loading rate of 40 $mg.L^{-1}.day^{-1}$. After residence time optimization, effluent perchlorate concentrations were consistently below detection for the remainder of the incubation (Fig. 6). Furthermore, one of the UFBER replicates was kept operational after the experiment was concluded at day 21 and while there was no concurrent negative control, this reactor successfully maintained non-detectable perchlorate concentrations in the effluent for a further 40 days without any fluctuations in efficiency (data not shown).

While successful for treating perchlorate, the presence of AQDS was not optimal for water treatment as it adds cost to the treatment and would require an additional step to remove it from the treated effluent. Furthermore, headspace gas analysis indicated that significantly more H_2 was produced (3.72 $\mu g.min^{-1}$) in the UFBER configuration than in the batch BERs, probably because of the much larger cathodic surface area, which would theoretically provide enough reducing equivalents to reduce all of the perchlorate at a volumetric loading rate of 66 $mg.L^{-1}.day^{-1}$. In order to determine the necessity of the mediator several UFBERs were constructed, inoculated with an active perchlorate reducing culture of strain VDY and amended with 59 $mg.L^{-1}$ acetate as before. An initial perchlorate volumetric loading rate of approximately 60 $mg.L^{-1}.day^{-1}$ was set. Where necessary AQDS was added to the influent to give a final concentration of 0.4 $mg.L^{-1}$ (1 μM). As predicted, immediate perchlorate removal was apparent in the presence and absence of the AQDS as well as in the open circuit control because of the initial amendment with acetate. However, the effluent perchlorate content of the open circuit control rapidly rebounded to levels approaching the influent concentration within 48 hours (Fig. 7). In contrast, perchlorate removal was consistent both in the presence and absence of AQDS in the closed circuit UFBERs for the remainder of the operation. Removal efficiencies were essentially identical regardless of the presence or absence of AQDS and both UFBERs continuously achieved removal efficiencies of greater than 95% (Fig. 7) indicating that AQDS was not necessary to achieve effective treatment efficiencies at high loading rates in these reactors.

Discussion

Electrodes as electron donors for microbial metabolism. These studies represent the first demonstration that electrodes can serve as a primary electron donor for microbial perchlorate reduction and have resulted in the isolation and characterization of a novel isolate, *Dechlorospirillum* strain VDY, that can exist stably in the BER for extended treatment periods. Previous studies have similarly demonstrated the use of an electrode as the primary electron donor for the dissimilatory reduction of nitrate by *Geobacter* species (30,31), fumarate by both *Geobacter* and *Actinobacillus* species (30,32), hexavalent uranium by *Geobacter* species (33), and CO_2 by an undefined enrichment (31). Furthermore, electrochemical reduction of soluble iron by a cathode has also been shown to support growth and CO_2 fixation by the iron-oxidizing *Acidithiobacillus* species (34,35). In contrast to those studies, however, electrochemically generated H_2 through the electrolysis of water at the cathode surface is likely to play a role in the microbial reduction of perchlorate observed in our BER's amended strain VDY in the absence of AQDS. This may also explain the conflicting results obtained with strain VDY and the other DPRB tested in this study none of which could reduce perchlorate in the BER in the absence of AQDS. This is because H_2 is not utilized as an electron donor by the *Dechloromonas* or *Azospira* species tested in this study (24,36-38) while, in contrast, physiological characterization revealed that strain VDY could readily use H_2 as an electron donor for respiration.

However, other alternatives to H_2 mediation may also exist. The previous *Geobacter*-based studies showed members of this genus were able to directly access electrons off the electrode surface (30) without the need for electrolytically produced H_2. *Geobacter* species are similarly known to pass electrons directly onto an anodic surface in the absence of electron shuttling compounds when oxidizing organic electron donors (39). Whether or not these organisms use a reversal of a single pathway to mediate these contrasting electron transfers is unknown. Additionally, microbially produced electron shuttles have also been shown to play an important role in electron transfer in the anodic chamber of microbial fuel cells (40). While strain VDY has been shown to utilize hydrogen for the reduction of perchlorate, the possibility still exists that it may also utilize electrons directly from the electrode surface or produce an electron shuttling compound to further supplement its metabolism of perchlorate in the cathodic chamber and this represents an important future direction for this research. Regardless of the mechanism of electron transfer off the electrode surface, the removal of AQDS or any other additional organic carbon source from the BER system is advantageous for treatment cost-effectiveness as well as effluent water quality.

Reactor effectiveness. The results obtained in this study indicate that the stimulation of microbial perchlorate reduction in the UFBER is already competitive with existing state of the art bioreactors without any electrochemical optimization. Current high-throughput treatment of concentrated wastestreams is over 400 $mg.L^{-1}.day^{-1}$ (41), however, average volumetric loads are one tenth of that. The UFBER in this study showed consistent removal of 60 $mg.L^{-1}.day^{-1}$, without addition of a chemical electron donor. This is significant given the inherent inefficiencies of the H-style electrochemical cell employed for these studies. The limitations to microbial stimulation stem from high internal resistance that can be overcome easily with design modifications already in use for a variety of microbial fuel cell (MFC) technologies. These modifications included increased cation-specific membrane surface area or removal of the cation-specific membrane altogether, as well as decreased distances between the anode and

cathode. Given the order of magnitude improvements in power production from these types of modifications in MFCs (42), it would be expected that similar modifications would improve UFBER performance for perchlorate treatment.

Significance. The results presented here indicate that microbial perchlorate reduction can be coupled to the removal of electrons off the surface of an electrode. This has important implications with regards to the continuous long-term treatment of perchlorate contaminated waters and wastestreams. Although previous studies have resulted in the development of various alternative bioreactor designs (13) all of these are limited by the requirement for a continuous addition of a suitable chemical electron donor. Because microbial perchlorate reduction is generally inhibited by the presence of O_2 and to some extents nitrate (29,43), excess electron donor must be added to biologically remove these components from reactor influents prior to the stimulation of perchlorate reduction. Such additions must be carefully monitored to prevent the presence of residual labile electron donor in the reactor effluent which may result in biofouling of distribution systems and the formation of trihalomethanes (14,15). This is especially true if the total electron accepting capacity of the perchlorate present in the contaminated stream is small relative to that of the nitrate and dissolved O_2 content which is the case for most contaminated waters (9). Bioelectrical reduction at the cathode surface overcomes many of these issues because no chemical electron donor is added to the bioreactor.

In conclusion, the results presented here demonstrate the exciting potential for the application of bioelectrical reduction for the treatment of perchlorate contamination without many of the limitations normally associated with bioreactor-based processes.

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Figures

Figure 1. Perchlorate reduction by *Azospira suillum* strain PS using acetate or the BER cathode as the primary electron donor.

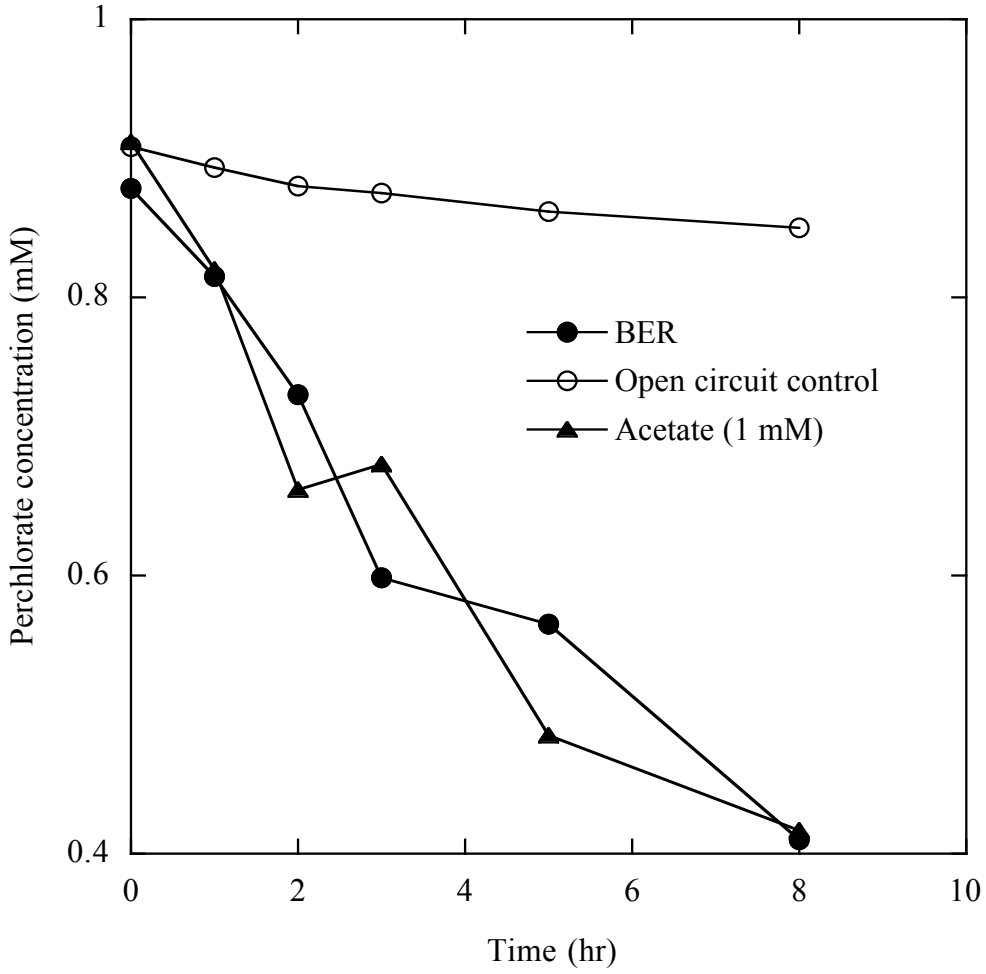


Figure 2. Perchlorate reduction coupled to AH_2DS oxidation by *Dechloromonas agitata* strain CKB. Data here are representative of triplicate incubations.

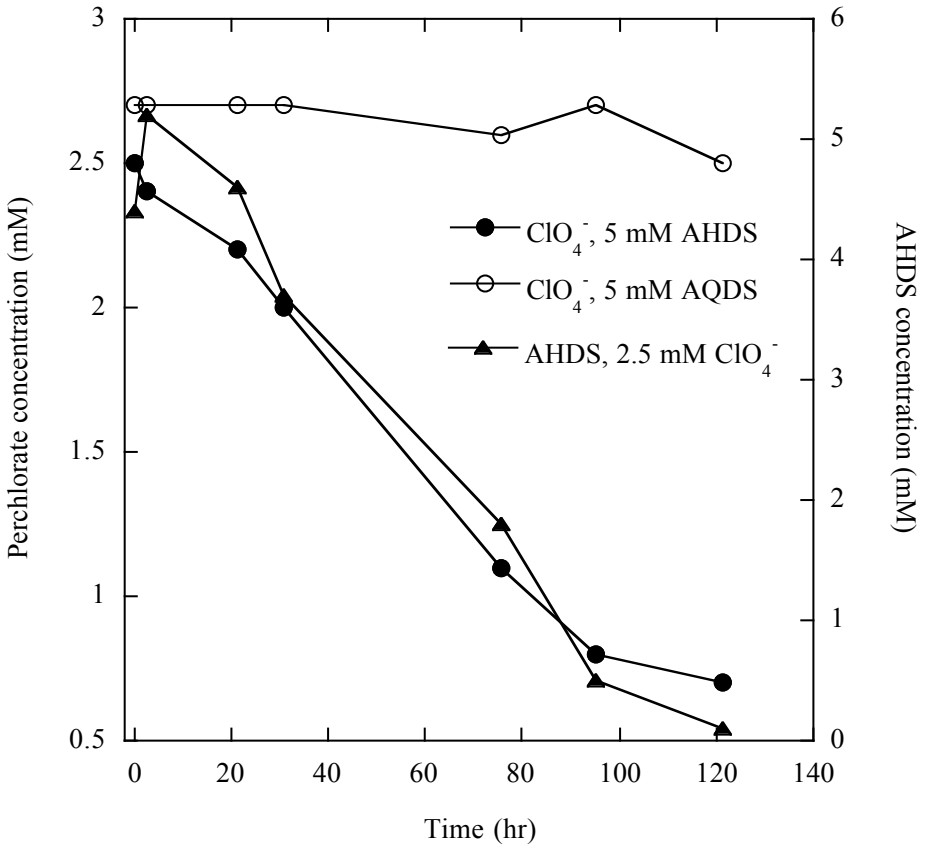


Figure 3. Cumulative perchlorate reduction in the BER and open circuit control by the indigenous DPRB population in Strawberry Creek waters collected from UC Berkeley campus. This data is representative of duplicate experiments.

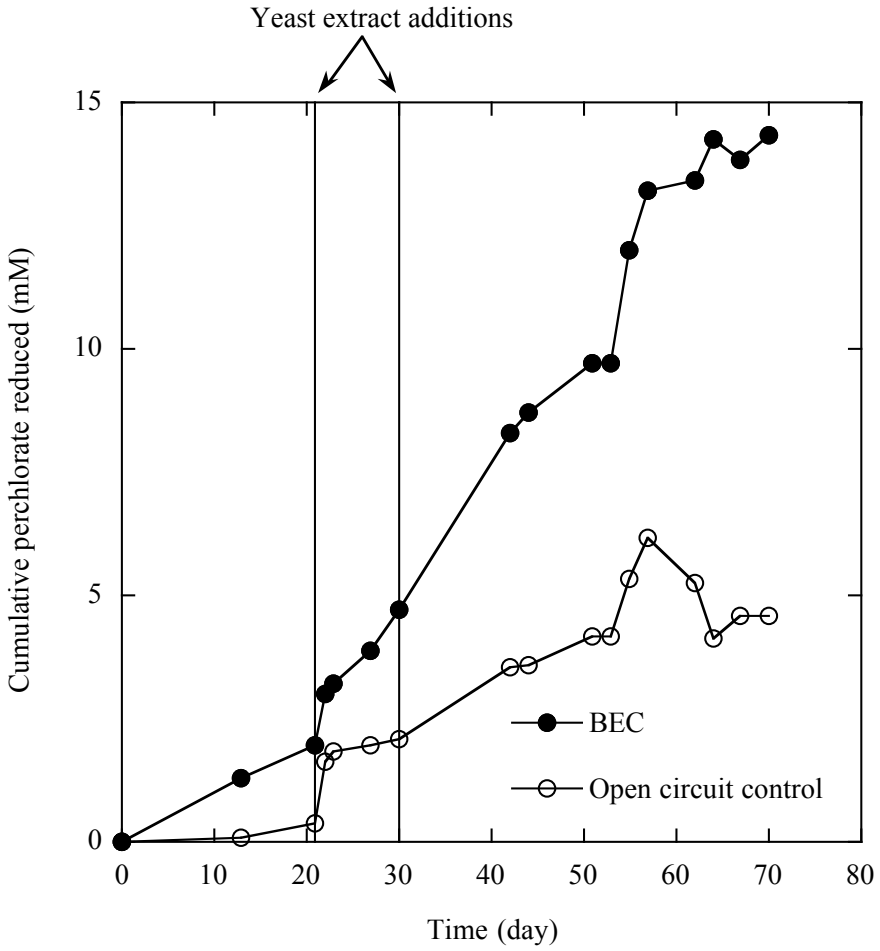


Figure 4. Phylogenetic tree resulting from heuristic analysis of a 16S rRNA dataset consisting of 1411 characters using the Kimura 2-parameter distance setting. Bootstrap values of >50% from 500 replicates are indicated near the relevant nodes.

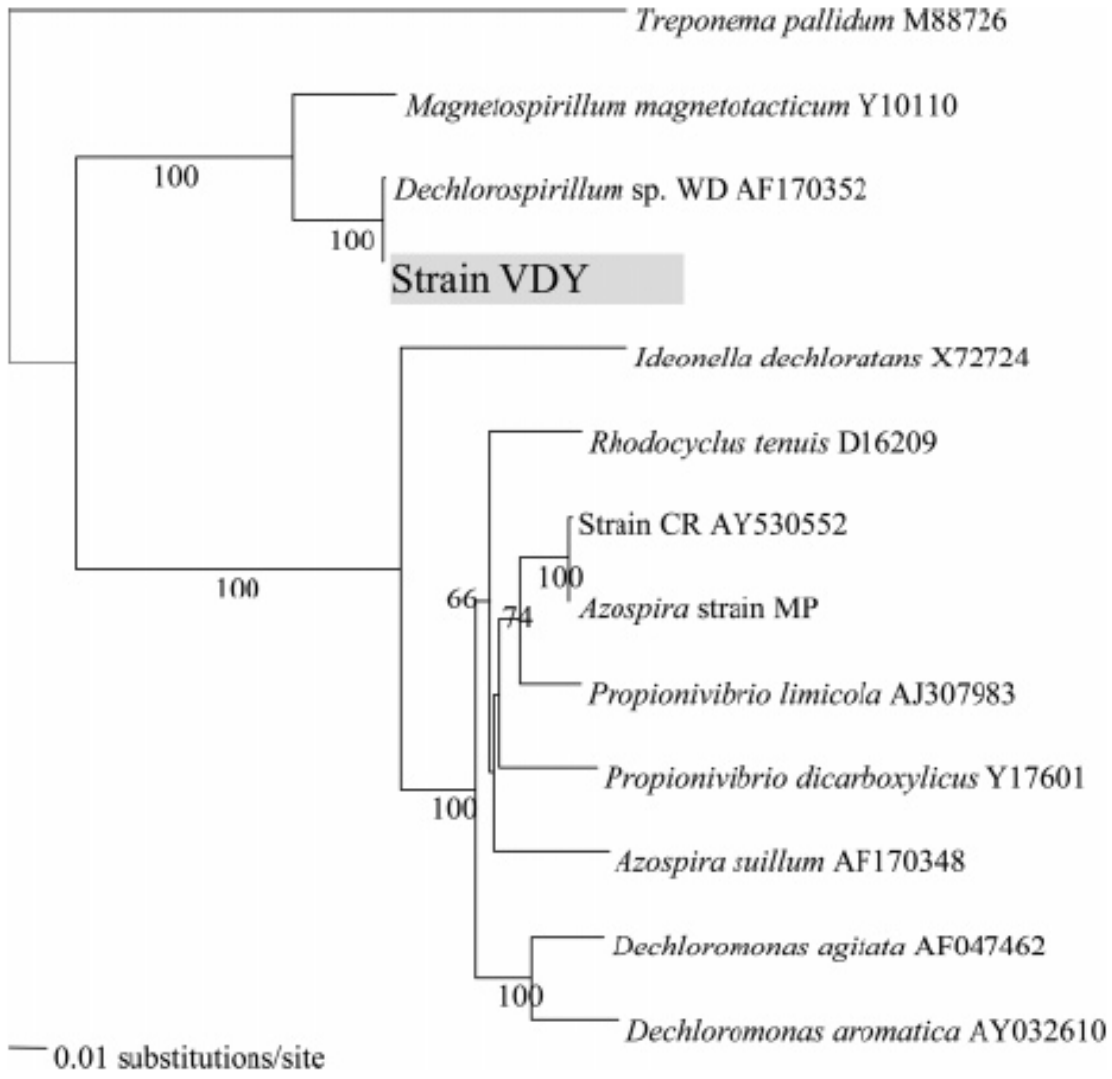


Figure 5. Perchlorate removal by strain VDY in a batch BER with and without AQDS.

