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Discoveries in the Biology of Oxidized Chlorine

By

Tyler P Barnum

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
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Abstract

Discoveries in the Biology of Oxidized Chlorine

by

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Doctor of Philosophy in Microbiology
University of California, Berkeley
Professor John D. Coates, Chair

Chlorine participates in a biogeochemical cycle that rivals that of elements like nitrogen and sulfur in chemical diversity. Nature produces a variety of organic and inorganic chlorine-containing molecules that participate in different biological, chemical, and geological processes. The most defining feature of chlorine is its high electronegativity, perhaps best illustrated by the very high reduction potential of molecules in which chlorine is at a higher oxidation state, such as hypochlorous acid (HOCl), chlorite (ClO_2^-), chlorate (ClO_3^-), and perchlorate (ClO_4^-).

Compared to research on the biogeochemical cycles for other elements, the mechanisms by which oxidized chlorine molecules are produced and consumed are relatively understudied. One reason is that descriptions of the biogeochemical chlorine cycle have been incomplete, omitting important processes involving inorganic chlorine molecules. Another reason is that the microbiology of oxidized chlorine has been studied almost exclusively through a reductionist approach that can preclude the discovery of ecological interactions. Yet another reason is that the genes known to be involved in the metabolism of oxidized chlorine have yet to be used to find new organisms and processes that metabolize oxidized chlorine. Here, a more holistic approach, enabled by improvements in genome sequencing, is used to better describe the biology of oxidized chlorine across several research projects.

The first chapter of this dissertation provides the first review of the entire biogeochemical chlorine cycle, emphasizing connections between the various biological, chemical, and geological processes that interconvert chlorine between different chemical forms.

The second chapter of this dissertation is a published research article describing the reduction of perchlorate in microbial communities for the first time by using bioinformatics techniques to obtain genomes from metagenomes. Instead of being dominated by the specific bacteria known to respire perchlorate, perchlorate-reducing communities contain diverse organisms that interact via the chemical intermediates of dissimilatory perchlorate reduction.

The third chapter of this dissertation is a published research article investigating the mechanism of one such interaction between perchlorate-reducing bacteria and chlorate-

reducing bacteria. A combination of genomics, strain isolation, genetics, metabolite measurements, and theoretical modeling are used to learn that these two metabolisms, which have been studied separately for several decades, have a conserved interaction due to the accumulation of chlorate by perchlorate-reducing bacteria.

The fourth chapter of this dissertation is a brief report characterizing a possible perchlorate reductase or chlorate reductase first identified in perchlorate-reducing communities. An organism with this reductase, which is always found in genomes adjacent to the chlorite-degrading enzyme chlorite dismutase (Cld), is capable of chlorate reduction but not perchlorate reduction, indicating the enzyme is a chlorate reductase.

The fifth chapter of this dissertation extends the above comparative genomics analysis to identify *any* gene or organism linked to the enzyme Cld. Because Cld is biomarker for chlorite and other chlorine oxyanions, this approach was able to expand the environments, organisms, and processes known to participate in oxidized chlorine biology beyond the organisms and genes described above. Specifically, more was learned about the reduction of perchlorate and chlorate in the environment; the potential oxidation of chloride beyond hypochlorous acid by chemical or biological activity; and the connection between chlorite and reactive chlorine stress response.

Together, this research has answered important questions about the reduction of chlorine while opening new questions about the oxidation of chlorine and the role of oxidized chlorine species in the environment.

Dedication

To the next generation of scientists, who cannot perform science for their own curiosity but must perform science for the selfless betterment of our planet.

Acknowledgements

I would like to thank those who made this research possible.

The National Science Foundation provided financial support for this work through its Graduate Research Fellowship Program. My development as a scientist and research was buoyed by dedicated staff in the Department of Plant & Microbial Biology and by broader programming available at U.C. Berkeley for better teaching, science, and programming. Such institutional support greatly enabled better scientific inquiry.

My thesis committee helped me prioritize research projects and charitably made themselves available to help me. Dr. Kara Nelson, my outside committee member, volunteered her time and attention to make sure my degree and research were advancing appropriately. Dr. Jill Banfield was the reason I applied to U.C. Berkeley and is a role model for using genomics techniques with scientific rigor. Dr. John Coates, my advisor, provided invaluable expertise in microbial physiology while learning new methodologies to help me pursue questions in different areas of microbiology.

Participants in this research from the John Coates's lab were many. Lauren Lucas, Dr. Charlotte Carlström, and Anna Engelbrektson performed the enrichments and isolations that initiated chapters two and three of this dissertation. Dr. Israel Figueroa helped design some of the bioinformatics methods used in chapter two. Kaisle Hill helped me plan and execute experiments in chapter three. An outside collaborator, Yiwei Cheng, provided expertise in modeling for chapter three. Other help came from my graduate student peers, Dr. Ouwei Wang, Dr. Maggie Stoeva, Victor Reyes-Umaña, Sophia Ewens, and Dr. Matthew Olm, who provided feedback on many chapters and general support. Yi Liu ensured a functioning laboratory for conducting research. Finally, in helping me critically think through different experiments and conclusions, Dr. Hans Carlson acted as a *de facto* fourth committee member.

Everyone has something to teach others, and the scientific and personal role models that I learned from have been too many to list here.

Many people have the potential to do what I have done but did not reach this stage. For that, I owe a stable early childhood in which my parents, family, and friends allowed my curiosity to blossom. I received the benefit of state-supported advanced curriculum in excellent public elementary, middle, and high schools staffed by many wonderful, caring, dutiful teachers. Johns Hopkins University accepted this boring Indiana boy, unlike other elite institutions, and offered me need-based tuition plus additional support through a Bloomberg Scholarship when I needed it most. I owe my intellectual heritage to my educators and peers at Johns Hopkins, who are truly world-class in character. In particular, Dr. Jocelyne DiRuggiero gave me the confidence and preparation for graduate school that every undergraduate student hopes for. I acknowledge these unearned advantages and promise to help others throughout my life.

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Preface

Because the first chapter of this dissertation does not serve as a traditional introduction, I would like to explain the motivation behind my research.

Perchlorate and chlorate have long been identified as pollutants addressed through biological remediation. When I began this research, how bacteria degrade perchlorate and chlorate through metabolic respiratory pathways had been adequately explained. Only a few questions remained about how it occurs: how other organisms in communities may affect the behavior of perchlorate-reducing bacteria and chlorate-reducing bacteria, and if there are a greater variety of organisms and enzymes that participate in this process than currently understood. Answering these questions motivated chapters two, three, and four of this dissertation and could only be enabled by recently developed metagenomic approaches to peer into microbial communities and predict metabolic activity in different organisms.

Underlying that research, though, were broader questions about the context of perchlorate and chlorate reduction. How do these metabolisms occur in the natural environment? How do they fit into the global biogeochemical cycle of chlorine? How are the enzymes used in these metabolisms also used in other biological processes? Those questions motivated the expansion of this dissertation's scope from the study of perchlorate and chlorate metabolism to the consideration of all oxidized chlorine molecules and their roles on Earth. In some ways, this undermines the position of perchlorate and chlorate reduction as a research subject, as it is seen as a small, relatively inconsequential part of a larger biogeochemical cycle. In other ways, this is an important step for the field, as any one metabolism *should* be seen as a part of something larger. The last chapter of this dissertation adopts this new perspective to link different aspects of oxidized chlorine biology using genomic data, and the first chapter of this dissertation synthesizes a broad set of literature to propose a biogeochemical cycle for chlorine.

One of the biggest motivations for adopting a broader scope was to empower future researchers. Instead of focusing on more minute aspects of a specific metabolism, I could identify entirely new areas that might be valuable to study. In particular, readers of this dissertation who are not interested by the metabolisms of perchlorate and chlorate reduction (despite their unique biology) will learn as I did that the metabolism of chlorite and hypochlorous acid – the lower oxidation states of chlorine – is broadly important yet understudied.

It is my hope that the contents of this dissertation have successfully integrated different processes of the chlorine cycle into a single, accessible, and actionable field of study.

Chapter 1
Unifying the biogeochemical chlorine cycle

Abstract

Chlorine's natural roles in the Earth system are astounding in scope and effect. Chlorine is a means for balancing the charge and osmotic potential of the cell, a source of energy and metabolites for microorganisms, a cause of oxidative stress, a mobilizer of metals in geologic fluids, and a mechanism of atmospheric ozone degradation. Because these topics span independent scientific disciplines, a holistic summary of a global biogeochemical "chlorine cycle" is lacking. Here, we provide a concise synthesis of the processes by which chlorine enters and exits the biosphere, is converted between chemical forms, and is used in different biological functions. Chlorine is found as a component of minerals, as an anion, as a gas, in oxidized states covalently bound to oxygen, and in reduced states covalently bound to carbon in organic molecules. The total amount of chlorine in the biosphere decreases with the deposition of evaporite sediments on continents and increases with the weathering of rocks and emission of volcanoes, with a net decrease since the early Earth. Life uses chlorine for various purposes and can alter chlorine through enzymatic oxidation, reduction, and chlorination and dechlorination of organic molecules. Chlorine is transported between environments as a highly soluble anion, as wind-blown sea salt spray, or as volatile organic molecules produced mostly by plants. Sunlight reacts with chlorine in the atmosphere and produces the highest oxidation states of chlorine on a global scale. Other oxidation states of chlorine, like that produced by the mammalian immune system, are highly reactive and rapidly oxidize or chlorinate biomolecules. Accordingly, life has evolved cellular systems to respond to the special type of oxidative stress caused by reactive chlorine species. In summary, the many forms and transformations of chlorine constitute a complex biogeochemical cycle that affects all of life on Earth.

Introduction

Chlorine is among the most abundant elements on Earth. In the ocean and most geologic fluids, chlorine is the most abundant element other than hydrogen and oxygen [1], and in cells, chlorine is the most common anion and a major control on osmotic potential [2, 3]. After oxygen and fluorine, chlorine is the most electronegative element. Only one additional electron is needed to complete chlorine's valence octet, a property that makes it highly electrophilic and able to form a large dipole moment in a covalent bond. Owing to its high electronegativity, chlorine is most often found in its reduced form as chloride (Cl^-) or organic chlorine (R-Cl), in which chlorine forms a single bond with carbon in a variety of organic molecules including gases [4-6]. Reduced chlorine also occurs as hydrogen chloride (HCl), which in water forms the strong acid hydrochloric acid (HCl, pKa -6.3). Oxidized chlorine is inorganic: the aqueous hypochlorous acid (HOCl), chlorite (ClO_2^-), chlorate (ClO_3^-), and perchlorate (ClO_4^-), and various gaseous chlorine species like chlorine dioxide (ClO_2) and molecular chlorine (Cl_2). Notably, hypochlorous acid and molecular chlorine exist in a pH-dependent equilibrium in which molecular chlorine is formed below pH 4.

All of the above chlorine molecules are found in nature (Table 1-1). The common misconception that chlorine-containing molecules are unnatural may be due to the unusually high reactivity (or relative inertness) of many of the most famous chlorine chemicals. Molecular chlorine was used as a chemical weapon in the first world war [2]. Hypochlorous acid, molecular chlorine, and chlorine dioxide are used as disinfectants in water treatment plants [2]. The oxyanions of chlorine constitute fireworks and propellants for spacecraft [7]. Small organic chlorine chemicals are used as solvents, like trichloroethylene (C_2HCl_3), or precursors to polymers, like vinyl chloride (C_2H_3Cl) for making polyvinylchloride ("PVC") [8]. Chlorofluorocarbon, a volatile organic chlorine compound, was widely used as a refrigerant until its international regulation following the discovery that it degrades the ozone layer [9]. Many pharmaceuticals are modified with chlorine to alter their activity in the human body [10]. Because of the preeminence of synthetic chlorine, even early researchers assumed that biochemical reactions involving chlorine were not natural but accidents of biology or newly evolved traits [11, 12].

Another reason for this confusion is that the understanding of how chlorine is transformed by natural processes occurred through independent lines of research. Geologists identified the origins and fates of chlorine on Earth's surface. Biochemists identified thousands of organic chlorine compounds produced across all domains of life. Microbiologists identified how and when microorganisms consume organic and inorganic chlorine. Atmospheric chemists and biogeochemists identified sources and sinks of volatile chlorine compounds. Immunologists and biologists identified oxidized chlorine production by immune cells and, in turn, an evolutionary pressure for bacteria to adapt to reactive chlorine species.

These processes are not themselves independent, of course, but interconnected in a single biogeochemical chlorine cycle. The processes of the chlorine cycle – the biology, chemistry, and geology of chloride, addition and removal of chlorine from organic molecules (chlorination and dechlorination), oxidation of chlorine, reduction of chlorine, and responses to reactive chlorine species – have been subject to various reviews (Table 1-2). Regrettably, however, no review considers all of these topics in a single unified treatise. That is problematic because separate consideration of these processes obscures the connections between them.

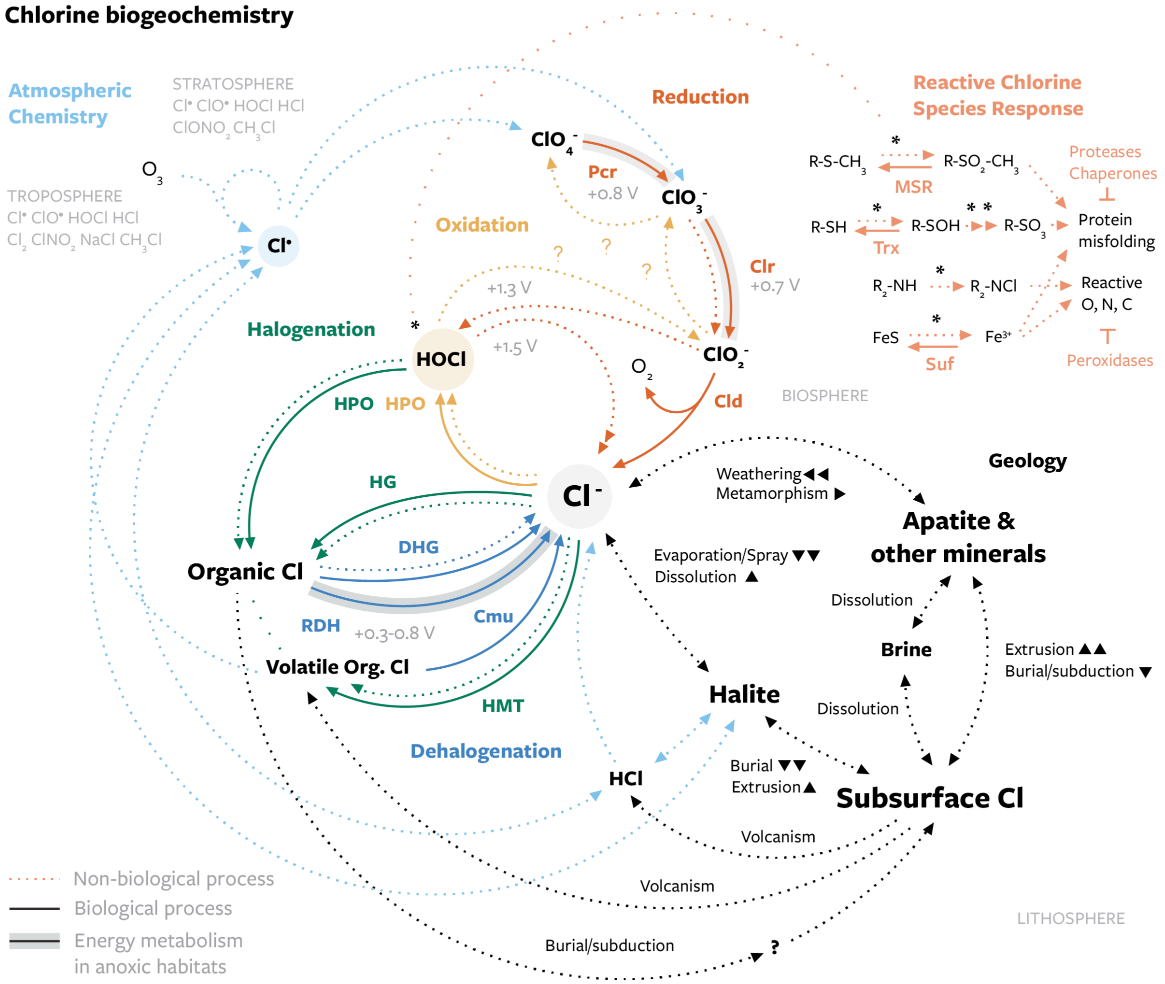


Figure 1-1. An overview of major transformations in the biogeochemical chlorine cycle. Dashed lines indicate physical and chemical processes, with the direction and flux of geological processes indicated by arrowheads. Solid lines indicate biological processes and are denoted by enzymes that can be involved in that process. Steps that yield energy in anaerobic respiratory metabolisms are highlighted gray, and the reduction potential of the redox couple is indicated by gray text. Question marks indicate processes that plausibly exist or are poorly understood. Not shown are subsurface geology, reactions involving chlorine gases and radicals, and all cellular components of RCS response. Enzyme abbreviations are as follows: Cmu, chloromethane methyltransferase; Pcr, perchlorate reductase; Clr, any chlorate reductase; Cld, chlorite dismutase; HPO, haloperoxidase; HG, halogenase; DHG, dehalogenase; RDH, reductive dehalogenase; HMT, halide methyltransferase; MSR, methionine sulfoxide reductase; Trx, thioredoxin; and Suf, iron-sulfur cluster synthesis.

The goal of this review is to provide a concise overview of the biogeochemical chlorine cycle that connects disparate disciplines and helps identify gaps for further study. The major biological, chemical, and geological processes of the chlorine cycle are covered, with special consideration given to biological processes (Figure 1-1). Key enzymes (Table 1-3) and the reduction potentials for different chlorine compounds (Table 1-4) are included. Estimated quantities for reservoirs and fluxes can be found in existing reviews (Table 1-2) and are not discussed in detail here. Each process is covered in a different section, with consideration of the roles of other halogens (F, Br, I) and broader lessons and suggestions for future research discussed at the end.

Chloride

Chloride is the most common form of chlorine in the biosphere. In the Earth's subsurface, chloride is a component of brines where, as an anion, it is highly mobile and helps transport metal cations [13]. It enters the biosphere from volcanic emission of hydrogen chloride and from the weathering of chlorine-containing minerals, primarily apatite, amphibole, and mica [14]. Chloride's high solubility and negative charge mean that it is highly mobile in soils, with its distribution largely controlled by the water cycle [15]. Streams eventually deliver most chloride to the ocean, and chloride is transported back to continents in salt spray [16]. Chloride is bioavailable in the environment at approximate concentrations of less than 0.2 mM in rain, 0.03-0.2 mM in soil water, 0.01-10mM in freshwater, about 500 mM in oceans, and up to 10,000 mM in evaporative water bodies [3, 16-18]. Many biochemical reactions involve chloride as a substrate or product (see below sections), and it can be an essential structural component for the function of some proteins, such as the oxidation of water to oxygen by photosystem II [19]. Chloride is even used, rarely, for its properties as an acid: nearly all vertebrates combine hydrogen ions and chloride in a stomach to form hydrochloric acid [20].

Life most frequently uses chloride as a solute. Chloride is present in mammalian cells at about 100 mM and in plant cells and prokaryotic cells at concentrations that vary greatly depending on the salinity of the environment [3, 17]. Cells transport chloride to balance osmotic potential, to control charge across the cellular membrane, or to import other substrates [17, 21, 22]. Uptake by plants and microorganisms is significant enough to be a major sink of chloride in soils [17, 23]. In evaporating bodies of water, some archaea use a powerful class of chloride importers driven by light, called halorhodopsins, to counteract the high osmotic potential [24]. Evaporation ultimately leads to the precipitation of chloride as a salt, predominantly halite (NaCl) [14]. Chlorine exits the biosphere when evaporite minerals are buried and when oceanic crust infiltrated by chloride is subducted [25-27]. Enough evaporites have been buried over geologic time to change the salinity of the oceans: Earth's early oceans may have been 1.5-2 times as salty as today's oceans [26, 28, 29]. Yet enough chloride remains in the biosphere to form the center of a significant biogeochemical cycle (Figure 1-1).

Chlorination

Chloride is converted to organic chlorine by the formation a single covalent bond between a chlorine atom and a carbon atom. Chlorine can be found in a diverse array of organic molecules from small gases like chloroform ($CHCl_3$) to large recalcitrant molecules in soil [5]. Organic chlorine production has been observed across all types of life, from bacteria to algae, plants, and animals, with the exception of archaea [5, 8, 30]. Chlorine metabolites are used for a variety of purposes, from chemical defense to intercellular signaling [16]. Chlorination typically occurs at the end of biosynthetic pathways, meaning chlorinated sites are rarely modified further [10]. The purpose of chlorination is that the chlorine atom, in replacing another group at a particular site, can change almost any chemical property of the molecule, including shape, solubility, charge density, or reactivity [16].

Biological chlorination is performed by enzymes called halogenases. Halogenases are classified by how they change the oxidation state of a halide ion (X^-) during catalysis (Table 2). A halogenase either performs a 2-electron oxidation of a halide to a halenium (X^+) or a hypohalous acid (HOX), a 1-electron oxidation of a halide to a halogen radical (X*), or no oxidation of the halide [10]. Each chlorinates a different type of chemical site in a molecule. Hypochlorous acid primarily chlorinates electron-*rich* carbon sites, like aromatic carbons, and is produced by heme- and vanadium- haloperoxidases and flavin-dependent halogenases [8, 10]. Chlorine radicals can chlorinate electron-*poor* aliphatic carbon sites and are produced by enzymes such as non-heme iron halogenases [10, 31]. Nucleophilic halogenases chlorinate only electron-*deficient* carbons by using chloride as a nucleophile. Therefore, halogenases have different roles in nature. Radical-producing halogenases provide great specificity for biosynthesis of secondary metabolites [10]. In contrast, some haloperoxidases release hypochlorite, which then chlorinates molecules without specificity [10], potentially causing oxidative stress. A nucleophilic halogenase in plants called halide methyltransferase is a substantial source of chloromethane (a.k.a. methyl chloride) [32], a minor greenhouse gas and the most common volatile organic chlorine in the atmosphere [33].

Organic chlorine is abundant and widespread. Chlorine accounts for 0.01 – 0.5% of the mass of organic matter in soils, making it the sixth most common element in organic molecules after sulfur and nitrogen – and as common as phosphorous [6, 15, 34]. Organic chlorine is even more common than chloride in some forest soils [15, 35], acting as a reservoir of chloride [23]. Leaves contain some organic chlorine, and its amount increases during decay [36, 37]. Similarly, the primary producers of the ocean synthesize organic chlorine, but it is a greater proportion of organic matter after decay – about 1 chlorine atom for every 500 carbon atoms [38]. Chemical reactions form some of this organic chlorine [5, 38, 39], yet the vast majority of chlorination is caused by biological activity [40, 41]. A key cause of chlorination in soils are the abundant and active extracellular halogenases produced by bacteria and fungi [42-44]. The combination of these reactions produces large amounts of organic chlorine, and the many sources of volatile organic chlorine molecules, from plants [45] to volcanoes [46], ensure that organic chlorine is distributed globally.

Dechlorination

Microorganisms use organic chlorine as substrates for growth and energy [10, 47]. Chloride is removed from organic chlorine by enzymes called dehalogenases. Several different biochemical classes are currently recognized [10], but overall dehalogenases are less well understood than halogenases. Hydrolytic dehalogenases replace chloride with a hydroxyl group from water or alcohol [10]. Oxidative dehalogenases instead use a hydroxyl group from oxygen or hydrogen peroxide to remove chloride, but these enzymes have dioxoygenase, monooxygenase, and peroxidase activity towards a range of organic molecules and do not appear specific to halogenated organics [10]. Reductive dehalogenases catalyze the 2-electron reduction of organic chlorine through either alpha-elimination or beta-elimination: the replacement of a single C-Cl bond with a C-H bond or of two adjacent C-Cl bonds with a C-C double-bond [10, 48]. Most but not all of the reductive dehalogenase are sensitive to oxygen [49]. Some organisms perform dechlorination inadvertently; for example, chloromethane is metabolized by a specialized methyltransferase (Cmu) but also by monooxygenase activity meant for methane and ammonia [33, 47].

The high electronegativity of chlorine makes carbon bound to chlorine more electron-deficient. Accordingly, many organic chlorine molecules have a reduction potential high enough to be an energetically favorable electron acceptor in oxidative phosphorylation (Table 4) [50, 51]. In this process, known as organohalide respiration, bacteria and archaea use reductive dehalogenases to conserve energy as proton motive force [52, 53]. Organohalide respiration is an optional metabolism in some bacteria but an obligate metabolism of others [54], emphasizing the importance of organic chlorine as an energy source in some environments. While the total energy of organohalide respiration is less than that of many other respiratory processes, the large reservoir of organic chlorine in sediments provides a natural niche for these organisms across anoxic terrestrial and marine habitats [55-57].

Chemical and geological processes are other important sinks for organic chlorine. Photolysis by sunlight can degrade organic chlorine [58]. Chloride can be released by simple hydrolysis from some organic chlorine molecules, like chloromethane [59]. The N-Cl bond in chlorinated primary and secondary amines, or organic chloramines (R_2-N-Cl / R-NH-Cl), can also undergo hydrolysis [60]. Organic chlorine in soluble or volatile molecules is subject to physical transport in water or air [15]. Organic chlorine can remain in sediments and has been detected in peat and coal [61], but the chemical transformations that occur to organic chlorine during early sedimentary rock formation and burial have largely been unexplored [38]. Ultimately, the burial of organic chlorine will subject it to enough heat and pressure to break the carbon-chlorine bond, releasing chloride [14]. In contrast, organic chlorine in the atmosphere is subjected to photochemical degradation and oxidation.

Oxidation

Photochemistry is the most significant source of higher oxidation states of chlorine. Chlorine photochemistry involves complex sets of rapid reactions involving high-energy photons, short-lived chlorine gases and radicals, and other gases and solids [62-68]. In the troposphere, sea salt spray is the major site of chlorine photochemistry and is responsible for the depletion of ozone (O_3) above the ocean surface [67-69]. Another set of reactions occurs in the stratosphere, where photolysis of inorganic and organic chlorine produces chlorine radicals that catalyze ozone degradation [62]. Most atmospheric chlorine returns to Earth in a reduced state, but a small fraction from stratospheric photochemistry yields stable oxidized chlorine molecules, chlorate and perchlorate, which are then deposited globally [63, 70]. Perchlorate and chlorate may also be formed by lightning and other photochemical reactions [65, 70, 71], but the atmospheric production of perchlorate and chlorate is enough that desert sediments, where low rain and limited biological activity mean less transport and depletion, have concentrations of perchlorate of about 1 mg kg^{-1} sediment (arid deserts) and up to 200 mg kg^{-1} sediment (hyperarid deserts) [72].

A stunning consequence of the chlorine cycle is that the degradation of stratospheric ozone by chlorine increases the amount of ultraviolet (UV) radiation that reaches the biosphere. UV radiation has enough energy to break covalent bonds DNA and other biomolecules [73]. Depletion of stratospheric ozone leads to greater transmission of UV, occurring fastest where aerosols are present, at the poles of the planet [9]. Complete destruction of stratospheric ozone appears to have occurred at least once in Earth's history, during the largest extinction event ever, the End-Permian extinction. During this period, volcanism through organic-rich halite deposits created enough chloromethane to destroy most of Earth's stratospheric ozone [74]. The global irradiation that resulted was such that fossilized pollen spores from that period are grossly deformed by mutations [73, 75]. In addition to depleting stratospheric ozone, today's added input of chlorine to the stratosphere has led to more deposition of perchlorate [76]. Possibly, past cataclysms also increased global perchlorate and chlorate concentrations.

Life, too, oxidizes chlorine, but inorganic chlorine species have such high reduction potentials that they cannot be oxidized for a source of energy (Table 4) – even by phototrophic metabolisms [77]. Presently, haloperoxidases, which catalyze the oxidation of chloride to hypochlorous acid by hydrogen peroxide, are the only known biological source of oxidized chlorine. Haloperoxidases used for intracellular biosynthesis pathways presumably produce only small amounts of hypochlorous acid, but other haloperoxidases produce much more. Fungi use haloperoxidase (vanadium haloperoxidase) for the degradation of lignin [78], and mammalian neutrophils use haloperoxidases (myeloperoxidase) to destroy bacterial pathogens during phagocytosis [79, 80]. Hypochlorous acid is also produced by widespread chemical reactions involving iron and oxygen [38, 39]. Thus, oxidation of chlorine is widespread. Hypochlorous acid can oxidize itself to chlorate [81], but any such oxidation of chlorine beyond hypochlorous acid has only been observed in atmospheric reactions. One hint these reactions may occur in microbial habitats is that various bacteria encode an enzyme for chlorite degradation

[82, 83]. Possibly, some combination of chemical and photochemical reactions produces higher oxidation states of chlorine in the biosphere [71, 84, 85].

Reduction

Chlorine oxyanions are used as electron acceptors in oxidative phosphorylation by some prokaryotes [7]. The oxyanions are reduced either by a perchlorate reductase (Pcr), which reduces perchlorate to chlorate and chlorate to chlorite, or by a chlorate reductase (Clr), which reduces only chlorate to chlorite [7]. In a remarkable act of biochemical creativity, chlorite is not reduced to hypochlorous acid but converted by a chlorite dismutase (Cld) into chloride, which generates molecular oxygen (O_2) that is then respired for energy or used by oxygenases [7, 82]. Because genes for these enzymes are horizontally transferred [86, 87] and perchlorate and chlorate are deposited globally [70], microorganisms that respire these compounds are widespread, although respiration occurs usually only during anoxia [88, 89]. Some anoxic habitats, like estuary sediment, have large amounts of metals and sulfur that can chemically consume the reactive intermediates of the respiratory pathway [90, 91]. Otherwise, the respiration of perchlorate produces moderate concentrations of chlorate, which can be used by chlorate-reducing bacteria [92], and very low concentrations of chlorite and hypochlorous acid [93].

Reduction of chlorine oxyanions occurs not only through specialized metabolisms. These anions can be mistaken for other substrates, leading to accidental transport into cells and reduction by oxidoreductases used in other metabolisms [94]. Inadvertent reduction of perchlorate or chlorate to chlorite by nitrate reductases may be one reason why Cld can be found in organisms that do not respire perchlorate or chlorate [95]. Perchlorate is chemically stable in solution, but chlorate, chlorite, and hypochlorous acid are reactive two-electron oxidants, with each reaction in the series producing a stronger reactive oxidant (Table 4). For that reason, chlorine oxyanions are a significant source of oxidative stress, especially hypochlorous acid.

Reactive Chlorine Species (RCS) Response

Hypochlorous acid (HOCl) is the most oxidizing chlorine oxyanion (Table 4) and lethal to bacteria at micromolar concentrations [96]. Unlike other chlorine oxyanions, HOCl can pass through cellular membranes unaided because it is protonated at physiological pH [97]. HOCl can oxidize or chlorinate various biomolecules but preferentially reacts with sulfur atoms in amino acids, followed by nitrogen atoms in primary and secondary amines [79, 98]. HOCl oxidizes methionine to methionine sulfoxide and cysteine to disulfide bonds or to sulfenic acid, sulfinic acid, and sulfonic acid sequentially [79, 98]. HOCl chlorinates amines as well as electron-rich molecules such as tryptophan [98]. Chloramines are themselves reactive and are converted in reactive aldehydes by hydrolysis [60, 98]. Reactive oxygen, nitrogen, and carbon species can be produced by HOCl, partly as a consequence the oxidation of iron-sulfur clusters leading to Fenton chemistry [80].