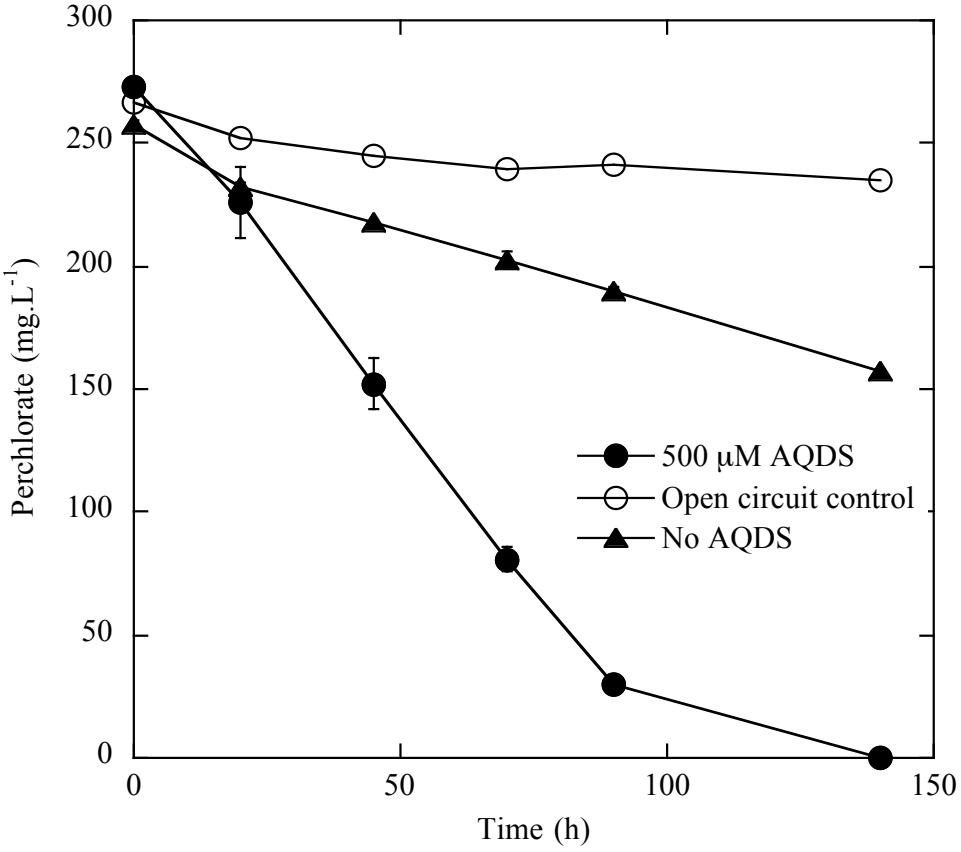


Figure 6. Continuous-flow BER treatment of perchlorate with AQDS. The results depicted are the mean of triplicate determinations.

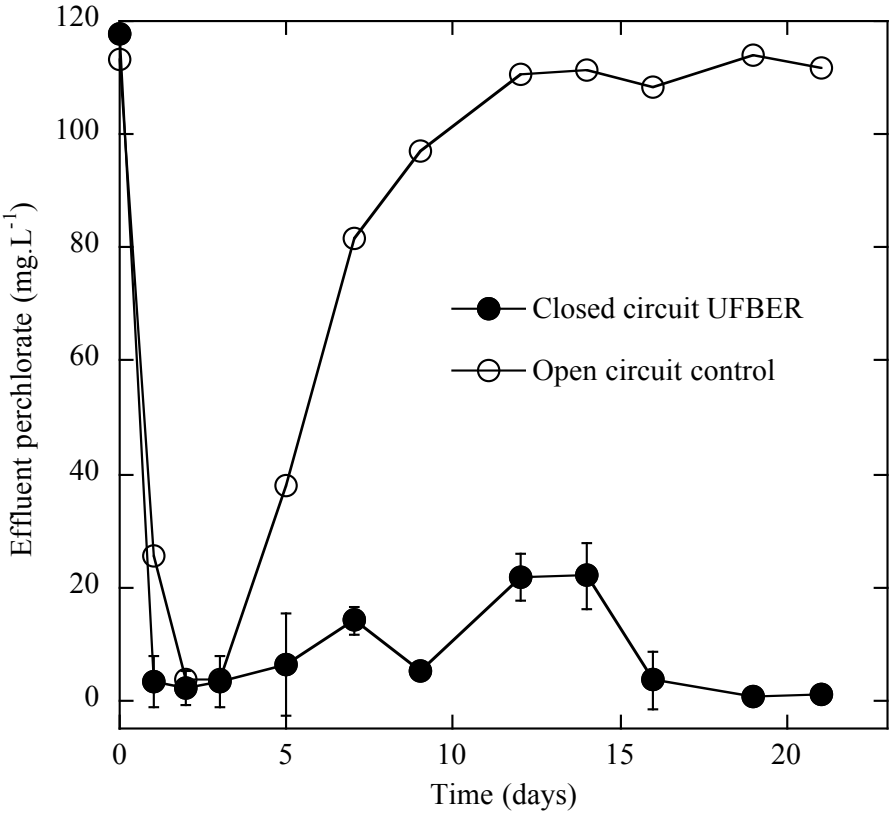
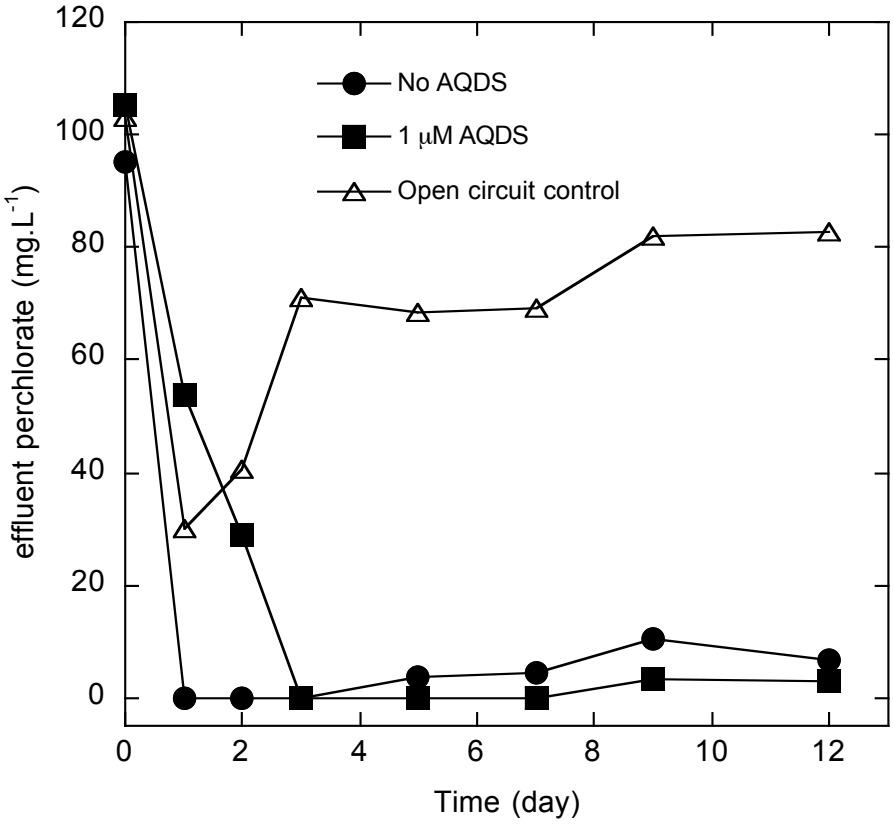


Figure 7. Continuous-flow BER treatment of perchlorate with and without AQDS. Concentrations were done as singlet representatives, with two shown here as examples.



This chapter was published in Environmental Science and Technology under the following reference:

Thrash, J. C., J. I. VanTrump, K. A. Weber, E. Miller, L. A. Achenbach, and J. D. Coates. 2007. Electrochemical Stimulation of Microbial Perchlorate Reduction. Environ. Sci. Technol. 41:1740-1746.

Chapter 3
Description of the novel perchlorate-reducing bacteria *Dechlorobacter hydrogenophilus* gen. nov., sp. nov., and *Propionivibrio militaris*, sp. nov.

Abstract

Novel dissimilatory perchlorate-reducing bacteria (DPRB) were isolated from enrichments conducted under conditions different from those of all previously described DPRB. Strain LT-1^T was enriched using medium buffered at pH 6.6 with 2-(N-morpholino)ethanesulfonic acid (MES), and had only 95% 16S rRNA gene identity with its closest relative, *Azonexus caeni*. Strain MP^T was enriched in the cathodic chamber of a perchlorate-reducing bioelectrical reactor (BER) and together with an additional strain, CR (99% 16S identity), had 97% 16S identity with *Propionivibrio limicola*. The use of perchlorate and other electron acceptors distinguished strains MP^T and CR from *P. limicola* physiologically. Strain LT-1^T had differences in electron donor utilization and optimum growth temperatures from *A. caeni*. Strains LT-1^T and MP^T are the first DPRB to be described in the *Betaproteobacteria* outside of the *Dechloromonas* and *Azospira* genera. On the basis of phylogenetic and physiological features, strain LT-1^T represents a novel genus in the *Rhodocyclaceae*; strain MP^T represents a novel species within the genus *Propionivibrio*. The names *Dechlorobacter hydrogenophilus* gen. nov., sp. nov, and *Propionivibrio militaris* sp. nov. are proposed.

Introduction

Perchlorate has been widely used as an oxidant in munitions propellant, as well as other applications and, due to its competitive inhibition of iodine uptake in the thyroid gland, can cause decreased levels of thyroid hormone production (49). As a result of unregulated disposal of the compound prior to 1997, perchlorate contamination is now widespread (11). DPRB are capable of perchlorate reduction completely to harmless chloride, and therefore represent an ideal means for remediating this compound (11). DPRB have been isolated from a variety of locales and perchlorate reduction genes have been found in areas as remote as Antarctica (4, 13). Their existence in such varied, and sometimes pristine, habitats has been puzzling in the context of DPRB research primarily focused on bioremediation of a contaminant perceived as anthropogenic. Recently, however, several reports have discovered natural perchlorate deposition in a variety of locales, indicating a potential natural source which would help explain the ubiquity of these organisms (35-37, 41).

In spite of their broad geographic distribution, DPRB have been phylogenetically limited to the Proteobacteria (11). Thus far, organisms in pure culture occur in only five genera in the *Alpha*-, *Beta*-, and *Gammaproteobacteria* classes. It is possible that perchlorate reduction may have only evolved in a common ancestor within the Proteobacteria and was subsequently lost in multiple genera, but a more parsimonious theory is that perchlorate reduction evolved recently and has been transferred horizontally. This theory is supported by a lack of phylogenetic synteny between the 16S rRNA genes and chlorite dismutase (a required perchlorate-reduction pathway gene) genes from the same organisms (4). The limited phylogenetic representation of DPRB given their ubiquity and the probability of horizontal gene transfer makes little sense without considering culture bias. Although a few of these organisms have been isolated with hydrogen as an electron donor (29, 31, 32, 43, 52), most have been isolated under mesophilic, chemoorganoheterotrophic conditions (6, 12, 13, 23, 28, 39, 47, 50).

A variety of studies have shown that even with the same starting inoculum, alteration of enrichment conditions can lead to isolation of novel organisms, both physiologically and phylogenetically (2, 8, 14, 19, 33). The isolation of two DPRB from the cathodic chamber of a bioelectrical reactor (BER) confirmed this, since no perchlorate-reducing organisms had been previously enriched under such circumstances (46). *Dechlorospirillum sp.* strain VDY had novel physiological characteristics compared to other tested DPRB (46). Further, enrichments carried out at 55˚C lead to the isolation of the first thermophilic microorganism capable of perchlorate reduction (3), although the presence of known perchlorate-reduction pathway genes was not confirmed, so it is unknown whether or not this organism was a dissimilatory or co-incidental perchlorate-reducer. Regardless, *Moorella perchloratireducens* was also the first bacterium isolated outside of the Proteobacteria capable of perchlorate reduction, providing additional evidence that alteration of enrichment conditions leads to novel physiologies and phylogenetic affiliations, and sometimes both in the same organism.

In the course of our continuing studies on the diversity of organisms capable of dissimilatory perchlorate reduction, three new isolates have been characterized and are reported here, two of which were enriched using conditions that were unique compared to that of previously described DPRB. Strain MP^T, the other organism isolated from the cathodic chamber of the same BER enrichment (46), its closest relative, strain CR, and strain LT-1^T, were mesophilic, strictly respiratory, facultative anaerobes that were phylogenetically distinct from all other previously described DPRB. Strains MP^T and CR are the first DPRB to be identified in the

genus *Propionivibrio*, and strain LT-1^T is the first from the new genus, proposed here as *Dechlorobacter* gen. nov. The unique phylogenetic affiliation of these strains supports previous observations that alteration of enrichment and isolation conditions can select for unique organisms capable of perchlorate reduction. The environmental role of these new species in the natural redox cycle of chlorine is still unknown. However, the results of these studies do indicate that the true phylogenetic diversity of organisms capable of perchlorate reduction still remains to be discovered.

Materials and Methods

Culture conditions, enrichments, and isolation

Unless otherwise mentioned, the basal medium used for cultures was the same as previously described (6, 46). Strain MP^T was enriched as previously described (46). One milliliter samples from the cathodic chamber were used to inoculate basal bicarbonate medium containing 5 mM anthrahydroquinone-2,6-disulfonate (AHDS) and 10 mM perchlorate amended with 0.1 mM acetate as a suitable carbon source. Medium showing growth was then streaked onto R2A (Difco, BD) plates. A single white colony was picked and transferred to liquid medium containing 10 mM acetate and perchlorate. Strains CR and strain $LT\text{-}1^T$ were isolated using the standard shake tube method previously described (6). For determining pH-dependent growth characteristics, anoxic phosphate-buffered medium was used. NaH_2PO_4 and Na_2HPO_4 were added in appropriate concentrations to establish stable pH at 6.0, 6.5, 6.8, 7.0, 7.2, and 7.5. These were boiled, cooled, and dispensed under a 100% N_2 headspace. Omitting sodium bicarbonate, all other medium components were identical with bicarbonate-buffered medium. Cultures were grown in triplicate at each different pH and monitored using optical density at 600nm. Electron donors and acceptors were added from sterile anoxic stocks. Alternative electron acceptors were tested in media amended with 10 mM acetate, alternative electron donors with 10 mM perchlorate. For determination of alternative electron acceptors and donors, substrate utilization was judged positive only after a minimum of three consecutive transfers under growth conditions.

Analytical methods

All experimental analyses were performed in triplicate to ensure reproducibility and the results are expressed as the mean of these determinations. Controls were complete in singlet unless otherwise stated. The concentration of perchlorate and other anions in cultures was determined using ion chromatography as previously described (9). Fe(II) concentration was determined using the Ferrozine assay (26). Culture purity was verified by microscopic inspection and 16S rRNA gene sequence analysis (below). Cell density increase was monitored by optical density changes at 600 nm ($O.D._{600}$).

Microscopy

Cells were observed using phase contrast microscopy on a Zeiss Axioskop 2 *plus*. Scanning electron microscopy (SEM) was carried out as described (48) by harvesting cells, washing twice with 0.1 M sodium cacodylate buffer pH 7.2, and fixing with glutaraldehyde. Cells were then resuspended in 1% osmium tetroxide in sodium cacodylate buffer for 2 h and rinsed in sodium cacodylate buffer. Cells were then dehydrated for 10 min in 35%, 50%, 70%, 80%, 95%, and 100% ethanol, followed with critical point drying, mounting onto stubs and sputter coated with palladium/gold, and viewing with a Hitachi S5000 scanning electron microscope at 20 kV.

Whole-cell fatty acid analysis

Whole-cell fatty acid content was determined by growing cells in basal medium as described above, harvesting by centrifugation, and re-suspending in 1 mL buffer. Fatty acids were identified using the Sherlock Microbial Identification System (v. 4.5, MIDI, Newark, DE) according to the manufacturer's protocol.

Molecular/Phylogenetic analysis

Genomic DNA of strain MP^T was isolated from pure cultures using the Power Soil Kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol and from strains CR and LT-1^T as previously described (46). 16S rRNA gene PCR on genomic DNA from strain MP^T was carried out as described previously (51), and from strains CR and LT-1^T as described previously elsewhere (46). PCR of the *cld* gene sequences was carried out as described by Bender et al. (4). 16S rRNA gene sequences were aligned with MUSCLE 3.6 (18) and analyzed with MrBayes 3.2 (24, 40) using 4 chains until the standard deviation of the split frequencies was stabilized below 0.01, in this case for 1,158,000 generations, with a sample frequency of 1000. The first 25% of the samples were discarded for accurate estimation of the posterior probability distribution of the summary tree.

Results

Both strains MP^T and LT-1^T were enriched under unusual conditions compared with previously described DPRB. Strain MP^T was enriched in the cathodic chamber of a BER in the presence of the electron shuttle anthroquinone-2,6-disulfonate (46) and isolated by inoculating medium containing 10 mM acetate and perchlorate, prior to streaking onto aerobic R2A plates after growth was observed. A single colony was picked in an anaerobic chamber and transferred to anoxic medium containing 10 mM acetate and perchlorate. Strain LT-1^T was enriched using slightly acidic (pH 6.6) MES-buffered medium inoculated with soil from the Longhorn Army Ammunition Plant, TX. Strain CR was enriched as previously described (13) with perchlorate-contaminated soil from Los Alamos National Laboratory as the inoculum.

Strains MP^T and CR grew singly as curved rods, approximately 2 μm long × 0.3 μm in diameter (Fig 1). Strain LT-1^T grew as a slightly-curved (Fig 2a) or short, straight rod (Fig 2b), 0.8-1.6 × 0.3 μm, either singly or in chains. Cells from all three strains were observed by phase-contrast microscopy to be motile when grown on acetate and perchlorate (10 mM). MP^T, CR, and LT-1^T were strictly respiratory, as judged by their inability to grow in the absence of an electron acceptor either with 10mM glucose, or in rich media (10mM glucose, 1g/L casamino acids, 1g/L yeast extract). None of the strains were capable of growth after Pasteurization at 80˚C and were therefore considered incapable of forming spores. All three strains grew by complete reduction of perchlorate to chloride, and all three strains contained copies of the chlorite dismutase gene (*cld*), previously reported for strains LT-1^T and CR (4), and confirmed in this study for strain MP^T (data not shown). The *cld* sequence for strain MP^T was most closely related to that of CR. Figure 3 shows typical growth curves for strain MP^T (a) and strain LT-1^T (b) on 10 mM acetate and perchlorate. Cell density increase was monitored by optical density changes at 600 nm. Strains MP^T and CR had maximum growth rates at 30˚C of 0.21 and 0.22 h^{-1}, respectively. Also for both strains, chlorate accumulated to ~2 mM (2.1 mM for strain MP^T-Fig 2) when grown on 10 mM perchlorate. In contrast, strain LT-1^T showed much less (maximum concentration ~0.1 mM) and more transient chlorate accumulation (Fig 3b). Strain LT-1^T had a maximum growth rate of 0.28 h^{-1} at 37˚C.

Table 1 describes the comparable substrate utilization range for the three strains. In general, they reduced a similar suite of electron acceptors and utilized similar electron donors. Strain CR was not capable of utilizing Fe(II) (as $FeCl_2$) or H_2, however, and exhibited a lower NaCl tolerance. It was capable of growing at 40˚C, whereas strain MP^T could not, and strain CR grew optimally at pH 7 as compared to 6.8 for strain MP^T. LT-1^T grew optimally at pH 6.5, which is unusual for DPRB, but is not unexpected since it was enriched in MES-buffered medium at pH 6.6. However, maximum growth rates in MES-buffered medium were considerably lower than those on bicarbonate-buffered medium (0.03 h^{-1} vs. 0.16 h^{-1} at 37˚C, respectively).