Reactive chlorine species therefore require responses to oxidative stress in general but also responses to the unique chemistry of HOCl [80, 98]. For example, HOCl is more effective at oxidizing cysteine and methionine, which causes sulfur starvation and protein misfolding [79, 98]. Protein chaperones are thus a key part of RCS response, as are enzymes that reduce oxidized sulfur species such as thioredoxin and methionine sulfoxide reductase [98, 99]. Iron-sulfur cluster biosynthesis proteins and peroxidases (catalase, peroxidase, organic hydroperoxide reductases) are important for repairing and mitigating RCS stress [98]. In addition, cells have been observed to synthesize methionine rich peptides and glutathione to scavenge HOCl, repair both intracellular and extracellular RCS damage, and have regulatory systems specific for HOCl [93, 98, 99]. This view of RCS response, however, is largely derived from studies of host-associated bacteria exposed to HOCl. Many more components to RCS response may be found in more diverse organisms tested with various reactive chlorine species (e.g. ClO_2^-, or Cl_2 from HOCl at low pH). For example, it is conceivable that dehalogenases would be useful for scavenging chlorinated organic molecules like chlorotyrosine.

Other Halogens

The chemical similarity between chlorine and other halogens means that iodine, bromine, and fluorine participate in many of the same reactions as chlorine and have likely influenced the evolution of chlorine metabolism. Halogens differ in ionic size ($I^- > Br^- > Cl^- > F^-$), electronegativity ($F > Cl > Br > I$), elemental abundance on Earth ($F > Cl > Br > I$), and higher oxidation states (F^- is oxidized only to F_2) [2, 10], which affects their distribution and consumption in different processes. F^- is common in minerals but rare in the biosphere [13], whereas I^-, Br^- are most abundant in oceans (0.0004 mM and 0.9 mM), though found at their highest ratio to Cl^- in terrestrial environments (about 1 : 5 : 100) [39]. Enzymes like halide methyltransferases can prefer one halide over another ($I^- > Br^- > Cl^-$), and any haloperoxidase that oxidizes Cl^- also oxidizes I^- and Br^- [10]. Sea salt aerosols contain both chloride and bromide, and tropospheric photochemistry involves chloromethane as well as iodomethane and bromomethane [69]. Organohalide-respiring prokaryotes use organic bromine in ocean sediment [56]. Complicating matters, many organic functional groups behave like halides. These "pseudohalides," like thiocyanate (SCN^-), are synthesized from common elements by diverse organisms and can be used by some halogenases [32, 100]. Halogens and pseudohalogens are therefore important factors in the processes and evolution of the chlorine cycle.

Concluding Remarks

The biogeochemical chlorine cycle is characterized by the high solubility of inorganic chlorine, the electrophilic behavior of chlorine in covalent bonds (leading to high reduction potentials), and the ability of chlorine to be volatilized and to participate in photochemistry. As a consequence of these chemical properties, a vast array of chlorine-containing molecules can be found across nature, from countless organic molecules to inorganic chlorine gases and radical species. Therefore, as argued elsewhere, biochemical activity towards anthropogenic chlorine should no longer be assumed first to be a recently evolved trait [6]. The chlorine cycle is not unaffected by human activity, of course: for example, volatile chlorine emissions increase the deposition of perchlorate and chlorate [76]; salt ponds expand the habitat available for halophilic microorganisms [101]; groundwater pollution organic and inorganic chlorine leads to localized microbial blooms [7, 102]; irrigation practices influence chloride concentration over large areas [17]; and disinfectants like hypochlorous acid, chlorine dioxide, or chloramine may increase selection for reactive chlorine species response systems [103]. But the use of a chlorine molecule by an organism should be assumed to have evolved naturally over geologic time unless evidence to the contrary [104].

A unified view of the biogeochemical chlorine cycle shows clearly that different processes are connected. Chlorination may be an important step in the geological chlorine cycle by slowing its removal from sediments by water. The evolution of chloromethane production in plants may have increased the global production of perchlorate and chlorate and abundance of perchlorate- and chlorate-reducing bacteria. Perhaps the most overlooked link between different processes is hypochlorous acid: this molecule is produced by intracellular haloperoxidases for chlorinating organic molecules, by extracellular haloperoxidases for the decomposition of plant matter, by mammalian immune cells for killing bacteria, as a byproduct of microbial reduction of perchlorate and chlorate, and by oxidative chemistry in the environment. Because of the centrality of hypochlorous acid, sensing and responding to reactive chlorine species seems important for most of the biological processes in the chlorine cycle. Thus, the evolution of new biochemical tools for chlorine metabolism in one process may be exchanged to another, as has been observed for molybdopterin methionine sulfoxide reductases. More broadly, all of the processes of the chlorine cycle affect one another through changes in the amounts and fluxes of chlorine between different reservoirs.

Research Priorities

Future research is expected to continue expanding knowledge on the chemical diversity and uses of organic chlorine by life, the speciation and quantity of chlorine across various environments, and the connections between the chlorine cycle and other processes on Earth. Immediate research should address the largest gaps of understanding in the chlorine cycle identified herein: cellular responses to reactive chlorine species, the sources and roles of oxidized chlorine in the biosphere, and the intersection of chlorine biology and geology. Answering the following questions in particular could provide better information on how different processes in the chlorine cycle are linked:

1. What are the causes of and responses to reactive chlorine stress in diverse organisms? For example, see Melnyk, Youngblut [93].
2. Are higher oxidation states of chlorine produced within natural habitats by sunlight, oxidative chemistry, or biological activity?
3. How are reactive chlorine species involved in ecological interactions other than mammalian immune defense? For example, see Bengtson, Bastviken [44].
4. How is organic chlorine altered during sediment formation? For example, see Selverstone and Sharp [105].

5. How and when did chlorine metabolisms originate in geologic time? For example, see Knauth [106].

Tables

Table 1-1. Chlorine-containing molecules commonly found in nature.

Molecule	Name	Ox. State	Phase	pKa	Note
ClO_4^-	Perchlorate	+7	Aqueous	-8.7	
ClO_3^-	Chlorate	+5	Aqueous	-1.0	
ClO_2^-	Chlorite	+3	Aqueous	2.0	
HOCl / ClO^-	Hypochlorous acid / hypochlorite	+1	Aqueous	7.5	Cl_2 below pH 4
Cl^- / HCl	Chloride / hydrochloric acid	-1	Aqueous	-6.3	
R-Cl	Organic chlorine (R=organic group)	-1	Aqueous		Various
CH_3Cl	Chloromethane (methyl chloride)	-1	Gas/Aqueous		
NaCl	Halite	-1	Solid		
ClO_2	Chlorine dioxide	+2	Gas		
Cl_2	Molecular chlorine	0	Gas		
HCl	Hydrogen chloride	-1	Gas		

Table 1-2. A selection of reviews and comprehensive reports that detail different processes in the chlorine cycle. More recent publications are prioritized, but some earlier, seminal publications are included.

Year	Publication	Overview	Cl Cycle Processes
1992	Gribble, G. W. Naturally occuring organohalogen compounds - a survey. Journal of Natural Products 55 (1992).	Early statement on natural organic chlorine	Halogenation
1995	Graedel, T. E. & Keene, W. C. Tropospheric budget of reactive chlorine. Global Biogeochemical Cycles 9, 47-77 (1995).	Early review of tropospheric chlorine chemistry, incl. estimated inventories and fluxes	Oxidation
2000	Winterton, N. Chlorine: The Only Green Element – towards a Wider Acceptance of its role in natural cycles. 173-225, (2000).	Early seminal review on the biogeochemical chlorine cycle, incl. estimated inventories and fluxes	Halogenation, Dehalogenation, Chloride, Oxidation
2002	Oberg, G. The natural chlorine cycle - Fitting the scattered pieces. Applied Microbiology and Biotechnology 58, 565-581, (2002).	Early seminal essay arguing that chlorine naturally cycles between chloride and organic matter	Halogenation, Chloride, Dehalogenation (minor)
2003	Öberg, G. M. in Natural Production of Organohalogen Compounds The Handbook of Environmental Chemistry Ch. Chapter 3, 43-62 (2003).	Early estimate of reservoirs and fluxes of chlorine in soils	Halogenation, Dehalogenation, Chloride
2007	Schafer, H., Miller, L. G., Oremland, R. S. & Murrell, J. C. Bacterial Cycling of Methyl Halides. Advances in Applied Microbiology 61, 307-346, (2007)	Chloromethane production and consumption and its role in the atmosphere	Halogenation, Dehalogenation, Oxidation (minor)
2008	Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. Nature Chemical Biology 4, 278-286, (2008).	The reactivity of hypochlorite and other reactive oxidants in human cells	Reactive chlorine species response, Oxidation
2009	Butler, A. & Sandy, M. Mechanistic considerations of halogenating enzymes. Nature 460, 848-854, (2009)	Halogenating enzymes that activate chlorine by oxidation	Halogenation
2010	Catling, D. C. et al. Atmospheric origins of perchlorate on Mars and in the Atacama. Journal of Geophysical Research 115, E00E11, (2010)	Atmospheric chemistry producing perchlorate and chlorate	Oxidation
2013	Gray, M. J., Wholey, W.-Y. & Jakob, U. Bacterial responses to reactive chlorine species. Annual review of microbiology 67, 141-160, (2013).	The chemical of reactive chlorine species and how bacteria respond	Reactive chlorine species response
2016	Youngblut, M. D., Wang, O., Barnum, T. P. & Coates, J. D. (Per)chlorate in Biology on Earth and Beyond. Annual Review of Microbiology 70, 435-459, (2016).	The reduction of perchlorate and chlorate to chloride by microbial respiration	Reduction, Reactive chlorine species response (minor), Oxidation (minor)
2016	Field, J. A. Natural Production of Organohalide Compounds in the Environment. (2016)	The formation of different organic chlorine species	Halogenation, Dehalogenation (minor)
2016	Weigold, P. et al. A metagenomic-based survey of microbial (de)halogenation potential in a German forest soil. Scientific Reports 6, 28958, (2016)	Summary of genes and taxa involved in halogenation and dehalogenation in forest soils	Halogenation, Dehalogenation

2017	Agarwal, V. et al. Enzymatic Halogenation and Dehalogenation Reactions: Pervasive and Mechanistically Diverse. Chemical Reviews, acs.chemrev.6b00571, (2017)	Halogenating and dehalogenating enzymes, incl. table of enzyme structures	Halogenation, Dehalogenation
2018	Atashgahi, S. et al. Microbial Synthesis and Transformation of Inorganic and Organic Chlorine Compounds. Frontiers in Microbiology 9, 1-22, (2018)	Processes in the biogeochemical chlorine cycle driven by microorganisms	Halogenation, Dehalogenation, Oxidation, Reduction
2018	Atashgahi, S., Haggblom, M. M. & Smidt, H. Organohalide respiration in pristine environments: implications for the natural halogen cycle. Environ Microbiol 20, 934-948, (2018).	Natural reductive dehalogenation	Dehalogenation, Halogenation (minor), Oxidation (minor)
2018	Harlov, D. E. & Aranovich, L. in The Role of Halogens in Terrestrial and Extraterrestrial Geochemical Processes (Springer, 2018).	The geology of chlorine and other halogens	Chloride
2019	Wang, X. et al. The role of chlorine in global tropospheric chemistry. Atmospheric Chemistry and Physics 19, 3981-4003, (2019).	Modeling of tropospheric chlorine chemistry, incl. estimated inventories and fluxes.	Oxidation

Table 1-3. An overview of enzymes and genes that produce or consume chlorine-containing compounds.

Category	Enzyme	Reaction	EC
MSR	MsrA, Peptide-S-Methionine sulfoxide reductase	Reduces peptide-methionine(S)-S-oxide to peptide-methionine(S)	EC:1.8.4.11
MSR	MsrB, Peptide-R-Methionine sulfoxide reductase	Reduces peptide-methionine(R)-R-oxide to peptide-methionine(R)	EC:1.8.4.12
MSR	MsrP, Protein-methionine-sulfoxide reductase, molybodpterin	Reduces methionine sulfoxide to methionine	EC:1.8.5.-
Trx	TrxA, Thioredoxin	Reduces oxidized thiols	var.
Trx	TrxB/TrxR, Thioredoxin reductase	Reduces thioredoxin	EC:1.8.1.9
Pcr	PcrA, Perchlorate reductase	Reduces perchlorate to chlorate, reduces chlorate to chlorite	EC:1.97.1.-
Clr	ClrA, Chlorate reductase	Reduces chlorate to chlorite	EC:1.97.1.-
Cld	Cld, Chlorite dismutase	Chlorite : molecular oxygen lyase	EC:1.13.11.49
HPO	CPO, Heme-dependent haloperoxidase	Oxidizes halide (X-) to hypohalite (XO-)	EC:1.11.1.10
HPO	V-HPO, Vanadium haloperoxidase	Oxidizes halide (X-) to hypohalite (XO-)	EC:1.11.1.10
HG	FDH, Flavin-dependent halogenases	Oxidizes halide (X-) to hypohalite (XO-)	EC:3.8.1.1
HG	NHFeH, α-ketoglutarate-dependent non-heme iron halogenase	Oxidizes halide (X-) to halogen radical (X*)	n.r.
HG	SalL, 5′-halo-5′-deoxyadenosine synthases	Nucleophilic substitution of halide	n.r.
HMT	S-adenosyl-L-methionine:halide methyltransferase (various)	Nucleophilic substitution of halide producing halomethane	EC 2.1.1.165
Cmu	CmuA, Chloromethane:corrinoid methyltransferase	Catabolic removal of chloride from chloromethane	EC:2.1.1.-
RDH	RdhA, Respiratory reductive dehalogenase	Reduction of organic halogens via alpha or beta elimination	EC:1.21.99.5
RDH	RdhA, Nonrespiratory reductive dehalogenase, NADPH-dependent	Reduction of organic halogens	n.r.
DHG	Haloalkane dehalogenase (various)	Halide removed by hydroxyl from water or alcohol	var.
DHG	Oxidative dehalogenases (various)	Halide removed by hydroxyl group from O2 or H2O2; may be co-metabolic	var.

Table 1-4. Reduction potentials for select redox couples involving chlorine.

Reactant	Product	Eo' (V) [a]	Reference
Molecular chlorine	Chloride	1.360	Youngblut et. al 2016
Hypochlorous acid	Chloride	1.280	Winterbourn
Chlorite	Hypochlorous acid	1.261	Youngblut et. al 2016
Molecular oxygen	Water	0.816	Youngblut et. al 2016
Perchlorate	Chlorate	0.813	Youngblut et. al 2016
Hypochlorous acid	Molecular chlorine	0.804	Youngblut et. al 2016
Chlorate	Chlorite	0.744	Youngblut et. al 2016
Tetrachloromethane	Trichloromethane	0.584	Dolfing et. al 1994
2,3,4-Trichlorophenol	3,4-Dichlorophenol	0.410	Dolfing et. al 2015
Chloromethane	Methane	0.380	Dolfing et. al 1994
Chloroacetate	Acetate	0.374	Dolfing et. al 1994
2-chlorophenol	Phenol	0.357	Dolfing et. al 2015
Monochlorobenzene	Benzene	0.239	Dolfing et. al 2015

[a] standard conditions: pH 7, 25 °C; solutes at 1 M

Chapter 2

Genome-resolved metagenomics identifies genetic mobility, metabolic interactions, and unexpected diversity in perchlorate-reducing communities

Abstract

Dissimilatory perchlorate reduction is an anaerobic respiratory pathway that in communities might be influenced by metabolic interactions. Because the genes for perchlorate reduction are horizontally transferred, previous studies have been unable to identify uncultivated perchlorate-reducing populations. Here we recovered metagenome-assembled genomes from perchlorate-reducing sediment enrichments and employed a manual scaffolding approach to reconstruct gene clusters for perchlorate reduction found within mobile genetic elements. *De novo* assembly and binning of four enriched communities yielded 48 total draft genomes. In addition to canonical perchlorate reduction gene clusters and taxa, a new type of gene cluster with an alternative perchlorate reductase was identified. Phylogenetic analysis indicated past exchange between these gene clusters, and the presence of plasmids with either gene cluster shows that the potential for gene transfer via plasmid persisted throughout enrichment. However, a majority of genomes in each community lacked perchlorate reduction genes. Putative chlorate- or sulfur-reducing populations were dominant in most communities, supporting the hypothesis that metabolic interactions might result from perchlorate reduction intermediates and byproducts. Other populations included a novel phylum-level lineage (*Ca.* Muirbacteria) and epibiotic prokaryotes with no known role in perchlorate reduction. These results reveal unexpected genetic diversity, suggest that perchlorate-reducing communities involve substantial metabolic interactions, and encourage expanded strategies to further understand the evolution and ecology of this metabolism.

Introduction

Perchlorate (ClO_4^-), the most oxidized chlorine oxyanion, is produced naturally through successive oxidation of chloride by photochemical reactions or electrical discharge [63, 70]. Natural concentrations of perchlorate are low in most environments but appreciable in many desert soils and in regolith on Mars [63, 107]. Perchlorate is utilized as an anaerobic respiratory electron acceptor by dissimilatory perchlorate-reducing microorganisms (DPRM), which obtain energy by coupling the reduction of perchlorate to the oxidation of various electron donors [7]. In the canonical perchlorate reduction pathway, perchlorate reductase (PcrAB) reduces perchlorate to chlorate (ClO_3^-) and then chlorite (ClO_2^-), chlorite dismutase (Cld) dismutates chlorite to chloride (Cl^-) and oxygen (O_2), and a terminal oxygen reductase reduces oxygen to water (Supplementary Figure 2-1) [7]. The reactive chlorine species hypochlorite (ClO^-), a spurious byproduct of chlorite dismutation, damages cells through oxidizing reactions, and in some DPRM a periplasmic methionine sulfoxide reductase (YedYZ) in combination with a methionine-rich peptide (MRP) subverts oxidative damage to proteins [93].

The genes for canonical perchlorate reduction are found within a horizontally transferred gene cluster referred to as the perchlorate reduction genomic island (PRI) [86]. PRI always contain chlorite dismutase (*cld*) and perchlorate reductase (*pcr*) and generally include genes for transcriptional regulation, oxidative stress response, molybdenum cofactor biosynthesis, and genetic mobility [86, 108]. The perchlorate reductase operon consists of catalytic subunits (*pcrAB*), a maturation protein (*pcrD*), and sometimes electron transfer subunits (*pcrCO*) and a quinol-oxidizing subunit (*pcrQ*) [108, 109]. Contemporary horizontal transfer of PRI complicates the identification of perchlorate-reducing bacteria in communities, though transfer seems to be restricted within specific families in the *Alpha-, Beta-, Gamma-*, and *Epsilonproteobacteria* [108]. In contrast, the genes involved in the closely related dissimilatory chlorate respiration, chlorate reductase (*clrABDC*) and *cld*, were recently exchanged between different *Protebacteria* classes [87]. Although a single ancestral PRI had been suggested based on monophyly in both PcrA and Cld [108], a broader extent of genetic exchange in chlorine oxyanion metabolism is hinted at by the polyphyletic nature of ClrA and the transfer of the *cld* gene from DPRM to dissimilatory chlorate-reducing microorganisms (DCRM) [87]. Further, the bias imposed by isolation techniques may mean that communities host a more diverse set of perchlorate-reducing microorganisms than previously identified.

Preliminary evidence suggests that the intermediates and byproducts of perchlorate reduction could potentially support other metabolisms in communities. For example, the intermediate chlorate transiently accumulated during perchlorate reduction by *Dechlorosoma* sp. HCAP-C, but was consumed when a DCRM was added in co-culture [110]. Similarly, chlorite, a toxic intermediate, was consumed by non-DPRM cells expressing Cld in an engineered mutualistic interaction with cells that reduced the perchlorate to chlorite [111] (denoted “symbiotic perchlorate reduction” [7]). An alternative mechanism of mutualism involves the sulfur redox cycle. Elemental sulfur (S^o) and polysulfide (S_n^{2-}) are produced as byproducts of perchlorate reduction through enzymatic oxidation of sulfide by PcrAB, and abiotic reaction with chlorite, hypochlorite, and oxygen (Supplementary 2-1) [112, 113]. This process, named perchlorate-dependent sulfide oxidation (PSOX), has been suggested to support elemental sulfur-reducing bacteria in multiple perchlorate-reducing communities based on the enrichment of taxa affiliated with isolated sulfur-reducing bacteria [114, 115]. In turn, the concurrent reduction of sulfur species to sulfide allows some organisms to grow by perchlorate reduction without chlorite dismutase [90, 116] (denoted "cryptic perchlorate reduction" [7]). None of the proposed metabolic interactions, however, have been demonstrated to occur in naturally enriched co-cultures or communities.

The goals of this study were to assess the genetic and phylogenetic diversity of uncultivated DPRM and to identify the possible extent of metabolic interactions occurring in perchlorate-reducing communities. Recently developed techniques to recover genomes from large metagenomic datasets enable the prediction of metabolic functions and interactions in complex communities [117-119]. A previous study using 16S rRNA gene sequencing showed that perchlorate-reducing communities from marine sediment enriched with acetate and perchlorate consisted mostly of phyla that do not contain known DPRM, such as *Deltaproteobacteria* and *Bacteroidetes* [120]. We reevaluated these same enrichments by using genomic DNA sequencing, *de novo* assembly, and binning to link perchlorate reduction genes to the genomes of specific populations. The high sequence similarity of perchlorate reduction genes led to incomplete assembly and binning, a common problem with genes subject to mobility or strain variation. We overcame this limitation by employing contig assembly graphs to manually construct genomic regions containing *pcr* and *cld* sequences. While most diversity in DPRM matched that of isolated organisms, by using *cld* as a biomarker for novel (per)chlorate reductase genes we identified a gene cluster containing *cld* and a putative alternative perchlorate reductase. That numerous genomes lacked any perchlorate reduction genes led us to conclude that much of the community may be supported by metabolic interactions with DPRM.

Materials and methods

Enrichment and sampling

Samples were selected from enrichments described in Carlström et. al 2016. Briefly, those enrichments utilized a sterile, anoxic, defined medium at pH 6.8 (bicarbonate-buffered) with 10 mM perchlorate (electron acceptor) and 10 mM acetate (non-fermentable electron donor and carbon source) [115, 121]. Inoculum consisted of sulfidogenic marine sediment collected from the San Francisco Estuary near the outlet of a freshwater stream (37.8629° N, 122.3132° W) in Berkeley, California, USA. Bottles with defined media and 5% (v/v) sediment inoculum were incubated at 30°C for 63 days, and biomass samples (20 mL) were collected weekly using 0.22-µm filters (MoBio, Carlsbad, CA) and preserved at -80°C. Partial removal of perchlorate (2.1 ± 0.4 mM) and complete removal of acetate occurred in the no-donor and no-acceptor controls, respectively, indicating the presence of alternative electron donors and acceptors from the sediment inoculum, although no sulfate was removed [115]. For this study, to obtain distinct perchlorate-reducing communities a single sample was chosen to represent each condition at a time point following substantial perchlorate removal: 14 days at 1% salinity (NaCl, w/v), 28 days at 3% salinity, 42 days at 5% salinity, and 42 days at 7% salinity (Supplementary Figure 2-2).

Metagenome sequencing and assembly

DNA was extracted using the MoBioRNAPowerSoil Kit with the DNA Elution Accessory Kit (MoBio Laboratories, Inc., Carlsbad, CA) as previously described [115]. DNA library preparation and sequencing were performed by the Vincent J. Coates Genomics Sequencing Laboratory at the California Institute of Quantitative Biosciences (QB3-Berkeley, Berkeley, CA) using an Illumina HiSeq 2000 platform with 100-bp paired-end reads. Prior to assembly, reads were trimmed using Sickle v. 1.33 with default parameters [122]. Reads from all samples were then co-assembled using MEGAHIT v. 1.0.2 with "meta-sensitive" presets [123]. Following assembly, reads were dereplicated by mapping reads to contigs using the Burrows-Wheeler Alignment Tool v. 0.7.10 (BWA) BWA-MEM algorithm [124] and selecting unique reads using Picard Tools v. 2.4.1 (http://picard.sourceforge.net/). Automated scaffolding of contigs was performed using SSPACE v. 3.0, which uses paired-end read information to join contigs by filling the length of the gap with the character "N" [125], without scaffold extension and with 25% variation from the estimated median insert size (275 bp) allowed. All computation was performed on the computer cluster at the QB3 Computational Genomics Resource Laboratory (CGRL) (UC Berkeley, Berkeley, CA).

Metagenome-assembled genome binning

Binning of scaffolds was performed using the default pipeline in Anvi'o v. 2.0.2 [126] as follows. Dereplicated reads from each sample were mapped to scaffolds using BWA-MEM and sorted and indexed using Samtools v. 0.1.19 [124] to provide differential abundance estimates across samples. Scaffolds greater than 2000 bp were binned using CONCOCT [127], which uses abundance, nucleotide composition, and read-pairing for automated binning, and the resulting bins were manually refined using abundance, GC content, taxonomic markers, and single copy gene counts to remove incorrectly binned genomic and viral sequences. Plasmid sequences, which sometimes can be maintained at a higher copy number than chromosomal sequences, were assigned to bins based on similar patterns of coverage between samples and the taxonomic identity of plasmid genes. Coverage for scaffolds and bins was calculated as follows: coverage = (number reads mapped to sequence) * (read length) / (total scaffold length).

The quality of each bin was assessed using CheckM v. 1.0.1 [128], which uses lineage-specific sets of single copy genes (SCGs) to estimate completeness and contamination and assigns contamination to strain heterogeneity if amino acid identity is great than 90%. Further analysis used only high-quality draft genomes, defined as bins that are near-complete (>90% SCGs present) with low contamination (<5% redundant SCGs), as well as medium-quality draft genomes that were more than moderately complete (>70% SCGs present) with medium contamination (<10% redundant SCGs) or less. The quality of bins belonging to candidate phyla radiation (CPR) bacteria, a broad lineage missing single copy genes found in other bacteria [129], was assessed using a set of CPR-specific single copy genes provided by the developers of Anvi'o.

Activity estimates were calculated using the index of replication (iRep), which measures genome replication as the ratio of coverage between the replication origin and terminus that are predicted from the distribution of coverage across a genome [130].

Resolution of sequences through individual assembly and manual scaffolding

Key genes for perchlorate reduction were subject to poor assembly and automated scaffolding and were not binned. Therefore, we applied contig graphs, which are visualizations of assemblies where unresolved contigs are displayed as branches between contigs, to identify, scaffold, and bin sequences containing perchlorate reduction genes. To reduce sequence complexity, each sample was assembled individually using MEGAHIT as above. Contig graphs (FASTG format) were generated using the "contig2fastg" tool in MEGAHIT. Bandage ("a Bioinformatics Application for Navigating De novo Assembly Graphs Easily") v. 0.8.0 [131] was used to visualize contigs, identify BLAST hits to specific gene sequences, manually merge contigs, and relate merged contigs to a genome bin. Criteria for merging contigs were that contigs (1) shared a similar abundance and (2) lay in the same contiguous path. Merged contigs were binned when contiguity with multiple contigs from the same genome bin was certain. This strategy was also used to recover some 16S rRNA genes.

Phylogenetic Analysis

The taxonomic position of metagenome-assembled genomes was determined using multi-locus sequence analysis (MLSA) as performed in PhyloPhlAn v. 0.99 [132], which constructs a phylogenetic tree based on 400 conserved protein sequences. Following initial placement of recovered genomes among 2013 IMG genomes [133], additional published genomes were added (Supplementary Table 1) to provide refined placement. As contamination in bins may lead to an aberrant MLSA phylogeny, taxonomic novelty was confirmed by comparing the identity of ribosomal protein amino acid sequences to the NCBI database and, when available, 16S rRNA gene sequences to the NCBI and SILVA databases [134, 135].

Functional Annotation of Metagenome-assembled Genomes

Functional annotation of each genome was performed using Prokka v. 1.11 [136], which employs a suite of sequence prediction software to annotate RNA and protein gene sequences [137-144]. Gene loci were plotted using Annotation Sketch as implemented by genometools v. 1.5.9 [145]. Metabolic functions were determined by the presence or absence of key genes using criteria defined in Supplementary Table 2-2. Specific genes were confirmed using a combination of BLAST searches, sequence alignment, motif analysis, and phylogenetic trees (Supplementary Figure 2-3). For phylogenetic tree construction, protein sequences were downloaded from protein families on the Pfam databases (Uniprot and NCBI), selected using custom HMM profiles, and supplemented with the top 100 IMG homology search results [133] and top 250 BLASTP search results for a representative protein. A reduced set of proteins was obtained by clustering proteins at either 80% or 100% identity using UCLUST v. 5.2.32 [146]. Novel metagenomic sequences were added to these sequences and aligned in MUSCLE v. 3.8.31 [147]. Alignments were edited in AliView v. 1.18 [148] to remove N- and C-terminal overhangs and positions consisting of greater than 95% gaps. Phylogenetic trees were generated in PhyML v. 3.0 [149] using 100 bootstraps and the appropriate evolutionary model as determined by ProtTest v. 3.0 [150]. Phylogenetics trees were visualized using the FigTree v. 1.4.3 software package (http://tree.bio.ed.ac.uk/software/figtree/).

Data availability

All reads and genome sequences will be available through the NCBI Bioproject accession PRJNA387015 at the time of publication.

Results and discussion

Community overview

Previously performed perchlorate-reducing enrichments [115] were subjected to metagenomic sequencing to identify perchlorate reduction genes, taxa, and potential interactions in communities. Samples selected from the marine sediment inoculum and the endpoint of four enrichments (1%, 3%, 5%, and 7% salinity) provided a baseline community and four distinct communities enriched for perchlorate-reducing microorganisms. The five samples were sequenced, yielding 303 million total 100-bp reads (Supplementary Table 2-3), then co-assembled and scaffolded to produce a metagenomic dataset of 603 Mbp of scaffold sequences (Supplementary Table 2-4). Automatic and manual binning yielded 36 high-quality and 12 medium-quality metagenome-assembled genomes totaling 145 Mbp (Table 2-1). Enrichment enabled a large proportion of communities to be recovered: an average of 68.8% of reads from enriched communities mapped to binned sequences, compared to only 3.2% of reads from the sediment inoculum (Supplementary Figure 2-4).

Among the recovered genomes were taxa related to previously isolated canonical DPRM as well as unrelated taxa, including multiple uncultivated lineages (Table 2-1; Supplementary Figure 2-5). While the enrichments contained considerable phylogenetic diversity, only a minority of genomes recovered from each community encoded both perchlorate reductase (*pcr* or *pnar*; see below) and chlorite dismutase (Figure 2-1A). Distinct sets of these DPRM were abundant (>5x coverage) at each salinity: bin Arcobacter BM102 at 1% salinity; bins Sedimenticola BM301, Sedimenticola BM503, and Rhizobiales BM303 at 3% salinity; bins Sulfurimonas BM502, Sedimenticola BM503, Arcobacter BM504, Rhizobiales BM505, Sulfurimonas BM702, and Rhizobiales BM303 at 5% salinity; and bin Sulfurimonas BM702 at 7% salinity (Figure 1B; Supplementary Table 2-5). Activity, as measured by the index of replication (iRep), varies within DPRM: Arcobacter BM102 (iRep = 1.42) and Rhizobiales BM303 (iRep = 1.33, 1.49) were actively replicating, whereas Sulfurimonas BM502 (iRep = 1.17), Arcobacter BM504 (iRep = 1.18), and Rhizobiales BM505 (iRep = 1.23) were likely not replicating and values for genomes Sedimenticola BM301, Sedimenticola BM503, and Sulfurimonas BM702 could not be determined (Figure 2-1C; Supplementary Table 2-5).