Fatty acid composition for strains MP^T, CR, and LT-1^T are shown in Table 2. The primary dominant fatty acid for strain MP^T was the summed feature 16:1 ω5c/15 iso 2-OH, for CR and LT-1^T it was 16:1c9. 16:0 was the second most abundant fatty acid in all three strains.

Analysis of the 16S rRNA gene sequences indicated that all three strains were members of the *Rhodocyclaceae* family in the *Betaproteobacteria* (Fig 4). Sequences for strains MP^T and CR were 99% identical, and both strains were most closely related to *Propionivibrio limicola* GolChi1^T (97% identity), making them probable members of the *Propionivibrio* genus. Strain LT-1^T was most closely related to the *Azonexus* genus, having 95% identity with *A. caeni*.

Discussion

These studies resulted in the isolation and characterization of three novel organisms that lie outside of the phylogenetic groupings of known DPRB. In spite of the close identity to *P. limicola*, both strains MP^T and CR had only 95% identity to *P. decarboxylicus* and *P. pelophilus*, the other described species in the genus. Furthermore while all members of the *Propionivibrio* were fermentative and formed acetate and propionate as endproducts (7, 22, 27, 44), strains MP^T and CR were strictly respiratory. *P. limicola* was incapable of utilizing external electron acceptors (oxygen, nitrate, thiosulfate, sulfur, amorphous ferric iron) whereas strains MP^T and CR utilized oxygen, nitrate, chlorate and perchlorate, and *P. limicola* also was unable to make use of carboxylic acids whereas MP^T and CR could use many (Table 1). It is unknown whether or not *P. limicola* can reduce perchlorate, but since the perchlorate reduction pathway involves production of oxygen and subsequent reduction of it by cytochrome oxidase (1, 39), and the *pcrC* gene encodes for a *c*-type cytochrome (5), it is unlikely that this organism could make use of perchlorate since it cannot reduce oxygen and contains no cytochromes (7). Strains MP^T and CR were also distinct from *P. limicola* morphologically and were curved rods whereas *P. limicola* is a straight rod (7). Given the combination of physiological and morphological distinction with the phylogenetic distance from *P. limicola*, strain MP^T should be considered a new species, and the name *Propionivibrio militaris* sp. nov. is proposed. Given the significant similarity to strain MP^T, CR is considered a strain of *P. militaris*.

Strain LT-1^T had only 95% 16S rRNA gene identity to its nearest relative, *Azonexus caeni*, which distinguished it as a novel genus. In addition, strain LT-1^T was morphologically distinct from both *A. caeni* and *A. fungiphilus*, the two described *Azonexus* species. LT-1^T was a short, straight rod, whereas *A. caeni* was more rounded and asymmetrical (34), and *A. fungiphilus* was a long, curved rod, which sometimes was capable of growing filamentously (38). Strain LT-1^T was also smaller than either of the *Azonexus* species. Physiologically, strain LT-1^T was distinct from *A. caeni* in that it could use perchlorate, chlorate, and Mn(IV) as terminal electron acceptors, and butyrate, iso-valerate, fumarate, hexanoic acids, ethanol, H_2, and AHDS as electron donors; *A. caeni* could not use any of these electron donors (34). *A. fungiphilus* was a strict aerobe (38) and *A. caeni* was a facultative anaerobe that was capable of denitrification (34). It is unknown if these organisms could use perchlorate. *A. caeni* had a growth optimum at between 25 and 30˚C, whereas strain LT-1^T had a growth optimum at 37˚C. Taken together, the phylogenetic, morphological, and physiological differences between strain LT-1^T and *A. caeni* indicate that strain LT-1^T represents a novel species of a new genus, and the name *Dechlorobacter hydrogenophilus* is proposed.

Chlorate accumulation in DPRB is not well understood. Strains MP^T and LT-1^T accumulated chlorate in much different amounts (Fig 3). However another DPRB, *Azospira* sp. strain PCC, had comparable chlorate accumulation to that of strains MP^T and CR as a result of higher relative affinity for perchlorate versus chlorate, making perchlorate a competitive inhibitor of chlorate reduction (16). A reasonable hypothesis is that the perchlorate reduction genes in MP^T and CR are more similar to those of PCC than to those of strain LT-1^T and other DPRB that do not exhibit this type of intermediate accumulation, but this needs to be tested.

The new appreciation for naturally-occurring perchlorate brought about by the work of Rajagopalan et al. (35, 36), Rao et al. (37), and Scanlon et al. (41) makes the search for organisms capable of using this compound for respiration much more than a means to understand and increase the operational parameters of perchlorate bioremediation schemes. Although DPRB

have been isolated from a variety of locales, the natural abundance, diversity, and ecology of DPRB has yet to be explored. The new evidence of perchlorate as a naturally occurring compound makes study of this metabolism important in understanding the biological processes involved in natural cycling of chlorine. Current isolates will be indispensable for understanding the physiology behind future culture-independent studies. Recent evidence for perchlorate on Mars (20, 21, 30) even presents the possibility of studying chemolithoautotrophic DPRB as model organisms for Martian astrobiology, and comparative cell biology will only be available with multiple organisms capable of these metabolisms.

However, in spite of the new horizons for examining DPRB in the environment, studies continue to demonstrate the prevalence of perchlorate as a contaminant, and one which has made its way to the top of the food chain, having been found in dairy and breast milk (15, 17, 25). As a result, the need for effective remediation technology for perchlorate continues to grow. With bioremediation as the most effective means to completely eliminate this compound, the development and testing of new strategies will most likely lead to the discovery of novel DPRB, since new treatment processes can lead to new enrichment conditions. BER technology (46) and hydrogen-based reactors (29, 32) are examples of treatment alternatives that have already enriched for novel organisms. A remarkable number of BER options exist (45), and new reactors using these as well as the application of microbial fuel cell technology (already shown capable of stimulating nitrate-reducing microorganisms (10)) for perchlorate bioremediation (42), should lead to even more disparate isolates.

Description of *Dechlorobacter* gen. nov.

Dechlorobacter [de.chlor.o.bac'ter L. pref. *de* from; Gr. adj. *chloros* green (chlorine); N.L. masc. n. *bacter* a short rod; N.L. masc. n. *Dechlorobacter* dechlorinating rod]. Rod-shaped cells, 0.8-1.6 × 0.3 μm, non-spore-forming, non-fermentative, facultative anaerobe. Cells are motile and occur singly or in chains. Gram negative. Strictly respiring, complete oxidizer that oxidizes acetate with O_2, ClO_4^-, ClO_3^-, Mn(IV), or NO_3^- as electron acceptors. Perchlorate and chlorate are completely reduced to Cl^-. Type species is *Dechlorobacter hydrogenophilus*.

Description of *Dechlorobacter hydrogenophilus* sp. nov.

Dechlorobacter hydrogenophilus [hy.dro.gen'o.phi'lus L. n. *hydrogen* the dihydrogen molecule H_2; phi'lus L. v. loving *hydrogenophilus* loving hydrogen, on which it can grow]. Optimum growth occurs at 37˚C, pH 6.5, in freshwater basal medium with 0% NaCl. Cells can grow over the temperature range 4-37˚C, but not at 42˚C, and between pH 6.0-7.2. Cells can use organic substrates such as acetate and malate and can grow mixotrophically with hydrogen as the electron donor. Cells could not utilize formate, lactate, citrate, heptanoic acid, Fe(II), thiosulfate, fumarate, ethanol, methanol, glycerol, glucose, lactose, sucrose, fructose, maltose, ribose, benzene, benzoate, toluene, p-Cresol, phenol, phenanthrene, or octane as electron donors, and could not utilize sulfate, sulfur, ferric citrate, ferric NTA, ferric pyrophosphate, fumarate or selenate as electron acceptors. Cells contain the fatty acids 10:0 3-OH, 12:0, 12:0 3-OH, 14:0, 16:1c9, 16:0, and 18:1c11/t9/t6. Type species is *Dechlorobacter hydrogenophilus*. The type strain is LT-1^T (ATCC BAA-1869) and was isolated from soil collected at the Longhorn Army Ammunition Plant, Longhorn, TX.

Description of *Propionivibrio militaris* sp. nov.

Propionivibrio militaris [mil'i.tar.is L. adj. *militaris* martial, war-like, referring to the ability of the organism to degrade perchlorate, frequently used in rocket propellants].

Curved rod-shaped cells, ~2 × 0.3 μm, non-spore-forming, non-fermentative, facultative anaerobe. Gram negative. Cells are motile and occur singly. Strictly respiring, complete oxidizer that oxidizes acetate with O_2, ClO_4^-, ClO_3^-, NO_3^- or NO_2^- as electron acceptors. Perchlorate and chlorate are completely reduced to Cl^- with chlorate accumulation during growth on perchlorate. Cells can use chemoorganotrophic substrates such as acetate and propionate as electron donors for growth as well as AHDS. Cells could not use formate, methanol, ethanol, catechol, glycerol, benzoate, citrate, glucose, sucrose, fructose, maltose, or urea as electron donors, and could not utilize sulfate, thiosulfate, fumarate, malate, ferric NTA, or AQDS as electron acceptors. Optimum growth occurs at 30˚C in freshwater basal medium with 0% NaCl. Cells contain the fatty acids 10:0 3-OH, 12:0, and 16:0. Type species is *Propionivibrio militaris*. The type strain is MP^T (DSM 21683, ATCC BAA-1728) and was isolated from the cathodic chamber of an active perchlorate-reducing BER enrichment inoculated with water from Strawberry Creek at UC Berkeley, Berkeley, CA.

The type strain, MP^T, has the following additional characteristics: cells can utilize the inorganic electron donors hydrogen and Fe(II). Cells grow between 10-37˚C, but not at 40˚C. Growth occurs at pH 6.0-7.5, with 6.8 optimum. Cells also contain the fatty acids 16:1 ω7c/15 iso 2OH, 17:0 cyclo, and 18:1 ω7c.

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Tables and Figures

Table 1. Physiological characteristics of strains MP^T, CR, and LT-1^T.

	Strain MP^T	Strain CR	Strain LT-1^T
Size (µm)	~2 × 0.3	~2 × 0.3	0.8-1.6 × 0.3
Morphology	Curved rod	Curved rod	Rod
Gram stain	Negative	Negative	Negative
Motility	+	+	+
Spore-forming	-	-	-
Fermentative	-	-	-
Electron acceptors utilized	ClO_4^-, ClO_3^-, NO_3^-, NO_2^-, O_2	ClO_4^-, ClO_3^-, NO_3^-, O_2	ClO_4^-, ClO_3^-, NO_3^-, O_2, Mn(IV)
Electron donors utilized	Acetate, propionate, butyrate, iso-butyrate, valerate, lactate, pyruvate, succinate, malate, fumarate, Fe(II), H_2, AHDS	Acetate, propionate, butyrate, isobutyrate, valerate, iso-valerate, lactate, pyruvate, succinate, malate, fumarate, heptanoate, hexanoate, Casamino acids, yeast extract, ethanol, AHDS	Acetate, propionate, butyrate, iso-valerate, pyruvate, succinate, malate, fumarate, hexanoate, Casamino acids, yeast extract, ethanol, H_2, AHDS
Optimum growth	pH 6.8, 30˚C	pH 7.0, 30˚C	pH 6.5, 37˚C
Salinity tolerance	1% NaCl	<1% NaCl	1% NaCl

Table 2. Fatty acid profiles for strains MP^T, CR, and LT-1^T. Values are given as percent content and reported for those fatty acids present above 1%.

Fatty acid	MP^T	CR	LT-1^T
10:0	< 1	-	-
10:0 3-OH	1.25	2.74	2.32
12:0	5.75	4.97	4.44
12:0 3-OH	-	-	1.36
14:0	< 1	< 1	1.54
16:1c9	-	50.38	47.51
16:1c11	-	1.10	-
16:1 ω5c	< 1	-	-
16:1 ω7c	-	-	-
16:0	26.92	21.05	33.29
17:1 ω6c	-	-	-
17:0 cyclo	1.13	-	-
18:1 ω7c	16.55	-	-
18:0	< 1	-	-
18:1 isomers	-	-	-
Summed features			
16:1 ω5c/15 iso 2-OH	46.15	-	-
18:1c11/t9/t6	-	19.07	9.55

Figure 1. SEM of strain MP^{T}, scale bar=1 μm.

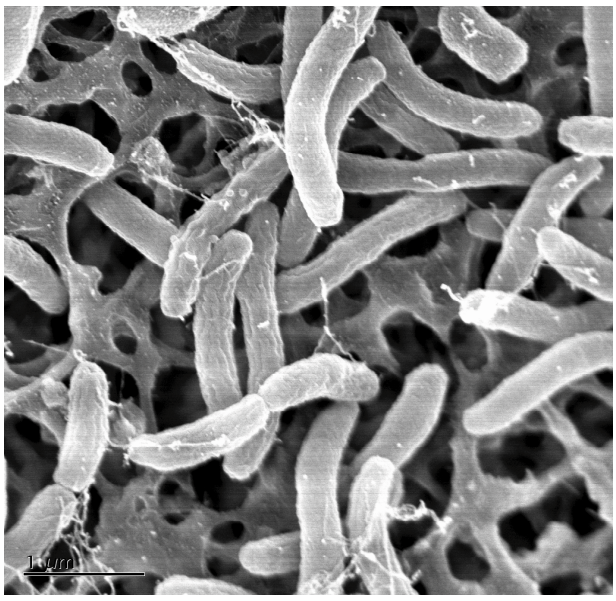


Figure 2. SEMs of strain LT-1^{T}, scale bars = 125 nm

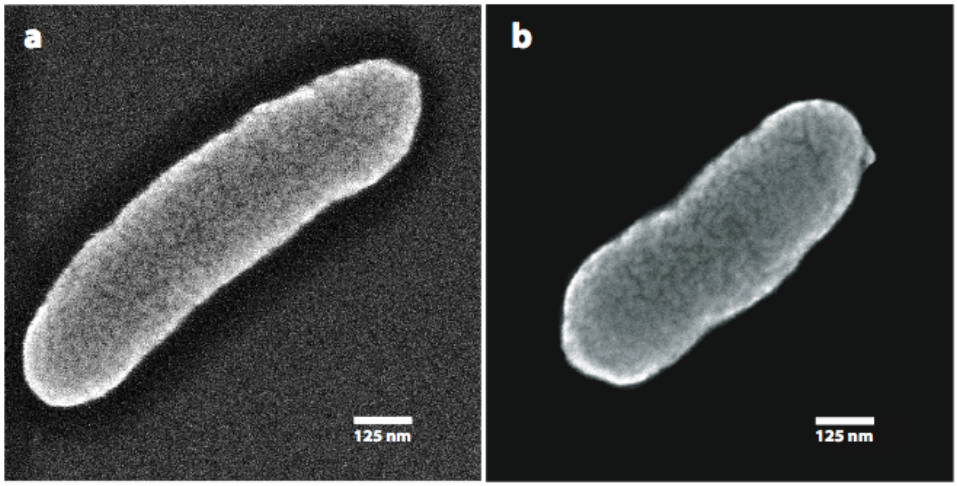


Figure 3. Growth curves of strain MP^T (a) and strain LT-1^T (b). Cell number increase was monitored with optical density (O.D.) at 600 nm. Closed circles, O.D.; open squares, [ClO_4^-]; open triangles, [Cl^-]; open diamonds, [ClO_3^-]; all concentrations in mM. Error bars represent the standard deviation of triplicate experiments.

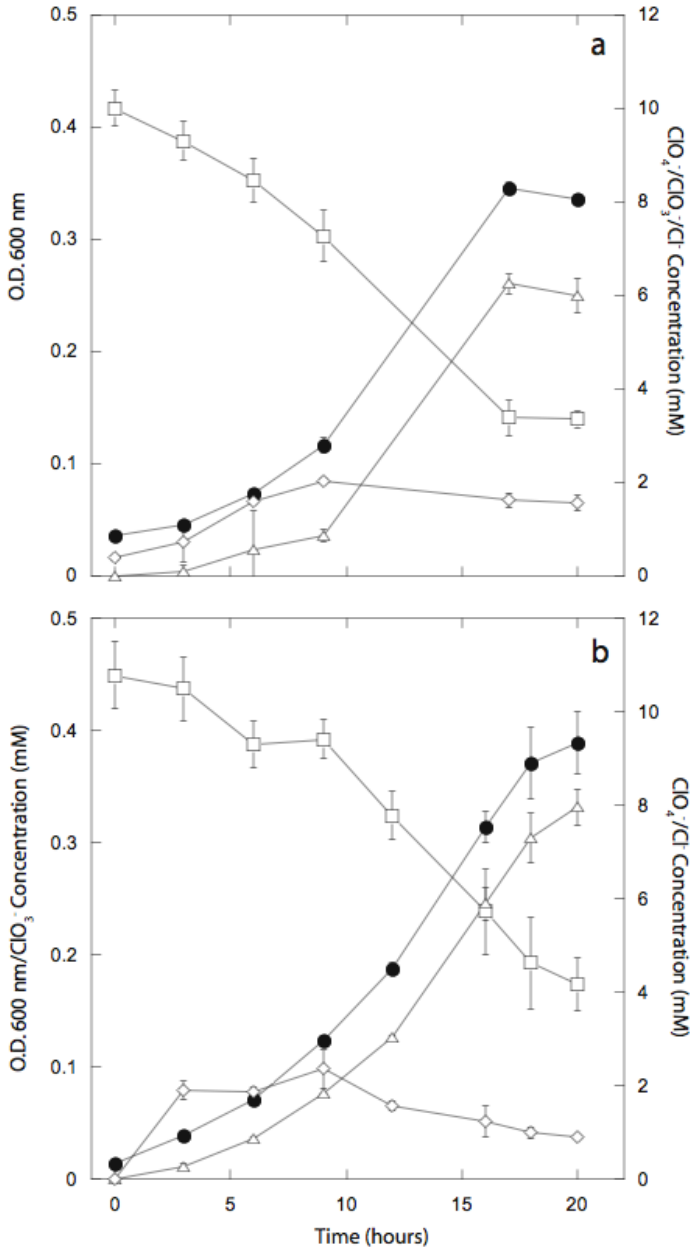
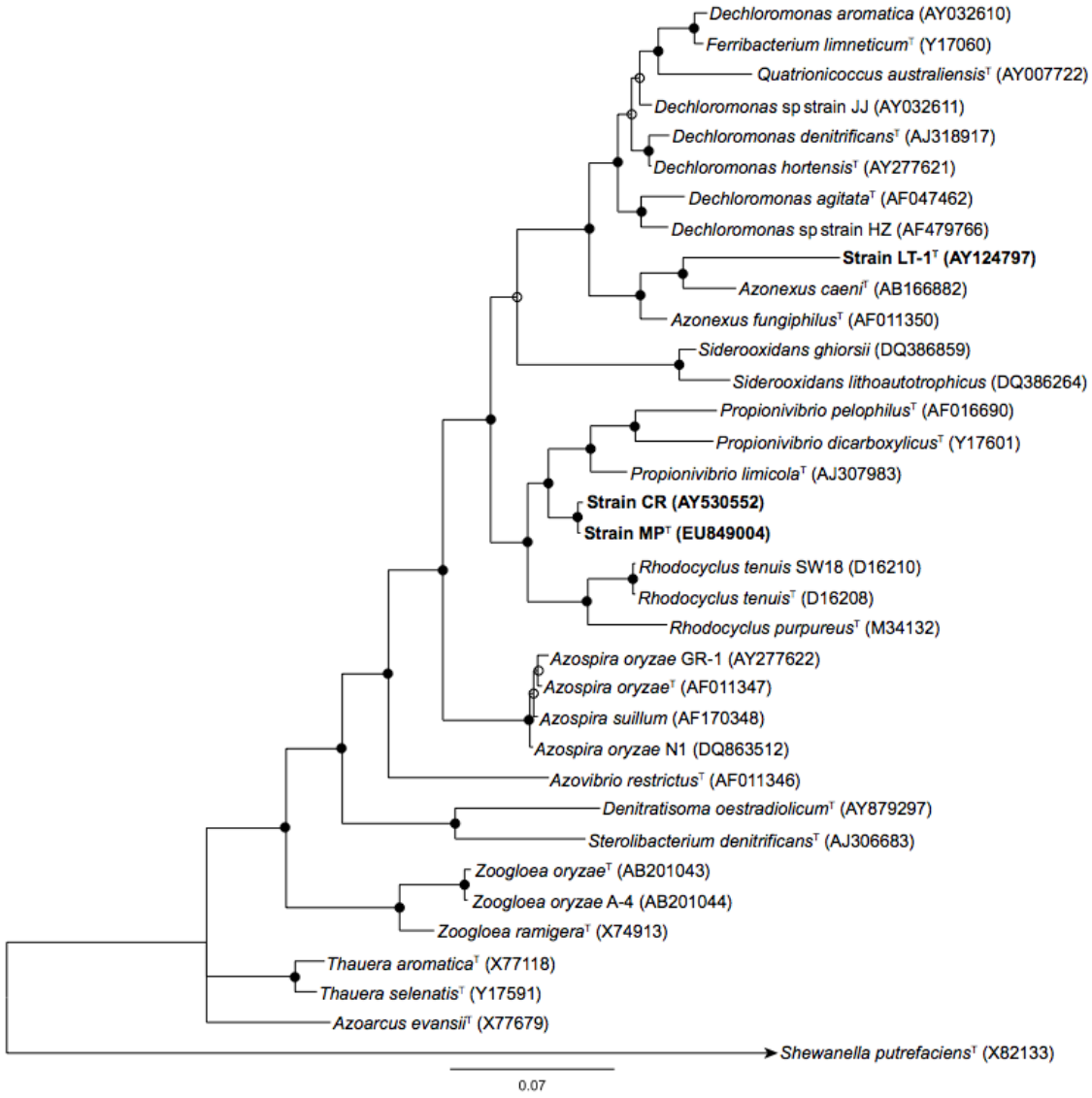


Figure 4. Bayesian 16S rRNA gene phylogenetic tree showing the position of strains MP^T, CR and LT-1^T in the *Rhodocylaceae*. Scale bar represents 0.07 changes per position. Circles at the nodes indicate posterior probabilities > 0.85 (closed) or < 0.85 (open).



This chapter has been published in Applied Microbiology and Biotechnology under the following reference:

Thrash, J. C., J. Pollock, T. Torok, and J. D. Coates. 2009. Description of the novel perchlorate-reducing bacteria *Dechlorobacter hydrogenophilus* gen. nov., sp. nov., and *Propionivibrio militaris*, sp. nov. Appl. Microbiol. Biotechnol. In press.

Chapter 4
Characterization of *Magnetospirillum bellicus* sp. nov., a novel dissimilatory perchlorate-reducing bacterium in the *Alphaproteobacteria* isolated from a bioelectrical reactor

Abstract

Previously isolated dissimilatory perchlorate-reducing bacteria (DPRB) have been primarily affiliated with the *Betaproteobacteria*. Enrichments from the cathodic chamber of a bioelectrical reactor (BER) inoculated from creek water in Berkeley, CA yielded a novel organism, most closely related to a previously described strain WD (99% 16S rRNA gene identity). Strains VDY^T and WD are related to *Magnetospirillum* with 96% 16S rRNA gene identity between them and both *M. gryphiswaldense* and *M. magnetotacticum*, and distinguish a clade of non-magnetosome forming "*Magnetospirillum*" species capable of respiring perchlorate in the *Alphaproteobacteria*. In spite of the phylogenetic location of this organism, VDY^T did not contain copies of key magnetosome-formation genes *mamI* and *mamL*. Strain VDY^T was motile, non-spore-forming, and in addition to perchlorate could use oxygen, chlorate, nitrate, nitrite, and nitrous oxide as alternative electron acceptors with acetate as the electron donor. Transient chlorate accumulation occurred during respiration of perchlorate. The organism made use of fermentation endproducts such as acetate and ethanol serving as carbon sources and electron donors for heterotrophic growth, and in addition strain VDY^T could grow chemolithoautotrophically with hydrogen serving as an electron donor. VDY^T contains a copy of the RuBisCO *cbbM* gene, which was expressed under autotrophic but not heterotrophic conditions. A 16S rRNA-specific fluorescence in situ hybridization (FISH) probe was developed to target both strains VDY^T and WD to specifically identify cells of this clade via microscopy. DNA-DNA hybridization with strain WD confirmed VDY^T as a separate species (46.2% identity), and the name *Magnetospirillum bellicus*, sp. nov. (DSM 21662, ATCC BAA-1730), is proposed.

Introduction

Dissimilatory perchlorate-reducing bacteria (DPRB) use perchlorate as a terminal electron acceptor during respiration, reducing it completely to harmless chloride. As a consequence, bioremediation of perchlorate has been identified as the most effective means of treating this harmful contaminant (12), which, due to historically unregulated release into the environment, has become widespread (16, 24, 44). Fortunately, DPRB are ubiquitous and can be readily isolated from a variety of environments (1, 12, 13, 42, 47), and a key gene in the pathway, chlorite dismutase (*cld*) has been detected in even more, including Antarctica (7). Much has been revealed about the biochemistry and genetics of microbial perchlorate reduction through the study of several model organisms, including *Dechloromonas aromatica* and *D. agitata*, by a variety of groups (6, 8, 9, 17, 29, 34, 35, 38, 47, 51, 56, 57).

Less is known about the variation in physiology between these organisms or the evolution of the perchlorate-reduction metabolism, highlighting a need for further isolation and characterization of pure cultures. The lack of phylogenetic synteny between *cld* and the 16S rRNA gene among tested DPRB suggests the gene may have been horizontally transferred (7). Given that at least elements of the pathway may be mobile, it is not unreasonable to expect that a wide phylogenetic diversity of organisms could acquire the ability to reduce perchlorate. As more enrichment conditions are tested (4, 41, 42), sometimes as a result of bioreactor development for treatment of perchlorate (43, 50), more novel DPRB are becoming known, supporting the hypothesis that the metabolism may be widespread within the tree of life, similarly to other respiratory processes such as the reduction of Fe(III) and nitrate. Comparative physiology and genetics of pure culture isolates could facilitate deeper understanding into the natural roles for these organisms, and therefore each new characterized isolate offers a valuable contribution.

Although perchlorate has been primarily regarded as a contaminant, a variety of studies are now showing that perchlorate is naturally occurring (34-36, 39), which provides a possible explanation for the selective pressure behind the evolution of perchlorate reduction genes. As we begin to understand more about the chlorine cycle on Earth, understanding the variety of organisms capable of interacting with the various oxyanions of chlorine is becoming more important. Further, recent discovery of perchlorate on Mars (20) now makes the study of this metabolism relevant to any burgeoning Martian astrobiology. Here we report the characterization of a unique DPRB in the *Alphaproteobacteria*. Strain VDY^T was isolated from the surface of a working electrode in an active, perchlorate-reducing BER which was inoculated with water from Strawberry creek on the UC Berkeley campus (43). This is only the second described DPRB in the *Alphaproteobacteria*, the other being the closely related strain WD (28). These strains compose a clade of non-magnetosome forming DPRB in the *Magnetospirillum*, and in spite of their phylogenetic affiliation with this genus, are distinguished by genetic, biochemical, and physiological differences.

Materials and Methods

Medium and Culturing Conditions

Culturing media was prepared as described previously (8). All cultures were grown and evaluated using freshwater 30mM bicarbonate-buffered basal media (pH 6.8) under a N_2-CO_2 (80%:20%) headspace except for testing optimum pH for growth where a phosphate-buffered media was utilized. NaH_2PO_4 and Na_2HPO_4 were added in appropriate concentrations to establish stable pH at 6.0, 6.5, 6.8, 7.0, 7.2, and 7.5. These were boiled, cooled, and dispensed under a N_2 headspace. Omitting sodium bicarbonate, all other media components were identical with bicarbonate-buffered media. Electron acceptors and donors were added separately from sterile, anoxic stock solutions. For testing of salinity growth optima, NaCl was added to culture media from a 5M sterile, anoxic stock. Acetate and perchlorate (10mM) were used for testing of alternative electron donors and acceptors, respectively, and together for testing optimum growth temperature, pH, and salinity.

Scanning Electron Microscopy

Cells for electron microscopy were grown anaerobically in freshwater basal media (described above) amended with acetate (10 mM) and perchlorate (10 mM), prepared and imaged as described previously (45).

16S rRNA Gene Sequencing and Analysis

Isolation of genomic DNA, 16S rRNA gene specific PCR, and sequencing of PCR products was completed as described previously (13, 43). 16S rRNA gene sequences were aligned with Muscle 3.6 (17) and Bayesian analysis of 16S rRNA gene phylogeny was completed with MrBayes 3.2 (21, 38). The program was run with four chains until the standard deviation of the split frequencies was stabilized below 0.01, in this case for 241,000 generations, with a sample frequency of 1000. The first 25% of the samples were discarded for accurate estimation of the posterior probability distribution of the summary tree. Organisms used in the *Acetobacteraceae* were: *Acetobacter pasteurianus*^{T} (X71863), *Acetobacter cerevisiae*^{T} (AJ419843), *Gluconobacter oxydans*^{T} (X73820), *Gluconobacter cerinus*^{T} (X80775), *Paracraurococcus ruber*^{T} (D85827), *Gluconacetobacter oboediens*^{T} (AJ001631), *Gluconacetobacter europaeus*^{T} (Z21936), *Gluconacetobacter hansenii*^{T} (X75620), *Roseococcus thiosulfatophilus*^{T} (X72908), *Acidomonas methanolica*^{T} (D30770), *Acidocella facilis*^{T} (D30774), *Acidiphilium aminolytica*^{T} (D30771), *Acidiphilium cryptum*^{T} (D30773); organisms used in the *Rickettsiales* were: *Rickettsia honei*^{T} (AF060705), *Rickettsia australis*^{T} (L36101), *Rickettsia aeschlimannii*^{T} (U74757), *Wolbachia pipientis* (AF179630), *Holospora obtusa* (X58198). All other accession numbers are shown in Figure 2.

DNA-DNA Hybridization

DNA-DNA hybridization was performed at the DSMZ. DNA was purified and hybridized as described (10, 16, 27).

DNA base composition

G+C content was determined by HPLC at the DSMZ according to (27).

Fatty acid determination

Cells were grown in basal media as described above, harvested by centrifugation, and re-suspended in 1mL buffer. Whole-cell fatty acid content was determined using the Sherlock Microbial Identification System (v. 4.5, MIDI, Newark, DE) according to the manufacturer's protocol.

mamI/mamL PCR

Genomic DNA was isolated using the Power Soil Kit (MoBio) as directed by the manufacturer's protocol. Primers specific to *mamI* and *mamL* (29) were used to amplify these genes under the following parameters: 95˚C for 5 minutes, then 32 cycles of 95˚C fro 30s, 55˚C for 30s, 72˚C for 30s, followed by a final incubation at 72˚C for 10 minutes. 50µL reactions contained 3µL (100ng) genomic DNA as the template, 1µL (20µM) each primer, and 1.25u Taq polymerase (TaKaRa).

RNA Isolation

Cultures were grown in freshwater basal media with an N_2-CO_2 (80%:20%) headspace under autotrophic (H_2 as the sole electron donor, CO_2 as the carbon source) and heterotrophic (acetate as the electron donor and carbon source) conditions with perchlorate as the sole terminal electron acceptor. Total RNA was isolated by trapping cells on a nylon membrane filter (Nalgene) via vacuum filtration, immersing the filter into 1mL Trizol reagent (Sigma-Aldrich) in Lysing Matrix E (MP Bio) tubes, and bead-beating for 30 seconds. After a 5 minute room temperature incubation, 200µL chloroform (Fisher Scientific) was added and the tubes shaken for 30 seconds and allowed to incubate at room temperature for a further 10 minutes. The tubes were then centrifuged (13,000 rpm) at 4˚C for 15 minutes and the supernatant was transferred to new tubes to which 500µL DEPC-H_2O was added. The tubes were mixed, supplemented with 1mL isopropanol (Sigma-Aldrich), mixed again, and allowed to incubate at room temperature for 10 minutes. The tubes were then centrifuged (13,000 rpm) at 4˚C for 15 minutes, supernatant decanted and replaced with 1mL 75% ethanol, vortexed and centrifuged (13,000 rpm) again at 4˚C for 5 minutes. After decanting the supernatant, pellets were allowed to air dry for 10 minutes and resuspended in 100µL DEPC-H_2O. The resuspended nucleic acids were then further purified using the AllPrep DNA/RNA Mini Kit (Quiagen) according to the manufacturer's protocol, including the optional DNase I steps (E1-E4).

cDNA Preparation

Total RNA (7µL) from the isolation protocol was added to 1µL of 3µM *cbbM*-specific primers (18, 46), 4µL 2.5mM each dNTPs (Invitrogen), heated at 65˚C for 5 minutes, and quickly chilled on ice. To this, 4µL of 5x First-strand buffer (Invitrogen), 2µL 0.1M DTT (Invitrogen), and 1uL RNAseOUT (Invitrogen) were added, mixed gently and incubated at 42˚C for 2 minutes. 0.5µL Superscript II Reverse Transcriptase (Invitrogen) was added, mixed gently, incubated at 42˚C for 50 minutes, and then again at 70˚C for 15 minutes.

RuBisCO cbbM and cbbL PCR

Primers designed to amplify form I (*cbbL*) and form II (*cbbM*) of the RuBisCO large subunit were utilized as previously described (18, 46). Briefly, amplifications involved the following parameters: 95˚C for 4 minutes, then 35 cycles of 95˚C fro 45s, 57˚C for 45s, 72˚C for 45s, followed by a final incubation at 72˚C for 10 minutes. PCR products were visualized by agarose gel electrophoresis and imaged using a NucleoTECH gel imaging system.

Whole-cell rRNA FISH analysis and probe design

Cells of strain VDY^T were harvested from an active chemolithoautotrophic culure oxidizing hydrogen coupled to perchlorate-reduction, fixed in 4% paraformaldehyde and washed in phosphate-buffered saline (PBS). Oligonucleotide probes were designed to target the 16S rRNA gene of VDY^T using methods reviewed by Hugenholtz et al. (22), and taking into consideration accessibility of the target region as reported by Behrens et al. (5). The FISH probe, named VDY1249, utilized had the following sequence: 5'-Cy3-GCGAGCTCGCAACCCATTG-3'. Hybridizations were performed as previously described (3), with incubation at 46˚C for 4.5 hours and washing at 48˚C for 15 minutes. Hybridization and wash buffers were made with 10% formamide. Cells were counter-stained with DAPI (4',6'-diamidino-2-phenylindole dihydrochloride) DNA stain for cell enumeration, and imaged with epifluorescence microscopy. No-probe controls were done and *E. coli* was used to check for non-specific binding, both were negative.

Analytical methods

All experimental analyses were performed in triplicate to ensure reproducibility and the results are expressed as the mean of these determinations. Controls were complete in singlet unless otherwise stated. The concentration of perchlorate and chlorate in cultures was determined using ion chromatography as previously described (10). Cell growth in active cultures was monitored by optical density at 600nm and by total direct cell count using phase-contrast microscopy (Petroff-Hausser counting chamber, 0.02-mm depth). Samples collected for counting were immediately fixed in 0.2µm filter-sterilized formaldehyde (final concentration 3.7%).

Results

Phylogenetic characterization

Strain VDY^T was spirillum-shaped, ~0.5µm × ~3µm (Fig. 1), typically with two helical rotations, though cells have been seen with as few as one or as many as five, and were highly motile, similar to strain WD (12, 28). Phylogenetic analysis of the 16S rRNA gene sequences from these organisms indicated that they were closely related to each other (99% 16S rRNA gene similarity) and placed them within the family *Rhodospirillaceae* of the *Alphaproteobacteria* (Fig. 2). They are most closely related to *Magenetospirillum gryphiswaldense* MSR-1, with 96% 16S rRNA gene identity between both VDY^T and WD and *M. gryphiswaldense*. DNA-DNA hybridization between strain VDY^T and strain WD showed 46.2% similarity, well below the 70% similarity required to distinguish these two strains as separate species.

Magnetosome formation

To further examine the distinction between strain VDY^T and *Magnetospirillum* spp., it was tested physiologically and genetically for the ability to produce magentosomes, via visual inspection of response to a magnetic field and molecular probing, respectively. Strain VDY^T was unable to produce magnetosomes in magnetospirillum-specific media (25) or in high ferrous-iron conditions in its typical culture media. PCR amplification of *mamI* and *mamL*, magnetosome-specific genes in *Magnetospirillum* (37) strains AMB-1, MS-1, and MSR-1, did not identify the genes in VDY^T (data not shown).

Fatty acid profiles

VDY^T contained a very similar fatty acid profile to that of WD when grown on perchlorate (10mM) and acetate (10mM) (Table 1). The major fatty acid present for both was 18:1ω7c. Secondary fatty acids were the summed feature 16:1ω7c/15 iso 2OH and 16:0. These three fatty acids made up 87.12% and 89.80% of the fatty acid content of strains VDY^T and WD, respectively, and were the only fatty acids present above 5% of the total.

Growth characteristics

Table 2 describes the physiological characteristics of strain VDY^T as compared to previously described DPRB, strain WD, *Dechloromonas agitata*, and *Azospira suillum*. Strain VDY^T grew between 10 and 42˚C, but not at 50˚C, with an optimum growth rate at 42˚C (0.29 hr^{-1}). It grew optimally at pH 6.8. It could grow with NaCl concentrations up to 1.5%, but not at 2%, and showed little growth defects in media containing at least 40mM perchlorate or chlorate. VDY^T was non-spore-forming, as indicated by the inability of cultures to grow in new media after incubation at 80˚C for three minutes. VDY^T was an obligate respiratory organism, being unable to ferment. The alternative electron acceptors and donors utilized by strain VDY^T are listed in Table 1. Strain VDY^T shared a similar electron donor and acceptor repertoire as strain WD, *D. agitata*, and *A. suillum*. Each could use oxygen, perchlorate, chlorate, and nitrate as alternative electron acceptors. VDY^T had a superior growth rate on nitrate compared to perchlorate, and different temperature optima for either compound, indicative of different enzymes involved (Fig 3a). Like many DPRB, strain VDY^T could use a range of fermentation endproducts, including acetate, lactate, and ethanol as elctron donors and carbon sources for heterotrophic growth. VDY^T could also utilize the electron donors $FeCl_2$, H_2, and the humic substances analog anthrohydroquinone-2,6-disulfonate (AHDS).

Chlorate accumulation

Similarly to strain WD, VDY^T transiently accumulated chlorate while growing on perchlorate. Figure 3b shows chlorate (0.14mM) accumulation during growth on perchlorate and acetate by strain VDY^T. After 4 hours, chlorate was removed concomitantly with perchlorate, however the temporary chlorate accumulation indicates a differentiation between the initial perchlorate reduction step in strain VDY^T and other DPRB. A recent study quantified the kinetic difference in another chlorate accumulating DPRB, *Azospira* strain PCC (15), and found that the chlorate accumulation by strain PCC was consistent with competitive inhibition of chlorate reduction by perchlorate. Two other recently reported strains also showed similar chlorate accumulation (42), generally around 20% of the initial perchlorate concentration, whereas in strain VDY^T the accumulation was closer to 1%. VDY^T therefore represents a third class of perchlorate-reduction physiology, intermediate between no accumulation and the considerable accumulation of chlorate seen in strains PCC, MP, and CR. Comparative analysis of the *pcr* genes and enzymes between organisms from these three classes could yield important information about the evolution of the perchlorate reduction pathway.