Enrichment proved sufficient and necessary to recover DPRM genomes from marine sediment. All perchlorate reductase genes recovered could be associated with binned genomes. Furthermore, the sediment inoculum dataset contained effectively none of the enriched DPRM genomes (<0.3x coverage) or scaffolds containing perchlorate reduction genes (<1x coverage; Supplementary Figure 6), and perchlorate reduction genes were not present in an individual assembly of the sediment inoculum. Similarly, perchlorate reduction genes are largely absent from published metagenomic sequences on the IMG database (results not shown), emphasizing the apparent low relative abundance of DPRM in natural ecosystems.

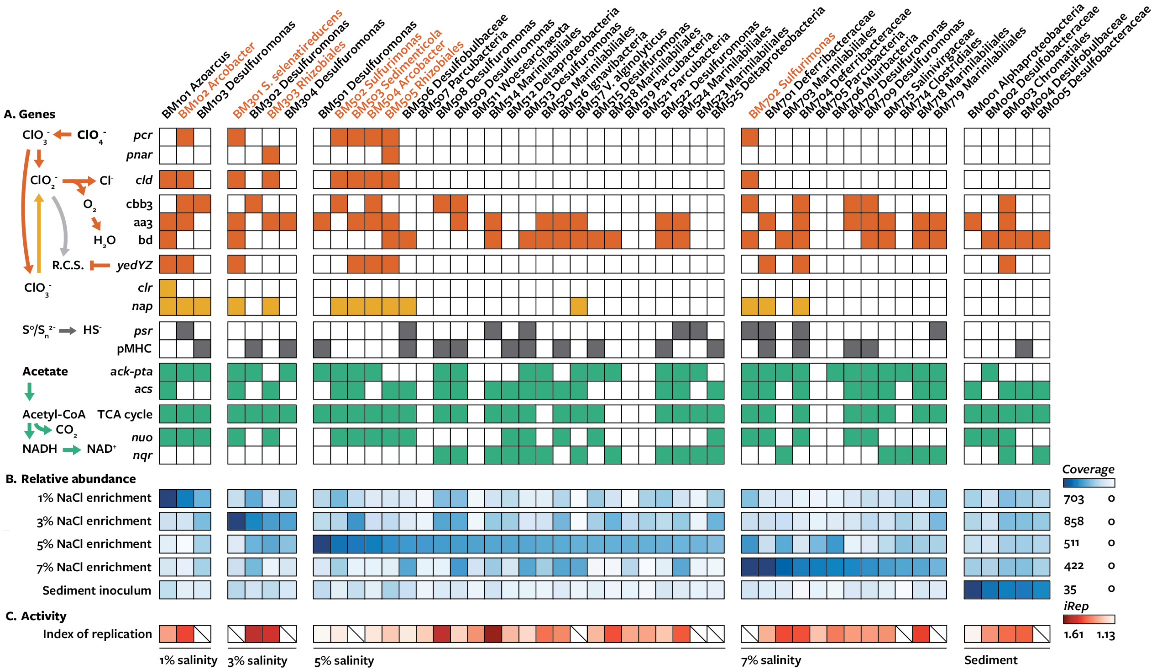


Figure 2-1. Summary of energy metabolism genes, abundance, and activity of metagenome-assembled genomes from perchlorate-reducing enrichments (1%, 3%, 5%, and 7% salinity) and sediment inoculum. Genomes predicted to be dissimilatory perchlorate-reducing bacteria are shown in red. (A). Gene content based on presence (filled squares) or absence (empty squares) of key genes involved different metabolisms. Genes in the same vertical division perform the same function. Gene abbreviations: pcr, perchlorate reductase; pnar, periplasmic Nar nitrate reductase; cld, chlorite dismutase; yedYZ, periplasmic methionine sulfoxide reductase; clr, chlorate reductase; nap, periplasmic nitrate reductase; cbb3, cbb3-type cytochrome c oxidase; aa3, aa3-type cytochrome c oxidase or cytochrome bo(3) ubiquinol oxidase; bd, cytochrome bd quinol oxidase; psr, polysulfide reductase; pMHC, periplasmic multi-heme cytochrome c; ack-pta, acetate kinase and phosphotransacetylase; acs, acetyl-CoA synthase; nqo, proton-translocating NADH:quinol oxidoreductase; nqr, sodium-translocating NADH:quinol oxidoreductase. (B) Relative abundance of metagenome-assembled genomes within samples (coverage / coverage of most abundant community member). (C) Relative activity across samples as measured by index of replication (iRep). Slash indicates the iRep value could not be determined.

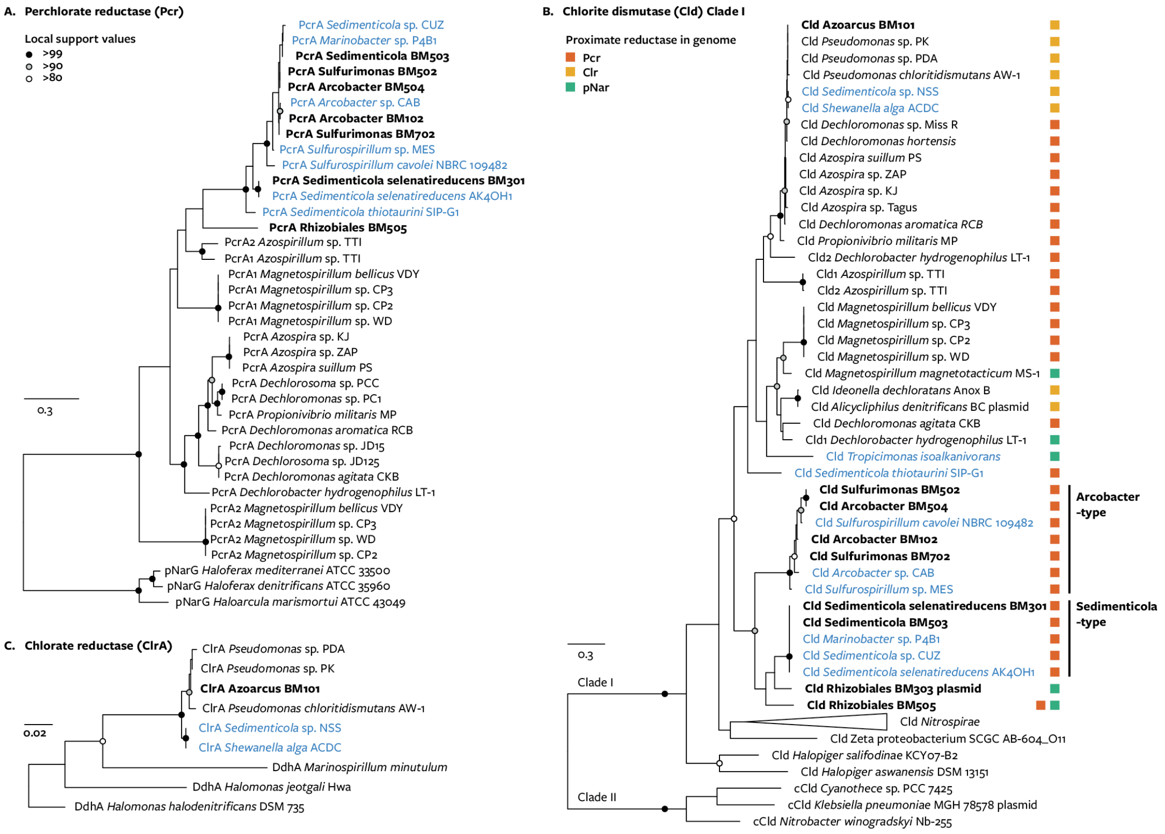
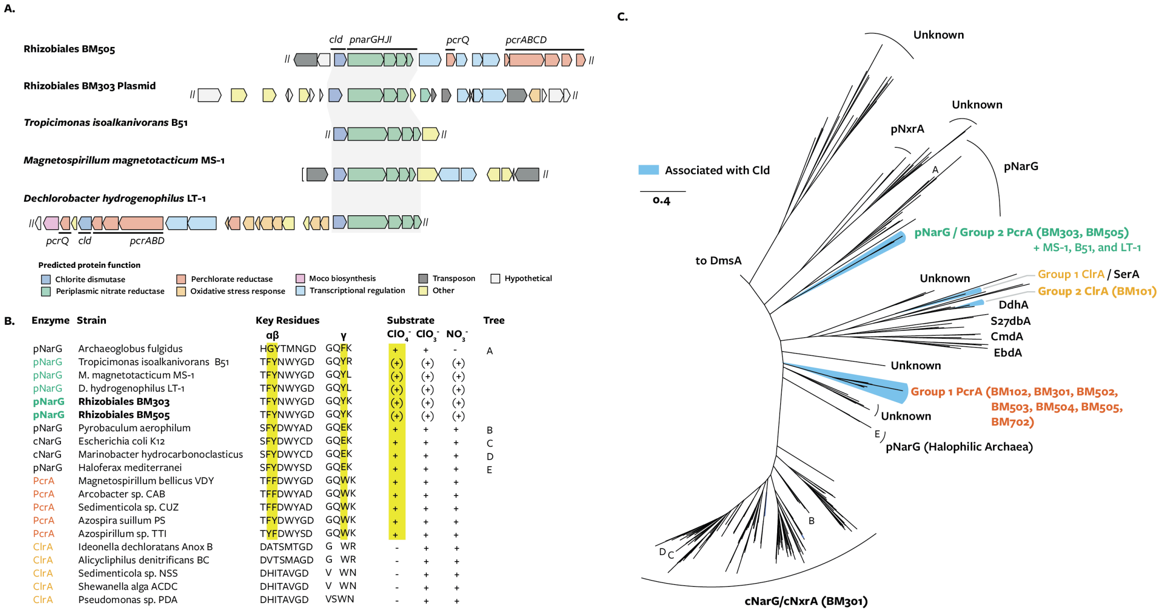


Figure 2-2. Maximum-likelihood tree of perchlorate and chlorate reduction enzymes obtained from metagenome-assembled genomes (bold) and the NCBI and IMG databases. Trees were constructed using MUSCLE and PhyML(100 bootstraps) and visualized with FigTree. Branch length and local support values provided by bootstrapping are indicated. Blue text indicates taxa isolated from marine environments. (A) Perchlorate reductase alpha subunit. (B) Chlorite dismutase; colored squares indicate the closest perchlorate or chlorate reductase gene to the chlorite dismutase gene. (C) Chlorate reductase alpha subunit.

Expanded diversity of perchlorate reduction genes and taxa in communities

Perchlorate reduction genes and taxa in the enriched communities mostly resemble those of previously isolated DPRM, suggesting that studied isolates are representative of active environmental perchlorate reducers. Phylogenetic analysis of *pcrA* and *cld* from the enriched populations indicated that most were closely related to *pcrA* and *cld* from characterized marine isolates (Figure 2-2). Correspondingly, these genomes were closely related to previously isolated DPRM (Table 2-1; Supplementary Figure 2-5). All recovered *pcrA* sequences were co-located in a perchlorate reduction genomic island (PRI) with *cld* and other perchlorate reductase subunits, and gene order within related PRI were conserved (Supplementary Figure 2-7): *Sedimenticola* genomes possessed the gene cluster *pcrABCDQ-cld*, and *Arcobacter* and *Sulfurimonas* genomes possessed *pcrABD-cld*. Our database search also detected *pcrA* and *cld* in previously sequenced strains *Sulfurospirillum* sp. MES and *Sulfurospirillum cavolei* NBRC 104982, which possessed a distinct PRI with *cld-pcrABDQ* (Supplementary Figure 2-7). The ability of *Sulfurospirillum* spp. to use perchlorate as an electron acceptor has not been tested [151, 152]. These results expand characterized or putative DPRM to four genera in the

Epsilonproteobacteria: *Arcobacter*, *Wolinella, Sulfurimonas*, and *Sulfurospirillum* [153, 154]. Additionally, Rhizobiales BM303 and Rhizobiales BM505 constituted a putative novel family in the order *Rhizobiales*, whereas all other alphaproteobacterial DPRM have been found in the order *Rhodospirillales* [7]. The *pcrA* and *cld* sequences of DPRM in the *Sulfurimonas, Sulfurospirillum*, and *Rhizobiales* differ somewhat from previously characterized sequences but generally cluster together with other marine organisms, a pattern that could be caused by unique selection pressures in the marine ecosystems, by decreased gene transfer between environments, or simply by sampling bias.



Enzyme	Strain	Key Residues αβ	Key Residues γ	Substrate ClO_4^-	Substrate ClO_3^-	Substrate NO_3^-	Tree
pNarG	Archaeoglobus fulgidus	HGYTMNGD	GQFK	+	+	-	A
pNarG	Tropicimonas isoalkanivorans B51	TFYNWYGD	GQYR	(+)	(+)	(+)	
pNarG	M. magnetotacticum MS-1	TFYNWYGD	GQYL	(+)	(+)	(+)	
pNarG	D. hydrogenophilus LT-1	TFYNWYGD	GQYL	(+)	(+)	(+)	
pNarG	**Rhizobiales BM303**	TFYNWYGD	GQYK	(+)	(+)	(+)	
pNarG	**Rhizobiales BM505**	TFYNWYGD	GQYK	(+)	(+)	(+)	
pNarG	Pyrobaculum aerophilum	SFYDWYAD	GQEK	+	+	+	B
cNarG	Escherichia coli K12	SFYDWYCD	GQEK	+	+	+	C
cNarG	Marinobacter hydrocarbonoclasticus	SFYDWYCD	GQEK	+	+	+	D
pNarG	Haloferax mediterranei	SFYDWYSD	GQEK	+	+	+	E
PcrA	Magnetospirillum bellicus VDY	TFFDWYGD	GQWK	+	+	+	
PcrA	Arcobacter sp. CAB	TFFDWYAD	GQWK	+	+	+	
PcrA	Sedimenticola sp. CUZ	TFFDWYAD	GQWK	+	+	+	
PcrA	Azospira suillum PS	TFYDWYGD	GQWK	+	+	+	
PcrA	Azospirillum sp. TTI	TYFDWYSD	GQWK	+	+	+	
ClrA	Ideonella dechloratans Anox B	DATSMTGD	G WR	-	+	+	
ClrA	Alicycliphilus denitrificans BC	DVTSMAGD	G WR	-	+	+	
ClrA	Sedimenticola sp. NSS	DHITAVGD	V WN	-	+	+	
ClrA	Shewanella alga ACDC	DHITAVGD	V WN	-	+	+	
ClrA	Pseudomonas sp. PDA	DHITAVGD	VSWN	-	+	+	

Figure 2-3. Characterization of periplasmic nitrate reductase (pNar) genes with possible perchlorate reductase activity. (A) Gene clusters containing both pnarGHJI and cld. (B) Alignment of selected Nar-type enzymes constructed using MUSCLE and edited using AliView. Perchlorate reductase residue positions α, β, and γ are structurally conserved and functionally significant [155]. Substrate utilization: +, reduced substrate in vivo or in vitro; -, did not reduce substrate; (+), predicted to reduce substrate. Tree: position in the phylogenetic tree at right. (C) Maximum likelihood phylogeny of Nar-type molybdoenzymes constructed using MUSCLE and PhyML with 100 bootstraps. Four lineages are found in gene clusters with chlorite dismutase (blue background): group 1 PcrA, group 1 ClrA (SerA-like), group 2 ClrA (DdhA-like), and group 2 PcrA (pNarG-like). Genome bins encoding proteins from each group are indicated.

In addition to observing the canonical PRI, genomic analysis identified a novel perchlorate reduction gene cluster distinguished by an alternative putative perchlorate reductase. In bins Rhizobiales BM303 and Rhizobiales BM505 and three previously sequenced genomes, *cld* is adjacent to the gene for a periplasmic Nar-like molybdopterin reductase (*pnarGHIJ*) (Figure 3A). Available evidence suggests that the pNar enzyme could function as an alternative perchlorate reductase: pNar from thermophilic bacteria and archaea reduce perchlorate to chlorite, and no pNar tested has lacked perchlorate reductase activity [90, 116, 156-158]. Additionally, in sequence alignments pNarG, like cNarG, had structurally similar amino acids in the key residues (positions α, β, and γ) that shape the PcrA active site and influence its affinity for perchlorate (Figure 2-3B) [155]. Whereas the lack of chlorite dismutase in other *pnar*-containing organisms suggests their perchlorate reduction is inadvertent, the conserved association of *pnar* with *cld* in this gene cluster strongly suggests it is specialized for perchlorate reduction. The gene clusters also resembled PRI by encoding, variously, genes for reactive chlorine stress response, signaling, plasmid replication, transposon mobility, and phage proteins. Furthermore, pNarG proteins from the *pnar-cld* gene cluster formed a deep-branching monophyletic clade basal to pNarG proteins from other organisms (Figure 2-3C), which supports a specialized function distinct from other pNar proteins. Available genomic and phylogenetic evidence support that pNar enzymes in this clade primarily function as perchlorate reductases, henceforth referred to as group 2 perchlorate reductases, although biochemical and genetic evidence are ultimately needed to confirm their function.

The physiological difference between group 2 perchlorate reductases and the previously characterized group 1 perchlorate reductases is uncertain. Two gene clusters encoded both reductases. Additionally, strains encoding only the alternative perchlorate reductase, *Magnetospirillum magnetotacticum* MS-1 and *Tropicimonas isoalkanivorans* B51, did not grow using perchlorate or chlorate as an electron acceptor with acetate as the electron donor (results not shown) [159]. However, Rhizobiales BM303, which also encoded only a group 2 Pcr, was enriched in two communities, suggesting that group 2 Pcr alone may be sufficient for dissimilatory perchlorate reduction in some conditions. The similarity of pNarG and cNarG could explain the failure to cultivate organisms with group 2 Pcr: both NarG enzymes lack a key tryptophan residue (position γ; Figure 2-3B) for the proposed gating mechanism in group 1 Pcr that limits the rate of perchlorate reduction at unnaturally high concentrations of perchlorate, such as those supplied in cultivation media [155]. Rapid reduction of perchlorate to chlorite by group 2 Pcr could be detrimental to cells unless chlorite is removed, as would occur by chemical reaction with sulfide present in the sediment inoculum but absent from most cultivation media.

Genetic mobility of perchlorate reduction genes

PRIs suffered from fragmentation into multiple contigs during co-assembly that initially prevented binning and characterization. We noticed that individually assembled samples had a higher average contig length than the co-assembled samples (Supplementary Table 2-4), which implied that co-assembly led to greater fragmentation. By assembling samples individually then using contigs graphs to manually combine contigs, we reconstructed complete PRI and linked them to specific genomes binned from the co-assembly. To our knowledge, this is the first study in which contig graphs have been used to associate mobile genetic elements with populations in microbial communities. Contig graphs revealed that even within individual samples, the same sequences (Figure 2-4A) and perchlorate reduction genes (Figure 2-4B) were associated with multiple genomes. In the 3% salinity community, a PRI was contiguous with both Sedimenticola BM301 and BM503, though *pcrA* and *pcrD* did not assemble as a consensus sequence. In the 5% salinity community, a PRI was contiguous with sequences from Arcobacter BM504 and Sulfurimonas BM502. Notably, the *pcrAB* sequence from that PRI was also contiguous with Sedimenticola BM503. In contig graphs, the presence of identical sequences in multiple metagenome-assembled genomes indicates either horizontal transfer during the enrichment or the assembly of slightly diverged sequences as a consensus sequence. The latter conclusion is supported by similarity of PcrA and Cld phylogenies between metagenome-assembled genomes and previously isolated strains (Figure 2-2).

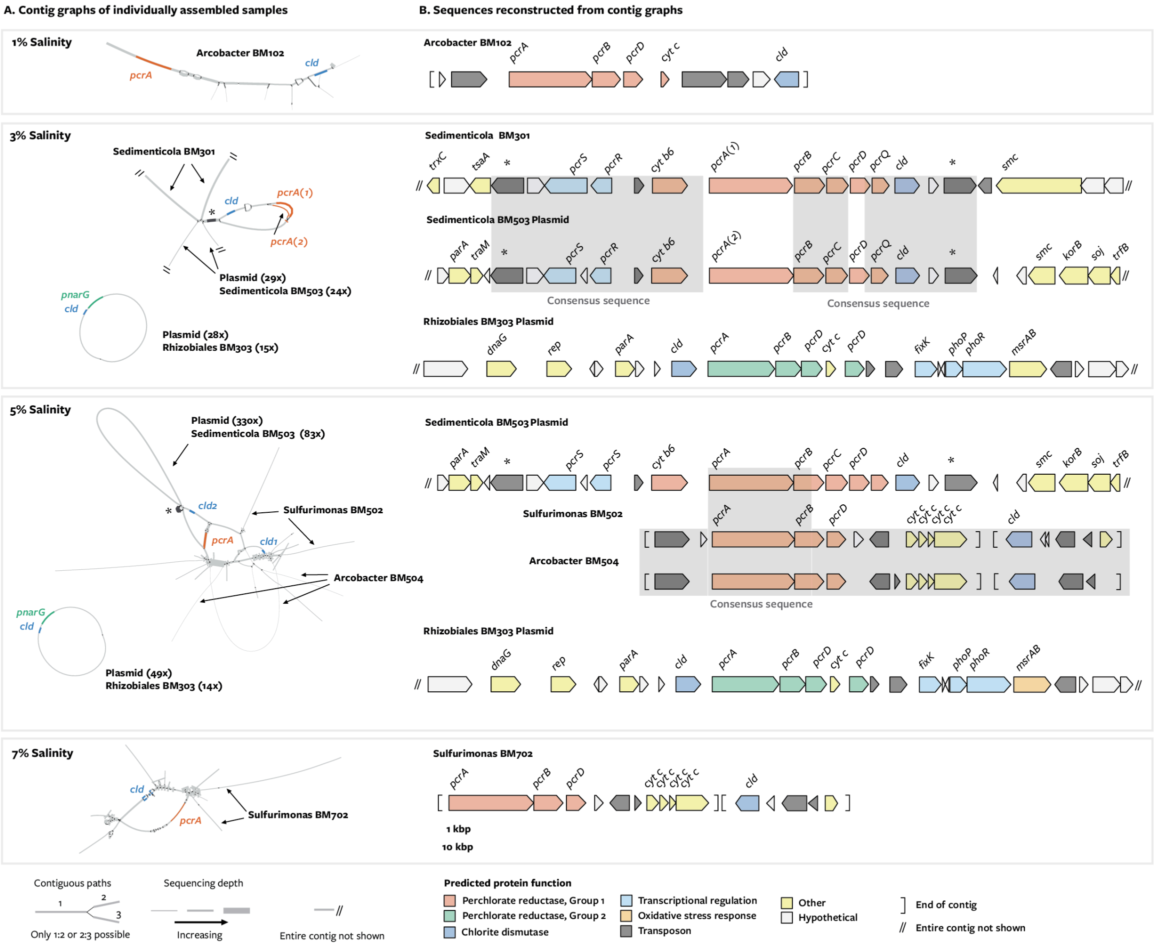


Figure 2-4. Use of contig graphs to manually assemble perchlorate reduction genes within mobile genetic elements. (A) Each sample was assembled individually and inspected for perchlorate reduction genes in contig graphs. Contig graphs depict possible connections between contigs produced by de Bruijn graph assembly. The real connections between contigs may be determined using information such as coverage (line weight) and gene content (line colors). PRI sequences including group 1 pcrA (red), group 2 pcrA ("pnarG", green), and cld (blue) that were clearly connected to contigs from genome bins (arrows) were added to those bins. Mean coverage for particular plasmid sequences and bins is indicated in parenthesis. For simplicity, only contigs within a few connections of PRI sequences are shown, and some contigs are not shown in their entirety. (B) Reconstructed PRI from contig graphs. Shaded genes indicate sequences that were highly similar and assembled as a consensus sequence from multiple strains. Each genome was annotated independently using Prokka.

Two plasmids encoding perchlorate reduction genes were identified in the metagenomic dataset. A transposon containing key perchlorate reduction genes *pcrABCDQ* and *cld* was integrated into the chromosome of Sedimenticola BM301 in 3% salinity community but into a conjugative plasmid in the 3% and 5% salinity communities. We assigned this plasmid to Sedimenticola BM503 on the basis of similar abundance to the plasmid in the 3% salinity condition (24x and 29x coverage, respectively) (Figure 2-4A) and the close taxonomic relationship of Sedimenticola BM503 to Sedimenticola BM301. The plasmid was four times more abundant than Sedimenticola BM503 in the 5% salinity community, however, in this community the plasmid was either maintained at high copy number or present in multiple organisms. A second plasmid, containing a PRI with group 2 *pcr* instead of group 1 *pcr*, was present in both the 3% and 5% salinity. While this plasmid initially binned with Sedimenticola BM503 (which is at two-fold greater coverage in one sample), we re-assigned the plasmid to Rhizobiales BM303 due to more similar patterns of abundance (15x:28x and 14x:49x coverage) (Figure 2-4A) and the close taxonomic relationship of Rhizobiales BM303 to Rhizobiales BM505, which has a closely related group 2 *pcr* on a 326,675-bp chromosomal scaffold. The plasmid encodes group 2 *pcrABD*, *cld*, and genes for cytoplasmic methionine sulfoxide reductase (*msrAB*), a sensor histidine kinase system, and a transposase. Perchlorate reduction genes have previously only been associated with evidence of prior integration, such as bordering transposases and insertion scars, or potential excision, in the case of the integrative conjugative element in *Dechloromonas aromatica* RCB [108]. Thus Sedimenticola BM503 and Rhizobiales BM303 provide the first examples of DPRM with perchlorate reduction gene clusters on intact plasmids. However, the available data is not sufficient to determine whether or not these plasmids served as vectors for gene transfer during enrichment.

Implications for the evolution of perchlorate reduction

The perchlorate reduction genes obtained from these communities make evident a substantial reorganization of perchlorate reduction gene clusters throughout their evolutionary history. Protein sequences from recovered genomes showed a distinct phylogenetic grouping of Cld between *Arcobacter*-type and *Sedimenticola*-type proteins (Figure 2-2B) that was not replicated for PcrA (Figure 2-2A). For example, Sedimenticola BM301 and Sedimenticola BM503 encoded identical Cld (100% identity) but distinct PcrA (89% identity). These discordant phylogenies support the past independent exchange of *cld* or *pcr* between PRI. The most likely evolutionary history is that a group of *Gammaproteobacteria* (Sedimenticola BM503, *Marinobacter* sp. P4B1, and *Sedimenticola* sp. CUZ) acquired *pcrA* from one of a group of *Epsilonproteobacteria* (*Sulfurospiriullum* spp., *Sulfurimonas* spp., or *Arcobacter* spp.). These results challenge the previous hypotheses that *pcrAB* and *cld* were propagated together following the formation of a single ancestral PRI and that the exchange of perchlorate reduction genes between phyla is rare [108]. The exchange of these genes may result from the need to optimize perchlorate reductase and chlorite dismutase activity when perchlorate concentrations or other geochemical conditions change.

The identification of gene clusters with group 2 perchlorate reductase challenge the very notion of a single ancestral PRI. While group 1 and group 2 Pcr formed distinct monophyletic groups (Figure 2-3C), the Cld proteins from the gene clusters showed no such phylogenetic clustering (Figure 2-2B). Therefore, *cld* has been exchanged between gene clusters with phylogenetically distinct perchlorate reductases. Clusters that included both group 1 and group 2 *pcr,* as found in *Dechlorobacter hydrogenophilus* LT-1 and Rhizobiales BM505, may represent an intermediate point in this exchange. The exchange may be driven by environmental perchlorate concentrations: it was recently demonstrated that group 1 PcrAB is a specialized member of the DMSO reductase superfamily with a relatively high perchlorate affinity (K_M = 6 μM), unlike the related cNarGH nitrate reductase which has a much lower affinity for perchlorate (K_M = 1.1 mM) [155]. In contrast to supporting a single ancestral PRI, these data support the formation of two perchlorate reduction gene clusters through the independent acquisition of chlorite dismutase. A similar history was inferred for the gene cluster responsible for dissimilatory chlorate reduction, the chlorate reduction composite transposon interior (CRI) [87]. Thus this study adds to empirical support for the idea that enzymes in the NarG subfamily of molydopterin oxidoreductases possess reductase activity towards various oxyanions but have been repeatedly adopted for perchlorate or chlorate reduction through the acquisition of the downstream enzyme Cld.

Metabolic interactions within perchlorate-reducing communities

Metagenome-assembled genomes lacking perchlorate reduction genes accounted for 35 of 43 enriched genomes (Figure 2-1A) and 10.5% to 91.5% of the recovered community (Figure 2-5A). Although we cannot dismiss the idea that unknown mechanisms for perchlorate reduction exist, it is not necessary to explain the growth of organisms in these communities. One explanation is that organisms use alternative electron acceptors or fermentable substrates that were present in the sediment inoculum [115]. A non-conflicting possibility of particular interest is that perchlorate reduction intermediates and byproducts provide growth substrates for non-DPRM. We identified genes for energy metabolism in every genome (Figure 2-1A) to determine whether interactions between metabolisms were possible in each community.

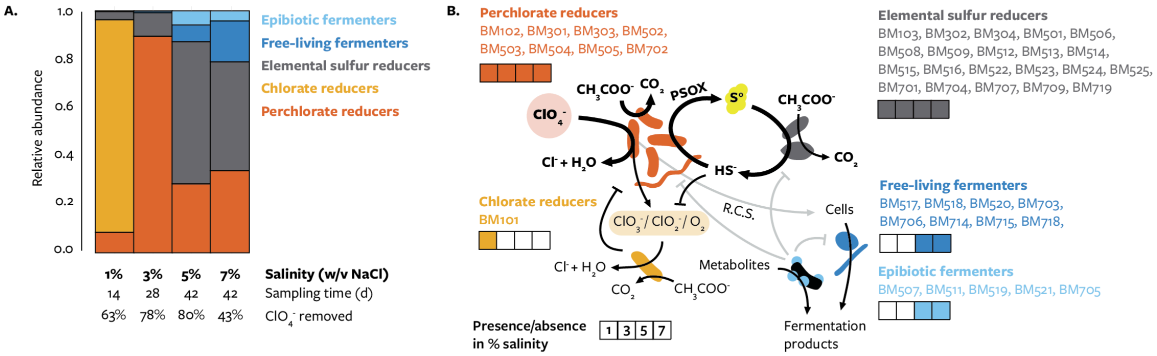


Figure 2-5. Metabolic potential of perchlorate-reducing communities. (A) The relative abundance, among high quality bins, of genomes with each predicted metabolism: dissimilatory perchlorate-reducing bacteria (red), dissimilatory chlorate-reducing bacteria (orange), dissimilatory elemental sulfur-reducing bacteria (grey), free-living fermentative bacteria (dark blue), and epibiotic fermentative bacteria (light blue). Communities differ in salinity (% NaCl w/v), sampling time (days), and perchlorate removed (%). (B). Possible metabolic interactions and their commonality among the four enriched communities.

Intermediates of perchlorate reduction may have supported the growth of non-DPRM in the 1% salinity community. The most abundant population in this community, Azoarcus BM101, lacked any perchlorate reductase yet encodes a complete chlorate reduction pathway consisting of genes for chlorate reductase (*clrABDC)*, chlorite dismutase (*cld*), and terminal oxygen reductases (Figure 1A). Azoarcus BM101 could therefore utilize any perchlorate reduction intermediate (ClO_3^-, ClO_2^-, or O_2), similar to the symbiotic perchlorate reduction metabolism previously identified [7, 111]. Unexpectedly, although this would have made it dependent on DPRM, Azoarcus BM101 was found at 10-fold higher abundance than the perchlorate-reducing population Arcobacter BM102 (Figure 2-5A). Specific growth characteristics of either strain that could explain this interaction are unknown. The most closely related isolated strain to BM102, *Arcobacter* sp. CAB, is not known to accumulate chlorate during perchlorate reduction, and though the chlorate reduction proteins in Azoarcus BM101 were nearly identical to those in previously characterized organisms (Figure 2-2C), it is the first chlorate-reducing bacteria to be described in the betaproteobacterial order *Rhodocyclales*. The consumption of perchlorate reduction intermediates by Azoarcus BM101 would likely limit the growth of DPRM due to energy losses, although which intermediates were involved in this interaction is uncertain (Figure 2-5B). While evidence for interaction between DPRM and chlorate-reducing microorganisms was only observed in one of four enriched communities, whether or not such interactions are a common feature of perchlorate-reducing communities is presently unknown.

Perchlorate-dependent sulfide oxidation (PSOX) to elemental sulfur and polysulfide could have supported dissimilatory elemental sulfur reducing microorganisms (DSRM) in every community at the expense of outgrowth of canonical DPRM [112, 113] (Figure 2-5). Populations in every community encoded genes for enabling elemental sulfur reduction: polysulfide reductase (*psr*) and periplasmic high molecular weight (HMW) multiheme cytochromes (Figure 2-1A) [160, 161]. Putative DSRM included *Denitrovibrio* (BM701, BM704), *Ignavibacteria* BM516*, Marinilabiliales* (BM514, BM524, BM523, BM719)*, Desulfobulbaceae* BM506, *Deltaproteobacteria* (BM512, BM525), and *Desulfuromonas* (BM103, BM302, BM304, BM501, BM508, BM509, BM513, BM515, BM522, BM707, BM709). At higher salinities DSRM dominated, as these populations accounted for 58.7% and 45.0% of the 5% and 7% salinity communities, respectively (Figure 2-5A). Many of these populations were actively replicating (iRep > 1.3; Supplementary Table 2-5) and showed equal or greater enrichment than DPRM populations (Supplementary Figure 2-8), demonstrating that DSRM were not only persisting through enrichment but actively growing. Because the inoculum is marine sediment, reduced sulfur species including elemental sulfur were likely present. Any sulfide present or produced by DSRM would be re-oxidized to elemental sulfur by PSOX (Figure 2-5B). The inhibition of DPRM by sulfide [112, 113] provides a possible mechanism by which elemental sulfur-reducing microorganisms could dominate communities without affecting the stoichiometric removal of acetate and perchlorate. Genomic evidence from these enrichments therefore supports the hypothesis that cryptic elemental sulfur cycling is a feature of perchlorate-reducing communities with sulfur-reducing microorganisms and reduced sulfur species.

Not all populations interacted directly with DPRM through perchlorate reduction intermediates or byproducts. Several populations from higher salinity communities lacked key genes for anaerobic respiration and likely obtained energy only through fermentation. Putatively fermentative populations include *Clostridiales* (BM714), *Vibrio alginolyticus* (BM517), and *Marinilabiliales* (BM518, BM520, BM703, BM715, BM718), Most of these genomes lacked NADH:quinone oxidoreductase, genes for acetate catabolism through the TCA cycle, and respiratory perchlorate, chlorate, oxygen, and sulfur reductases (Figure 2-1A). Previously isolated *Marinilabiliales* organisms have indeed been specialized for the fermentation of dead cells present in hypersaline marine sediments [162]. In contrast, like previously described members of the CPR *Bacteria* and DPANN *Archaea* superphyla [129, 163-165], *Ca.* Woesearchaeota and *Ca.* Parcubacteria/OD1 bins have a small size and limited gene content and were likely obligate epibiotic symbionts of other populations. One putatively fermentative population, BM706, constituted a distinct phylum-level lineage in our multi-locus sequence analysis (Supplementary Figure 2-5). BM706 is abundant in both the 5% and 7% salinity communities, totals 2.92 Mbp of sequence, and is predicted to be near-complete (96.6%) with low contamination (3.5%) (Table 2-1). BM706 is distinct from other genomes as determined by comparison ribosomal protein S3 (63% identity) and 16S rRNA gene sequences (84% identity) using BLAST. The only closely related (>84% identity) environmental 16S rRNA clones were found in hypersaline microbial mats in Guerrero Negro, Baja California Sur, Mexico, (all >94% identity) [166]. Indeed, the genome recovered from our enrichment appears to be specialized for high salinity environments, encoding a sodium/proton-translocating ATP synthetase, several sodium transporters, and numerous extracellular enzymes for the degradation of lysed biomass, and we suggest that the energy metabolism of BM706 relies on the production of a sodium motive force powered by the fermentation of dead cells (a graphical depiction of the metabolism can be found in Supplementary Figure 2-9). For this candidate taxon we propose a genus and species name *Ca.* Muirbacterium halophilum BM706 and the phylum name “Muirbacteria” in recognition of the contributions of naturalist John Muir (1838-1914) to the protection of natural areas in California. While the growth of *Ca.* Muirbacteria halophilum BM706 and other putative free-living fermenters may be attributable to fermentable substrates from the sediment, the reactive chlorine species produced by perchlorate-reducing bacteria could accelerate cell lysis and provide additional substrates for fermentative bacteria (Figure 2-5).

Conclusions

The metagenomic dataset obtained here resolves long-standing questions regarding the genetic and metabolic composition of perchlorate-reducing communities and opens exciting new questions about the evolution and ecology of this unique metabolism. The discovery of a second specialized perchlorate reductase, if confirmed, fundamentally reorders the evolution of this metabolism. Further attempts to cultivate and characterize the organisms with this gene cluster may identify ecologically important differences from the canonical perchlorate reductase. In these communities, gene clusters containing either perchlorate reductase could be found on plasmids, which may provide a genetic tool to study the exchange of perchlorate reduction genes between organisms. The high sequence identity of perchlorate reduction genes and their association with mobile genetic elements required manual assembly using contig graphs – a technique that may be widely applicable for binning key genes and identifying possible gene transfer in simple metagenomic datasets. In addition to DPRM, the typical perchlorate-reducing community appears to involve interactions with other organisms that could substantially influence ecosystem functions. Together, these results suggest a model of perchlorate reduction involving the combined activity of distinct perchlorate reductases and multiple interacting metabolisms.

Supplementary Material

Supplementary figures and tables can be found accompanying the published version of this chapter: https://doi.org/10.1038/s41396-018-0081-5

Tables

Table 2-1. Summary of metagenome-assembled genome bins.

Phylum	Genome Bin	16S[a]	GC (%)	Size (Mbp)	CDS	Compl. (%)[b]	Contam. (%)[b]	Scaffolds (#)	N50 (bp)[c]	Coverage[d]
Bacteroidetes	Marinilabiliales BM514	-	39.0	3.14	2677	91.49	4.43 / 50	478	8468	15
	Marinilabiliales BM518	-	39.7	3.35	2758	95.61	2.15 / 25	259	20566	13
	Marinilabiliales BM520	-	41.5	3.49	2732	97.85	1.08 / 0	348	13857	12
	Marinilabiliales BM523	-	35.4	3.92	3206	79.8	3.9 / 0	751	5963	7
	Marinilabiliales BM524	-	34.4	3.60	2910	93.23	1.08 / 0	223	30104	15
	Marinilabiliales BM703	(+)	32.9	4.24	4035	96.15	3.49 / 0	27	265205	113
	Marinilabiliales BM718	-	32.4	3.57	3157	91.08	1.61 / 20	544	7945	10
	Marinilabiliales BM719	-	33.9	3.29	2740	94.3	1.14 / 0	324	15276	10
	Salinivirgaceae BM715	-	35.1	4.32	3493	94.23	3.33 / 25	364	18792	11
Ignavibacteria	Ignavibacteria BM516	+	56.7	3.93	3264	92.35	3.01 / 33.33	436	13823	22
Ca. Muirbacteria[e]	Ca. Muirbacterium halophilum BM706	(+)	32.9	2.92	2669	96.56	3.5 / 20	147	33603	56
Ca. *Parcubacteria*	Parcubacteria BM507	+	35.4	1.27	1204	71.78	0.99 / 0	49	76005	21
	Parcubacteria BM519	+	37.2	0.97	948	72.94	2.09 / 0	59	28615	9
	Parcubacteria BM521	+	39.0	1.06	983	83.15	1.12 / 0	67	30243	10
	Parcubacteria BM705	+	33.5	1.43	1424	76.81	0 / NA	15	156199	46
Ca. Woesearchaeota[f]	Woesearchaeota BM511	+	31.1	1.59	1702	85.05	1.87 / 66.67	70	31020	16
Deferribacteres	Denitrovibrio BM701	+	41.1	2.88	2811	95.35	0 / NA	29	155259	324
	Denitrovibrio BM704	-	38.8	2.78	2700	93.97	0 / NA	13	410371	44
Firmicutes	Clostridiales BM714	-	44.6	1.93	1911	92.13	3.9 / 75	370	5971	13
Alphaproteobacteria	Alphaproteobacteria BM001	+	42.8	1.13	1175	97.85	0	10	189894	28
	Rhizobiales BM303	-	61.4	3.50	3308	96.12	2.17 / 44.44	428	11363	24
	Rhizobiales BM505	+	63.6	4.04	3609	98.58	0.79 / 0	7	859782	31
Betaproteobacteria	Azoarcus BM101	+	62.9	4.90	4690	98.86	3.63 / 20	160	59964	515
Deltaproteobacteria	Deltaproteobacteria BM512	-	54.2	2.53	2404	92.11	1.68 / 50	67	65871	15

	Deltaproteobacteria BM525	-	54.5	2.60	2497	94.21	2.52 / 0	192	22954	9
	Desulfobacteraceae BM002	-	50.0	3.80	3595	94.35	3.25 / 33.33	638	7626	11
	Desulfobacteraceae BM005	-	43.3	3.05	2788	84.52	1.81 / 0	577	6310	9
	Desulfobulbaceae BM004	-	47.5	2.66	2487	98.13	1.64 / 25	189	21028	12
	Desulfobulbaceae BM506	+	56.3	3.71	3194	98.81	0 / NA	83	74437	31
	Desulfuromonas BM103	-	55.9	2.93	2860	90.07	6.13 / 7.69	426	9781	9
	Desulfuromonas BM302	-	55.8	2.57	2473	94.03	1.51 / 85.71	216	19147	52
	Desulfuromonas BM304	-	56.7	3.30	3091	100	0.32 / 0	84	56769	15
	Desulfuromonas BM501	-	62.4	3.70	3348	98.06	0.65 / 0	56	94881	437
	Desulfuromonas BM508	-	55.0	2.66	2434	97.74	1.64 / 25	109	43575	31
	Desulfuromonas BM509	-	54.7	3.29	3141	96.02	0.65 / 100	219	24493	40
	Desulfuromonas BM513	-	52.5	3.12	2851	95.38	1.94 / 33.33	186	26287	20
	Desulfuromonas BM515	-	56.1	2.39	2258	74.23	1.61 / 33.33	138	24721	11
	Desulfuromonas BM522	-	56.3	3.12	2941	90	2.1 / 0	203	22928	10
	Desulfuromonas BM707	-	53.3	3.08	2941	99.35	0.65 / 0	121	56077	23
	Desulfuromonas BM709	-	58.3	3.03	2916	93.44	7.74 / 42.86	226	20005	20
Epsilonproteobacteria	Arcobacter BM102	+	28.1	3.48	3530	98.78	1.56 / 0	42	139512	49
	Arcobacter BM504	-	28.9	2.67	2847	97.76	0.95 / 0	12	347029	58
	Sulfurimonas BM502	-	36.6	2.38	2554	95.03	0.2 / 0	46	90685	96
	Sulfurimonas BM702	-	34.4	1.88	1999	97.14	3.22 / 60	122	26298	291
Gammaproteobacteria	Chromatiales BM003	-	60.6	3.62	3404	93.35	5.53 / 25.93	337	14686	15
	S. selenatireducens BM301	-	56.4	4.26	4076	98.72	1.46 / 0	31	238438	675
	Sedimenticola BM503	-	53.9	3.86	3805	99.65	0.64 / 0	109	74804	89
	Vibrio alginolyticus BM517	-	44.9	3.94	3460	84.18	2.94 / 73.33	725	6657	16

[a] 16S rRNA gene presence in genome bin: +, presence; -, absence; and (+), recovered through manual assembly using contig graphs

[b] CheckM estimates of completeness (Compl.), contamination (Contam.; left), and fraction of contamination from related strains (Contam.; right)

[c] Assembly contiguity: 50% of the total genome length is accounted for by contigs of this length or greater.

[d] Sum of mean read depth from each sample

[e] Proposed candidate phylum from this study

[f] Misassigned to Parvarchaeota in Carlstrom et. al 2016

Chapter 3
Identification of a respiratory symbiosis in the biogeochemical chlorine cycle

Abstract

A key step in the chlorine cycle is the reduction of perchlorate (ClO_4^-) and chlorate (ClO_3^-) to chloride by microbial respiratory pathways. Perchlorate-reducing bacteria and chlorate-reducing bacteria differ in that the latter cannot use perchlorate, the most oxidized chlorine compound. However, a recent study identified a bacterium with the chlorate reduction pathway dominating a community provided only perchlorate. Here we confirm a metabolic interaction between perchlorate- and chlorate-reducing bacteria and define its mechanism. Perchlorate-reducing bacteria supported the growth of chlorate-reducing bacteria to up to 90% of total cells in communities and co-cultures. Chlorate-reducing bacteria required the gene for chlorate reductase to grow in co-culture with perchlorate-reducing bacteria, demonstrating that chlorate is responsible for the interaction, not the subsequent intermediates chlorite and oxygen. Modeling of the interaction suggested that cells specialized for chlorate reduction have a competitive advantage for consuming chlorate produced from perchlorate, especially at high concentrations of perchlorate, because perchlorate and chlorate compete for a single enzyme in perchlorate-reducing cells. We conclude that perchlorate-reducing bacteria inadvertently support large populations of chlorate-reducing bacteria through the release of the intermediate chlorate. An implication of these findings is that undetected chlorate-reducing bacteria have likely negatively impacted efforts to bioremediate perchlorate pollution for decades.

Introduction

The chlorine cycle consists of the biological, geological, and chemical processes that interconvert organic and inorganic chlorine compounds [8]. Chlorine oxyanions are a group of inorganic chlorine compounds of particular interest in biology due to their high reduction potentials ($E^{0'} > 0.7$ V) [7, 79, 167, 168]. Hypochlorite (ClO^-) and chlorite (ClO_2^-) are highly reactive compounds that damage cells through oxidative chemistry [93, 98, 169], while chlorate (ClO_3^-) and perchlorate (ClO_4^-) are used as electron acceptors in respiration by some bacteria and archaea [7]. Uniquely among chlorine oxyanions, perchlorate is chemically stable in solution, and a necessary step in the chlorine cycle is the reduction of perchlorate to chloride by microbial respiration [7, 170]. Where this microbial activity is absent, geochemical reactions in the atmosphere lead to the accumulation of perchlorate and, to a lesser degree, chlorate [7, 108, 171]. Both atmospheric deposition of chlorine oxyanions and microorganisms respiring chlorine oxyanions appear to be widespread [89, 107], yet the biogeochemistry of this key part of the chlorine cycle is not well understood [7].

An important unresolved question is whether the microbial respiration of chlorine oxyanions in the environment is performed by individual cells or by groups of cells with different parts of the biochemical pathway [91, 111]. Many redox metabolisms from other elemental cycles have been found to occur through pathways that can be divided between different cells, including nitrate reduction [172]; ammonia oxidation [173, 174]; sulfur oxidation and reduction [175, 176]; and organic chlorine reduction [177]. Complete pathways might even be rare in environmental systems: a recent description of metagenome-assembled genomes from aquifer sediment found that only a minority of organisms with genes for nitrate reduction or sulfur oxidation had the complete pathway [117]. In many cases, respiratory metabolisms have been observed to involve both cells with complete pathways and cells with partial pathways, a form of symbiosis that can range from mutualistic to antagonistic [178-181].

Chlorate reduction could be considered a partial pathway of perchlorate reduction, as the two pathways share substantial similarities [7]. The key difference is whether or not the initial step of the pathway is catalyzed by a perchlorate reductase (Pcr), which reduces both perchlorate and chlorate, or by a chlorate reductase (Clr), which can only reduce chlorate (Figure 3-1A) [182]. Both metabolisms occur in the bacterial periplasm, where perchlorate and/or chlorate are reduced to chlorite, chlorite is converted to chloride and oxygen by a chlorite dismutase (Cld) [89, 183-185], and oxygen is reduced to water by one or more terminal oxidases [111, 186, 187]. Energy is conserved by the reduction of perchlorate, chlorate, and oxygen but not in the conversion of chlorite to oxygen and chloride (Figure 3-1A) [88]. Genes for these enzymes are found together within horizontally transferred genomic DNA or plasmid DNA, typically with accessory genes for signaling and regulation, reactive chlorine stress response, protein and cofactor assembly, and genetic mobility [87, 108, 188]. Some bacteria and archaea have been experimentally observed or engineered to reduce perchlorate or chlorate to chlorite, relying on a second organism or chemical reactions to remove chlorite [90, 111, 116, 157]. However, selection for perchlorate- or chlorate-reducing microorganisms from the environment has only yielded bacteria with the canonical pathways described above [7, 91].

Though the pathways for chlorine oxyanion respiration have been studied in parallel for decades [88, 189], research on interactions between them is sparse. One set of studies explored how unusually high accumulation of chlorate by the perchlorate-reducing bacterium *Dechlorosoma* sp. HCAP-C (PCC) could support chlorate-reducing bacteria [110, 190, 191]. Addition of a chlorate-reducing bacterium in co-culture with strain HCAP-C decreased the concentration of chlorate, and while models of the system suggested growth of the chlorate-reducing bacterium, the community structure *in situ* was not determined [110, 190]. Accumulation of chlorate by strain HCAP-C was proposed to occur because a single enzyme (Pcr) catalyzes two sequential reactions in the pathway (reduction of perchlorate to chlorate, and chlorate to chlorite) [191, 192]. As that trait is shared by all known perchlorate-reducing bacteria, and several perchlorate-reducing bacteria have been reported to accumulate chlorate, albeit at much lower concentrations [155, 193, 194], it was speculated that chlorate-reducing bacteria may be a common feature of natural perchlorate-reducing communities [190, 192].

No subsequent research examined the possibility of interaction between chlorine oxyanion reduction pathways in communities until recently, when we observed a genome with chlorate reduction genes in a perchlorate-enriched community [91]. Surprisingly, the putative chlorate-reducing population was 10-fold more abundant than the perchlorate-reducing population. Because no chlorate had been added to the cultures, the chlorate-reducing population either had unknown perchlorate reduction genes or was metabolizing intermediates of the perchlorate reduction pathway [91].

In the present study, we investigate the interaction between perchlorate-reducing bacteria and chlorate-reducing bacteria. After sequencing the genomes of perchlorate-reducing cultures obtained from estuary sediment enrichments [120], we detected contaminating bacteria that had not been completely removed during isolation. We discovered that several cultures were not predominantly perchlorate-reducing bacteria, as expected, but dominated by chlorate-reducing bacteria. We therefore used a combination of co-cultures, genetics, and modeling to confirm the interaction, define its mechanism, and explain how such a community structure could be produced. We conclude that the environmental chlorine cycle involves the interaction of a complete pathway and a partial pathway in the reduction of perchlorate to chloride.

Materials and Methods

Genome sequencing, assembly, binning, and annotation

Genomic DNA was extracted using a MoBio PowerSoil DNA Extraction Kit with a cell lysis protocol consisting of vortexing and heating at 70 °C for 5 min, repeated twice (MoBio Laboratories, Inc., Carlsbad, CA). DNA library preparation and DNA sequencing were performed by the Adam Arkin Laboratory or the Vincent J. Coates Genomics Sequencing Laboratory at the California Institute of Quantitative Biosciences

(QB3, Berkeley, CA) using an Illumina MiSeq V2 (150PE or 250PE) and Illumina Hiseq4000 (100PE), respectively. Paired-end reads from each sample were trimmed using Sickle v. 1.33 with default parameters [122], error-corrected using SGA v. 0.10.15 [195], and assembled using MEGAHIT v. 1.1.2 with the parameters --no-mercy and --min-count 3 [123]. After assembly, reads were mapped back to each assembly using the Burrows-Wheeler Alignment Tool v. 0.7.10 (BWA) BWA-MEM algorithm [196]. All manipulation of reads was performed on high-performance computing clusters administered by the Computational Genomics Resource Laboratory (CGRL).

Genome assemblies were screened for contamination using Anvi'o v. 3.1 [126]. Briefly, contigs >2,000 bp were manually binned into genomes using the hierarchical clustering generated from sequence characteristics and read coverage. When multiple genomes were present in a single assembly, contigs were binned into metagenome-assembled genomes (MAGs). Because perchlorate and chlorate respiration involve horizontally transferred genes that are subject to poor assembly, the BLAST feature in Bandage v. 0.8.0 [131] was used to identify key genes and confirm their presence and absence in genomes as previously described [91]. The completeness and contamination of each genome and metagenome-assembled genome was measured using CheckM [128], which measures the single copy genes expected within a lineage and defines contamination as redundant genes with less than 90% amino acid identity. Gene prediction and annotation was performed using Prokka v. 1.11 [136], and key genes were identified using custom profile Hidden Markov models (HMMs) trained on previously confirmed proteins using HMMER v. 3.1b2 [143]. All reads and genome sequences are available through the NCBI Bioproject accession PRJNA387015 [91].

Strains, media, and culture conditions

A complete set of strains and cultivation conditions are included in Supplementary Table 3-1. Growth medium for perchlorate-reducing cultures consisted of either a freshwater defined medium [89] or a marine defined medium [197] at pH 7.2 with, unless noted otherwise, 10 mM acetate as the electron donor and carbon source and 10 mM perchlorate as the electron acceptor. All media and stocks were made anoxic by sparging with N_2. Growth experiments were performed at 30 C in crimp-sealed tubes with an N_2 atmosphere. Concentrations of perchlorate, chlorate, and acetate were measured using ion chromatography. Cells were quantified by optical density at 600 nm (OD600). Isolation of chlorate-reducing strains was performed by streaking twice onto oxic solid media and confirmed by Sanger sequencing of individual colonies' 16S rRNA genes.

Quantification of perchlorate- and chlorate-reducing microorganisms

Primers to measure the model perchlorate-reducing bacterium *Azospira suillum* PS and model chlorate-reducing bacterium *Pseudomonas stutzeri* PDA were designed to bind variable regions of their respective small ribosomal subunit gene (16S rRNA) sequence and amplify ~150 bp sequence. Primers to measure all chlorate-reducing bacteria were designed to bind the chlorate reductase gene (*clrA*). The *clrA* gene consists of two phylogenetic groups (here termed groups 1 and 2) with highest similarity to the alpha subunits of selenate reductase or dimethylsulfide dehydrogenase, respectively [87]. Specific primer selection involved identifying highly conserved sequence positions within each *clrA* group but not across closely related genes. Related genes were identified by searching the NCBI NR database with BLASTP [134]. Primer-BLAST used Primer Pair Specificity to check against select genomes in the NCBI non-redundant database (Supplementary Table 3-2). Template DNA was quantified using qPCR with three technical replicates; a standard curve of known concentration; and SYBR qPCR Master Mix (Thermo Fisher Scientific) on a StepOnePlus qPCR machine (Applied Biosciences). Measurements were performed on four biological replicates sampled at the time of inoculation and at the last timepoint preceding stationary phase. Quantification of total extracted DNA used the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific).

The relative abundance of isolated chlorate-reducing strains in the enriched communities was determined from previous 16S rRNA gene amplicon data under Sequence Reads Archive accession SRP049563 [120]. We obtained amplicon sequence variants (ASVs) to differentiate between closely related taxa by using DADA2 v.1.10 with default settings and without pooling [198, 199]. 16S rRNA gene sequences from representative DPRM and DCRM were compared to ASVs using BLASTN [134]. Each ASV was assigned the taxonomy of the sequence with the highest percent identity above a threshold of 95% (approximately genus-level similarity). Relative abundance was calculated from the number of reads composing each ASV and the total reads per sample.

Genetics

Genetic deletions and insertions in *Pseudomonas stutzeri* PDA were performed using protocols, strains, and plasmids from previous work [111]. All primers, plasmids, and strains are included in Supplementary Tables 3-1 and 3-3. Vectors were introduced into *Pseudomonas stutzeri* PDA via conjugation with *Escherichia coli* WM3064. These vectors had regions of homology allowing allelic exchange for a clean deletion, which were obtained by selection on kanamycin and counter-selection on sucrose.

Modeling

Modeling of perchlorate and chlorate reduction used the Equilibrium Chemistry Approximation [200], a modification of Michaelis-Menton kinetics that can account for competition between organisms for substrates and competition between substrates for an enzyme's active site. The reaction rate for perchlorate reduction to chlorate by perchlorate-reducing bacteria is provided by Equation 1:

$$V_{PRB}^{ClO_4} = V_{\max}^{ClO_4} \cdot [B_{PRB}] \cdot \frac{[ClO_4]}{K_M^{ClO_4} \cdot \left(1 + \frac{[ClO_4]}{K_{PRB,O}^{ClO_4}} + \frac{[ClO_3]}{K_{PRB,M}^{ClO_3}} + \frac{[B_{PRB}]}{K_{PRB,M}^{ClO_4}}\right)} \cdot \frac{[DOC\ ]}{K_{PRB,M}^{DOC} \cdot \left(1 + \frac{[DOC\ ]}{K_{PRB,M}^{DOC}} + \frac{[B_{PRB}]}{K_{PRB,M}^{DOC}} + \frac{[B_{CRB}]}{K_{CRB,M}^{DOC}}\right)} \qquad (1)$$

Where V (concentration time^{-1}) is reaction rate and V$_{\max}$ (time^{-1}) is the maximum growth rate; B, [ClO$_4^-$], [ClO$_3^-$], and [DOC] (concentration) are, respectively, the density of cells and the concentration of perchlorate, chlorate, and dissolved organic carbon (acetate). K$_m$

(concentration) is the half-saturation concentration for each substrate. Together, these terms define how maximum reaction rate is limited by the concentration of perchlorate and acetate, as well as the competition of Pcr for chlorate and perchlorate.

The reaction rate for chlorate reduction was described similarly for perchlorate-reducing bacteria (Equation 2) and chlorate-reducing bacteria (Equation 3). To simplify modeling, we present chlorate reduction as one step instead of three steps (involving the intermediates chlorite and oxygen). We included the assumption that chlorate-reducing bacteria are unaffected by perchlorate.

$$V_{PRB}^{ClO_3} = V_{PRB,\max}^{ClO_3} \cdot [B_{PRB}] \cdot \frac{[ClO_3]}{K_{PRB,M}^{ClO_3} \cdot \left(1 + \frac{[ClO_4]}{K_{PRB,M}^{ClO_4}} + \frac{[ClO_3]}{K_{PRB,M}^{ClO_3}} + \frac{[B_{PRB}]}{K_{PRB,M}^{ClO_3}} + \frac{[B_{CRB}]}{K_{CRB,M}^{ClO_3}}\right)} \cdot \frac{[DOC\ ]}{K_{PRB,M}^{DOC} \cdot \left(1 + \frac{[DOC\ ]}{K_{PRB,M}^{DOC}} + \frac{[B_{PRB}]}{K_{PRB,M}^{DOC}} + \frac{[B_{CRB}]}{K_{CRB,M}^{DOC}}\right)} \tag{2}$$

$$V_{CRB}^{ClO_3} = V_{CRB,\max}^{ClO_3} \cdot [B_{CRB}] \cdot \frac{[ClO_3]}{K_{CRB,M}^{ClO_3} \cdot \left(1 + \frac{[ClO_3]}{K_{CRB,M}^{ClO_3}} + \frac{[B_{PRB}]}{K_{PRB,M}^{ClO_3}} + \frac{[B_{CRB}]}{K_{CRB,M}^{ClO_3}}\right)} \cdot \frac{[DOC\ ]}{K_{CRB,M}^{DOC} \cdot \left(1 + \frac{[DOC\ ]}{K_{CRB,M}^{DOC}} + \frac{[B_{PRB}]}{K_{PRB,M}^{DOC}} + \frac{[B_{CRB}]}{K_{CRB,M}^{DOC}}\right)} \tag{3}$$

The biomass yield and stoichiometry were calculated using the framework provided by Rittmann and McCarty [201]. Calculations required redox potentials and balanced half-reactions for the reduction of perchlorate to chlorate, the reduction of chlorate to chloride (via an oxygen intermediate), and the oxidation of acetate to carbon dioxide. Detailed methods and Python code are available at https://github.com/tylerbarnum/perchlorate-and-chlorate-reduction-2019. Unless otherwise noted, simulations of the model involved a theoretical case with all values equal for perchlorate-reducing bacteria and chlorate-reducing bacteria except for the ability to use perchlorate as a substrate (Table 3-1).

Results

Infiltration of perchlorate-reducing cultures by chlorate-reducing bacteria

Genomic sequencing of perchlorate-reducing cultures revealed large populations of chlorate-reducing bacteria, hinting to a metabolic interaction between perchlorate and chlorate reduction. The cultures had been obtained previously by selecting colonies from perchlorate-reducing enrichments and confirming their isolation with Sanger sequencing of the 16S ribosomal RNA gene [120]. Despite appearing axenic, the nine cultures produced a total of 16 genomes after assembly and binning: four draft genomes from axenic cultures, and 11 high-quality draft metagenome-assembled genomes (MAGs) and one medium-quality draft MAG from mixed cultures (Figure 1B) (Supplementary Table 3-4). Every less-abundant MAG was either at low relative abundance (0.9-9.5%) or in the same taxonomic family as the most-abundant MAG, which likely caused the failure to detect contaminating strains through 16S rRNA gene sequencing. Annotation of MAGs and assembly graphs identified genes for perchlorate reduction (*pcr*, *cld*, and a terminal oxidase) in only nine genomes. Unexpectedly, while perchlorate and acetate, a non-fermentable carbon source, were the only energy substrates available in the growth medium, MAGs lacking *pcr* were the most abundant organisms in several cultures

(Figure 3-1B). Instead, these three MAGs contained a complete chlorate reduction pathway (*clr*, *cld*, and a terminal oxidase) (Supplemental Figure 3-1).