Chemolithotrophy

Strain VDY^T could grow with hydrogen as an electron donor coupled to perchlorate reduction, which has only been described for three other pure cultures (42, 50). Figure 4 shows chemolithotrophic growth of strain VDY^T with hydrogen as the electron donor and perchlorate as the sole terminal electron acceptor in the bicarbonate-buffered media described above. Cells were grown in electron donor-limited media (10 mM acetate and 15 mM perchlorate) and allowed to reach late stationary phase to reduce the possibility of acetate carryover. Growth was monitored by direct cell counts. Cells reduced 2.2mM perchlorate over 48 hours relative to a no electron donor control, and increased in number from $6.5\times10^7 \pm 1.2\times10^7$ to $1.1\times10^8 \pm 2.1\times10^7$ cells.ml^{-1} in the first 24 hours. The no electron donor control showed no significant growth (Figure 4). Interestingly, although the cultures entered stationary phase after twenty-four hours, the organisms continued to respire the perchlorate to completion over 48 hours.

Genetic screening demonstrated the presence of the RuBisCO *cbbM* large subunit (form II) gene (Fig. 5, lane 3), but not the *cbbL* (form I) gene (data not shown) and differential expression of *cbbM* RNA under heterotrophic and autotrophic conditions. *cbbM* was expressed when cells were grown on hydrogen and perchlorate as the sole electron donor and acceptor, respectively, under an N_2-CO_2 (80%:20%) headspace (Fig. 5, lane 6), whereas cells grown with acetate as the electron donor under identical conditions showed no *cbbM* expression (Fig. 5, lane 4). Since up-regulation of RuBisCO under autotrophic conditions would be expected if this gene were present and differentially expressed, this data supports the involvement of RuBisCO in carbon fixation by strain VDY^T. Together with the growth data, these experiments support the conclusion that autotropic growth by VDY^T on hydrogen and perchlorate is possible.

Flourescence in-situ hybridization (FISH)

rRNA gene-specific fluorescence microscopy has proven effective for identification, enumeration, and examination of structural configuration and association of microorganisms in both pure culture and mixed communities (2, 23, 33). In an effort to build tools appropriate for detection of this DPRB clade, a 16S rRNA gene FISH probe was designed to target strain VDY^T. The probe has a single mismatch with strain WD and three and four mismatches with *M.*

gryphiswaldense MSR-1 and *M. magneticum* AMB-1, respectively (Table 3). Based on these mismatches, the probe was not expected to hybridize with *M. gryphiswaldense* MSR-1, and in fact did not (data not shown). However, the probe successfully hybridized with strains VDY^T and WD (Fig. 6a and 6b, respectively) in our optimized protocol, and can therefore serve as an effective means of identifying at least closely related strains of the clade. To selectively target strain VDY^T in future studies, a competitor probe (specific for strain WD, 5'-GCGAGCTCGCCACCCATTG -3') would need to be utilized in concert with probe VDY1249. As strain VDY^T was isolated from a BER enrichment inoculated with water taken from Strawberry Creek on the UC Berkeley campus (54), it was hypothesized that this organism would be detectable there using the FISH probe. Fresh water samples taken from the original sampling site as well as points both upstream and downstream were investigated with FISH without any sample concentration. Although generic 16S rRNA gene probes for Bacteria were positive (data not shown), VDY-type cells could not be detected with the specific probe. Even when cells from 1L water samples were concentrated onto 0.2µm filters the VDY-specific FISH probes failed to yield any positive results although bacterial cells could be detected with a universal Bacteria probe mix (EUBmix) (14). In no cases were spirillum-shaped cells observed in samples illuminated with the probes. To confirm this result, in parallel we checked that the general bacterial probes did in fact bind to pure cultures of VDY^T, WD, and *E. coli* (data not shown).

Discussion

These studies examined the phylogeny and physiology of strain VDY^T, and included molecular probing to assess the presence of magnetosome-forming genes and those responsible for autotrophic growth. As an important organism for the functioning of a perchlorate-reducing BER (43) it was deemed valuable to thoroughly investigate these characteristics and develop tools that would aid in further development of the BER technology. Remediation efforts for perchlorate have resulted in a wide array of bioreactor studies (31, 40, 43, 48), yet little is known about the organisms which dominate these systems or whether the systems select for specific community arrangements independent of inoculum source. The chosen electron donor may have much to do with the types of organisms that become dominant in treatment systems, whether that be organic compounds, hydrogen, or charged working electrodes, which have become useful for a variety of bioremediation efforts (11, 19, 41). In turn, the microbiological makeup of a treatment system could have significant influence over the efficiency of the process, and therefore the ability to identify individual taxa will be informative for diagnosing effectiveness.

Ecological study of DPRB, either in natural settings or in bioreactors, is nascent (30, 49). The characterization of novel isolates, including the successful design of molecular probes based on traits of interest, either phylogenetic or physiological (32), will aid in pursing these investigations. Information about distribution, dominance of particular members in situ, community structure (if any), and association of DPRB with other organisms will provide insight into the importance and prevalence of this metabolism, and the members responsible for it, in various environments. The development of the FISH probe described herein, together with previously developed immunoprobe (32) and functional gene probes (6), represents a contribution to this effort. Although strain VDY^T was enriched from a Strawberry Creek inoculum, FISH probing with the specific VDY-type probe was negative. The inability to detect this organism was most likely because of very low natural abundance in the water system, at least at the time of sampling. Considerably higher volumes of water would most likely be needed to detect this organism, or use of additional tools, such as quantitative PCR. However, in bioreactors where this organism may become significantly enriched, as in (54), this probe will be useful in understanding the contribution of VDY-type cells to the overall treatment community.

The taxonomic characterization of strains VDY^T and WD has demonstrated the unique nature of these organisms compared with those to which ostensibly they are most closely related- the *Magnetospirillum*. By most metrics, these organisms show very little similarity to their magnetotactic neighbors. Neither strain VDY^T or WD form magnetosomes under the conditions tested, and strain VDY^T doesn't contain copies of necessary magnetosome-formation genes *mamI* or *mamL*. Both use a range of electron acceptors, unlike other *Magnetosprillum* species. Although they are 96% similar based on 16S rRNA gene identity to MSR-1, VDY^T and WD have clearly distinct physiologies and constitute a very unique clade within the *Magnetospirillum*. Based on DNA-DNA hybridization identity strain VDY^T and WD can be called separate species within the *Magnetospirillum*.

Further investigation of the range of habitats and roles DPRB may play in uncontaminated settings will help reveal needed information for understanding natural biogeochemical cycles. For example, several known isolates, including strains VDY^T and WD, were also capable of oxidizing ferrous iron (8, 9, 13, 26, 42). The presence of naturally occurring perchlorate could offer an alternative electron acceptor for iron oxidation in anoxic environments, adding another pathway to the biologically mediated cycling of this important

element. Mesophilic, anaerobic, non-photosynthetic ferrous iron oxidation, similar to that described by Weber et al. coupled to nitrate reduction (45, 46), may also be possibly coupled to perchlorate reduction. Future studies are needed to illuminate the variety of roles DPRB occupy in nature and the full extent of this metabolism across the tree of life. Knowledge accumulated through characterization of the various isolated organisms will be instrumental in orienting these studies and generating hypotheses.

Description of *Magnetospirillum bellicus* sp. nov.

Magnetospirillm bellicus [bel'li.cus L. adj. *bellicus* martial, war-like, referring to the propensity of the organism to degrade perchlorate, a principle component of munitions and solid-rocket fuel].

Spirillum-shaped cells, 0.5 × 3µm, non-spore-forming, non-fermentative, facultatively anaerobic. Cells are motile and may occur singly or in chains. Strictly respiring, complete oxidizer that oxidizes acetate with O_2, ClO_4^-, ClO_3^-, NO_3^-, or N_2O as electron acceptors. Perchlorate and chlorate are completely reduced to Cl^- with transient chlorate accumulation during growth on perchlorate. Cells can use substrates for chemoorganotrophic growth such as carboxylic acids including acetate, propionate, and ethanol as electron donors for growth and hydrogen for lithotrophic growth. Cells contain the RuBisCO *cbbM* gene. Optimum growth occurs at 42˚C, pH 6.8, in freshwater basal medium with 0% NaCl. The G+C content of the genomic DNA of the type strain is 64.8 mol% (determined by HPLC). The type strain is VDY^T (DSM 21662, ATCC BAA-1730) and was isolated from the cathodic chamber of an active perchlorate-reducing BER enrichment.

Acknowledgements

I would like to gratefully acknowledge Dr. Jillian F. Banfield for contribution of reagents and equipment in helping with FISH analysis, Dr. Arash Komeili, Dr. Meghan E. Byrne, and Dr. Dorothee Murat for assistance with physiological and genetic testing of magnetosome formation, and Dr. Jason E. Stajich and Dr. Thomas J. Sharpton for assistance with MrBayes.

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Tables and Figures

Table 1. Comparative fatty acid composition of strains VDY^T and WD. Values are given as percentages of total membrane content.

Fatty acid type	VDY^T	WD
12:0	3.25	3.14
14:0	0.37	0.43
16:1ω7c	-	-
16:0	12.01	9.87
16:0 3OH	0.75	0.69
18:1ω9c	-	-
18:1ω7c	**64.64**	**68.51**
18:1	-	-
18:0	1.89	0.80
11 methyl 18:1ω7c	0.83	1.36
18:1 2OH	1.23	-
18:0 3OH	0.33	-
Summed features		
14:0 3OH/16:1 iso i	3.10	2.86
16:1ω7c/15 iso 2OH	10.47	11.42
18:1c11/t9/t6	-	-
19:1 2OH/cy 19:0	-	-
Unknown	3.59	3.28

Table 2. Physiological characteristics of strains VDY^T and WD compared to *Dechloromonas agitata* and *Azospira suillum*, DPRB in the *Betaproteobacteria*.

	VDY^T	**WD**	***D. agitata***	***A. suillum***
Cell type	Spirillum	Spirillum	Rod	Rod
Size(µm)	~0.5 × ~3	~0.2 × ~7	0.5 × 2	0.5 × 1-2
Motility	+	+	+	+
Temperature range	<10-42˚C	25-37˚C	25-40˚C	25-42˚C
Temperature optima	42˚C	35˚C	35˚C	35˚C
pH range	6.0-7.5	6.5-7.5	6.5-8.5	5.0-8.0
pH optima	6.8	7.2	7.5	7.2
NaCl tolerance	1.5%	1%	2%	1%
Spore-forming	-	-	-	-
Fermentative	-	-	-	-
G+C content	64.8%	66.1%	63.5%	65.8%
Electron acceptors				
Oxygen	+	+	+	+
Perchlorate	+	+	+	+
Chlorate	+	+	+	+
Nitrate	+	+	-	+
Nitrite	+	n/a	n/a	n/a
N_2O	+	n/a	n/a	n/a
Sulfate	-	-	-	-
Thiosulfate	-	-	-	n/a
Selenate	-	-	-	-
Arsenate	-	n/a	n/a	n/a
Fumarate	-	-	-	-
Malate	-	-	-	-
Fe(III) NTA	-	-	-	-
AQDS	-	-	-	-
Electron donors				
Acetate	+	+	+	+
Propionate	+	+	+	+
Isobutyrate	+	+	+	+
Butyrate	+	+	n/a	+
Valerate	+	+	n/a	+
Formate	-	-	-	-
Methanol	-	-	-	-
Ethanol	+	+	-	+
Catechol	-	-	n/a	-
Glycerol	-	-	n/a	-
Benzoate	-	-	-	-
Pyruvate	+	n/a	n/a	+
Citrate	-	-	-	-

Succinate	+	+	+	+
Lactate	+	+	+	+
Glucose	-	-	-	-
Sucrose	-	n/a	n/a	n/a
Fructose	-	n/a	n/a	n/a
Maltose	+	n/a	n/a	n/a
Casamino acids	+	+	-	+
Fumarate	+	+	+	+
Malate	+	+	+	+
Hydrogen	+	-	-	-
$FeCl_2$	+	+	+	+
AHDS	+	n/a	+	n/a
H_2S	-	n/a	n/a	n/a
H_2	+	-	-	-
Methane	-	n/a	n/a	n/a
Urea	-	n/a	n/a	n/a

Data for strain WD, *D. agitata*, and *A. suillum* were taken from Michaelidou et al. (28), Bruce et al. (8), and Michaelidou et al. (28), respectively, except G+C content, which was taken for *D. agitata* and *A. suillum* from Achenbach et al. (1), and electron donor data for WD, which was taken from Michaelidou et al. (28) and Coates et al. (13). G+C content for strain WD was reported in this study for the first time. All electron donors and acceptors were tested under growth conditions. A positive score was indicative of a minimum of three transfers on the substrate. Strain VDY^T could reduce a limited number of alternative electron acceptors, namely, perchlorate, chlorate, nitrate, nitrite, nitrous oxide, and oxygen- typical of most DPRB. It was non-fermentative, non-spore-forming and capable of oxidizing many fermentation endproducts such as acetate and ethanol, while also capable of chemolithoautotrophic growth on hydrogen.

Table 3. Specific 16S rRNA sequence for strain VDY^T and alignments with strain WD and the close relatives, *M. gryphiswaldense* MSR-1 and *M. magneticum* AMB-1.

Strain	Aligned 16S rRNA sequence
VDY^T	CAAUGGGUUGCGAGCUCGC
WD	CAAUGGGU**G**GCGAGCUCGC
MSR-1	CAAUGGGUUGC**T**A**A**C**C**CGC
AMB-1	CA**G**UGGGU**G**GC**T**A**A**CUCGC

The 16S rRNA region of strain VDY^T to which the FISH probe was designed showed only one mismatch with strain WD, but three mismatches with *M. gryphiswaldense* MSR-1 and four with *M. magneticum* AMB-1. The probe showed specificity to both strains VDY^T and WD in spite of the mismatch, but was not able to hybridize with either MSR-1 or AMB-1.

Figure 1. SEM of strain VDY^{T}. Cells are ~0.5µm × ~3µm and typically have two helical rotations, though they can have as many as five. Bar, 0.5µm.

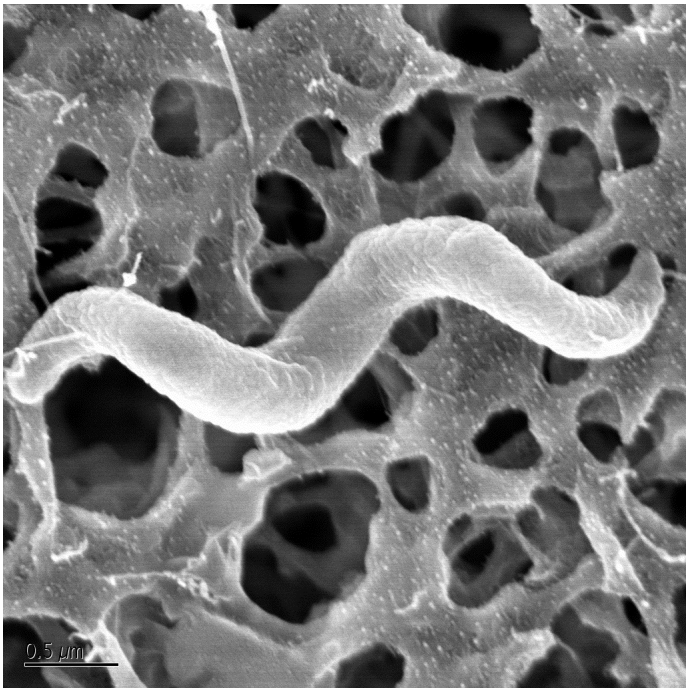


Figure 2. Phylogenetic placement of *Dechlorospirillum* among the *Rhodospirillales* in the *Alphaproteobacteria* according to 16S rRNA gene analysis. Tree was constructed using Bayesian analysis (MrBayes); bar represents 0.04 expected changes per site. Posterior probability values are indicated at nodes. The outgroup is *Pelobacter carbonolicus* in the *Deltaproteobacteria*.

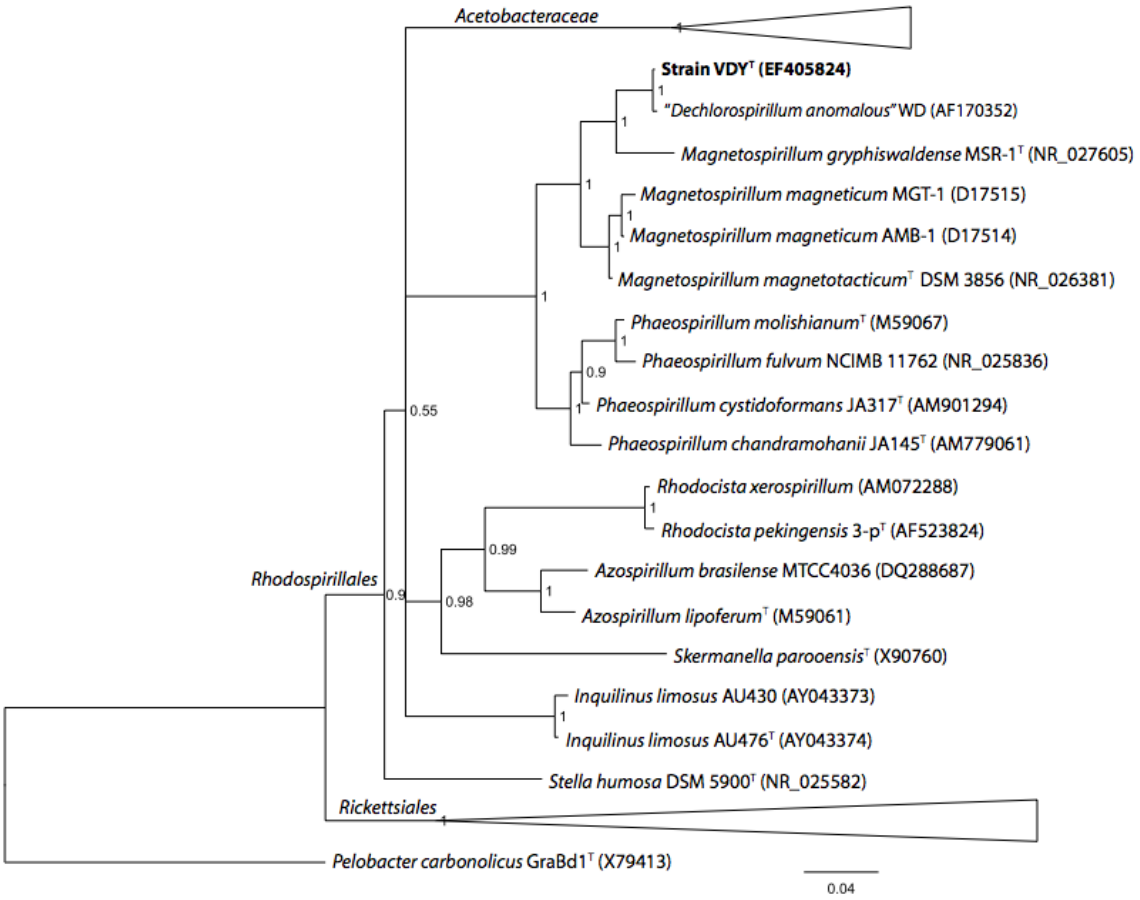


Figure 3. a) Comparative temperature-dependent growth optima of VDY^T on nitrate (open squares) and perchlorate (closed circles). All rates are averages of triplicates. b) Transient chlorate accumulation by strain VDY^T while growing on perchlorate. Chlorate accumulated to 0.14mM within 4 hours, but was removed concomitantly with perchlorate thereafter. O.D. 600nm, closed circles; ClO_4^-, open squares; ClO_3^-, open triangles. Error bars represent the standard deviation of triplicate samples.