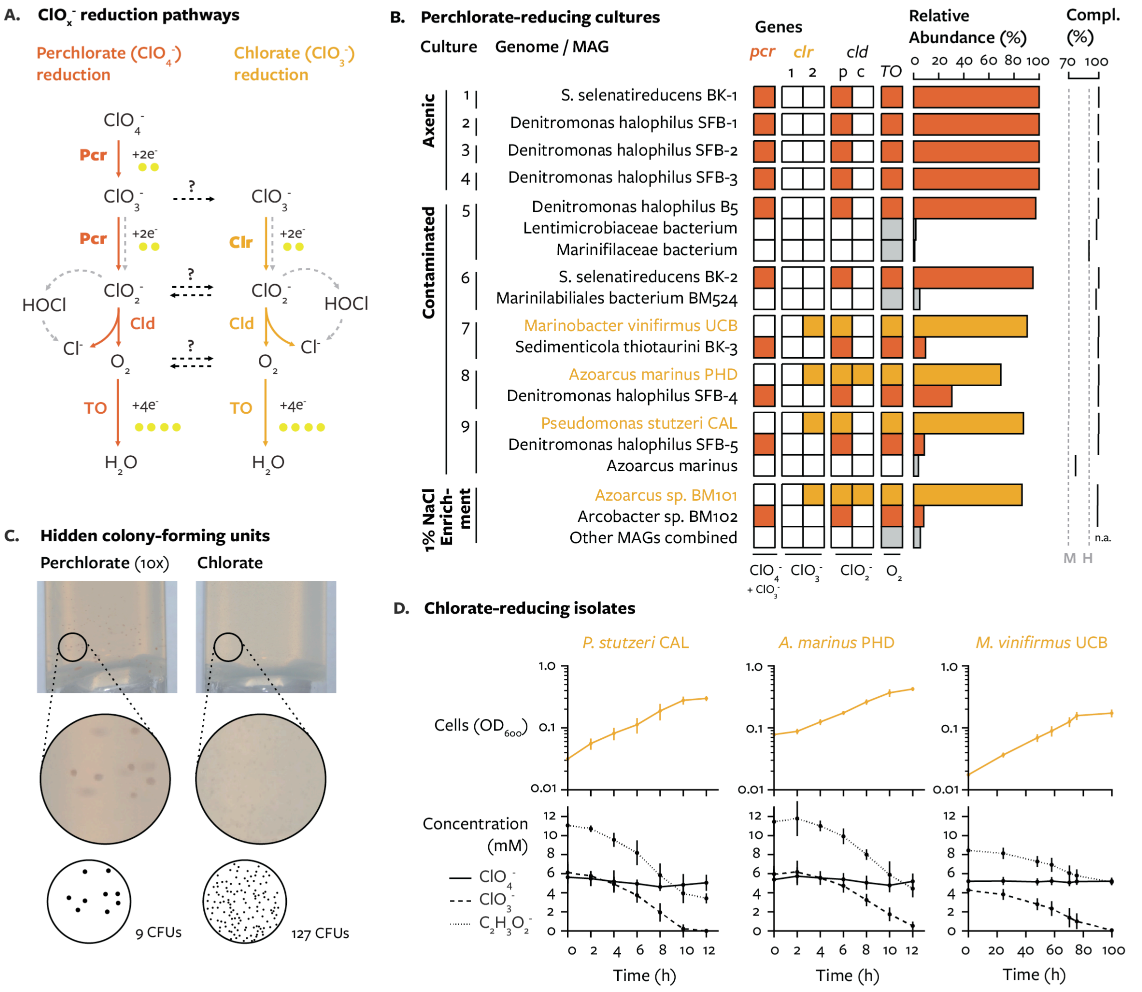


Figure 3-1. Isolation of chlorate-reducing bacteria from perchlorate-reducing cultures. (A) Pathways for the respiration of perchlorate (red) and chlorate (orange) involve the enzymes perchlorate reductase (Pcr) or chlorate reductase (Clr), chlorite dismutase (Cld), and a terminal oxidase reducing oxygen to water (TO). (B) Binning and key genes of genomes from perchlorate-reducing cultures. A previously sequenced perchlorate-reducing enrichment is included for comparison ("1% NaCl Enrichment"). Filled squares indicate gene presence. Relative abundance (%) was calculated as normalized coverage divided by total coverage for all genomes. Compl. (%) refers to percent completeness (single copy genes); dashed lines indicate medium quality (M) and high quality (H) completeness. All genomes had negligible contamination (<3%). (C) Magnified image of colonies that developed in agar media supplied perchlorate or chlorate from a co-culture of Denitromonas halophilus SFB-1 and Pseudomonas stutzeri CAL. The inoculum for perchlorate agar media was 10-times more concentrated. (D) Dissimilatory reduction of chlorate and not perchlorate by isolated chlorate-reducing bacteria.

The putative chlorate-reducing bacteria accounted for 69-90% of cells in the perchlorate-reducing cultures (Figure 3-1B), which is similar to what we previously observed in a perchlorate-enriched community [91]. The dominance of putative chlorate-reducing bacteria could be visually confirmed by comparing the number of colonies that develop on anaerobic tubes containing chlorate or perchlorate as the sole terminal electron acceptor (Figure 3-1C). Subsequent isolation and characterization of *Marinobacter vinifirmus* UCB, *Azoarcus marinus PHD*, and *Pseudomonas stutzeri* CAL confirmed the strains to be strictly chlorate-respiring microorganisms, as no perchlorate was consumed after two weeks of incubation (data not shown) or co-metabolized during dissimilatory chlorate reduction by any strain (Figure 3-1D). Because these strains cannot consume perchlorate themselves, the most parsimonious explanation of the observed community structure is that a perchlorate-reducing population supported a larger chlorate-reducing population.

Perchlorate reduction supports chlorate-reducing bacteria in simple and complex communities

The interaction between perchlorate- and chlorate-reducing bacteria was validated using defined co-cultures. Perchlorate- and chlorate-reducing strains were inoculated at equal cell densities (OD600) into anoxic media with perchlorate as the sole electron acceptor, and the relative number of chlorate-reducing cells between inoculation and the start of stationary phase was measured using the copy number of chlorate reductase alpha subunit (*clrA*) determined by qPCR. Chlorate-reducing strains grew in every co-culture with perchlorate-reducing bacteria (Figure 3-2A). No growth was observed in control media that lacked an electron acceptor (Supplementary Figure 3-2). The fitness of the chlorate-reducing bacteria was dependent on the partner perchlorate-reducing bacterium, with *Denitromonas halophilus* SFB-1 supporting the most growth of chlorate-reducing bacteria and *Dechloromonas agitata* CKB supporting the least growth (Figure 3-2A). Thus, all tested perchlorate-reducing strains supported some growth of chlorate-reducing bacteria.

To determine if this interaction occurs in more complex communities, we quantified the abundance of isolated strains in the original perchlorate-reducing enrichments using previously published 16S rRNA gene amplicon data [120]. Indeed, amplicon sequence variants (ASVs) corresponding to isolated chlorate-reducing strains in the genera *Azoarcus* and *Pseudomonas*, which are not known to contain perchlorate-reducing species, were highly abundant (>20%) in six of ten communities at low salinity (Figure 3-2B). In those communities, the ASVs affiliated with chlorate reduction accounted for 23-46% of total bacteria and archaea in the community and 40-84% of putative chlorate- and perchlorate-reducing taxa. Chlorate-reducing bacteria and perchlorate-reducing bacteria were found in various combinations in communities. That many different perchlorate-reducing bacteria can support the growth of chlorate-reducing bacteria, in both co-cultures and communities, demonstrated the interaction is based not on strain-specific traits but on conserved features of the metabolic pathways involved.

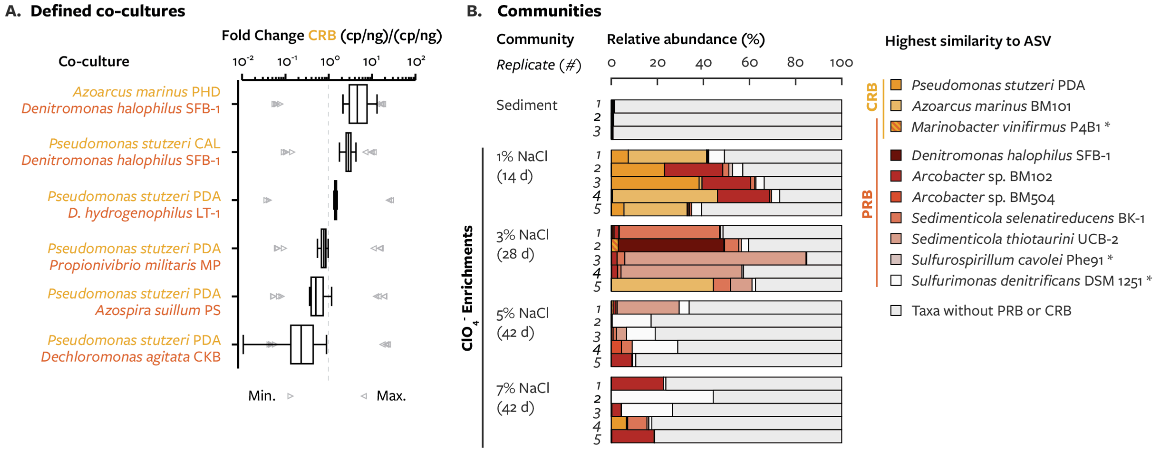


Figure 3-2. Co-cultivation of perchlorate-reducing bacteria (red, PRB) and chlorate-reducing bacteria (orange, CRB) in defined and undefined communities (A) Fold change of clrA in defined co-cultures between lag phase and late exponential phase batch growth. For the co-culture consisting of P. stutzeri PDA and A. suillum PS, primers for 16S rRNA genes was used. Arrows indicate the upper and lower bounds of fold change estimated from the initial and final OD600 of the co-culture. Boxplots indicate quartiles in the sample. (B) Relative abundance of 16S rRNA gene amplicon sequence variants grouped by similarity to the 16S rRNA genes of perchlorate- and chlorate-reducing taxa. Stacked bars are in the same order as the legend. *, strains most closely related to perchlorate-reducing MAGs for which 16S rRNA genes were not available.

Chlorate-reducing bacteria require the perchlorate reduction intermediate chlorate

Chlorate-reducing bacteria are able to use all components of the perchlorate reduction pathway except perchlorate (Figure 3-1A), so we sought to determine which intermediates were responsible for the metabolic interaction. We deleted different steps of the chlorate reduction pathway in the model chlorate-reducing bacterium *Pseudomonas stutzeri* PDA (PDA). Measuring the fitness of each of these mutants in co-culture with the model perchlorate-reducing bacterium *Azospira suillum* PS (PS) would demonstrate which steps of the chlorate reduction pathway were essential for growth from perchlorate reduction intermediates. Genes encoding enzymes for reactions upstream of an exchanged intermediate are non-essential for growth, whereas genes encoding enzymes for reactions downstream of the exchanged intermediate are essential. For example, if chlorite were the exchanged intermediate, PS growing by perchlorate reduction would support growth of PDA strains lacking *clrA* but not PDA strains lacking *cld* (Figure 3A).

In the co-cultures inoculated with equal cell densities of PS and PDA, growth of wild type PDA was characterized by a final relative abundance of 27% (final ratio PDA/PS = 0.37) (Figure 3-3B). In contrast, deletion of any steps of the chlorate reduction pathway prevented growth of PDA: the final abundance of PDA deletion strains ($PDA_{del}/PS < 0.048$) was equivalent to that expected with no growth of PDA ($PDA/PS < 0.060$) (Figure 3-3A). While all chlorate reduction genes were necessary, the particular necessity of chlorate reductase (*clrA*) demonstrated that chlorate is the only intermediate exchanged in enough quantity to support measurable growth. That is, chlorite dismutase and terminal oxidases are present in the PDA *clrA* deletion strain, yet any release of chlorite or oxygen by PS during perchlorate reduction was not sufficient to support growth of PDA. Thus, the basis of the metabolic interaction is the transfer of chlorate from perchlorate-reducing cells to chlorate-reducing cells.

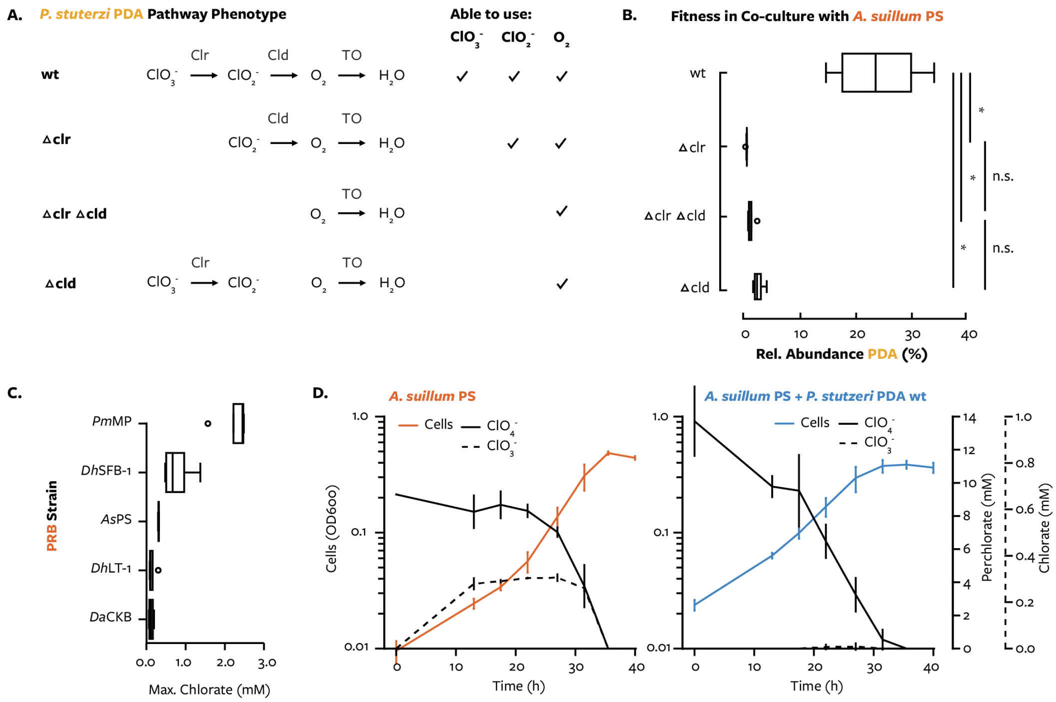


Figure 3-3. Determination of the perchlorate reduction intermediate that supports growth chlorate-reducing bacteria in defined co-cultures. (A) Genotype and phenotype (in pure culture) of chlorate reduction pathway mutants constructed in Pseudomonas stutzeri PDA (PDA). A chlorate reduction mutant would be unable to grow unless it can use the intermediate produced by perchlorate-reducing bacteria as a respiratory electron acceptor. (B) Fitness of chlorate reduction mutants in co-culture with Azospira suillum PS (PS) provided 10 mM perchlorate and 40 mM lactate, which PDA does not ferment. Relative abundance was calculated from qPCR measurements of both the PS and PDA 16S rRNA genes. *, significance of $p < 0.05$ (two-sided T-test); n.s., $p > 0.05$. Boxplots indicate quartiles in the sample with outliers as circles. (C) Maximum concentration of chlorate during dissimilatory perchlorate reduction by different strains of perchlorate-reducing bacteria (PRB) supplied 10 mM perchlorate. (D) Concentrations of perchlorate and chlorate during dissimilatory perchlorate reduction by PS or PS and PDA. Errors bars represent standard deviation of at least three replicates.

Other observations supported chlorate as the exchanged intermediate. Perchlorate-reducing bacteria accumulated chlorate at concentrations between 1% and 22% mol/mol of initial perchlorate (~10 mM) (Figure 3-3C), as reported previously [193, 202]. Additionally, chlorate accumulated in pure cultures of PS (<0.3 mM) but was consumed in co-cultures of PS and wild type PDA (Figure 3-3D). However, there was no clear relationship between the maximum concentration of chlorate that accumulated in pure cultures of perchlorate-reducing bacteria (Figure 3-3C) and the fitness of chlorate-reducing bacteria in co-culture (Figure 3-2A). Notably, kinetics differed between PS cultures with and without wild type PDA (Figure 3D, Supplemental Figure 3-3). When

PDA was present, the maximum growth rate and maximum perchlorate reduction rate by PS decreased and the onset of growth and perchlorate reduction was earlier when compared to the pure PS culture (Figure 3-3D). Similar changes in growth kinetics were observed in other co-cultures (Supplemental Figure 3-2). The kinetics of chlorate production and consumption thus seemed to be an important factor in the interaction.

Specificity for chlorate enables chlorate-reducing cells to exploit perchlorate-reducing cells

An understanding of the kinetics of the interaction was necessary to understand how chlorate release could produce the observed community structure. For example, how can accumulation of chlorate to only 3% of initial perchlorate concentration support chlorate-reducing bacteria at 27% of the community (Figure 3-3)? More generally, how can a population with the partial pathway outcompete a population with the complete pathway up to a factor of nearly ten (Figures 3-1 and 3-2)? To answer these questions, we used simulations of an Equilibrium Chemistry Approximation kinetics model, which included the effects of substrate competition within and between cells. We focused on the theoretical case where (1) the kinetics of chlorite and oxygen are ignored and (2) chlorate- and perchlorate-reducing populations were identical (maximum growth rate, yield, etc.) except for substrate utilization (Table 3-1, Equations 1-3): populations could use both perchlorate and chlorate or only chlorate. Therefore, the model's salient features were the yields and rates from the production and consumption of chlorate, as well as the competition of perchlorate and chlorate for Pcr. In simulations with the perchlorate-reducing population alone, these parameters led to the accumulation of chlorate during perchlorate reduction (Figure 3-4A). Importantly, growth rate was lower while the ClO_3^-:ClO_4^- ratio was low (Figure 3-4B). Chlorate influenced growth rate so strongly because chlorate reduction to chloride provided more energy (622.9 kJ/mol chlorate) than perchlorate reduction to chlorate (211.7 kJ/mol perchlorate). At low ClO_3^-:ClO_4^- ratios, the perchlorate-reducing population was less likely to reduce chlorate and more likely to reduce perchlorate because the concentration of ClO_4^- was much higher (Figure 3-4C).

Accordingly, we hypothesized that a population that could only reduce chlorate would have a higher growth rate at low ClO_3^-:ClO_4^- ratios than the perchlorate-reducing population. We tested this by adding the chlorate-reducing population to the simulation at equal initial concentration. The chlorate-reducing population outcompeted the perchlorate-reducing population and decreased the concentration of chlorate (Figure 3-4D), consistent with experimental observations. In support of our hypothesis, at low ClO_3^-:ClO_4^- ratios the chlorate-reducing population consumed almost all of the chlorate and had a higher growth rate (about 2-fold) than the perchlorate-reducing population (Figure 3-4E). With chlorate-reducing cells present, the consumption of chlorate delayed the increase of the ClO_3^-:ClO_4^- ratio (Figures 3-4B and 3-4E). Thus, in this simple theoretical case, chlorate-reducing cells had a growth advantage because they, unlike perchlorate-reducing cells, could consume chlorate at high perchlorate concentrations (Figure 3-4F). Additionally, the consumption of chlorate by chlorate-reducing bacteria created a positive feedback by maintaining a low ClO_3^-:ClO_4^- ratio (Figures 3-4C and 3-4F).

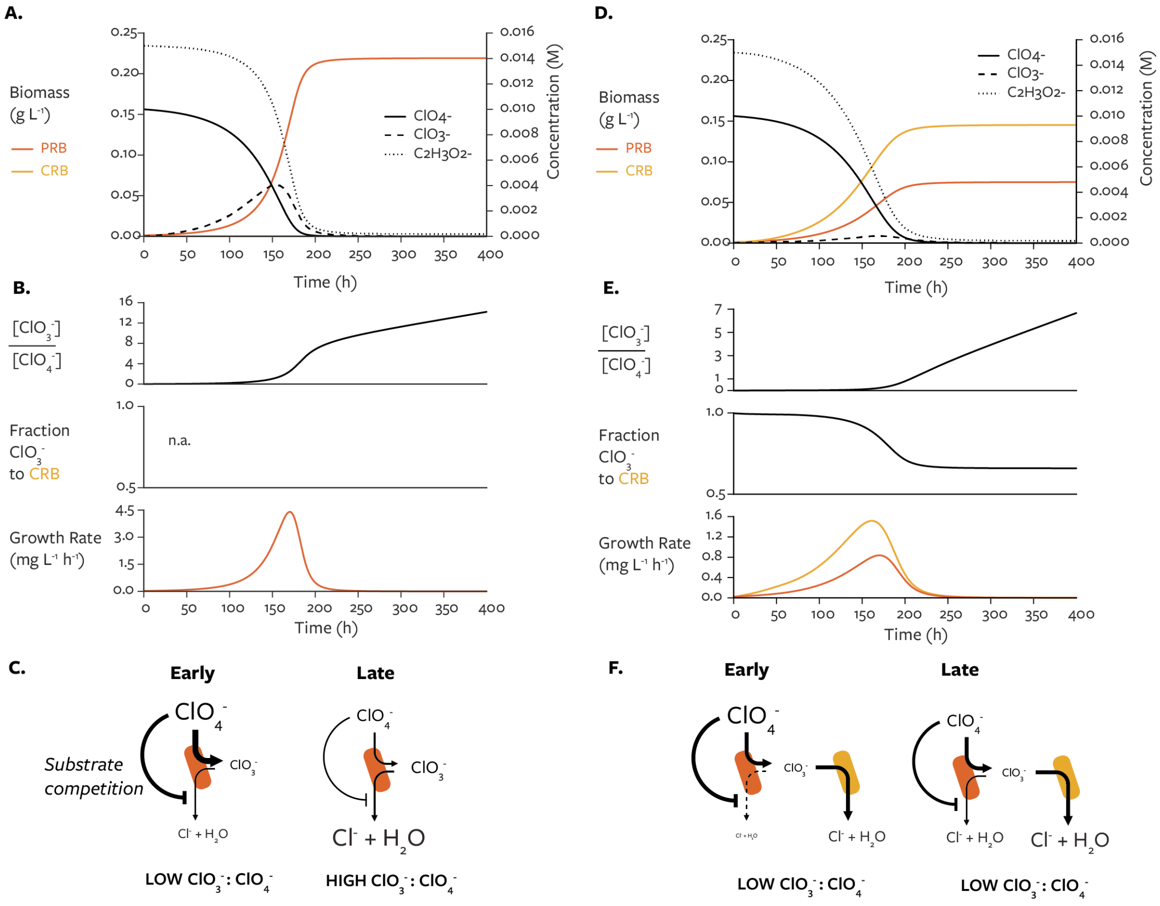


Figure 3-4. Modeling of perchlorate reduction and chlorate reduction. Simulated growth curves for perchlorate-reducing bacteria (A-C) alone and (D-F) with chlorate-reducing bacteria. [ClO3-] / [ClO4-], the ratio between chlorate concentration and perchlorate concentration; fraction ClO3- to CRB indicates the relative amount of chlorate consumed by the chlorate-reducing population at each time step; growth rate, the change in cell concentration between each time step.

We used additional simulations to observe how initial conditions affect the interaction. Varying the initial ratio of chlorate-reducing cells to perchlorate-reducing cells did not alter the ecological success or the fraction of chlorate acquired by the chlorate-reducing population (Supplemental Figure 3-4A-B); Chlorate-reducing cells ultimately dominated by acquiring a large percent of chlorate unless initially outnumbered 100-fold (Supplemental Figure 3-4C). Varying perchlorate concentration, however, did alter the success of chlorate-reducing cells (Supplemental Figure 3-4D-F). Even when chlorate-reducing cells outnumbered perchlorate-reducing cells, the perchlorate-reducers consumed nearly all available chlorate except at perchlorate concentrations above ~1 mM (Supplemental Figure 3-4E). Also, varying the affinity of different populations for perchlorate or chlorate altered the ecological success of chlorate-reducing cells (Supplemental Figure 3-5). While not necessarily predictive of behavior in the environment or over different temporal and spatial scales, these simulations provide an intuitive description of the interaction: chlorate-reducing cells exploit a niche made available by differences in enzyme kinetics and substrates.

Discussion

This study confirms and further interrogates the interaction between perchlorate reduction and chlorate reduction. Here we clearly demonstrate that bacteria with the perchlorate reduction pathway supported – and could be outcompeted by – bacteria with the chlorate reduction pathway. The interaction between perchlorate and chlorate reduction occurred in both controlled (i.e. co-cultures) and uncontrolled systems (i.e. enrichment and isolation) and in both freshwater and marine conditions. The basis of the interaction was the exchange of chlorate from perchlorate-reducing cells to chlorate-reducing cells. Chlorate was available for consumption likely due to competition of perchlorate and chlorate for a single enzyme in the periplasm of perchlorate-reducing cells [191]. Simulations showed that the chlorate-reducing cells are successful because chlorate can be reduced even at a low ClO_3^-:ClO_4^- ratio, a state that chlorate consumption perpetuates. In summary, chlorate-reducing bacteria were a common feature of perchlorate reduction and had a large effect on the structure and function of perchlorate-reducing communities.

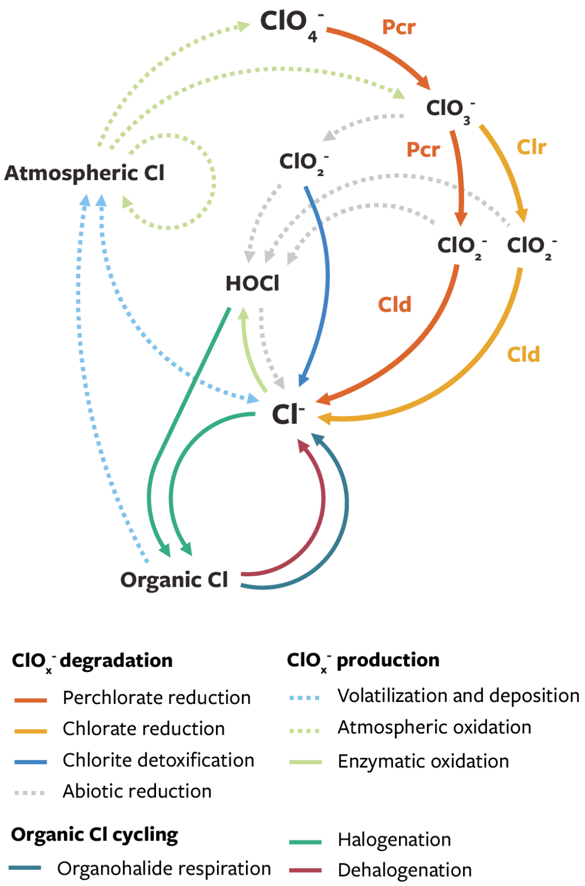


Figure 3-5. Model for the production and degradation of chlorine oxyanions. The perchlorate reduction pathway (red) accumulates chlorate, which can react with reductants and generate reactive chlorine species (gray) or be consumed by the chlorate reduction pathway (orange). We did not find evidence for the release of chlorite and oxygen by the perchlorate and chlorate reduction pathways, though both chemicals can react with any reductants in the periplasm. Perchlorate and chlorate reduction remove the products of atmospheric oxidation of chlorine (dashed yellow). Co-metabolic or inadvertent enzyme activities are not shown.

The basis of the interaction alters our understanding of the chlorine cycle. Perchlorate reduction involves the combined activity of perchlorate-reducing microorganisms, chlorate-reducing microorganisms, and any chemical reduction of their intermediates

(Figure 3-5). A role for chlorite-consuming or oxygen-consuming partial pathways in perchlorate reduction was not observed here (Figure 3-3), and an interaction based on the exchange of chlorite has been engineered [111] but not yet observed in nature. This is likely due to the high activity (k_{cat}/K_M) of chlorite dismutase (10^6-10^8 M^{-1} s^{-1}) relative to perchlorate reductase (~10^5 M^{-1} s^{-1}) [155, 203]. Because chlorate is less reactive than chlorite, cells that inadvertently reduce chlorate to chlorite would experience greater reactive chlorine stress; chlorite dismutase (Cld) can detoxify chlorite produced in this manner [95]. Additionally, the exchange of chlorate is less constrained by the reducing state of the environment than the exchange of chlorite. But chlorate does react with common environmental reductants such as reduced iron minerals [114, 204], and the reactivity of chlorate with iron increases with salinity [204], which may contribute to the lower frequency of chlorate-reducing bacteria in higher-salinity perchlorate-reducing enrichments (Figure 3-2B). The concentrations of reductants, chlorate, and perchlorate may all influence the relative contribution of perchlorate-reducing microorganisms and chlorate-reducing microorganisms to chlorine oxyanion respiration.

Interactions like that described here, where low accumulation of an intermediate supports large populations with a partial respiratory pathway, may be common across elemental cycles. Some evidence exists for the importance of these interactions in denitrification, for example. *Pseudomonas* strain G9, which contains a complete denitrification pathway producing inhibitory concentrations of nitrite, could grow only in co-culture with *Alcaligenes faecalis* strain TUD, which only reduces nitrite to dinitrogen, and the two strains were found at steady state at approximately equal cell densities [172]. Nitrite accumulation caused by inter-enzyme competition in *Pseudomonas stutzeri* strain A1501 was decreased by dividing nitrite production and consumption between different strains [179]. In denitrifying communities, then, it may be beneficial for some organisms to *lack* steps in the denitrification pathway. This was the case for a pooled transposon mutant library of *Azospira suillum* PS, where mutants with insertions in nitrite reductase, which is deleterious in pure culture, outcompeted cells with intact denitrification pathways [93]. Much remains to be learned about community structure impacts resulting from pathway distribution across different populations.

The importance of studying metabolic interactions in biogeochemical transformations is to learn how such interactions influence concentrations and rates. Previous studies that added chlorate-reducing bacteria to cultures of the perchlorate-reducing bacterium HCAP-C, which accumulates far more chlorate than the typically observed [155, 193, 194], had conflicting results where chlorate-reducing bacteria either slightly accelerated [190] or substantially decelerated the rate of perchlorate reduction [110]. We observed that adding chlorate-reducing bacteria to cultures of the model perchlorate-reducing bacterium *Azospira suillum* PS decreased the concentration of chlorate, maximum growth rate, and maximum perchlorate reduction rate (Figure 3-3D). Similar effects on growth rate were observed with perchlorate-reducing bacteria that accumulated varying concentrations of chlorate (Supplemental Figure 3-2, Figure 3-3C), and we directly demonstrated success of chlorate-reducing populations at the expense of perchlorate-reducing populations (Figure 3-2), though the success of chlorate-reducing populations differed between cultures for unknown reasons. Because chlorate reduction appears to substantially influence concentrations and rates during perchlorate reduction, chlorate-reducing bacteria could affect efforts to bioremediate perchlorate. For example, a description of perchlorate-reducing bioreactors with gene-centric metagenomics identified *Azoarcus* and *Pseudomonas* as among the most abundant genera, yet the *Azoarcus* isolate did not reduce perchlorate [205]. Our results predict that those organisms are chlorate-reducing bacteria that persisted in the bioreactors for over 10 years [205]. Understanding how this metabolic interaction affects perchlorate reduction kinetics in different communities and systems, and how it could be controlled, may be a promising line of future research.

A close interaction between metabolisms also has evolutionary implications, as co-occurrence can influence gene evolution and exchange. For example, in nitrifying microorganisms, niche differentiation led to high affinity and low affinity ammonia-oxidizing enzymes that function best at different pH [206] and to the exchange of ammonia monooxygenase to nitrite-oxidizing bacteria [207]. Not enough chlorate reductases and perchlorate reductases have been evaluated to draw general conclusions from their substrate affinities and catalytic rates. However, several chlorate-reducing bacteria contain genes or gene fragments of perchlorate reductase components (*napC* and *pcrD*) adjacent to the chlorite dismutase (*cld*), and these genes were most likely acquired from perchlorate-reducing bacteria [87]. Because environmental reduction of perchlorate or chlorate will likely involve both perchlorate-reducing microorganisms and chlorate-reducing microorganisms, a history of gene exchange between the two metabolisms is unsurprising.

Conclusions

Perchlorate reduction supports chlorate reduction through the release of the intermediate chlorate. The fundamental cause of the interaction is that the perchlorate reductase enzyme catalyzes both perchlorate reduction to chlorate and chlorate reduction to chlorite – therefore chlorate competes with perchlorate for perchlorate reductase, limiting subsequent steps of the perchlorate reduction pathway. Chlorate reduction, despite being a partial pathway, is ecologically successful because it can consume chlorate unabated and, in doing so, exacerbates the imbalance between perchlorate and chlorate. As for several other respiratory metabolisms, the respiration of chlorine oxyanions in the environment should be expected to involve cells performing complete and partial respiratory pathways. These findings have clear implications for understanding the evolution and the kinetics of chlorine oxyanion reduction.

Supplementary Material

Supplementary figures and tables can be found accompanying the preprint version of this chapter, found below, or the final published version when available:
https://doi.org/10.1101/781625

Tables

Table 3-1. Parameters for growth kinetics model. Populations were identical except for enzyme affinity for perchlorate, which for population 2 was set to be negligible (*).

Parameter	Population 1	Population 2
Km ClO4- (mM)	0.006	10000 *
Km ClO3- (mM)	0.007	0.007
Km C2H3O2- (mM)	1	1
Max. growth rate (h-1)	0.5	0.5
Death rate (h-1)	0	0
Type	Perchlorate reducer	Chlorate reducer
* *Set to be negligible*		

Chapter 4
An uncharacterized clade in the DMSO reductase family of molybdenum oxidoreductases is a new type of chlorate reductase

Abstract

The dimethyl sulfoxide (DMSO) reductase family of molybdopterin enzymes has many subfamilies catalyzing unique biogeochemical reactions. It also has many uncharacterized subfamilies. One such subfamily was predicted from comparative genomics to participate in a key step of the chlorine cycle: enzymes in this subfamily have a conserved genetic association with chlorite dismutase, which suggests they produce chlorite by reducing chlorate or perchlorate. Here we determined the activity of the uncharacterized enzyme by comparing strains in the phototrophic genus *Rhodoplanes* that encode either a canonical perchlorate reductase or the uncharacterized enzyme. *Rpl. piscinae* and *Rpl. elegans*, which encode perchlorate reductase, grew by using perchlorate as an electron acceptor. In contrast, *Rpl. roseus*, which encodes the uncharacterized enzyme, could grow by chlorate reduction but not by perchlorate reduction. When both chlorate and perchlorate were present in the growth medium, *Rpl. roseus* consumed only chlorate. Highly concentrated *Rpl. roseus* cells showed some perchlorate consumption, but chlorate consumption occurred at a ten-fold higher rate. Together, these genomic and physiological data define a new group of chlorate reductases. Some organisms encode both this chlorate reductase and a perchlorate reductase, raising new questions about the physiology and evolution of chlorine oxyanion respiration.

Report

The dimethyl sulfoxide reductase family consists of enzymes with a bis-molydopterin guanine dinucleotide cofactor, many of which catalyze key reactions in biogeochemical cycles, such as the reduction of dimethyl sulfoxide, arsenate, nitrate, selenate, polysulfide, thiosulfate, perchlorate, chlorate, and the oxidation of dimethyl sulfide, arsenite, nitrite, and formate [208]. Several subfamilies within this superfamily have unknown activity, including several clades in the functionally diverse type II DMSO reductase family enzymes [209]. Bacteria that respire perchlorate (ClO_4^-) or chlorate (ClO_3^-) use enzymes from that group: a perchlorate reductase (Pcr) that reduces perchlorate to chlorate and chlorate to chlorite, or a chlorate reductase (Clr) that reduces only chlorate to chlorite [7]. A heme-containing chlorite dismutase (Cld) converts reactive chlorite into chloride and oxygen. In a recent publication describing genomes from perchlorate-reducing communities enriched from estuary sediment [91], we identified a gene most closely related to periplasmic nitrate reductase (pNar), a distant homolog of Pcr and Clr, that was adjacent to the gene for Cld in the genome. Similar pNar-like genes were always associated with *cld* and formed a monophyletic clade, suggesting a specialized activity producing chlorite. We hypothesized that this enzyme was a second type of perchlorate reductase, or group 2 Pcr, based on the conservation of most active site residues with Pcr and the growth of a population encoding no other perchlorate reductase in enrichments provided only perchlorate. However, no organisms with group 2 Pcr alone have been directly observed to grow by perchlorate reduction, several genomes encode both group 1 and group 2 Pcr in an apparent redundancy [91], and the enrichments also supported organisms reducing chlorate produced by perchlorate-reducing bacteria [92]. The enzyme's function remains uncharacterized.

We identified a gene for the uncharacterized DMSO reductase family enzyme in the publicly available genome of the purple non-sulfur bacterium *Rhodoplanes roseus* DSM 5909 (Figure 4-1) [210]. The genus *Rhodoplanes* contains four sequenced strains, all of which are capable of anoxygenic phototrophy [211-213]. No bacterium in the genus *Rhodoplanes* has yet been observed to grow by dissimilatory perchlorate or chlorate reduction. However, every sequenced *Rhodoplanes* genome contains a DMSO reductase family enzyme adjacent to *cld*. Cld from these genomes formed a monophyletic clade in a phylogenetic tree, but their associated reductases differ (Figure 4-1). Only *Rpl. roseus* encodes the uncharacterized DMSO reductase family enzyme, which is most similar (83% amino acid identity) to that of *Magnetospirillum magnetotacticum* MS-1 [214]. *Rpl. elegans* DSM 11970, *Rpl. piscinae* DSM 19946, and *Rpl.* sp. strain T2.26MG-98 encode a canonical perchlorate reductase most closely related (73% and 74% amino acid identity, respectively) to that of *Azospirillum* sp. TSO22-1 [215]. The presence of different reductases in similar strains provided a natural experiment for determining the uncharacterized enzyme's function.

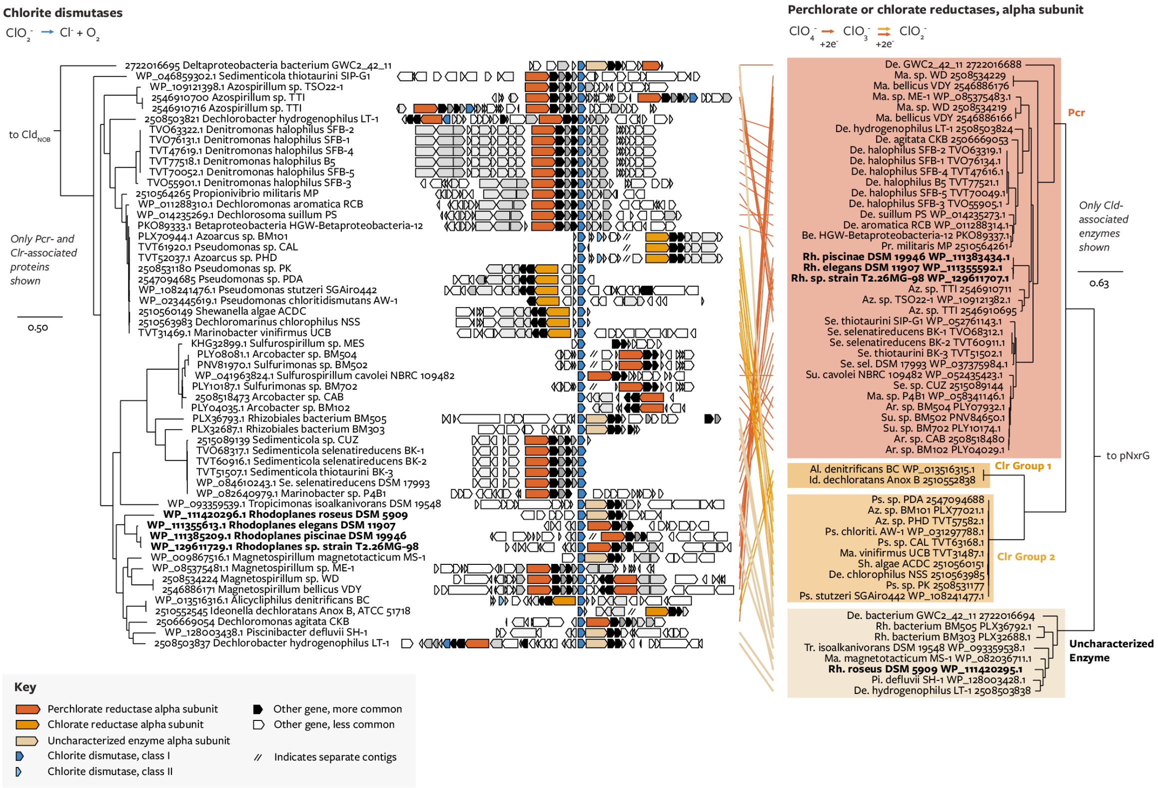


Figure 4-1. One member of the genus *Rhodoplanes* encodes an uncharacterized DMSO reductase family enzyme**.** Genes for dissimilatory perchlorate reduction or chlorate reduction (center) and maximum phylogenetic trees for chlorite dismutase (left) and the closest encoded reductase (right). Proteins from *Rhodoplanes* genomes are colored purple. Proteins not known to be involved in perchlorate or chlorate reduction were not included. The Cld tree was rooted to Cld proteins from *Nitrospira* spp., and the (per)chlorate reductase tree was rooted to periplasmic nitrite oxidoreductases from *Nitrospira* spp.

We tested the hypothesis that the uncharacterized enzyme has perchlorate reductase activity by comparing the capability to grow by perchlorate reduction between *Rpl. roseus* and two other *Rhodoplanes* strains, *Rpl. elegans* DSM 11970 and *Rpl. piscinae* DSM 19946. All strains were kindly provided by Breah Lasarre and James B. Mckinlay at Indiana University, Bloomington. Like all previously characterized organisms with perchlorate reductase and chlorite dismutase, *Rpl. elegans* and *Rpl. piscinae* were able to couple the reduction of perchlorate to the oxidation of acetate and growth in the dark (data not shown). *Rpl. roseus*, which encodes the uncharacterized enzyme, grew in dark with chlorate and nitrate as the electron acceptor, but limited growth was observed in the no acceptor control and in the perchlorate condition (Figure 4-2A). The limited growth could be explained by residual chlorate from the inoculum, and no perchlorate was reduced over the course of 400 hours (Figure 4-2B). Previously characterized bacteria with perchlorate reductases reduce both perchlorate and chlorate throughout growth [7], while bacteria with chlorate reductases reduce only chlorate [92]. When *Rpl. roseus* was provided with both ~10 mM of both chlorate and perchlorate, it reduced chlorate but not perchlorate (Figure 4-2C-D). Thus, at the concentrations tested, perchlorate reduction was neither a metabolism nor a co-metabolism of *Rpl. roseus*.

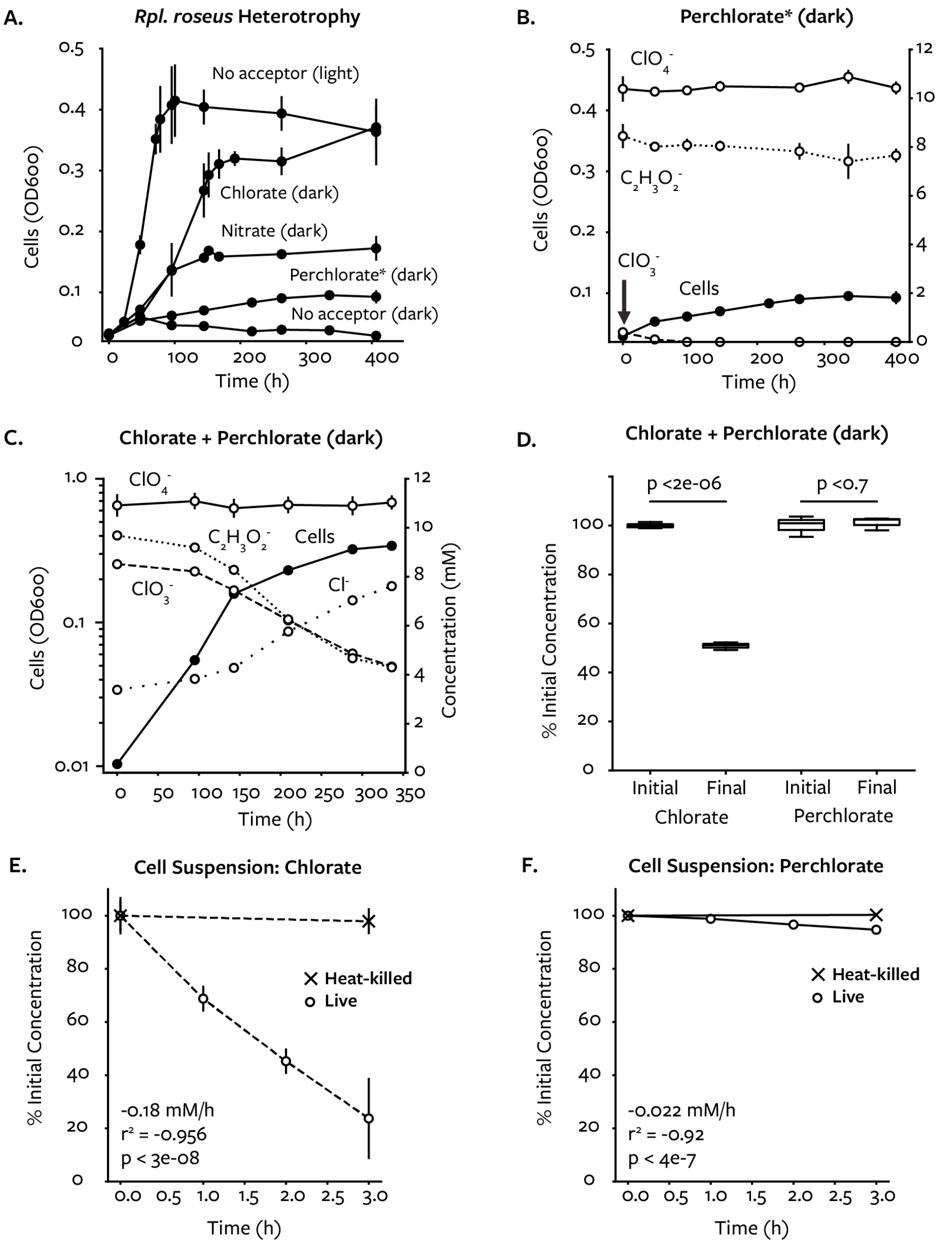


Figure 4-2. Patterns of cellular growth and enzymatic activity in *Rhodoplanes roseus* establish the uncharacterized DMSO reductase family enzyme as a chlorate reductase. (A-B) Growth of Rhodoplanes roseus DSM 5909 with different conditions of anaerobic respiration (dark) or anaerobic phototrophy (light) with acetate as the electron donor and carbon source. Concentration of ions in the perchlorate growth condition specifically is shown. Error bars represent the standard deviation of three biological replicates. The growth medium was modified from RM2 medium [216] and consisted of (g/L): ammonium sulfate (1.0), potassium phosphate monobasic (1.0), magnesium chloride hexahydrate (0.2), sodium chloride (0.2), calcium chloride dihydrate (0045), and sodium bicarbonate (2.5). Vitamins and minerals were added as described previously [89]. Medium was made anaerobic by sparging with a gas mixture consisting of 80% N2 and 20% CO2. Yeast extract (0.01% w/v) was added to stimulate growth [210]. Inoculum consisted of a 1:10 dilution of cells in late-log phase grown anaerobically with chlorate. Cultures were incubated at 30 C without shaking either in the dark or in the light. Concentrations of anions were measured using ion chromatography and cell density was measured by absorbance at 600 nm. (C-D) Growth of *Rpl. roseus* in the dark with both chlorate and perchlorate present in the growth medium. The percent of initial chlorate or perchlorate consumed between the initial (o h) and final (336 h) time points was compared using an

independent t-test. (E-F) Chlorate and perchlorate reduction by washed whole cell suspensions of *Rpl. roseus*. Error bars represent the standard deviation of four biological replicates. A linear regression for chlorate reduction and perchlorate reduction by live cells is shown. Cultures in the late-log phase were supplied 100 μg/ml chloramphenicol to inhibit gene expression then centrifuged at 4C at 8000 rpm for 30 minutes, washed with media lacking donor or acceptor, centrifuged again, and re-suspended at 10-fold their original concentration in media. To these washed cell suspensions were added approximately 1 mM acetate and 1 mM of either perchlorate or chlorate and monitored ion consumption during incubation at 30 C in the light and an N_2 atmosphere. A control for abiotic reactions was produced by autoclaving cells for 20 minutes.

Activity towards chlorate and perchlorate was further characterized using washed whole cell suspensions of chlorate reduction-grown *Rpl. roseus*, concentrated to an optical density (600 nm) of 4.51 (Fig. 4-2E-F). Live *Rpl. roseus* cells reduced chlorate at a rate of 0.18 millimolar per hour (linear least-squares regression, $r^2 = 0.956$, $p < 3e^{-8}$) (Fig. 4-2E). In contrast, perchlorate was reduced at a dramatically slower rate: 0.02 millimolar per hour (linear least-squares regression, $r^2 = 0.92$, $p < 4e^{-7}$) (Fig. 4-2F). No reduction of chlorate or perchlorate was observed in heat-killed controls (Fig. 4-2E-F). Chlorate reductase activity was approximately 10-fold higher than perchlorate reductase activity. These data are consistent with the uncharacterized enzyme in *Rpl. roseus* functioning as a chlorate reductase at high densities and laboratory concentrations of chlorate or perchlorate.

From these results we argue that the uncharacterized DMSO reductase family enzyme should be categorized as a chlorate reductase. That classification is supported by the presence of this reductase near perchlorate reductase in some organisms (Fig. 4-1) – a redundant arrangement if this reductase had the same function. While further evidence may show the enzyme's apparent limited perchlorate reductase activity to be sufficient to provide energy over long periods of time or at lower concentrations of perchlorate, its function at experimentally relevant durations and concentrations is as a chlorate reductase. We propose the term group 3 chlorate reductase to distinguish this protein and its orthologs from the phylogenetically dissimilar group 1 (serA-like) and group 2 (ddhA-like) chlorate reductases (Fig. 4-1).

The new chlorate reductase has other substantial differences to previously characterized chlorate reductases. Bacteria isolated by *selection* for chlorate reduction so far encode only group 1 Clr or group 2 Clr, suggesting some of difference are consequential for the metabolism. Group 3 ClrA has active site residues that more resemble those of PcrA than ClrA, with the main difference to PcrA being the substitution of a key nonpolar aromatic tryptophan residue with a polar aromatic tyrosine residue [91]. Group 3 ClrA proteins have lower shared amino acid identity (Figure 4-1) and are not closely related to other types of reductases [91], whereas other ClrA are very similar within groups and are closely related to selenate reductase (SerA) or dimethyl sulfide dehydrogenase (DdhA) [87]. Similarly, Cld proteins associated with group 3 ClrA have much greater phylogenetic diversity than those associated with group 1 or group 2 ClrA, which tend to be nearly identical within each group (Figure 4-1). Group 3 *clrA* can be found in genomic regions lacking markers of recent recombination, whereas group 1 and group 2 *clrA* are contained within chlorate reduction composite transposon islands [87]. Group 3 *clr* has conserved synteny with *cld*, with *cld* always preceding group 3 *clrA*, while group 1 and 2 *clr* do not (Figure 4-1). In summary, group 3 Clr is unique in its active site composition, phylogenetic diversity, and more conserved relationship with Cld and host genome.

This is the first report of dissimilatory perchlorate or chlorate reduction by organisms capable of phototrophy. Whether or not the ability of *Rhodoplanes* strains to perform both these metabolisms is related is unclear. A potentially analogous system is a nitrite oxidoreductase, also in the DMSO reductase family, that enables the phototrophic oxidation of nitrite to nitrate by providing electrons to photosystem II [77, 217]. *Rhodoplanes* spp. encode photosystem II, but it is unlikely that chlorate and chlorite serve as electron donors for phototrophy because the reduction potential of the half-couple reactions are higher than that of photosystem II [77]. Additionally, while some DMSO reductase family enzymes have been observed to perform reactions in reverse (e.g. oxidation of nitrite to nitrate instead of reduction of nitrate to nitrite), the enzyme Cld only performs the forward reaction of separating chlorite into chloride and oxygen, which would compete with the use of chlorite as an electron donor. A more likely reason for *Rhodoplanes* spp. to encode chlorate and perchlorate reduction genes is that anoxygenic phototrophs experience large fluctuations in oxygen concentrations and light [218]. Those environmental conditions would select for the ability to respire diverse substrates available at the Earth’s surface, which includes perchlorate and chlorate [70].

With the identification of the new chlorate reductase gene, it is clear that genes for perchlorate reduction and chlorate reduction can be found in the same genome, for example *Dechlorobacter hydrogenophilus* LT-1. Cells with both Pcr and Clr might express the reductases in different conditions, alternating between perchlorate reduction and chlorate reduction, or perhaps the presence of both reductases in the same genomic region is an intermediate state in horizontal gene transfer in which the organism transitions completely from perchlorate reduction to chlorate reduction or vice versa. Some evidence in support of the latter hypothesis is that the group 3 *clr* in *Rhizobiales* bacterium BM505 is co-located with a *pcrA* pseudo gene containing a premature stop codon (locus tag 0606_13310). Yet another explanation is that Clr helps perchlorate-reducing bacteria compete for respiratory pathways intermediates. Because Pcr can reduce both perchlorate and chlorate, large amounts of perchlorate lead to the accumulation of chlorate [192]. Perchlorate-reducing bacteria must compete with other strains and with chlorate-reducing bacteria, which can grow to large population sizes in perchlorate-reducing cultures by stealing chlorate [92]. Encoding a chlorate reductase in addition to a perchlorate reductase may help perchlorate-reducing bacteria minimize the loss of chlorate to competing cells. Studying bacteria capable of both perchlorate reduction and chlorate reduction will likely provide additional insights the physiology, regulation, and evolution of perchlorate and chlorate metabolism.

Identification of this new chlorate reductase was made possible by the observation of a conserved association of an enzyme with an accessory enzyme across a relatively small number of genomes [91]. We predict that as many more genomes are sequenced, more uncharacterized subfamilies in the DMSO reductase family will be associated with specific accessory genes, providing further opportunities for targeted characterization of these biogeochemically important oxidoreductases.

Chapter 5
Insights into the chlorine cycle through comparative genomics of chlorite dismutase (Cld)

Abstract

Only a limited number of genes are known to participate in chlorine redox chemistry or in repairing damage caused by reactive chlorine species. A key enzyme in the chlorine cycle is chlorite dismutase (Cld), which detoxifies chlorite (ClO_2^-) by converting it to chloride (Cl^-) and molecular oxygen. Because chlorite is an intermediate oxidation state of chlorine, Cld can be used as a biomarker for other oxidized chlorine species. Here, we used a comparative genomics approach to identify Cld in genomes and metagenomes and to predict genes involved in the chlorine metabolism. About 5% of bacterial and archaeal genera encoded Cld. Cld has been subject to extensive horizontal gene transfer but was not conserved within most genera, indicating selection by chlorite is strong yet episodic. Cld was detected in environments from soils and freshwater to water treatment systems. Cld was co-located in genomes with various enzymes with perchlorate (ClO_4^-) or chlorate (ClO_3^-) reductase activity, demonstrating that in the environment perchlorate and chlorate reduction occurs both through respiratory metabolism and through co-metabolism. The presence of Cld in oxic habitats and obligate aerobes without co-metabolic reductases suggested that chlorite might also be produced from oxidative chemistry or photochemistry. Association with Cld was also used to predict new types of proteins that respond to reactive chlorine species like hypochlorous acid (HOCl). The genetic linkage to Cld of transporters, regulators, and other proteins with unknown function presents several targets for future research in chlorine metabolism. In summary, the comparative genomics of Cld has provided an atlas for a deeper understanding of the biology of chlorine oxidation and reduction.

Introduction

The biogeochemical chlorine cycle involves the chlorination and dechlorination of organic matter and the oxidation and reduction of inorganic chlorine [8]. The latter processes are only partially understood. Chlorine can be oxidized by photochemical reactions in the atmosphere where it forms perchlorate and chlorate, but the outcome of aqueous photochemistry in the environment is unclear [63, 70, 71]. Perchlorate (ClO_4^-) and chlorate (ClO_3^-) are reduced by dissimilatory perchlorate- and chlorate-reducing bacteria, though the extent of inadvertent reduction by various organisms is unknown [7]. Some organisms can oxidize chloride (Cl^-) to hypochlorous acid (HOCl) but no biological oxidation of chlorine to chlorite (ClO_2^-), chlorate, and perchlorate has been observed. Reactive chlorine species, particularly HOCl, are widespread – produced by the mammalian immune system, by diverse organisms for chlorinating organic molecules, and by enzymes and chemical reactions in various environments [10, 39, 44, 80] – but microbial responses to reactive chlorine species have only been studied in a small number of species [98].

One approach to better understand chlorine redox chemistry is to identify a biomarker for chlorine oxyanions. Chlorite dismutase (Cld), the enzyme that degrades chlorite, can be just such a biomarker: the reduction of perchlorate and chlorate produces chlorite, and the reduction of chlorite produces HOCl. Cld catalyzes a chlorite:oxygen lyase reaction wherein a single molecule of chlorite is cleaved into chloride and molecular oxygen, which both detoxifies chlorite and provides oxygen [82]. Cld was first described as an essential enzyme in dissimilatory (per)chlorate-reducing bacteria [7, 88]. Inexplicably, Cld has since been observed in organisms not known to produce chlorite [83, 95, 219, 220]. Yet the amino acid residues for Cld activity are conserved across its phylogeny [169], and Cld has no activity towards other compounds including nitrite, nitric oxide, hydroxylamine, and thiocyanate, aside from low hydrogen peroxidase activity [83, 221]. Therefore, many more organisms have the ability to degrade chlorite than are known to produce chlorite by respiring perchlorate and chlorate.

Cld may be used to detoxify chlorite produced by inadvertent perchlorate and chlorate reduction. Due to the structural and chemical similarity of chlorate and perchlorate to oxyanions like nitrate, perchlorate reductase or chlorate reductase activity has been observed in various enzymes in the biogeochemically important DMSO reductase superfamily of molybdopterin oxidoreductases [208, 222-225]. In fact, nitrate reductases in the DMSO reductase superfamily were first identified by selecting for resistance to high concentrations of chlorate [226], and Cld has been experimentally shown to decrease inhibition by chlorate under nitrate-reducing conditions [95]. However, it is unclear if inadvertent perchlorate and chlorate reduction occurs at the lower concentration of those molecules found in natural environments. Respiratory (per)chlorate-reducing bacteria use specialized perchlorate reductases (Pcr) and chlorate reductases (Clr) with a high affinity for perchlorate and chlorate [7, 155]. These reductases, also in the DMSO reductase family, are some of the only genes known to be genetically linked to Cld, forming distinct genomic islands and composite transposons with additional genes for this metabolism [87, 108]. As perchlorate and chlorate reduction has only been studied in cultivated respiratory (per)chlorate-reducing bacteria, it remains to be seen if different organisms or sets of enzymes are responsible for reducing these chlorine oxyanions in nature.

Aside from reduction of perchlorate and chlorate, the only other source of chlorite would be the oxidation of chloride or HOCl. Such chemistry would seem unlikely, as the redox couples ClO_2^-/Cl^- and $ClO_2^-/HOCl$ have high reduction potentials under biological standard conditions (>1.1 V). Because electron acceptors in respiration and phototrophy have lower reduction potentials [77], the biological oxidation of chloride or HOCl is not energetically favorable. Yet the chemical oxidation of chloride or HOCl to chlorite coupled to the reduction of redox couples with higher reduction potentials would be, in theory, energetically favorable, and oxidation of HOCl to higher chlorine oxyanions has been observed through some chemical and photochemical reactions [71]. For example, HOCl is auto-oxidized to chlorite and chlorate over long periods of time [81]. However, that particular reaction occurs at unnaturally high concentrations, and because the oxidation of HOCl to chlorite is much slower than the oxidation of chlorite to chlorate, chlorite is only a trace intermediate in the reaction. An enzyme that catalyzes the oxidation of HOCl to chlorite would be beneficial to many organisms. HOCl reacts rapidly with various biomolecules[80, 98] and is a much stronger oxidant than chlorite and chlorate. An analogous system would be nitric oxide dioxygenase, which uses oxygen to oxidize nitric oxide to less-toxic nitrate [227]. In tandem with Cld, oxidation of HOCl to chlorite would yield harmless chloride and oxygen. Furthermore, the removal of HOCl would limit the inhibition of Cld by HOCl [228]. However, evidence for the oxidative chemical, photochemical, or biochemical production of chlorite in nature has not been reported.

Chlorite causes significant oxidative stress by oxidizing certain chemical groups (e.g. Fe in heme) and by producing the more-reactive HOCl [93, 109]. Compared to reactive oxygen and nitrogen species, reactive chlorine species (RCS) cause a greater degree of protein misfolding and sulfur starvation because of the greater reactivity of HOCl with the amino acids methionine and cysteine [79, 98]. HOCl oxidizes methionine to methionine sulfoxide much faster than peroxynitrite or hydrogen peroxide do [79], and HOCl can oxidize cysteine to sulfonic acids [98]. HOCl readily chlorinates primary and secondary amines and also aromatic carbons (e.g. tryptophan). Microorganisms respond to RCS using protein chaperones, regulatory systems, methionine and cysteine repair and biosynthesis, and scavenging of reactive byproducts like peroxides, aldehydes, and glyoxals [98]. RCS response has primarily been studied in a small number of bacteria that primarily experience HOCl produced by mammalian immune systems [98]. Low concentrations of HOCl are common in natural environments due to the broad distribution of haloperoxidases and chemical reactions [39, 44, 98], and high concentrations of HOCl and other RCS are a feature of the built environment due to the use of chlorine-based disinfection products, such as molecular chlorine/hypochlorous acid and chlorine dioxide [229, 230]. The components of RCS response in environmentally diverse microorganisms remain to be understood.

Thus, significant gaps remain in our understanding of the evolution of chlorite dismutase, perchlorate and chlorate reduction, the potential for chlorine oxidation, and microbial responses to reactive chlorine species. Here, we expand what is known about the biology of chlorine oxyanions by using Cld as a biomarker for chlorite. This comparative genomics approach adopts two assumptions: that organisms with Cld experienced chlorite, and that proximity to Cld means a gene is more likely to function in chlorite production or reactive chlorine stress response. By identifying Cld and its neighboring genes in genomes and metagenomes, we were able to describe the distribution of Cld across taxa and environments, describe the evolutionary history of Cld, and predict genes that are functionally related to Cld in different aspects of chlorine oxyanion biology. Additionally, we determine that the reduction of perchlorate and chlorate is insufficient to explain the distribution of Cld in the environment. From the observation that Cld is present in approximately 5% of bacterial and archaeal genera, we conclude that chlorite can be found in many environments and is a significant, episodic source of reactive chlorine stress.