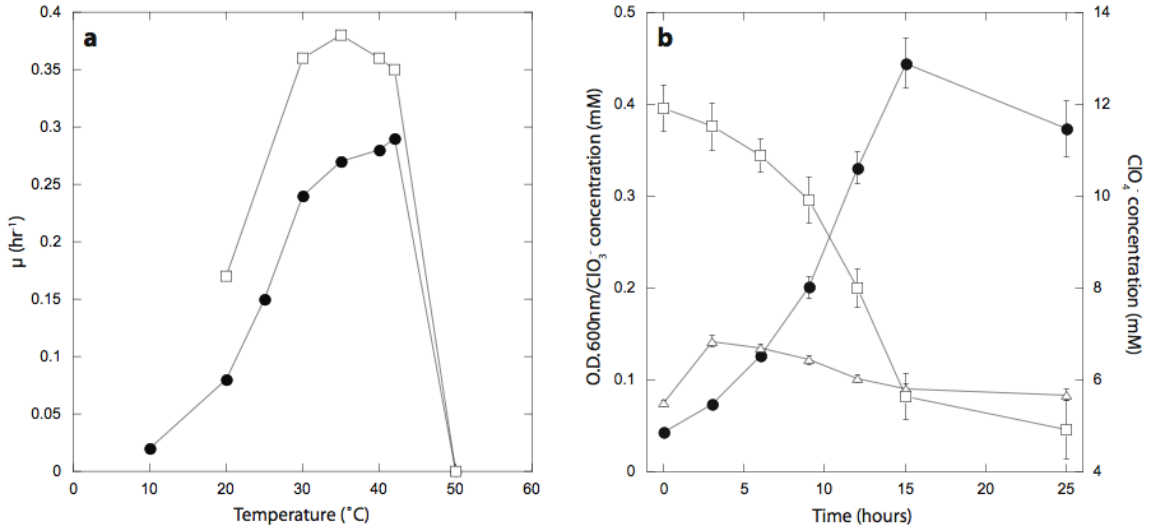


Figure 4. Chemolithoautotrophic growth of strain VDY^T (closed squares) compared to that of a control with no added electron donor (open squares), showing concomitant perchlorate reduction (closed circles- experimental; open circles- control). Cells were monitored with direct cell counts. Error bars represent the standard deviation of triplicate samples.

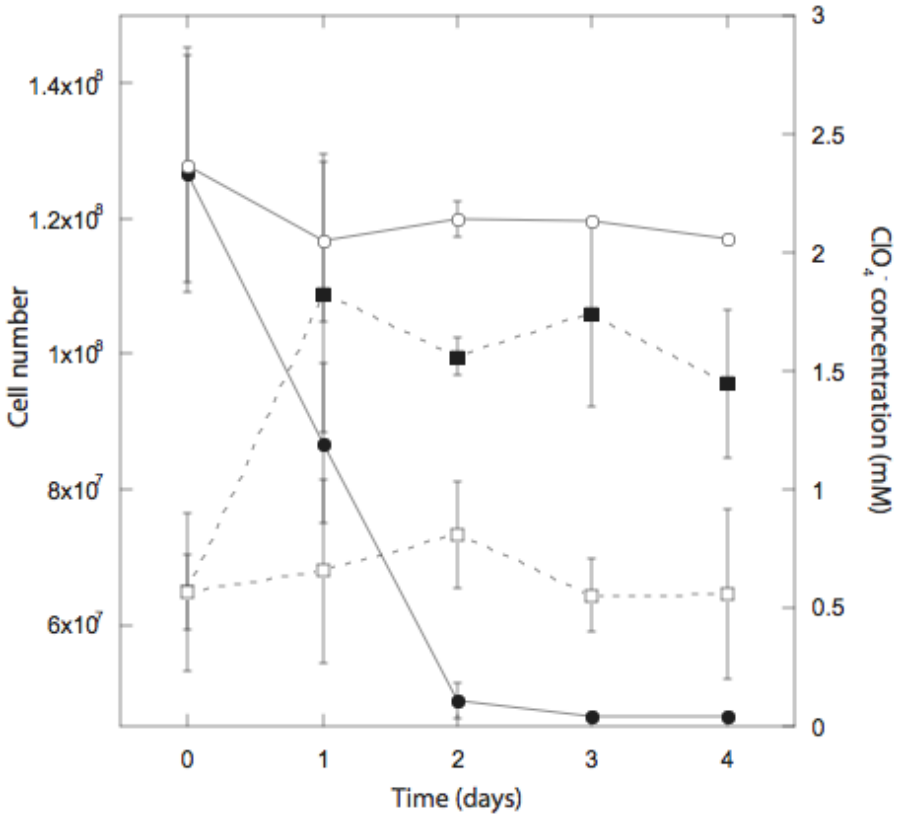


Figure 5. RT-PCR of the RuBisCO *cbbM* gene. Strain VDY^{T} contains a genomic copy of the *cbbM* gene (Lane 3), which is differentially expressed during chemolithoautotrophic growth (Lane 6), but not chemoorganoheterotrophic growth (Lane 4). Lane 1, 100bp ladder. Lane 2, negative control (water). Lanes 5 and 7 are RNA controls to discount genomic DNA contamination.

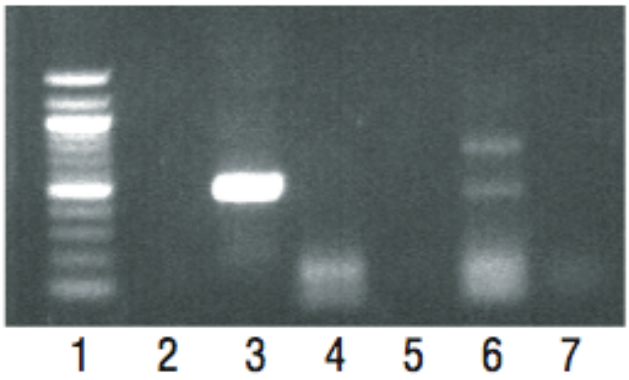
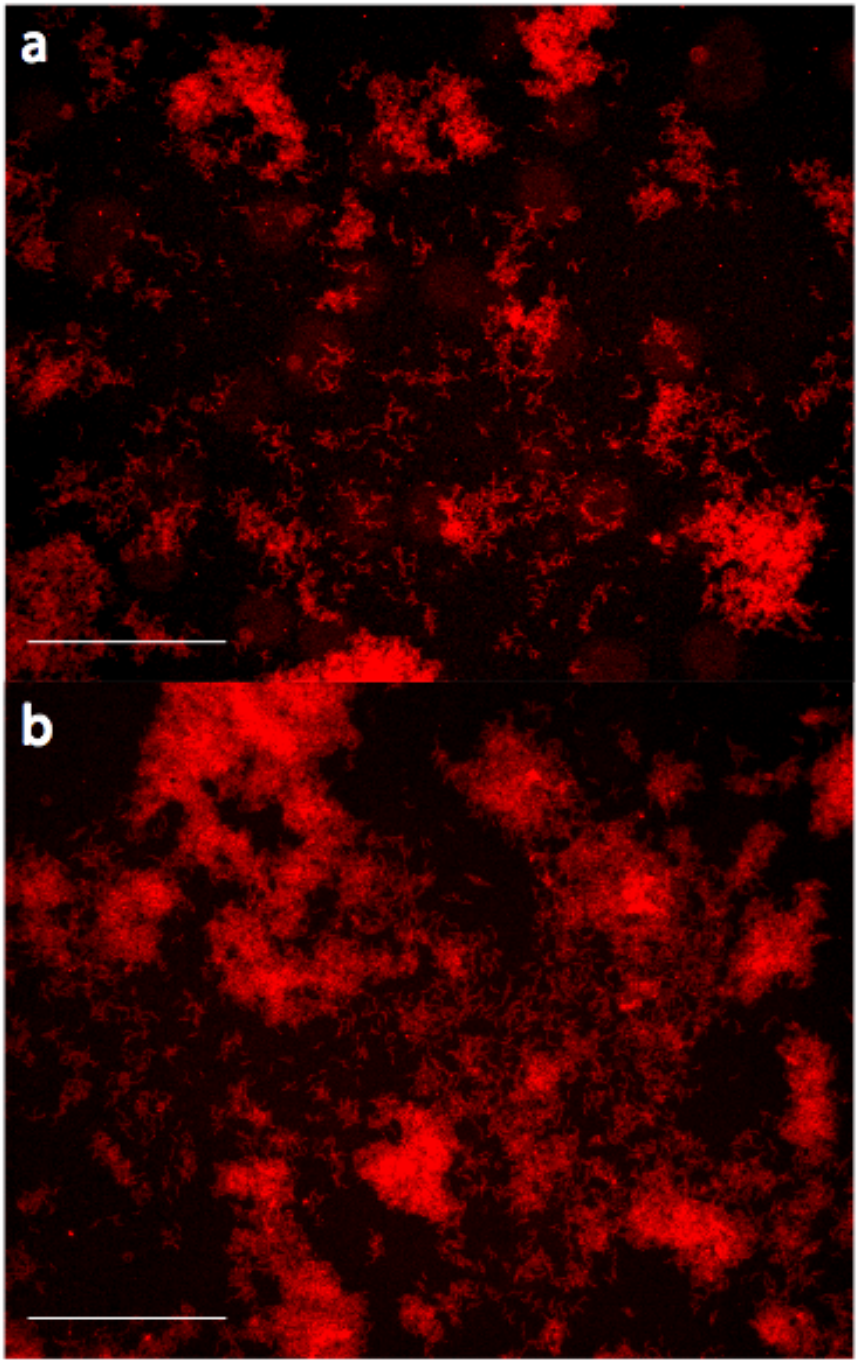


Figure 6. Fluorescence In-Situ Hybridization (FISH) tagging of strains VDY^{T} (a) and WD (b). Magnification = 630x. Bar, 50µm.



Chapter 5
Perchlorate-dependent iron(II) oxidation by strain VDY

Abstract

Previous work on bioelectrical reactors (BERs) for perchlorate reduction resulted in isolation of the novel dissimilatory perchlorate reducing bacteria (DPRB) strain VDY. The potential for this organism to oxidize iron(II) coupled to perchlorate reduction was tested in cell suspensions and growth conditions. Washed cell suspensions of strain VDY were capable of iron oxidation coupled to both perchlorate and nitrate, although electron balances for perchlorate-dependent iron oxidation indicated the presence of stored reducing equivalents. Attempts to culture VDY in electron donor-limited conditions did not eliminate these stored reducing equivalents. Spectrophotometric analysis of washed whole cells demonstrated the presence of reduced *c*-type cytochromes even after incubation with perchlorate. Under chemolithoautotrophic growth conditions with perchlorate as the electron acceptor, iron oxidation was observed, but without any increase in cell number. Perchlorate reduction was statistically similar to a control grown without iron(II), indicating that oxidation of iron(II) was not metabolically coupled to perchlorate reduction. When cultures were grown with 10 mM acetate and perchlorate as the electron donor and acceptor, respectively, in the presence of either 2 mM iron(II) or iron(III), VDY was unable to grow, compared to a no-iron control. Therefore, although strain VDY is capable of oxidizing iron(II) during perchlorate reduction, iron inhibits growth. However, the ability of the organism to reduce perchlorate and concomitantly oxidize iron(II) has implications for biogeochemical cycling of these compounds in environments where they occur together.

Introduction

Perchlorate has been widely used as a solid oxidant and, due to unregulated disposal prior to 1997, has become widespread throughout groundwater in the United States (22). Dissimilatory perchlorate reducing bacteria (DPRB) have been studied with considerable detail in response to the need for bioremediation of this important emerging contaminant (5). As such, much is known about the perchlorate-reduction pathway, and many DPRB have been isolated and cultured (5, 11)(Chapter 3). DPRB have been enriched from a wide range of environments, and evidence for their pervasive existence comes from functional gene detection (1, 6). Further, elements of the perchlorate-reduction pathway appear to have been horizontally transferred (1), but almost nothing of the natural habitats, distributions, and functional roles of these organisms is understood. Naturally occurring perchlorate has recently been found in a variety of locales, presenting a possible means for the selective pressure needed for this metabolism to evolve and move throughout the tree of life (17-19), and also justifying investigation of the roles these organisms may play in the natural environment. If perchlorate is naturally available as an electron acceptor, even at low levels, the range of electron donors these organisms may use coupled to reducing perchlorate will be informative to the types of biogeochemical processes DPRB may be involved with.

Research on the electron donors used to stimulate perchlorate reduction has primarily concentrated on bioremediation efforts. Pure culture investigations have revealed a large number of potential donors for the metabolism (2, 9, 13, 21, 25, 26). As a result, a range of organic and inorganic electron donors have been tested in bioremediation strategies, including hydrogen (15) and electrodes (21), and the humic substances analog, anthrahydroquinone-2,6-disulfonate (AHDS) (23). However, since understanding of the natural distribution of DPRB has been limited (1, 6), knowledge of what electron donors these organisms may use in nature is nascent. Since iron(II) exists in some solid forms which are readily available in the environment (e.g. pyrite), the identification of iron(II) as an electron donor for perchlorate reduction could be important for understanding global iron cycling as well as the naturally available electron donors for perchlorate-reduction in the environment. Perhaps more enticing is the possibility of this metabolism in extraterrestrial environments. Perchlorate has recently been discovered on Mars (8) and reduced iron is known to exist there as well (14).

DPRB have been shown capable of oxidizing iron(II) coupled to nitrate reduction (3, 10). However, this metabolism required an added carbon source such as acetate and growth has not been observed in association with the oxidation of the iron(II), even though cytochrome data indicated that electrons from the oxidation of iron(II) were capable of entering the electron transport chain (10). Further, when DPRB have been tested using chlorate as the electron acceptor instead of nitrate, the organisms were unable to survive (2, 10). Under such chlorate-reducing conditions, iron(II) was inhibitory to growth by acetate oxidation, even with 10mM acetate and only 1mM $FeCl_2$ (10). These data indicated that although some DPRB may be able to use iron(II) as an electron donor, this physiology was electron acceptor dependent.

Strain VDY was isolated from the cathodic chamber of a perchlorate-reducing bioelectrical reactor (BER) enrichment (21). This demonstrated the possibility of direct electrode oxidation, for which iron could possibly serve as a proxy. Characterization of the organism revealed it capable of chemolithoautotrophic growth on hydrogen coupled to

perchlorate reduction and growth cultures of VDY incubated with $FeCl_2$ and perchlorate showed characteristic oxidation products (Chapter 4). VDY was shown to be capable of surviving in the cathodic chamber of a BER and to continuously reduce perchlorate there under growth conditions, even though previously tested DPRB were incapable of doing so. Given the unique physiology of this organism, the lack of available data on iron(II) oxidation coupled specifically to perchlorate reduction in other DPRB, and the observation of iron(II) oxidation products in preliminary characterization, it was hypothesized that strain VDY was capable of perchlorate-dependent iron(II) oxidation. If true, this novel physiology would be valuable not only for the development of new bioremediation strategies for perchlorate, but would also aid in understanding the range of natural habitats available to DPRB and establish VDY as a good starting model organism for emerging Martian astrobiology.

Materials and Methods

Culture medium and conditions

Culturing media was prepared as described previously (2). Growth cultures were conducted using freshwater 30mM bicarbonate-buffered basal media (pH 6.8) under a N_2-CO_2 (80%:20%) headspace. Washed cell suspensions were carried out by centrifuging 1L of cells grown in minimal media and washing twice with 20mM PIPES buffer, pH 7, and resuspending after a third centrifugation in the same buffer. Cell suspensions were conducted using 20mM PIPES buffer (pH 7) under an N_2 headspace. Electron acceptors and donors were added separately from sterile, anoxic stock solutions. Iron(II) was added in the form of $FeCl_2$, and iron(III) as $FeCl_3$.

Reduced minus oxidized spectra

Reduced minus oxidized spectra were carried out as described (24) and carried out with a Cary 50 UV-Vis Spectrophotometer in an anaerobic chamber. Oxygen, perchlorate, nitrate, and chlorite were used as oxidants. Dithionite, $FeCl_2$, and acetate were used as reductants. All were added in concentrations of 5-10 mM in 20 mM PIPES buffer, pH 7. Cells were grown in the same manner as for cell suspensions, with 10 mM acetate and 15 mM perchlorate, harvested, centrifuged and washed twice in 20 mM PIPES buffer pH 7, and then pre-incubated in the same buffer with 5 mM perchlorate only for one hour. After centrifugation, concentrated cells were examined spectrophotometrically. Data was reported as the difference between the reduced and oxidized absorption spectra.

Analytical methods

All experimental analyses were performed in triplicate to ensure reproducibility and the results are expressed as the mean of these determinations. Controls were complete in singlet unless otherwise stated. The concentration of perchlorate and chlorate in cultures was determined using ion chromatography as previously described (4). Cell growth in active cultures was monitored by optical density at 600nm and by total direct cell count using phase-contrast microscopy (Petroff-Hausser counting chamber, 0.02-mm depth). Samples collected for counting were immediately fixed in 0.2µm filter-sterilized formaldehyde (final concentration 3.7%). For cell counts in cultures containing iron precipitate, oxalate was used as a chelator as described. Iron was measured as described (12).

Electron balances

Calculations determining relative reduced vs. oxidized equivalents were carried out using the following half reactions:

1) $ClO_4^- + 2e^- + 2H^+ = ClO_3^- + H_2O$
2) $ClO_4^- + 4e^- + 4H^+ = ClO_2^- + 2H_2O$
3) $ClO_4^- + 8e^- + 8H^+ = Cl^- + 4H_2O$
4) $NO_3^- + 2e^- + 2H^+ = NO_2^- + H_2O$
5) $NO_3^- + 5e^- + 6H^+ = 1/2N_2 + 3H_2O$
6) $Fe(III) + e^- = Fe(II)$
7) $CO_2 + 8e^- + 7H^+ = CH_3COO^- + 2H_2O$

Data is reported as the ratio of the means of the species being reduced (electron acceptors) to the species being oxidized (electron donors). Error of the ratios was estimated by multiplying the coefficient of variation with the ratio of the mean.

Results and Discussion

Washed cell suspensions

To assess the potential of strain VDY to oxidize iron(II) coupled to perchlorate reduction, washed cell suspensions were inoculated with cultures grown in stoichiometrically-balanced electron donor/acceptor replete media (10 mM acetate/perchlorate) according to:

$ClO_4^- + CH_3COO^- + H^+ \rightarrow Cl^- + 2CO_2 + 2H_2O$

These cells were then harvested in late log-phase. When incubated with 5 mM $FeCl_2$ and 5 mM ClO_4^-, cells oxidized 1.17 mM Fe(II) with concomitant reduction of 1.58 mM ClO_4^- over an 8-hour period (Fig 1). No iron was oxidized in heat-killed controls indicating that this process was dependent on enzymatic activity. Unexpectedly, 0.43 mM iron was oxidized by live cells without added electron acceptor. For the cultures with perchlorate and iron(II), complete reduction of perchlorate to chloride yielded a reduced electron acceptor to oxidized donor ratio of 1.35 ± 0.52, which is significantly different that the predicted ratio of 0.125 according to:

$8Fe(II) + ClO_4^- + 8H^+ \rightarrow 8Fe(III) + Cl^- + 4H_2O$

If reduction of perchlorate was assumed to only go to chlorite, the predicted reduced electron acceptor to oxidized donor ratio would have been 0.25 according to:

$4Fe(II) + ClO_4^- + 4H^+ \rightarrow 4Fe(III) + ClO_2^- + 2H_2O$

which is still significantly different than the measured ratio. If only partial reduction of perchlorate to chlorate is assumed, the total electron acceptor reduced vs. electron donor oxidized ratio would be 0.5 according to:

$2Fe(II) + ClO_4^- + 2H^+ \rightarrow 2Fe(III) + ClO_3^- + H_2O$

This value is still outside of the measured value, even taking experimental error into account. Thus, regardless of the assumed endproduct of perchlorate reduction, it was evident that a considerable amount of perchlorate was inexplicably reduced, implying an ability of VDY to store reducing equivalents (e.g. ß-hydroxybutyrate or glycogen).

To take into account the potential for VDY to store reducing equivalents when grown in 10 mM acetate/perchlorate, the washed cell suspension experiments were repeated using different starting incubation conditions. Cells were grown with 10 mM acetate and 15 mM perchlorate to ensure excess electron acceptor, and instead of harvesting during late log-phase, cells were allowed to progress into an extended stationary phase before harvesting after 36 hours. Were VDY to store reducing equivalents, excess electron acceptor relative to electron donor plus extended incubation time should ensure depletion of these cellular reserves. Again, prepared cell suspensions were incubated with either 5 mM $FeCl_2$/ClO_4^-, or 5 mM $FeCl_2$/ClO_4^- amended with 1 mM acetate to assess the relative contribution of abiotic iron(II) oxidation by biogenic chlorate accumulation, a phenomenon that was previously observed under growth conditions (Chapter 4). If iron oxidation were being carried out completely by an abiotic reaction with chlorate, the additional reducing equivalents present in acetate would allow for a significantly greater amount of iron oxidation in suspensions with acetate compared to those without it.

Unexpectedly, there was no statistical difference in iron oxidation between the suspensions with or without acetate (Fig 2). Suspensions with acetate oxidized a total of 0.57 ± 0.24 mM, (n=3) Fe(II), whereas those without oxidized a total of 0.80 ± 0.12 mM (n=3). Both suspensions also reduced statistically similar amounts of perchlorate: 1.38 ± 0.26 mM ClO_4^- and 1.08 ± 0.26 mM ClO_4^-, for incubations with and without acetate respectively. Furthermore, the overall rate of iron oxidation in the suspensions containing acetate was slower than those without

acetate, consistent with the competitive utilization of the two electron donors. In the absence of perchlorate cells oxidized 0.3 mM Fe(II) while 0.07 mM Fe(II) was oxidized in the heat-killed control.

Perchlorate-reduced to Fe(II) oxidized ratios were 2.42 ± 0.45 for incubations with acetate and 1.35 ± 0.28 for incubations without acetate. Assuming only one-step reduction of perchlorate to chlorate, with the ratio of 0.5 according the equation above, both of these incubations were again significantly different the theoretical value. The greater discrepancy between perchlorate reduced and Fe(II) oxidized in the suspensions containing acetate is consistent with competitive use of both electron donors for reduction of perchlorate. The balance for cultures without acetate was identical to that of the previous experiment, even though in this case the cells were grown under electron donor-limiting conditions. Strain VDY was therefore able to maintain stored reducing equivalents in spite of the excess electron acceptor and extended incubation time.

Growth cultures

Under chemolithoautotrophic growth conditions, VDY was capable of iron oxidation and perchlorate reduction, though no growth was observed (Fig 3). Cells were inoculated from a starting culture grown under electron donor-limited conditions as described above. The majority of iron oxidation took place in the first 48 hours, however perchlorate reduction was maintained for at least five days. Iron oxidation in the control without perchlorate was negligible compared to the cultures with the electron acceptor. Overall, 1.51 mM Fe(II) was oxidized, and 0.57 mM perchlorate was reduced. Measured chlorate accumulation was 0.057 mM, and with this taken into account, the overall calculation for milliequivalents reduced is as follows: 0.057 x 2 = 0.114 meq in the form of chlorate; 0.57 - 0.057 = 0.51 mM remaining. If this perchlorate is assumed to have been reduced to chlorite, 0.51 x 4 = 2.04 meq. 2.04 + 0.11 = 2.15 meq total reduced, vs. 1.51 meq oxidized, giving a ratio of 1.42. If all the perchlorate reduction were attributed to accessing the reducing equivalents from the Fe(II), this ratio should have been 1. This, combined with the observation that perchlorate reduction in the control with no added iron was the same as that in the cultures with $FeCl_2$, again demonstrated the presence of additional reducing equivalents.

Since iron had no effect on the total perchlorate reduced, the metabolism was hypothesized to be incidental, either a result of some abiotic reaction with chlorate or another intermediate produced during microbial perchlorate reduction. Measured chlorate never exceeded 57 μM, which was not enough oxidizing equivalents to explain the full extent of the iron(II) oxidized, but this may also be indicative of a steady state level of chlorate that was produced and subsequently oxdized the iron(II). Regardless of the mechanism, however, it can be concluded that iron(II) oxidation by strain VDY is dependent on active perchlorate-reduction, but perchlorate-reduction is not dependent on iron(II) oxidation.

Cytochrome scans

Consistent with the data from washed cell suspensions and growth cultures, reduced minus oxidized spectra of whole cells demonstrated the presence of reduced cytochromes in native cells, even after pre-incubation with perchlorate in the absence of an electron donor (Fig 4). Difference spectra show characteristic peaks for *c*-type cytochromes: 424, 521, and 553 nm. However, dithionite-reduced minus chlorite-oxidized cells show an almost identical difference spectrum to that of chlorite oxidized cells that had not been subjected to additional reductant.

Therefore, in spite of growing the culture under electron donor-limited conditions and incubating the cells with only perchlorate prior to spectrophotometric testing, strain VDY retained reduced *c*-type cytochromes. This supported the idea that VDY may have the capacity to maintain a reduced electron transport chain for extended periods of time. A previous study indicated that *c*-type cytochromes in *Geobacter sulfurreducens* could potentially serve as biological capacitors (7). Strain VDY may be capable of similar electron storage and enhanced energy reserves.

Iron toxicity for heterotrophic growth

Previously, iron(II) was observed to inhibit heterotrophic growth of strain PS on acetate when chlorate was the electron acceptor, even though growth was unaffected when that electron acceptor was replaced with nitrate (11). To assess whether this phenomenon was common to strain VDY, cultures were incubated in growth media containing 10 mM acetate/perchlorate with either 2 mM $FeCl_2$ or $FeCl_3$, and compared to a control without added iron. $FeCl_3$ was included as a control for the effect of valence state on the potential toxicity of iron. Figure 5 shows growth inhibition by both iron(II) and iron(III) compared with a no-iron control. In both cases, the presence of 2 mM iron inhibited growth and perchlorate reduction, regardless of the valence state, similarly to previous observations of strain PS.

Postulated model for growth inhibtion by iron during perchlorate reduction

The observed data have been summarized in a model describing the specific effect of iron on the perchlorate-reduction pathway (Fig 6). Since perchlorate reduction was possible, the perchlorate reductase (Pcr) enzyme complex was functional, however, because no growth was observed, it was hypothesized that the iron was directly inhibiting the chlorite dismutase (Cld). Further, $FeCl_3$ forms insoluble precipitates in the basal growth medium, making it unlikely to penetrate the outer membrane of the cells. Since the precipitated iron(III) was equally effective as iron(II) at inhibiting growth and the Cld is predicted to be located on the outer membrane (5, 16), the enzyme is in an ideal position to be the mechanism of growth inhibition. It has been shown that most energy conservation for growth during perchlorate-reduction occurs at the cytochrome oxidase step, not at the perchlorate reductase step (20). Electron transport chain inhibitor studies have confirmed the presence of complex 1, the bc_1 complex, and cytochrome oxidase in VDY (data not shown), and the cytochrome scans confirmed the presence of *c*-type cytochromes. Thus, if iron was inhibiting the Cld, no oxygen would be formed and subsequently reduced, thereby preventing the establishment of a proton-motive force. Further, inhibition of the chlorite dismutase would lead to a buildup of chlorite, which is toxic to the cells and could react abiotically with the iron(II) in solution thus producing Fe(III). For this mechanism to work, VDY must have a way of reducing perchlorate initially, but without an electron donor this would not be possible. The stored reducing equivalents indicated by the data, however, could provide this required electron donating capacity.

Since the model specifically implicates Cld in the mechanism of growth inhibition by iron, it was predicted that iron(II) oxidation coupled to nitrate reduction would be a complete, coupled metabolism. True to this model, cell suspensions with nitrate as the electron acceptor showed a very different, balanced trend compared to those with perchlorate. When grown with 10mM nitrate/acetate and harvested at late log-phase, cells were capable of oxidizing 2.01 mM Fe(II) and reducing 0.80 mM NO_3^- over 8 hours (Fig 7). No iron was oxidized in the heat-killed control, and again, 0.43 mM iron was oxidized by live cells without added electron acceptor.

Measured nitrite accumulation was 0.66mM after 8 hours. If complete reduction of the remaining nitrate to N_2 is assumed after taking into account this partial reduction to nitrite, a total of 2.02 meq were reduced. This gives a stoichiometry of 2.02 meq reduced to 2.01 meq oxidized, or 1, which is the same as predicted. Thus, in contrast to perchlorate-dependent iron oxidation, nitrate-dependent iron oxidation by strain VDY was most likely a coupled metabolic process, similar to that reported for an alternative perchlorate reducing organism strain PS (3). The significant physiological differences associated with the two alternative electron acceptors therefore indicate a different mechanism for iron oxidation in each.

Significance

Although these studies demonstrate that perchlorate-dependent iron(II) oxidation by strain VDY is an incidental reaction that inhibits growth of the organism, the fact that the organism has a mechanism for conserving reducing equivalents that can be utilized for perchlorate reduction means that in an environment where iron(II) and perchlorate co-exist, this organism may be capable of catalyzing this reaction and thus contributing to the biogeochemical cycling of iron. Further studies of the natural distribution of these organisms should aid in understanding this potential role in the environment. In addition, biochemical studies will be able to elucidate the sensitivity of the Cld to various forms and concentrations of iron so that the possible effects on perchlorate reduction in natural settings may be better anticipated.

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Figures

Figure 1. Perchlorate-dependent iron(II) oxidation by VDY. Concentrations are shown as the total change of ion concentration for ease of comparison. Closed squares, [Fe(II)] for cells with $FeCl_2$ and ClO_4^-; open circles, [Fe(II)] for cells with $FeCl_2$ only; open squares, [Fe(II)] for killed cells with $FeCl_2$ and ClO_4^-; closed triangles, [ClO_4^-] for cells with $FeCl_2$ and ClO_4^-; open triangles, [ClO_4^-] for killed cells with $FeCl_2$ and ClO_4^-.

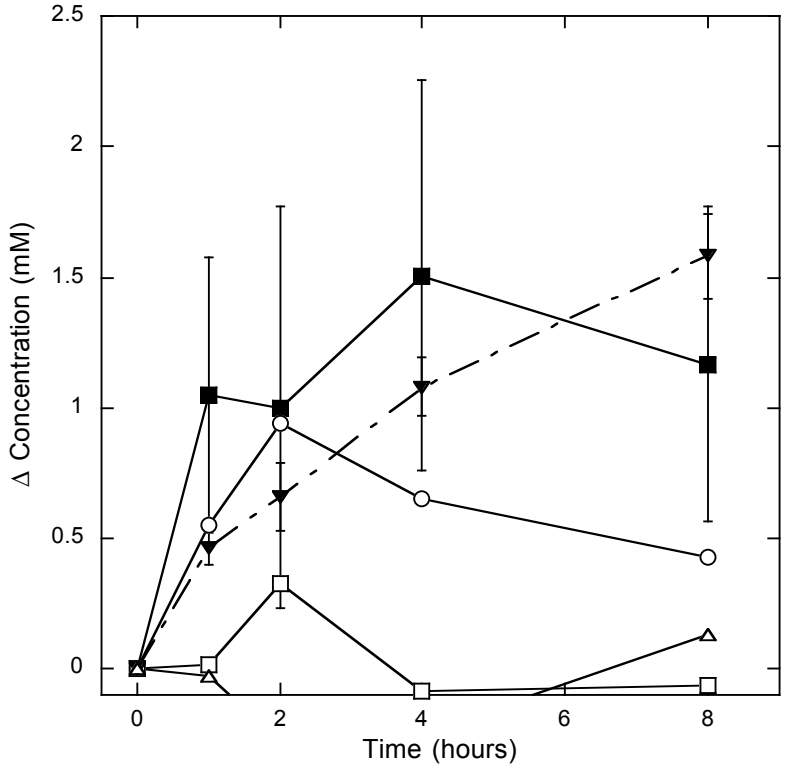


Figure 2. Perchlorate-dependent iron(II) oxidation by VDY cells grown in electron donor-limited conditions. Concentrations are shown as the total change of ion concentrations. Closed circles, [Fe(II)] for cells with acetate, $FeCl_2$, and ClO_4^-; closed squares, [Fe(II)] for cells with $FeCl_2$ and ClO_4^-; open circles, [Fe(II)] for cells with $FeCl_2$ only; open squares, [Fe(II)] for killed cells with $FeCl_2$ and ClO_4^-; closed diamonds, [ClO_4^-] for cells with acetate, $FeCl_2$, and ClO_4^-; closed triangles, [ClO_4^-] for cells with $FeCl_2$ and ClO_4^-; open triangles, [ClO_4^-] for killed cells with $FeCl_2$ and ClO_4^-.

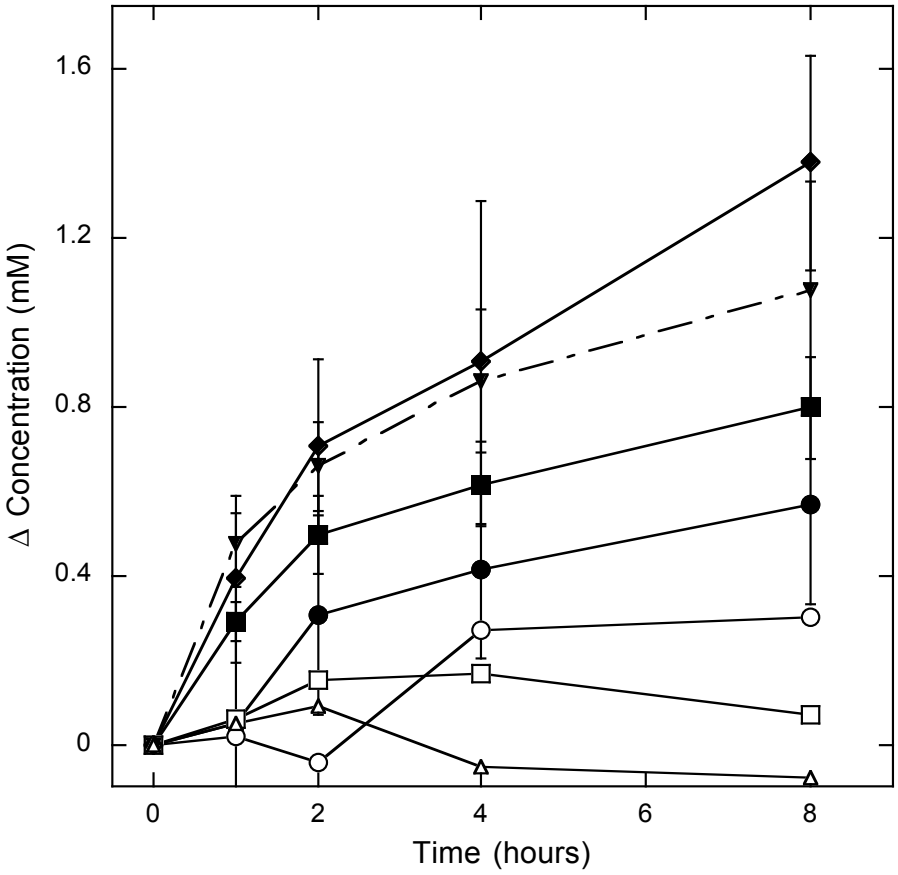


Figure 3. Perchorate-dependent iron(II) oxidation under growth conditions. Closed circles, [Fe(II)] for live cells with $FeCl_2$ and ClO_4^-; open circles, [Fe(II)] for live cells with $FeCl_2$ only; closed squares, [ClO_4^-] for live cells with $FeCl_2$ and ClO_4^-; open squares, [ClO_4^-] for live cell with ClO_4^- only; closed diamonds, total cells with $FeCl_2$ and ClO_4^-.

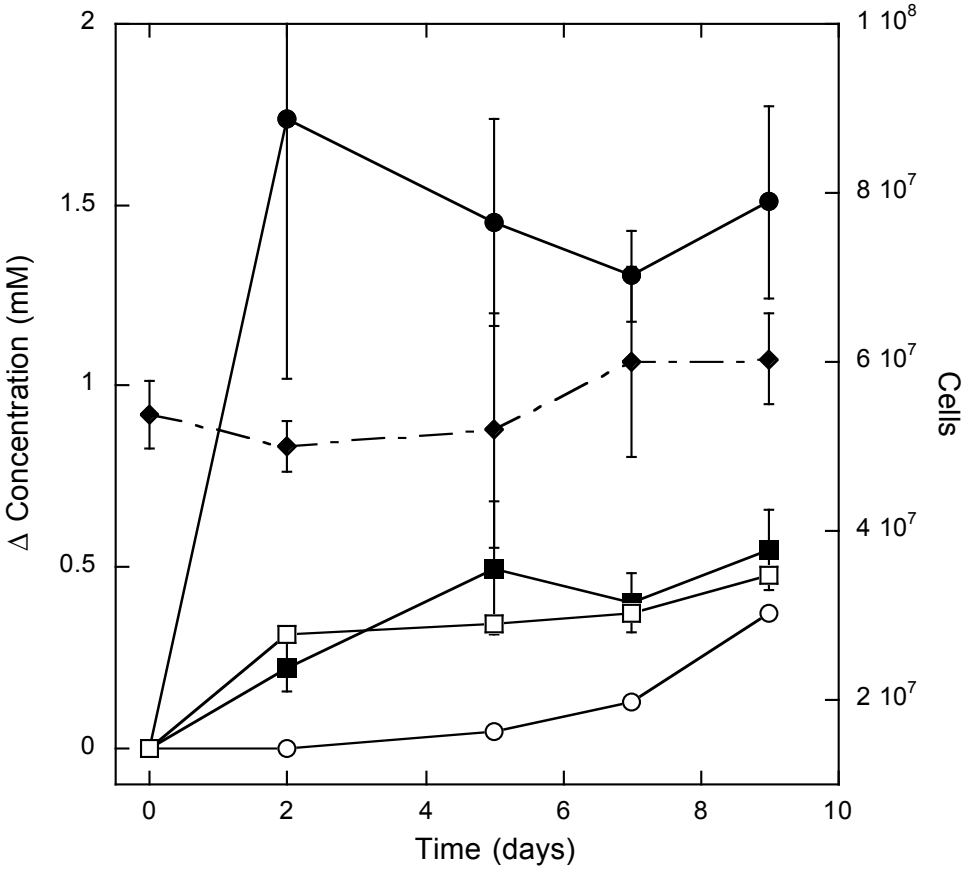


Figure 4. Comparative reduced-oxidized spectra for strain VDY. The top line is the reduced-oxidized spectra for untreated cells that were then oxidized with ClO_2^- (10 mM). The bottom line is the spectra for cells first reduced with dithionite (10mM) and then oxidized with ClO_2^- (10 mM).