Methods

Identification and distribution of chlorite dismutase (Cld)

A maximum likelihood phylogenetic tree of the protein family containing Cld was constructed using FastTree from the Pfam 06778 alignment of representative proteomes, at the 15% comembership threshold to limit the number of redundant proteins [231-233]. The presence of key residues for Cld activity were identified by comparing the positions in the alignment corresponding to the distal heme arginine (R127) and proximal heme lysine (K92), histidine (H114), and glutamic acid (E167) in Nitrobacter winogradskyii Nb-255 [169]. The diversity of Pfam 06778 led to some misalignment of proteins, so Cld proteins were defined as any protein in the monophyletic clade where most proteins had key residues. Proteins in the two major lineages of Cld were used to construct profile-hidden Markov models (HMMs) [143]. A concise distribution of Cld across bacterial genomes was obtained by plotting Cld and non-Cld proteins from Pfam 06678 against a precomputed bacterial tree of life [234].

Cld proteins were identified in genomes and metagenomes through an initial homology-search against a panel Cld proteins followed by confirmation with profile-HMMs. BLASTP was used to identify genomes in JGI IMG/M, NCBI GenBank, and NCBI RefSeq containing Cld, with RefSeq genomes being preferred for each taxon [133, 134, 235]. BLASTP was used to identify metagenomic contigs in JGI IMG/M using a search strategy including the largest metagenomes needed to search 90% of proteins in each “Ecosystem Category” (Built Environment, Aquatic, etc.). Metagenome-assembled genomes in the Uncultivated Bacteria and Archaea (UBA) dataset were searched directly with profile-HMMs [236]. Genomes from IMG/M, UBA, and GenBank were removed if it was determined that a contig with *cld* might be present as a methodological error (e.g. UBA1553, GCA_003243385.1). Genomic data and metadata from different sources were merged for using custom scripts.

The fraction of a taxonomic group with Cld was determined by comparing the number of RefSeq genomes with the *cld* gene to the total number of RefSeq genomes available to download within each taxonomic group (https://github.com/kblin/ncbi-genome-download). The detection of Cld in different environments was compared using the number of *cld* copies per million total coding domain sequences (CDS) obtained from IMG/M metagenome metadata. We advise that this calculation may be biased towards datasets with low genetic diversity and does not indicate the abundance of organisms with Cld in these environments. Due to inconsistent definitions of environments in IMG/M, metadata were using to assign each metagenome were assigned to a custom environmental category.

Phylogeny and composition of Cld

Cld proteins identified above were aligned using MUSCLE v3.8.1551 [147] and built into a maximum likelihood phylogenetic tree using FastTree [233]. The Python package ETE v. 3 was used to plot trees and to form clades of proteins at trees nodes in which the average distance to a protein was less than a selected value [237]. The source (e.g. environment, class, and genus) and length of a protein was obtained from associated metadata, and key residues for activity were defined as above. N-terminal and C-terminal extensions were defined as amino acids in the alignment beyond the positions within which the average Cld protein had amino acids. Signal peptides were assigned using SignalP v. 5, accepting a positive result from any type of organism (gram-negative, gram-positive, or eukaryotic) [238].

Comparative genomics of Cld gene neighborhoods

Genes within +/- 10 positions of *cld* on the same contig were defined as part of the Cld gene neighborhood. To compare neighborhoods, similar proteins in the neighborhood were clustered into protein subfamilies using MMSEQs v.7-4e23d set to a coverage of 0.5 and an E-value of 0.001 [239]. To aid analysis, the two major lineages of Cld were each defined as a separate subfamily, and subfamilies were numbered in order of their size in this dataset.

A simple statistic for gene linkage to *cld* was obtained by representing the co-occurrence of protein subfamilies across Cld gene neighborhoods as a network using the Python package networkx. In the network, each subfamily is a node and edges connect subfamilies found in the same gene neighborhood. A network statistic known as the clustering coefficient describes the fraction of a node's neighbors with an edge over the total number of edges possible between a node's neighbors. A protein subfamily that is only found in one genomic context with Cld – one set of genes – will have a clustering coefficient of 1. The more distinct genomic contexts a protein subfamily shares with Cld, the lower its clustering coefficient will be.

To simplify analysis, gene neighborhoods with 10+ genes were grouped by similar gene content using unsupervised machine learning methods in the Python package SciKit-learn. The features of the data were the presence (1) or absence (0) of each subfamily in the neighborhood. An initial linear dimensional reduction was performed with Principle Components Analysis, and the resulting 50 dimensions per neighborhood were subject to a non-linear dimensional reduction using t-Distributed Stochastic Neighbor Embedding (t-SNE) with a perplexity of 50 and 5,000 iterations. Neighborhoods close to each other in two dimensions were then clustered with the Density-Based Spatial Clustering of Applications with Noise (DBSCAN) algorithm.

Phylogenetic analyses of proteins associated with Cld

Proteins in the DMSO reductase family of molybdopterin enzymes, which might function as perchlorate and chlorate reductases, were identified using a profile-HMM built from the seed alignment of Pfam 00384 [143, 231]. A maximum likelihood phylogenetic tree was constructed from those proteins found near Cld and a curated set of proteins from Pfam 00384 proteins in representative proteomes at the 15% comembership threshold. Incomplete proteins were excluded using a size threshold of 300 amino acids, the size of dataset was reduced while maintaining diversity by clustering proteins at 50% amino acid identity using CD-HIT. Only positions in the alignment where a majority of proteins had residues were kept. The tree was constructed, plotted, and grouped into clades as described above. Profile-HMMs representing possible perchlorate and chlorate reductases were constructed from the alignment of proteins from five large clades in the phylogenetic tree.

A phylogenetic tree of the sulfite oxidase family of molybdopterin enzymes, which includes some methionine sulfoxide reductases, was constructed from Pfam 00174 as above but without the creation of clades and using positions in the alignment where more than 80% of proteins had amino acids. Methionine rich peptides were defined as proteins with a methionine composition so high that a subfamily would have less than a 0.01% chance of that methionine composition, assuming a normal distribution. A sequence similarity network (SSN) of methionine rich peptides and methionine sulfoxide reductases was created in which proteins are connected if they had a significant BLASTP similarity (E-value 0.00001, percent identity >50%). Other reactive chlorine stress response genes were identified by mapping a custom list of enzyme commission (EC) numbers against KEGG orthology annotations [240].

Results and Discussion

Distribution of Cld across genomes and metagenomes

Chlorite dismutase proteins are just a subset of the protein family Pfam 06778, which is part of the CDE superfamily [241]. Here, Cld was defined as proteins in Pfam 06778 that contain the key residues required for Cld activity [82, 169]. Cld proteins were only 6.7% of a representative set of Pfam 06778 and were found in only one part of the phylogenetic tree (Figure 5-1A), confirming previous analyses with smaller datasets. Most non-Cld proteins in Pfam 06778 are iron-coproporphyrin oxidative decarboxylases (HemQ) that are required for heme biosynthesis in monoderm bacteria. Reflecting that conserved role, non-Cld proteins were 93% of Pfam 06778 (Figure 5-1A) and were conserved across large groups of the bacterial tree of life (Figure 5-1B), primarily in the monoderm phyla *Firmicutes* and *Actinobacteria*. In contrast, Cld proteins were primarily found in the *Proteobacteria*, followed by other diderm phyla *Nitrospirae*, *Nitrospinae*, *Cyanobacteria, Planctomycetes,* and *Bacteroidetes* (Figure 5-1A), and they were sparsely distributed across the tree of life (Figure 5-1B). All further investigations of Cld refer only to such proteins, which are further divided into two lineages, group 1 and group 2 Cld [219].

Significantly more taxa have Cld than previously described. Profile-HMMs for both lineages of Cld were constructed and used to identify 2411 Cld in 2297 genomes/metagenome-assembled genomes and 6469 Cld in 1575 metagenomes (Figure 5-1C, Supplementary Data). Previous descriptions and phylogenies of Cld have involved only the small number of Cld from the bacterial phyla discussed above [82, 87, 219]. Here, Cld was identified in 143 genera across 14 phyla, including some *Archaea* and *Eukarya*. Metagenome-assembled genomes in *Euryarchaeota, Crenarchaeota*, and *Bathyarchaeota* encoded Cld, as did a metagenome-assembled genome from the *Nanoarchaeota*, which are ultra-small obligate symbionts [164], from a groundwater community with nitrifying bacteria (PRJNA514088). While metagenome-assembled genomes may be contaminated by sequences from other organisms, the low percent identity to other Cld sequences and similarity of neighboring genes to archaeal genes supported their assignment to archaea. The sole eukaryote with Cld was *Monoraphidium neglectum*, a unicellular green alga. Cld was previously reported in a different eukaryote, the poplar tree (Populus), but this was later determined to be contamination and removed (JGI, personal communication). In *Monoraphidium neglectum* the *cld* gene is fragmented, likely due to poor sequence assembly, but the one other gene on the contig was most similar to a protein from green algae (41% amino acid identity). Cld was also identified in the several bacterial phyla not previously reported to contain Cld: Actinobacteria, Verrucomicrobia, Firmicutes, Chloroflexi, and Spirochaetes.

Overall, Cld was observed in approximately 1% of genomes, 5% of genera and 15% of phyla among prokaryotes. While most copies of Cld were found in the phylum *Proteobacteria*, that was a consequence of bias in which organisms have been sequenced. Normalizing by the number of available genomes indicates that genomes from the phyla *Nitrospirae, Planctomycetes,* and *Nitrospinae* are most likely to contain Cld, followed by *Proteobacteria* and *Cyanobacteria* (Figure 5-1D). At the genus level, usually only a fraction of genomes had Cld, but it could be conserved (Figure 5-1E). Cld did not appear to be enriched in host-associated organisms, though Cld was relatively common in the *Enterobacteraceae*, which have both host-associated and saprophytic lifestyles, and was detected in specific pathogenic isolates such as *Acinetobacter baumanii* OIFC074 [242]. Cld is particularly common in organisms with specific traits, even among distantly related genera: the formation of nitrogen-fixing root nodules, such as *Bradyrhizobium* (81%, n= 184), *Rhizobacter* (55%, n=6), and *Rhizobum* (3%, n=21); the oxidation of one-carbon compounds, such as *Methyloversatilis* (78%, n=7) and *Methylobacterium* (11%, n=14); and the oxidation of nitrite, such as *Nitrotoga* (100%, n=4), *Nitrospira* (32%, n=17), *Nitrobacter* (50%, n=3), and *Nitrospina* (4%, n=1). Even the 1219-gene genome of *Nitrotoga* UBA7399_v2 contains Cld, despite streamlining being an adaptation to oligotrophy that removes unnecessary genes [243]. This extent to which Cld can be found outside of perchlorate- and chlorate-reducing bacteria far exceeds that described previously.

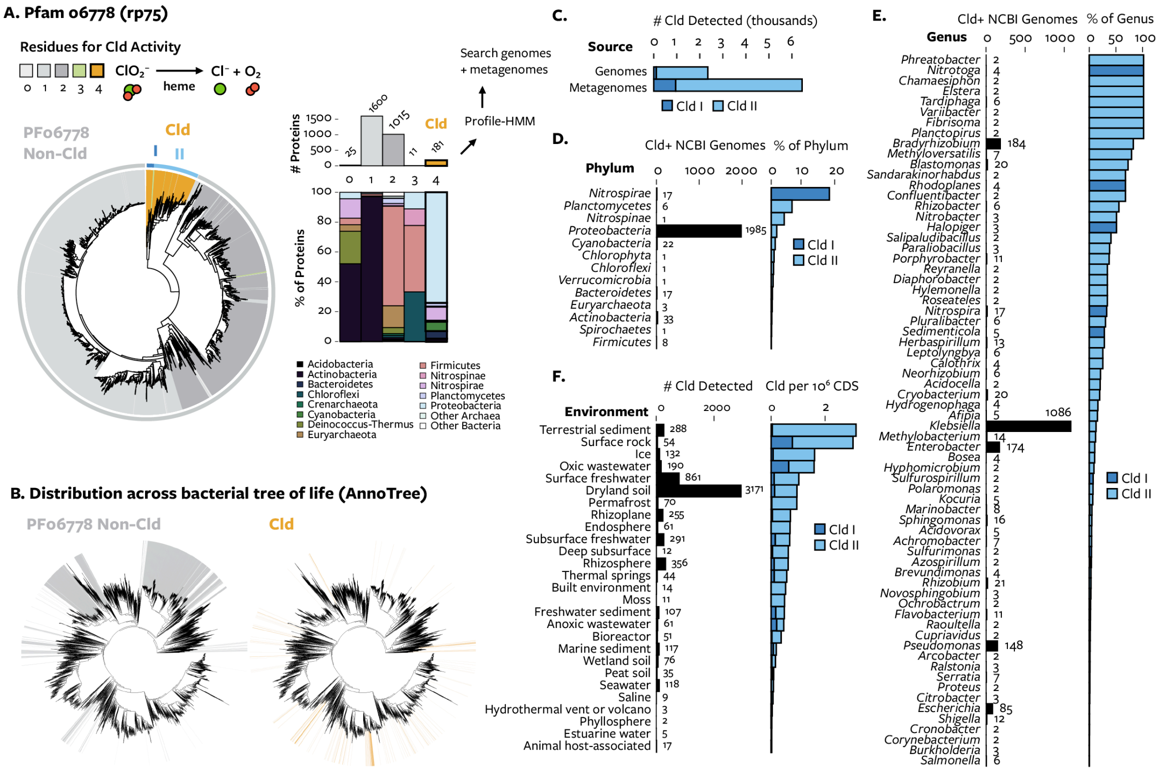


Figure 5-1. The distribution of Cld across genomes and metagenomes. (A) A maximum-likelihood phylogenetic tree of Pfam 06778. Color indicates the number of the 4 key residues for Cld activity in each protein. The number of proteins with each fraction of key residues, and the phylogenetic distribution of those proteins, is summarized at right. (B) A tree of all bacterial genomes annotated with the presence Pfam 06778 proteins, comparing the distribution of non-Cld proteins (left, gray) and Cld proteins (right, orange). (C) The total number of each Cld lineage detected in genomes and metagenomes. (D-E) The number (left) and percent (right) genomes within a given RefSeq phylum or genus. For simplicity, only genera with more than one Cld and 20+ genomes or >20% Cld are shown. (F) The number of Cld (left) and fraction of Cld per million genes (right) in different environments. Only environments with a sample size of more than 10 million genes are shown.

Thus, instead of being a tool of specialized anaerobic metabolism in anoxic sediments, Cld appears important for various organisms from a range of oxic and anoxic habitats. The distribution of Cld across a dataset of 6961 IMG/M metagenomes encoding 10.8 billion genes supports that determination. Cld were a low proportion of genes in host-associated systems and a greater proportion in freshwater and soil systems (Figure 5-1F). Comparing metagenomes with greater than 10 million genes with this metric indicated that Cld was most enriched in environments such as oligotrophic rocks, sediment, and ice followed by oxic wastewater, surface freshwater, and dryland soils. Within aquatic environments, Cld was more enriched in freshwater than marine metagenomes; within wastewater environments, Cld was much more enriched in oxic than anoxic metagenomes; and within soil environments, Cld was approximately equally common in endosphere, rhizoplane, rhizosphere and dryland soil metagenomes and less common in wetland soil. The shared features of environments where Cld is most frequently detected is that they are predominantly oxic, and many are exposed to high amounts of sunlight [244, 245]. Possibly, Cld is abundant in those environments because one or more of those oxidizing processes produces chlorite. Due to the atmospheric deposition of perchlorate and chlorate, quantifying the production of chlorite from oxidation or from aerobic (per)chlorate reduction (see below) will be challenging. Yet the broad distribution of Cld in genomes and metagenomes suggests that, whatever the source, many organisms in many environments encounter chlorite.

Cld phylogeny and evolution

A detailed phylogeny of Cld from genomes and metagenomes provides a better understanding of the selective pressure on this enzyme (Figure 5-2). 8924 Cld were grouped programmatically by branch length into 60 clades, with a maximum clade size of 2936 proteins and a median clade size of 11 proteins. The previously reported division between group 1 and group 2 Cld was reproduced in this tree, and a division of group 2 Cld into subgroups, which we term 2A and 2B, was strongly supported (>99% bootstraps) (Figure 5-2A). Key residues for chlorite:O2 lyase activity were conserved in all clades except the under-sampled clades 10 (n=2) and 32 (n=1) and clade 38 (n=21); many of the largest clades contained Cld that have been biochemically verified (Figure 5-2B). Most of the largest clades were in group 2A, followed by group 1 and group 2B. Only about half of the sequence diversity of Cld is present in cultured organisms, and the diversity of group 2 was especially expanded by inclusion of metagenomic sequences (Figure 5-2B). That some of largest clades are almost entirely metagenomic sequences indicates there are other environmentally abundant organisms with Cld yet to be discovered.

The expanded phylogeny revises the previous dogma wherein group 1 Cld are larger, periplasmic proteins and group 2 Cld are smaller, cytoplasmic proteins [82, 246]. Though that is generally true, signal peptide prediction (Figure 5-2C) suggested that several clades of group 1 Cld are not periplasmic, while two clades of group 2B Cld are typically periplasmic (incl. *Nitrospina* Cld) and some Cld from various group 2A clades are periplasmic. Assuming all Cld in clades 4 through 9 should have signal peptides, there was an 11% false negative rate for signal peptide detection, meaning the number of Cld that acquired signal peptides is likely underestimated. While the acquisition of signal peptides suggests that extracellular chlorite has been a strong, periodic selective pressure, a majority of Cld is not secreted (84% of metagenomic Cld), perhaps indicating the predominant need is for detoxification of intracellular chlorite. The average length of group 1 and 2A Cld did differ significantly at, respectively, 265 and 186 amino acids. But group 2B proteins were at an intermediate length of 229 amino acids. Considerable variation in protein size was observed within group 2A Cld (Figure 5-2C). Shorter proteins were likely artifacts of sequence assembly, but longer proteins appeared to result of N- and C-terminal extensions. About 4% of Cld had larger (>20 aa) extensions that must be either annotation errors or fusion proteins that might augment the function of Cld [247]. While maintaining key functional residues, the structure of Cld has evolved through gradual variation in protein size and punctuated acquisition of transmembrane signal peptides.

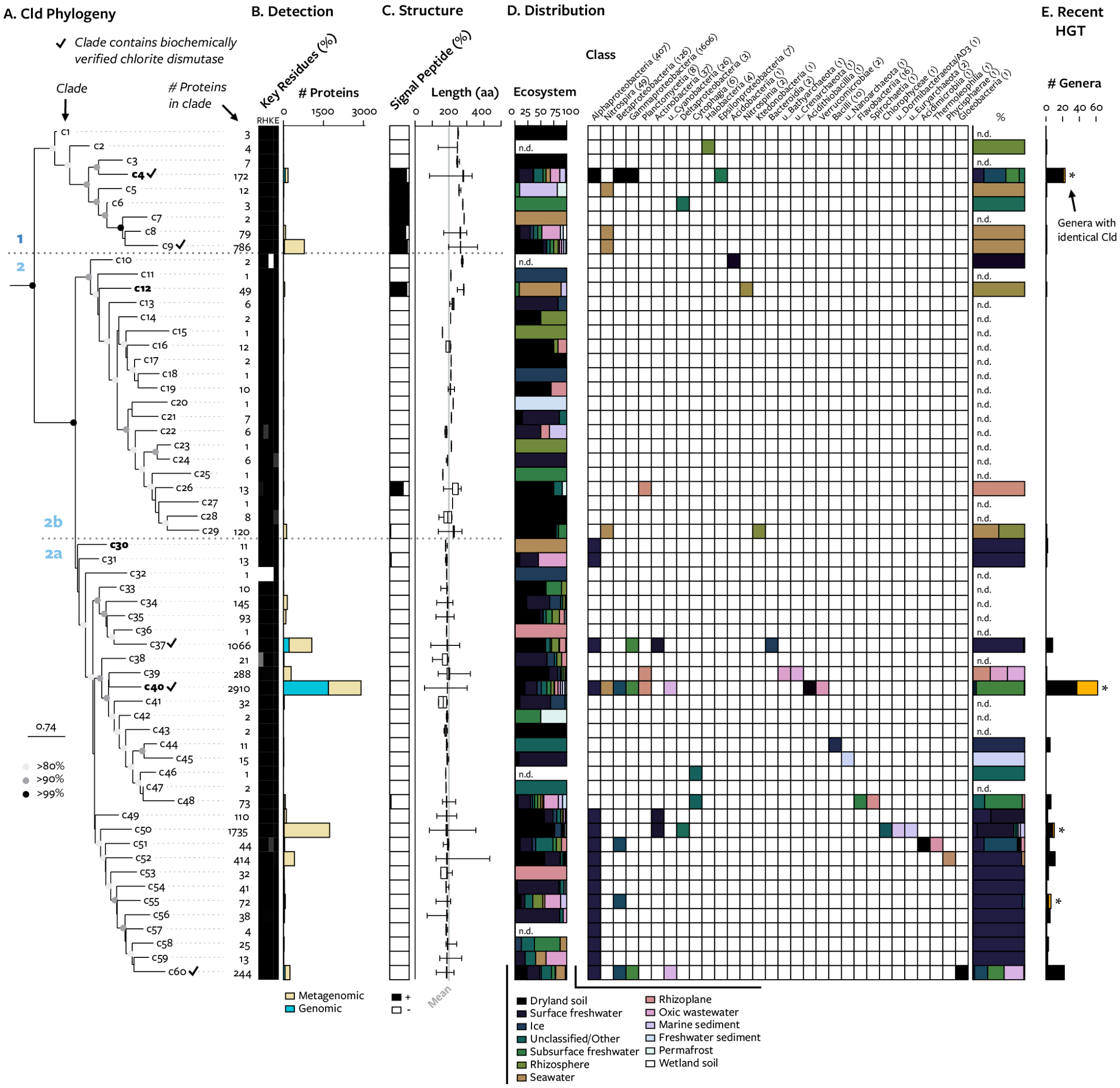


Figure 5-2. The phylogeny of Cld proteins and attributes of each lineage. (A) A maximum likelihood phylogenetic tree of Cld, with clades formed by phylogenetic distance and node support values indicated by color. The number of Cld per clade is listed at right. Clades containing biochemically verified Cld proteins are indicated by arrows, and clades containing sequences used in the initial search are bold. Major lineages of Cld are demarcated by dashed lines. (B) Additional metrics to assess Cld clades: (left) the proportion of each Cld clade with each key residue from 0% (white) to 100% (black), and (right) the number of Cld from genomic and metagenomic datasets. (C) Differences in Cld structure, either the proportion of the clade predicted to have a signal peptide (left, black) or the length of proteins in the clade represented as a boxplot where the box represents the interquartile range and whiskers represent maximum and minimum values. (D) Stacked barplots represent the proportion of each Cld clade in each environment (left) or each taxonomic class (right), with detection of each taxonomic class indicated by filled squares. (E) The number of genera found in each clade, with genera that can share an identical copy of Cld colored orange.

Cld did not differ in environment beyond the scale of individual clades, but the broader groups of Cld appeared to differ significantly in important aspects. Group 1 consisted almost entirely of Proteobacteria and Nitrospira that use periplasmic oxidoreductases to perform ether dissimilatory (per)chlorate reduction (2 clades, ~100 Cld) or nitrite oxidation (4 clades, ~900 Cld). Group 2 contained much greater phylogenetic diversity, including multiple instances of horizontal gene transfer across phyla and even domains of life, both of which suggest that cytoplasmic Cld has a much broader physiological role. The largest clades of group 2A Cld included cultivated organisms in the Enterobacteraceae, Bradyrhizobiaceae, and Actinobacteria (Figure 5-2B). In contrast, the only group 2b Cld in a cultured organism is from Nitrospina gracilis [248], and the group consists mostly of metagenome-assembled genomes from the taxa typically associated with slow growth, including the phyla Acidobacteria, Planctomycetes, Chloroflexi (class Ktedonobacteria), Nitrospirae, and Nitrospinae. Therefore, each of the different groups of Cld can be found in many environments but differ in their structure, localization, rarity, physiological roles, host taxa, and extent of horizontal gene transfer.

Cld has been subject to both ancient horizontal gene transfer and recent transfer between genera, despite being rarely conserved within a single genus. Such a phylogenetic pattern suggests that the ability to degrade chlorite has been subject to a strong yet episodic selection. Multiple clades consisted of Cld from different genera (Figure 5-1E). Alone, this metric reflects the combined signal of vertical and horizontal inheritance, but a detailed view shows that horizontal inheritance is a large component. For example, within clade 4, Cld from (per)chlorate-reducing proteobacteria is intertwined with Cld from nitrite-oxidizing *Nitrotoga* [209], indicative of recurrent exchange. Another indication of recent horizontal gene transfer is that some genera, even the genus Bradyrhizobium, have Cld from different clades (data not shown). Representing the most recent horizontal gene transfer: genomes from different genera possessed identical Cld proteins (Figure 5-1D). In one case the same Cld protein (WP_011514928.1) was found in genomes from 18 genera and three classes (Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria). Strong, episodic selection for Cld is ongoing.

Cld gene neighborhoods

No gene evolves in isolation. Cld likely evolved in close association with genes involved in the production of chlorite and response to reactive chlorine stress. Because genes located near each other across genomes are more likely to be functionally related [249, 250], co-location of genes with Cld across genomes can be used to identify such genes. We defined the set of genes within 10 genes upstream and downstream of Cld as a gene neighborhood and clustered all 61,215 proteins from 8,751 gene neighborhoods into 11,081 subfamilies. A protein subfamily is more likely to have a function related to chlorite if it is found with Cld in many different gene neighborhoods, a property that is indicated by a low "clustering coefficient" (Methods, Figure 5-3A). The vast majority of protein subfamilies in Cld gene neighborhoods were not genetically linked to Cld (Figure 5-3B). Subfamilies with the lowest clustering coefficients (< 0.1) include genes expected to occur near Cld, such as (per)chlorate reductases, oxidative stress response, genetic mobility, and signaling (Table 5-1) [86, 87, 108]. Non-heme chloroperoxidase (subfamily 122), which chlorinates organic molecules using hypochlorous acid, had a very low clustering coefficient of 0.13, indicating a possible link between chlorite and chlorination.

Many uncharacterized subfamilies with the lowest clustering coefficients, such as the cupin domain-containing protein (subfamily 3), SDR family NAD(P)-dependent oxidoreductase (subfamily 8), and the NADPH:quinone reductase-like Zn-dependent oxidoreductase (subfamily 85), belong to protein families that contain oxidoreductases. While these subfamilies' functions remain unknown, their genetic linkage to Cld means they are likely important in chlorine oxyanion biology.

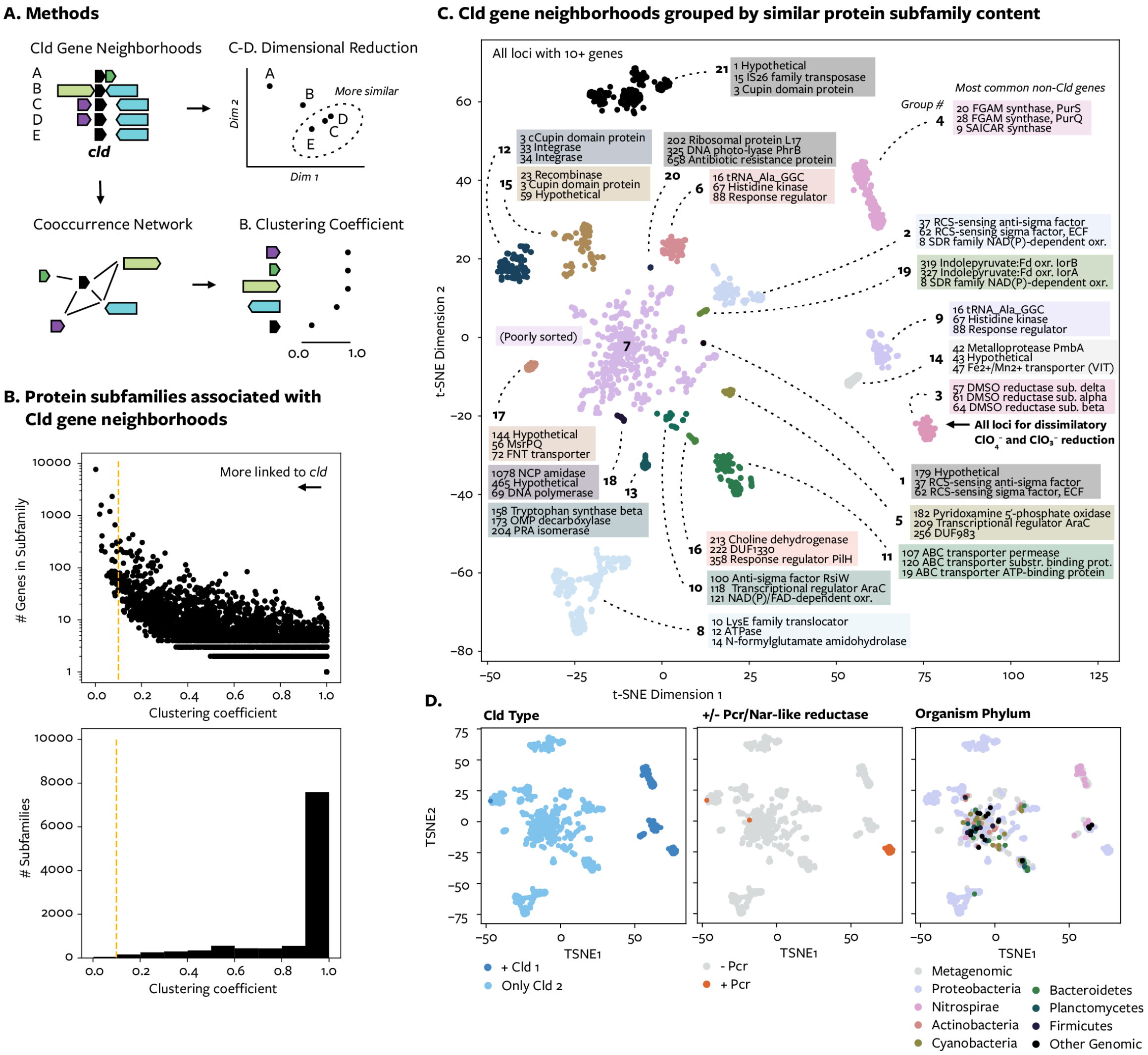


Figure 5-3. Statistical analysis of Cld gene neighborhoods. (A) Schematic diagram explaining analyses. Gene neighborhoods are compared using proteins clustered into protein subfamilies. In the gene-centric analysis, the co-occurrence of genes in different neighborhoods is used to construct a network, from which a clustering coefficient for each gene is derived. In the neighborhood-centric analysis, neighborhoods with more similar gene content are plotted through several dimensional reduction steps and clustered. (B) The distribution of protein subfamilies by their clustering coefficient, a measure of linkage to *cld.* The threshold value for defining "hits" is indicated. (C) Cld gene neighborhoods colored by group, indicating thee top three most common proteins subfamilies in each group (D). Cld gene neighborhoods colored by the presence of group 1 Cld (left), by the presence or absence of reductases closely related to Pcr, Clr, and Nar (center), or by the phylum of the host organism (right).