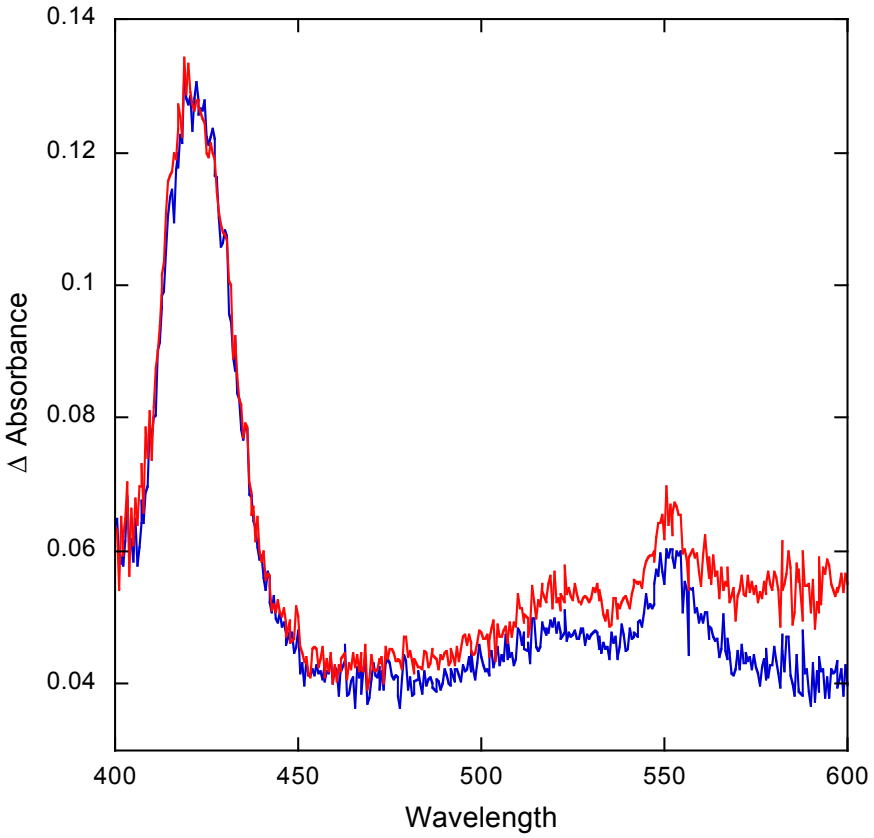


Figure 5. Effect of iron(II) and iron(III) on heterotrophic growth of strain VDY. Closed squares, cells with added Fe(II); closed circles, cells with added Fe(III); open circles, cells with no added iron; closed triangles, [ClO_4^-] for cells with added Fe(II); closed diamonds, [ClO_4^-] for cells with added Fe(III); open squares, [ClO_4^-] for cells with no added iron.

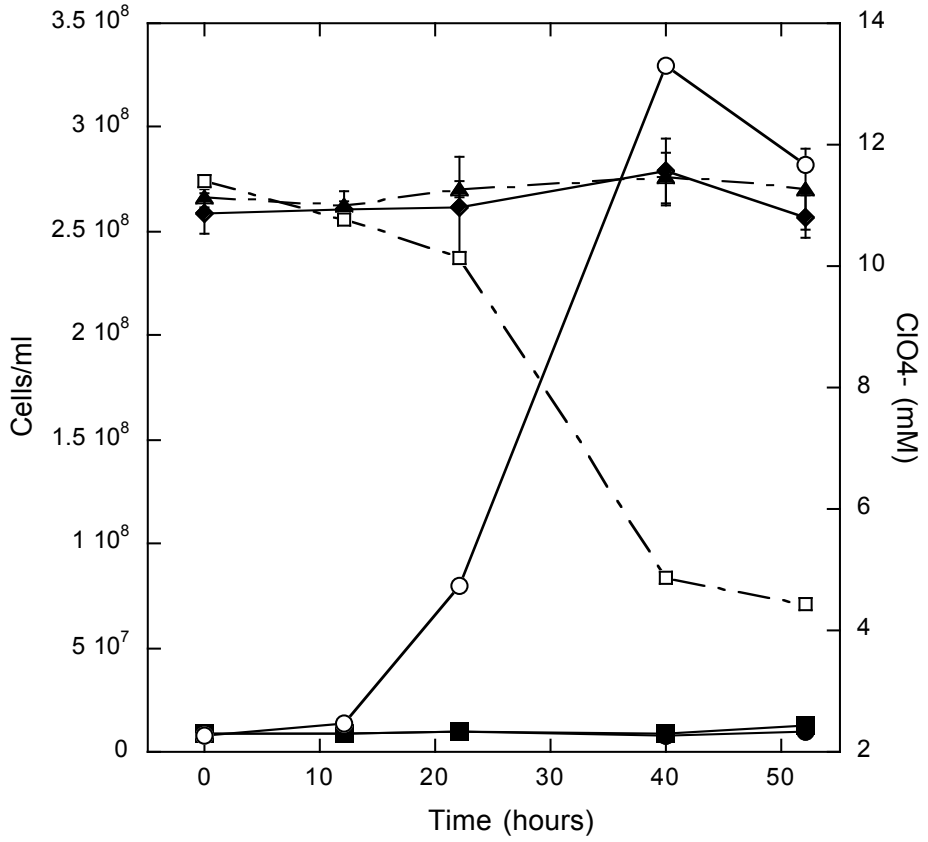


Figure 6. Model for possible interations of iron with the perchlorate-reduction pathway of strain VDY. Both iron(II) and iron(III) are postulated to inhibit chlorite dismutase (Cld). This prevents the formation and subsequent reduction of oxygen, disabling the creation of a proton motive force. Cld inhibition would also cause a buildup of chlorite, which is toxic to the cell and can also abiotically react with iron(II). OM, outer membrane; PM, periplasmic membrane; Pcr, perchlorate reductase.

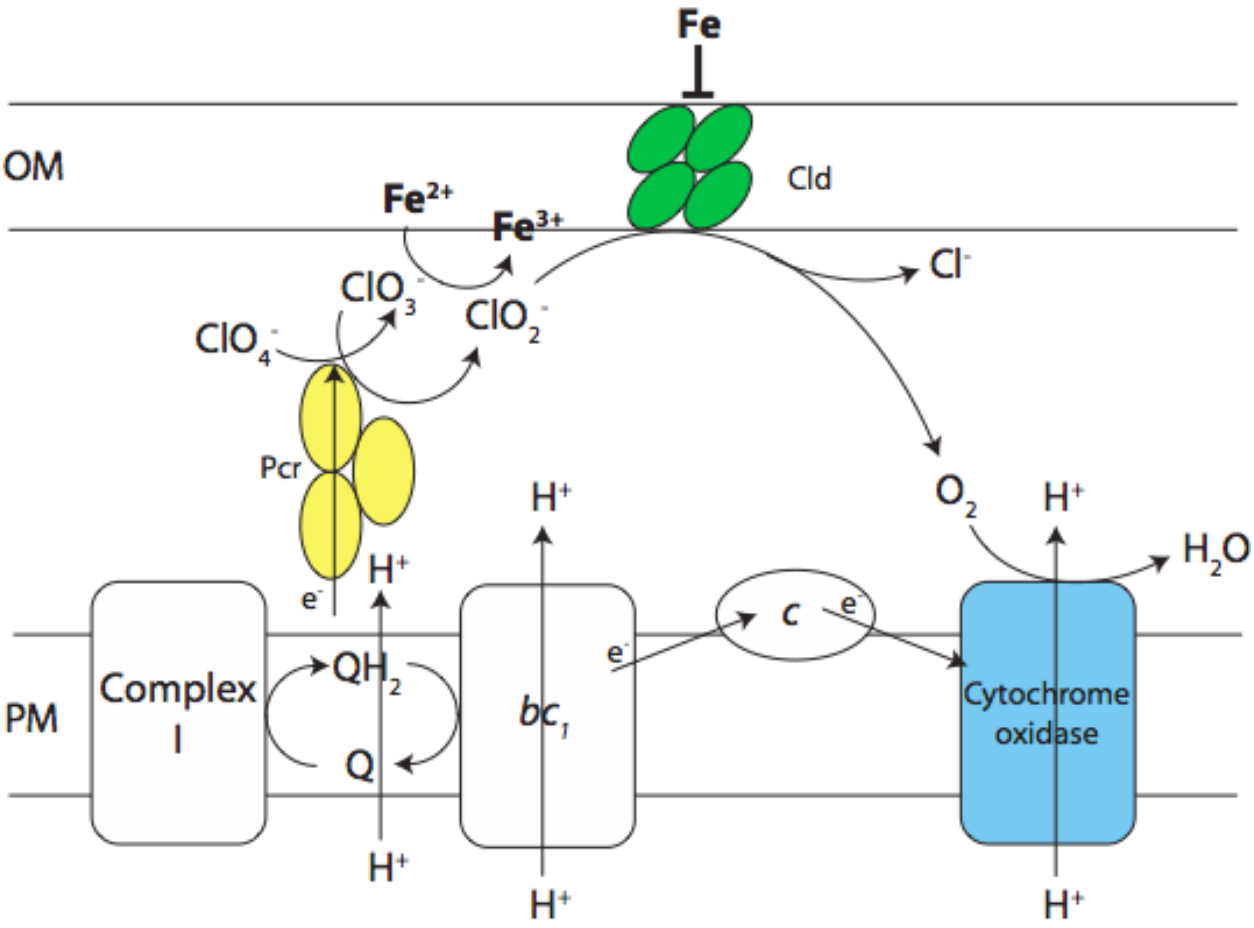
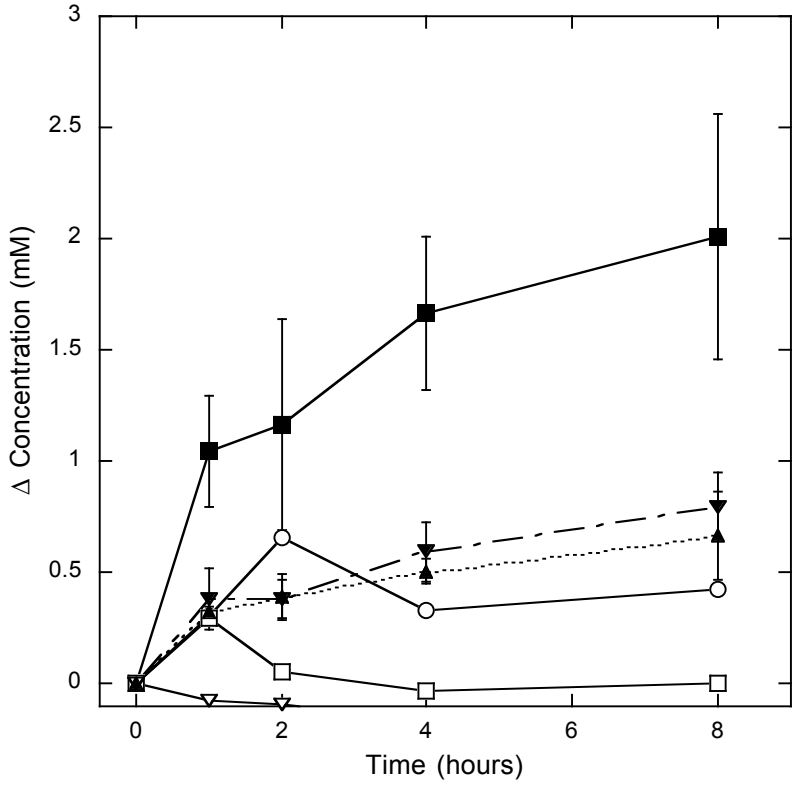


Figure 7. Nitrate-dependent iron(II) oxidation by VDY. Concentrations are shown as the total change of ion concentration for ease of comparison. Closed squares, [Fe(II)] for cells with $FeCl_2$ and NO_3^-; open circles, [Fe(II)] for cells with $FeCl_2$ only; open squares, [Fe(II)] for killed cells with $FeCl_2$ and NO_3^-; closed inverse triangles, [NO_3^-] for cells with $FeCl_2$ and NO_3^-; open inverse triangles, [NO_3^-] for killed cells with $FeCl_2$ and NO_3^-; closed upright triangles, [NO2-] for cells with $FeCl_2$ and NO_3^-.



Chapter 6
Summary and Future Directions

The study of DPRB crosses multiple fields and motivations. While much of the research on these organisms has been driven by bioremediation efforts, the discovery of naturally-occurring perchlorate on Earth and Mars in recent years (2-4) has begun to stimulate basic research for understanding the distribution and evolution of microbial perchlorate reduction and DPRB in their own right. This bifurcated focus has been exemplified in the above chapters. The original goal of the project was to develop a means of stimulating DPRB in an ex-situ system that would overcome the problems of biofouling and feedback control associated with labile organic donor addition. The use of electrochemical systems for the purpose of stimulating microbial metabolism had been applied in a variety of ways prior to this project, and in the course of developing the BER system for perchlorate reduction, these systems were studied and are the subject of the introductory Chapter 1.

In Chapter 2, stimulation of DPRB with a bioelectrical reactor (BER) was demonstrated as a viable means of overcoming biofouling and thus provided an alternative to traditional bioremediation schemes that make use of addition of cheap carbon sources such as ethanol or acetate. The study demonstrated the need for specifically-selected DPRB in such a system, but also the ready availability of such organisms in the nearby environment. Isolation of strain VDY allowed further development of continuous-flow BERs that could effectively remove perchlorate over week- and month-long time periods without interruption. This system functioned effectively both with and without the mediator AQDS, removed influent perchlorate over a range of concentrations, and could remove mixed-waste contamination with nitrate and perchlorate. As a direct result of the success of this system, patents were applied for: # 60/867,393 “Bioelectrical Treatment of Perchlorate and Chlorate” and # 60/975,584 “Bioelectrical Treatment of Xenobiotics." As of the date of this filing, these patents are available for licensing through the Lawrence Berkeley National Laboratory office of technology transfer, and one company has begun developing a pilot-scale reactor for testing and optimization of the technology on a larger scale.

While the BER system was successfully demonstrated at the bench scale, this system has yet to be optimized for pilot- and full-scale implementation. Data collected in the above work suggest that the system could be competitive with existing technologies in terms of volumetric treatment of perchlorate-contaminated influent per unit time, however, it is unknown whether this system will scale linearly with increased size. The H-cell configuration is unlikely to be utilized for larger systems because of the inefficiencies associated with this design, including footprint, internal resistance, and the cost of the proton-exchange membrane. Thus, optimization of the BER for increased size will include redesigning the basic architecture of the reactor. Further, no work has been done to assess the cost of implementing one of these systems on the scale needed to compete with existing technologies. If the system can be successfully scaled to the volumetric loading rates of current perchlorate-reducing reactors, cost analysis per unit treated perchlorate will need to be completed to bring this technology to market. While initial reactor startup includes inoculation with strain VDY, any reactor operated in the field with inevitably enrich for a community of DPRB. Nothing is known about the dynamics of a mixed community in a perchlorate-reducing BER. Studies carried out to assess the development and continuity of such a community will aid in optimizing these reactors and contribute to overall knowledge of the ecology of these organisms.

During development of the BER, two novel organisms were isolated from the community enrichment. While strain VDY was investigated further in the BER context, little was known about other aspects of their physiology. Both were novel DPRB for different reasons: VDY was novel physiologically in that it could survive in the cathodic chamber, unlike previously isolated members of the *Dechloromonas* and *Azospira* genera. The other organism, strain MP was a member of the *Propionivibrio* genus, which had no previously known DPRB. The complete characterization of this and two other DPRB was the subject of Chapter 3. Phylogenetic recharacterization of strain MP, strain CR, and strain LT-1 demonstrated these organisms represented two novel clades of DPRB in the *Betaproteobacteria*, and as a result, this study effectively doubled the number of known genera in this phylum which have organisms capable of perchlorate reduction. Chapter 4 details the complete characterization of strain VDY, which was closely related to another DPRB in the *Alphaproteobacteria*. These two organisms are phylogenetically (with respect to the 16S rRNA gene) nested within the genus *Magnetospirillum*, and yet are not capable of making magnetosomes and have numerous other unique physiological features relative to their closest neighbors. Strain VDY does not contain key magnetosome-forming genes. In addition, this study also developed a genus-specific FISH probe for the identification and quantification of these DPRB in the *Alphaproteobacteria*.

The isolation and characterization of new DPRB lends itself to comparative studies of these organisms and the similarities and differences in the perchlorate-reduction dynamics between them. DPRB occupy several unique clades in the Proteobacteria (Figure 1). Previous work has shown that elements of the metabolism may be horizontally transferred between organisms (1), and therefore it is tempting to speculate that the perchlorate reduction phenotype could be found in organisms from any phylogenetic affiliation. Further alteration of enrichment conditions and isolation techniques should bring more organisms into pure culture for study. Comparative analysis of the genes involved in perchlorate reduction between different organisms will help in understanding the evolution of the metabolism. Strain MP, similarly to strains CR and PCC, was shown to accumulate much more chlorate as an intermediate in perchlorate reduction than other DPRB, including for example strain VDY. Since different phenotypes exist within observed DPRB, it should be possible to compare the *pcrA-D* and *cld* between individuals to determine the responsible sequence changes. This kind of analysis may also help determine the evolutionary timeline for the genes in question and aid in estimating the origin of the metabolism.

Since strain VDY was capable of perchlorate in the cathodic chamber of the BER and could make use of hydrogen as an inorganic electron donor, the possibility of this organism oxidizing iron(II) coupled to perchlorate reduction was investigated in Chapter 5. It was determined that VDY was in fact capable of oxidizing iron(II) in cell suspensions and growth media, but this oxidation was not coupled to perchlorate reduction and in fact inhibited growth of the organism. In addition, it was discovered that strain VDY has the ability to store reducing equivalents, in an as yet undetermined manner, that can be used to reduce perchlorate.

The energy storage of strain VDY is a unique phenotype for DPRB, and with regards to perchlorate reducers has only been observed in this organism and its closest relative, strain WD. It is possible these organisms make and store well-established storage molecules such as glycogen or ß-hydroxybutyrate. However, it is also possible that the stored reducing equivalents observed in VDY take a different form. Regardless, understanding this storage mechanism is important since it implies that organisms like VDY are capable of perchlorate reduction, to some degree, in the absence of added electron donor. In addition, the BER studies in Chapter 2

indicate that this did not happen over the observed time spans in the bioelectrical reactors, since no perchlorate reduction occurred in the absence of applied current in these systems. However, due to the applied charge, the environment in the cathodic chamber of the BER is different than that in regular liquid culture. It is therefore not unreasonable to hypothesize that VDY is capable of making use of stored reducing equivalents for perchlorate reduction only in certain environments.

Overall, the work circumscribed by this dissertation accomplished several things. A successful new bioremediation strategy was developed and brought to the licensing stage of development as a marketable technology. Two new DPRB were isolated, adding to the collection of these organisms available for investigation, and the distribution of DPRB in the *Betaproteobacteria* was overhauled with the identification of two new clades of organisms capable of perchlorate reduction. As described above, however, there are several points of departure for continuing studies based on this work.

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Figure 1. Bayesian 16S rRNA gene tree showing representatives of all current DPRB clades. Stars indicate nodes with a posterior probability of 1, filled circles posterior probablity 0.8 – 0.99, open circles posterior probability < 0.8. Scale bar indicates 0.08 changes per position.