To understand which genes may be involved in related biological processes, gene neighborhoods with greater than 10 genes were grouped by gene content (Figures 5-3A and 5-3C). Unsupervised dimensional reduction was used to prioritize important genes in clustering: for example, neighborhoods clustered by the type of Cld present and the presence or absence of Pcr-like reductases but not necessarily by taxonomy of the host genome (Figure 5-3D). Cld was found in many distinct genomic contexts with the exception of group 7, which consists of poorly sorted neighborhoods that contain a mix of genes linked to Cld as well as various other proteins. Group 3 was typified by subunits of dissimilatory perchlorate and chlorate reductases. Groups 12, 15, and 21 contained the cupin domain protein (subfamily 3) and transposases, which helps explain the high linkage of this protein and Cld. Regulatory proteins were typical of groups 5, 6, 9, and 10, and group 2 contained reactive chlorine-sensing regulatory elements (subfamilies 37 and 62) with an SDR family NAD(P)-dependent oxidoreductase (subfamily 8) [251]. No specific transporters for chlorine oxyanions are known, but transporters characterized two neighborhood-groups. Group 11 contained ABC transporters, some of which are ATP-driven nitrate transporters that might alternatively be involved in chlorate transport. Group 17 contained a formate-nitrite transporter (FNT) family protein as well as a periplasmic RCS response protein and cytoplasmic Cld. As formate (HCO_2^-) and nitrite (NO_2^-) are structural analogues of chlorite (ClO_2^-) and this gene cluster is found in metagenomic *Mycobacteria* from anoxic lakes across the world, these genes might function in importing extracellular chlorite to be converted to oxygen inside the cell. Such an activity is supported by the deleterious nature of FocA formate transporter and NirC nitrite transporter in chlorite stress conditions [252]. In summary, beyond simply being associated with dissimilatory (per)chlorate reduction, the ability to degrade chlorite is associated with the transport of chlorine oxyanions, sensing and response to reactive chlorine species, and mobile genetic elements containing uncharacterized enzymes.

Reduction of perchlorate and chlorate to chlorite

Cld gene neighborhoods from genomes and metagenomes provided an answer to an important question: are characterized dissimilatory (per)chlorate-reducing bacteria responsible for perchlorate and chlorate reduction in the environment? Neighborhoods were searched for the presence of proteins in the DMSO reductase family of molybdopterin oxidoreductases (Pfam 00384) [208], which includes respiratory perchlorate reductase (Pcr), respiratory chlorate reductases (Clr), and various enzymes that inadvertently reduce perchlorate or chlorate. 105 genes from two major groups of the DMSO reductase family were detected in Cld gene neighborhoods (Figure 5-4A-C). Cytoplasmic Cld was found with enzymes known to have perchlorate and chlorate reductase activity: a tetrathionate reductase (TtrA) in *Diaphorobacter*, cytoplasmic dissimilatory nitrate reductase (Nar) in *Pseudomonas* and *Cryobacterium*, and several assimilatory nitrate reductases (Nas) in multiple *Flavobacterium* genomes, including metagenomic sequences from lake water and marine sediment. Periplasmic dissimilatory nitrate reductase (Nap) were not observed. The co-occurrence of Cld with formate dehydrogenase (FdhN) and an unknown Fdh-like protein (YdeP) cannot be presently explained but could be somehow related to the structural similarity between chlorite and formate. Cld was also found on soil metagenome contigs with uncharacterized enzymes most similar to – yet distinct from – periplasmic nitrite oxidoreductases (pNxr) of nitrite-oxidizing bacteria (*Nitrospira* and Nitrotoga), anammox bacteria, and other organisms [209]. This uncharacterized reductase can be found with either periplasmic or cytoplasmic Cld, so it is not likely to function in dissimilatory (per)chlorate reduction. The association of diverse enzymes capable of perchlorate or chlorate reductase activity with Cld confirms that inadvertent reduction of perchlorate and chlorate occurs in the environment – and at such a degree that can select for Cld to degrade the chlorite produced.

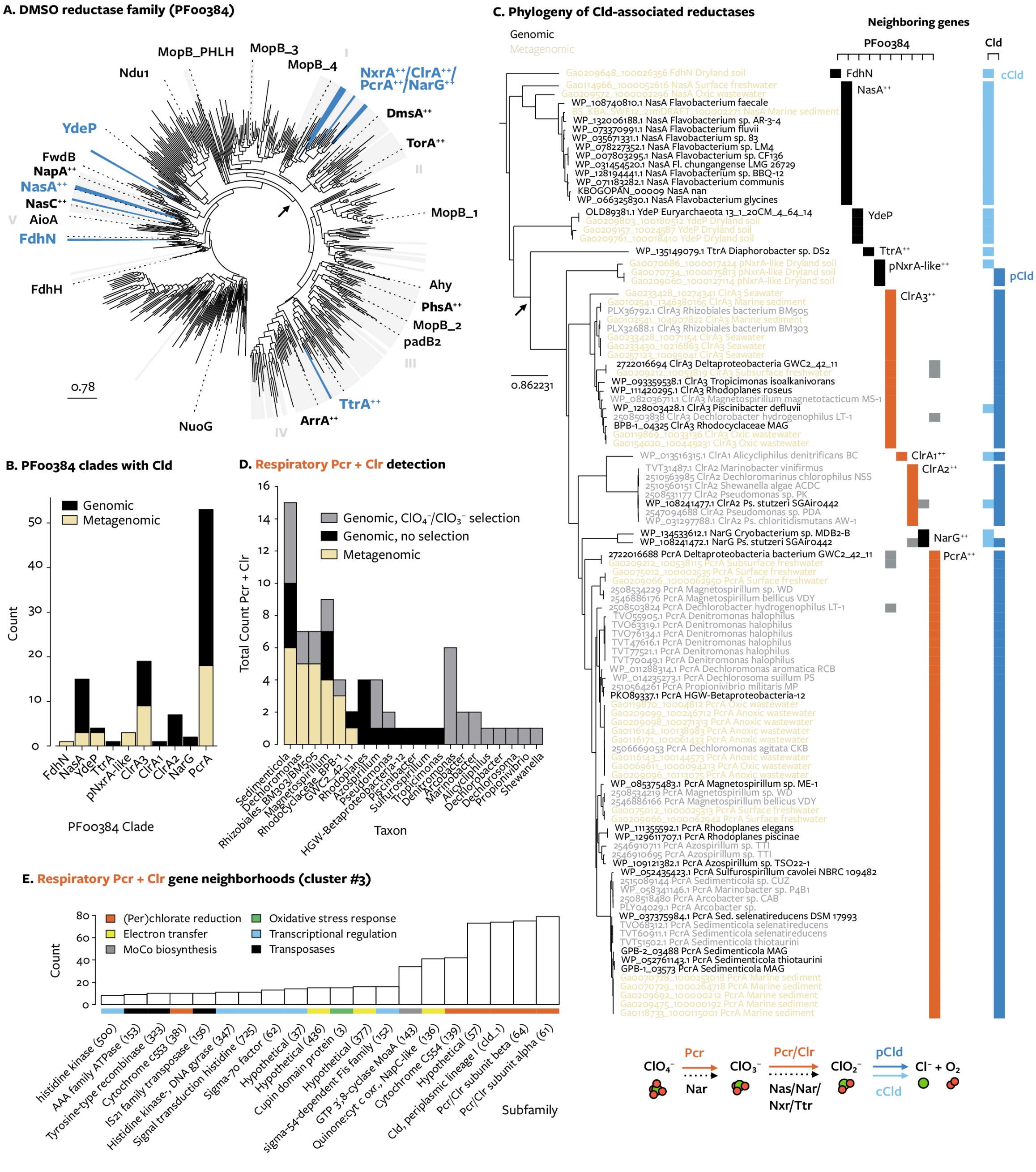


Figure 5-4. The distribution of Cld among possible perchlorate and chlorate reductases in the DMSO reductase family. (A) An unrooted maximum likelihood phylogenetic tree of representative proteins from

the DMSO reductase family. Clades containing proteins from Cld gene neighborhoods are highlighted in red, and two major groups are shaded. (B) The number of Cld-associated proteins in each clade of the DMSO reductase tree and whether they were obtained from genomes and metagenome-assembled genomes (blue) or metagenomes (yellow). (C) An unrooted maximum likelihood phylogenetic tree of only Cld-associated proteins, their source (genomic or metagenomic), and the presence or absence or different types of genes in their gene neighborhood. (D) The number of genomes per genus or other taxon with proteins from Pcr, Clr1, Clr2, or Clr3, and whether or not the organisms were subjected to selection for those genes (i.e. providing perchlorate or chlorate as respiratory electron acceptors). Metagenomic proteins were assigned to the closest genomic relative's taxon. (E) The number of subfamilies found in gene neighborhoods with both Cld and Pcr/Clr.

More work is needed to quantify the contribution of co-metabolic perchlorate and chlorate reduction in different environments. In this dataset, metagenomic Cld genes were less often found with inadvertent reductases than with respiratory Pcr and Clr: 19 Pcr and 9 Clr genes were detected in metagenomes, whereas all other DMSO reductase family enzymes were detected only 7 times (Figure 5-4B). Association of Cld and inadvertent reductases was detected in metagenomes of dryland soil, surface waters, and oxic wastewater, whereas Pcr and Clr were observed in subsurface freshwater, marine sediments, seawater, and anoxic wastewater (Figure 5-4C). Dissimilatory (per)chlorate reduction may even occur in the deep subsurface: Pcr and Clr were present in the genome of *Rhodoplanes* sp. strain T2.26MG-98 from a 490-m borehole in Iberia, as noted in [212], and in the metagenome-assembled genome Betaproteobacteria bacterium HGW-12 from a 150-m to 200-m borehole in Japan [253]. In agreement with previous isolated dissimilatory (per)chlorate-reducing bacteria [89, 154], this broad distribution shows that (per)chlorate reduction occurs in a variety of environments.

The fraction of environmental perchlorate and chlorate reduction that does occur through respiration appears to be performed by previously characterized dissimilatory (per)chlorate-reducing bacteria. Metagenomic reductases largely resemble those already characterized (Figure 5-4C-D), and relatives of (per)chlorate-reducing bacteria have been isolated without selecting for the ability to respire perchlorate or chlorate (Figure 5-4D). Such isolates show that the association of Cld with Pcr and Clr is not an artifact of selection, and they account for some of the most common reductases from metagenomes (Figure 5-4D). However, there is some novelty among these organisms. One of these bacteria, *Piscinibacter defluvii*, encodes group 3 Clr and both cytoplasmic and periplasmic Cld (Figure 5-4C). Pcr has not been found with cytoplasmic Cld, whereas group 1 and 2 Clr had been previously found with cytoplasmic Cld acquired in the same mobile genetic element as periplasmic Cld. The independent acquisition of cytoplasmic Cld by yet another type of chlorate reductase – but not perchlorate reductase – suggests that chlorate reduction can involve more intracellular chlorite stress than perchlorate reduction. Another bacterium, the genome GWC2_42_11 assembled from an aquifer sediment metagenome [117], encodes phylogenetically divergent copies of both Pcr and group 3 Clr (Figure 5-4C), Cld, and cytochrome c oxidase. As a member of the phylum Deltaproteobacteria/GWC2-55-46 (NCBI/GTDB), GWC2_42_11 is the most evolutionary distinct putative (per)chlorate-reducing bacterium, and its reductases may help understand the earliest forms of (per)chlorate respiration.

In general, however, the 71 gene neighborhoods with Pcr or Clr match the functions identified in a study of only 13 genomes [108]: (per)chlorate reduction, electron transfer, oxidative stress response, transcriptional regulation, molybdenum cofactor biosynthesis, and transposases (Figure 5-4E). The only unknown function is the cupin domain protein, which has been predicted to be involved in reactive chlorine stress response due to a fitness benefit at high concentrations of chlorite [93]. The congruence between genomic and metagenomic sequences is unexpected because culturing selects for only fast growing, independent populations. Perhaps growth by dissimilatory (per)chlorate reduction in the environment occurs in short intervals, requiring a similar growth strategy.

Oxidation of chloride or hypochlorite to chlorite

The prevalence of Cld in aerobic organisms and oxic habitats (Figure 5-1) strongly suggested that chlorite is formed from oxidizing processes. However, as shown above, Cld is also used to degrade chlorite formed from inadvertent perchlorate and chlorate reduction in oxic habitats. If chlorite is formed from the oxidation of chlorine, Cld should be present in organisms unable to reduce perchlorate or chlorate. Using profile-HMMs representing the broad parts of the DMSO reductase family phylogeny that have perchlorate or chlorate reductase activity (Figure 5-4A), we identified genomes with Cld that are unlikely to reduce perchlorate or chlorate enzymatically (Figure 5-5A). Despite the commonality of enzymes such as assimilatory nitrate reductase, this search identified 27 such genomes in isolated strains. These putative “non-(per)chlorate reducers” represented 6 of the 19 phyla with Cld (Figure 5-5B) and 15 of 151 genera. All are aerobes, and none were reported to be facultative anaerobes or anaerobes (Figure 5-5B). They were isolated from diverse habitats, often characterized by high sunlight (lakes and ponds, desert rocks and sediments, growing with diatoms, cyanobacteria, or mosses) or by high amounts of reactive chlorine species (human body, wastewater treatment plant, swimming pool, showerhead biofilm) (Figure 5-5B).

Despite Cld gene neighborhoods of non-(per)chlorate reducers being only 0.3% of the total dataset, the subfamilies found in them contain 8% of all subfamilies with a clustering coefficient below 0.25 (Figure 5-5C). Many neighborhood-groups contained at least one neighborhood from a non-(per)chlorate reducer (Figure 5-5C). An even greater number of subfamilies and neighborhood-groups meet this condition with the inclusion of metagenome-assembled genomes, which were initially excluded due to possible of being incomplete. Therefore, genes associated with Cld are enriched in these organisms, and they represent a fair amount of the diversity of gene neighborhoods where Cld is found. This suggests that many of the genes found with Cld respond to environmental chlorite or the oxidizing conditions that might produce it.

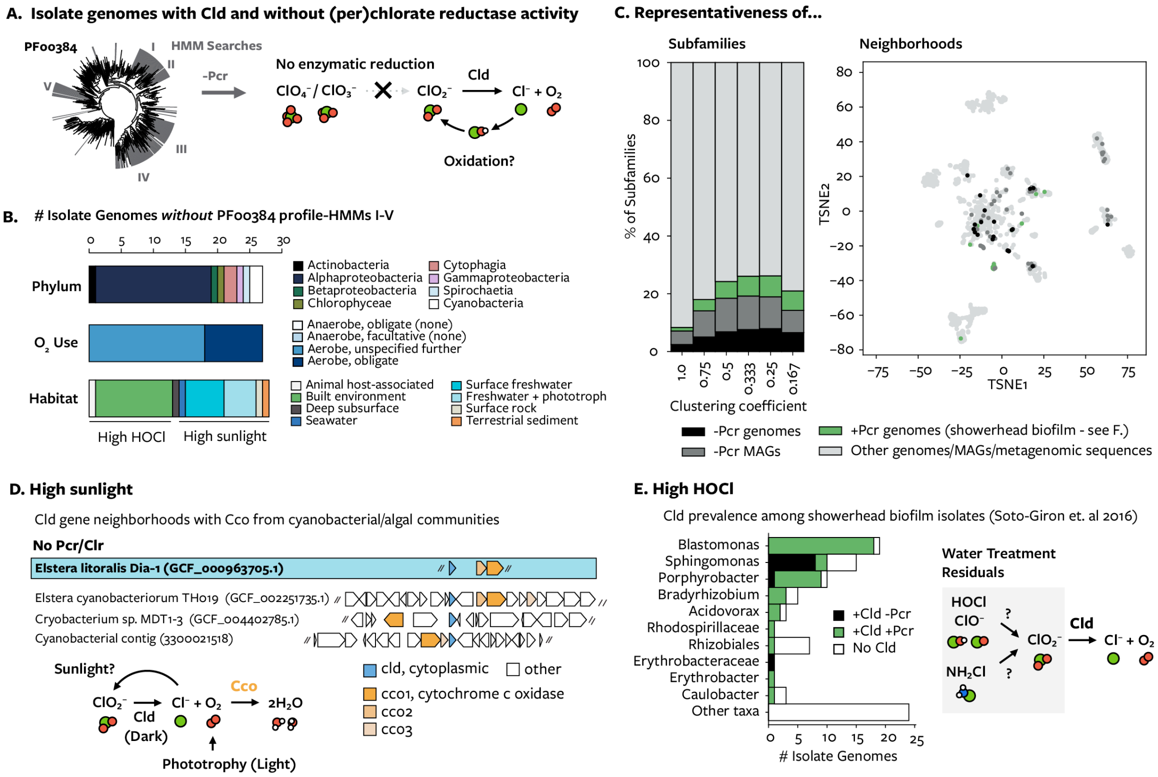


Figure 5-5. Genomes without respiratory and co-metabolic perchlorate/chlorate reductases. (A) Profile-HMMs were used to find isolated microorganisms without enzymes from the broad parts of the DMSO reductase family that might have (per)chlorate reductase activity. These organisms may experience chlorite produced from oxidative chemistry. (B) The number of isolate genomes with respiratory or co-metabolic reductases grouped by phylum, relationship with oxygen, and the habitat they were isolated from. (C) The representativeness of organisms without (per)chlorate reductases compared to the entire dataset of subfamilies below each clustering coefficient threshold (left) and neighborhoods (right). (D) Gene neighborhoods encoding cytochrome c oxidase in organisms from communities with oxygenic photosynthesis. (E) The number of organisms isolated from showerhead biofilm communities impacted by free chlorine residuals.

High sunlight may be one such condition. Habitats with high sunlight are very oxidizing due to the combined effects of UV radiation and oxygenic phototrophy [254, 255]. The non-(per)chlorate reducer *Elstera litoralis* was isolated from a high sunlight habitat [256], a diatom biofilm, and encodes a cytochrome c oxidase near Cld. Cld and cytochrome c oxidase co-occur in diverse bacteria from high sunlight habitats: alphaproteobacterium *Elstera cyanobacteriorum* from freshwater cyanobacterial aggregates [257]; the actinobacterium *Cryobacterium* sp. MDT1-3 from a glacier [258], which are highly affected by sunlight; and a metagenomic contig from a photosynthetic biofilm on mafic rock belonging to a cyanobacterium (80% amino acid identity to CoxA in *Phormidium ambiguum*) [259]. Oxygen can be limiting in phototrophic communities during dark periods or at depth from the surface [259]; if chlorite were available, Cld and cytochrome c oxidase would enable aerobic respiration even when gaseous oxygen is limiting. Other organisms from high sunlight communities have Cld gene neighborhoods with genes for tolerating UV radiation. Four of 34 MAGs from a sunlight photobioreactor metagenome have Cld (UBA7691, UBA7681, UBA7678, UBA7677), including Planctomycetaceae bacterium UBA7678 in which a periplasmic group 2a Cld is co-located with carotenoid biosynthesis genes for limiting UV photodamage [260]. Deoxyribodipyrimidine photo-lyase, which is a light-activated protein that repairs UV-damaged DNA, is found in 21 gene neighborhoods with Cld (groups 2, 20, and 7), including MAGs from *Limnobacter* spp. in the upper ocean (UBA6514, UBA7229) [261] and from a betaprotebacterium in culture with *Leptolyngbya glacialis* TM1FOS73 (GCA_003242045.1) [262]. These genomic data suggest that chlorite is coincident with sunlight.

Possibly, reactive nitrogen species also produce chlorite. Molecules like nitric oxide and peroxynitrite have high reduction potentials and can oxidize various molecules, producing other reactive species such as carbonate radicals [263, 264]. One of the types of organisms most frequently found with Cld is the nitrite-oxidizing bacterium (Figure 5-5-1E), which experiences high amounts of reactive nitrogen species [265]. Nitrite-oxidizing bacteria also encode nitrite oxidoreductase (Nxr), which can reduce chlorate [266]. The predicted co-localization of Cld and Nxr across nitrite-oxidizing bacteria led to the hypothesis that Cld is for responding to inadvertent chlorate reduction by Nxr [83]. However, the presence of Cld in non-(per)chlorate reducers invites reconsideration of that hypothesis. Some *Nitrospira* MAGs in this dataset have a cytoplasmic Cld, as did a *Nitrospira* contig from one of the metagenomes most enriched in Cld, a biofilm in a cave made of mafic rock (contig Ga0187892_10000042) [267], even though *Nitrospira* encode periplasmic Nxr. The production of chlorite by oxidative chemistry involving reactive nitrogen species is an attractive alternative hypothesis, as it is consistent both with the correlation of Nxr and Cld in genomes with localization of Nxr and Cld in different cellular compartments. A supporting datum is that Cld can be found co-located genes that respond to reactive nitrogen species, such as peroxiredoxins that reduce peroxynitirite [268] and nitrotyrosine monooxygenases that repair nitrated tyrosine.

HOCl is another potential source of chlorite and is common in nature and in the built environment. All 12 non-(per)chlorate reducers from the built environment were isolated from water treated with HOCl, and 10 were present in showerhead biofilms. Surprisingly, 47 genomes in our dataset are from 89 strains isolated from those showerhead biofilms (Figure 5-6E), and together organisms with Cld could account for up to 15-50% of those five communities [269]. Drinking water distribution systems are characterized by oligotrophic conditions and by residual chlorine in the form of HOCl and/or chloramine (NH_2Cl). In the showerhead water in question, free residual chlorine was 0.8 mg/liter and the pH was 8.4 [269], meaning both HOCl and hypochlorite (ClO^-) were present.If chlorine dioxide was used to treat the water, it is possible that chlorite was present as a byproduct. As many of those strains are non-(per)chlorate reducers, chlorate and perchlorate reduction is not the source of chlorite for any of the strains in that community. Cld gene neighborhoods from these organisms were prevalent in neighborhood-group 2, which most commonly contains an unknown SDR family protein (subfamily 8) and HOCl-sensing proteins. Curiously, a *Mycobacterium*-like organism was the majority of most communities but does not encode Cld. Mycobacteria are more resistant than other bacteria to HOCl [270]. The oxidation of extracellular HOCl/ClO^- to chlorite would decrease both its reactivity and its permability – a major selective benefit.

One possible hypochlorite-oxidizing enzyme is suggested by a special case of genetic linkage with Cld. Some genomes were found to have identical Cld despite belonging to different genera, which is indicative of recent horizontal gene transfer (Figure 5-2E). 90% of gene neighborhoods with such identical Cld contained a cupin domain-containing protein (subfamily 3). A cupin domain is a conserved structural fold found in proteins of extremely diverse functions, including oxidoreductase activity, and has been implicated in reactive chlorine stress response in (per)chlorate reducing bacteria (Figure 5-4E) [93]. The cupin domain protein is one of the most common subfamilies in the dataset and, being found with Cld in 1487 genomes among 40 genera, has one of the lowest clustering coefficients (0.026). Five of the seven identical Cld found in multiple genera were encoded in transposons with the cupin domain protein, including the three most common Cld proteins: WP_001097412.1, found in 1221 genomes among 12 genera mostly in the *Enterobacteraceae*; WP_002718009.1, found in 112 genomes, 11 genera, and two classes; and WP_011514928.1, found in 32 genomes across 19 genera and three classes. A number of hypothetical proteins linked to Cld belong to families containing oxidoreductases (Table 5-1) and might therefore function in the oxidation of HOCl. Because of its association with Cld in mobile genetic elements, the cupin domain protein is the most likely of these proteins to be a HOCl oxidase. Such an enzyme would be more likely to occur in mobile genetic elements with Cld because of the synergism between HOCl oxidation to chlorite and chlorite degradation. While such activity is purely hypothetical, it is just one of a number of different processes suggested by genomic data that could produce chlorite from lower oxidation states of chlorine.

Chlorite and reactive chlorine species (RCS) response

Chlorite and the product of its two-electron reduction, hypochlorous acid (HOCl), cause broad cellular damage, particularly due to the oxidation of cysteine, methionine, and primary and secondary amines [98]. Because Cld prevents oxidative stress from chlorite, Cld gene neighborhoods likely encode other RCS response genes (Figure 5-6A). One of the most important components of RCS response is the reduction of methionine sulfoxide (MetO) [98]. Cells reduce MetO to Met using methionine sulfoxide reductases (MsrA, MsrB, and MsrP/YedY) and an electron donor such as thioredoxin (Trx) or transmembrane cytochrome b (MsrQ) [99]. Some cells co-express Msr and Met rich peptides (Mrp) to scavenge HOCl [93]. Despite the importance of MetO reduction in RCS response, the Mrp, MsrP, and MsrQ components of this system have been poorly defined.

First, new Mrp were defined by fitting a normal distribution to the mean Met content of all protein subfamilies and selecting proteins with exceptional Met content ($p< 0.00001$, >8.7% Met) (Figure 5-6B). We focused on Mrp genes found with MsrA, MsrB, or MsrP. These Mrp-Msr systems were found in (per)chlorate-reducing bacteria, soil and rhizosphere metagenomes, and *Methylorubrum* spp. (Figure 5-6C). The phylogenetic relationships between proteins in the MetO reductase system were investigated using a network analysis and prediction of signal peptides (Figure 5-6D). MsrA and MsrB proteins each formed one group and were occasionally found as protein fusions, as previously reported [99]. In contrast, Mrp formed multiple distinct groups had no significant sequence similarity to one another. Thus, short HOCl-scavenging peptides seem to have evolved independently multiple times, possibly *de novo* from non-coding DNA. A search for methionine rich peptides without the requirement of Cld will likely identify even more groups of Mrp.

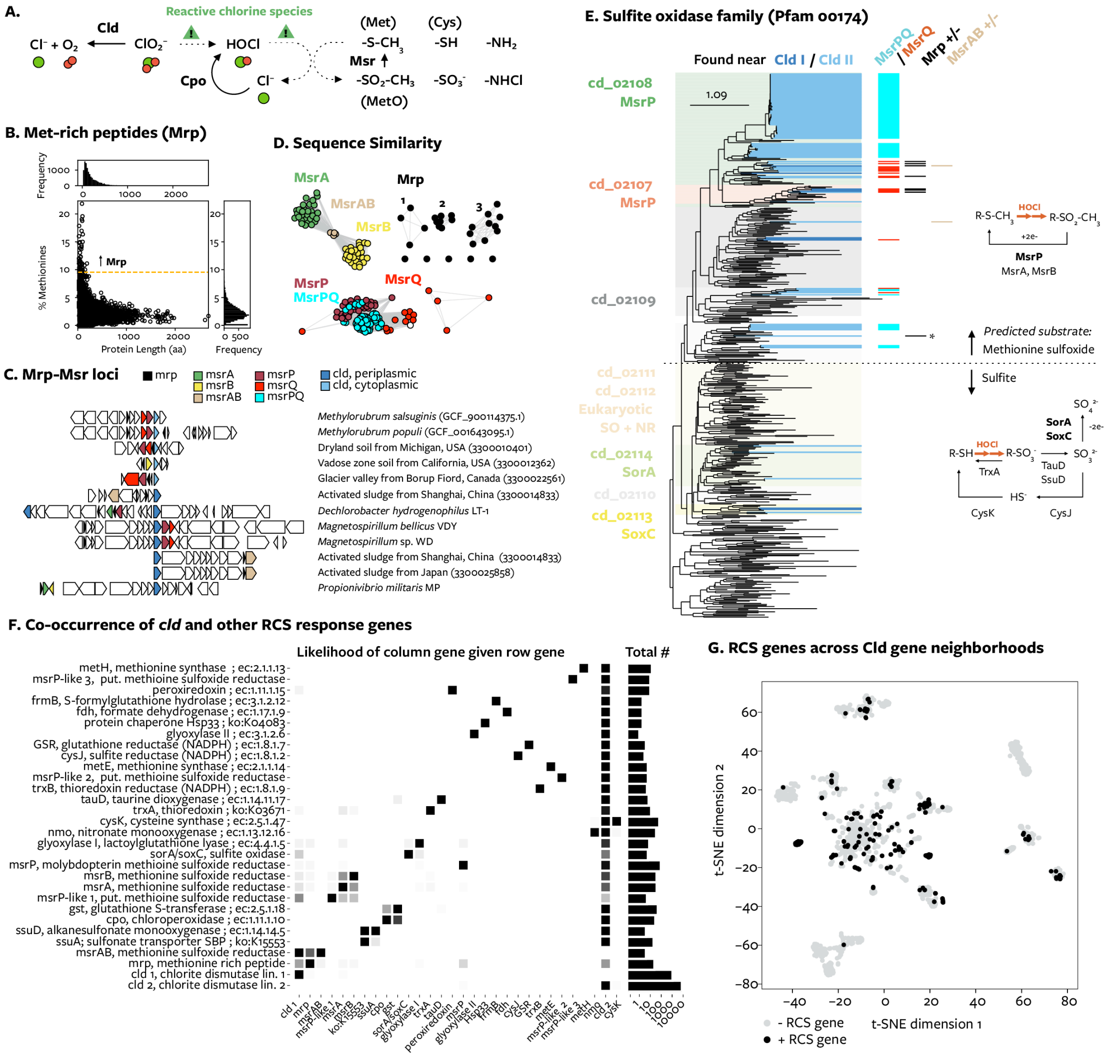


Figure 5-6. Association of Cld with genes for reactive chlorine species response. (A) Schematic diagram indicating why Cld, having chlorite as a substrate, would be associated with HOCl and genes for repairing HOCl-mediated oxidative damage. (B) Distribution of the mean methionine content in protein subfamilies (% methionine) used to define methionine rich peptides (Mrp). (C). Gene loci with both methionine rich peptides and methionine sulfoxide reductases (Msr). (D) A sequence similarity network indicating the relatedness of different proteins annotated as Msr or Mrp, with a complicated network topology for MsrP and MsrQ. (E) A maximum likelihood phylogenetic tree of Pfam 00174 to delineate MsrP from its related proteins. Proteins found with Cld are highlighted blue and further annotated by the presence of a fused or adjacent transmembrane subunit (MsrQ) and a nearby Mrp, MsrA, or MsrB. Clades are highly by their conserved domain annotation. (F) The coccurrence of reactive chlorine species response genes found with Cld. Shading indicates the frequency that the row gene is found with the column gene, from 0 (white) to 1 (black). The column at right is the number of genes detected. (G) Cld gene neighborhoods including reactive chlorine species response genes.

The characterization of MsrP and MsrQ was complicated by the network topology of these proteins consisting of multiple subgroups and fusions (Figure 5-6D). Any member of the sulfite oxidase family of molybdopterin enzymes (Pfam 00174) near Cld had been assumed to be MsrP, which would permit the inclusion of non-MsrP proteins [271]. To define which proteins in the sulfite oxidase family reduce methionine sulfoxide, a phylogenetic tree of Pfam 00174 was constructed from reference proteins and proteins found with Cld (Figure 5-6E). Proteins with Cld were further annotated with the presence of Mrp, other Msr proteins, and transmembrane subunits, indicating a source of chlorite, methionine, methionine sulfoxide, and a possible electron donor, respectively. Clades cd_02107 and cd_02108 had large numbers of proteins with these signatures of methionine sulfoxide reductase activity, which has only been described in proteins from these clades: MsrP from *Azospira suillum* PS (cd_02108) and subsequently MsrP from *Escherichia coli* and *Rhodobacter sphaeroides* (cd_02107) [93, 272, 273]. One subclade of cd_02108 consisted of a fusion between MsrQ and MsrP and was co-located with a formate/nitrite transporter family enzyme and cytoplasmic Cld on metagenomic contigs (neighborhood-group 17, referenced earlier). That clade, belonging to *Actinobacteria*, was the type of MsrP most commonly detected near Cld in metagenomes.

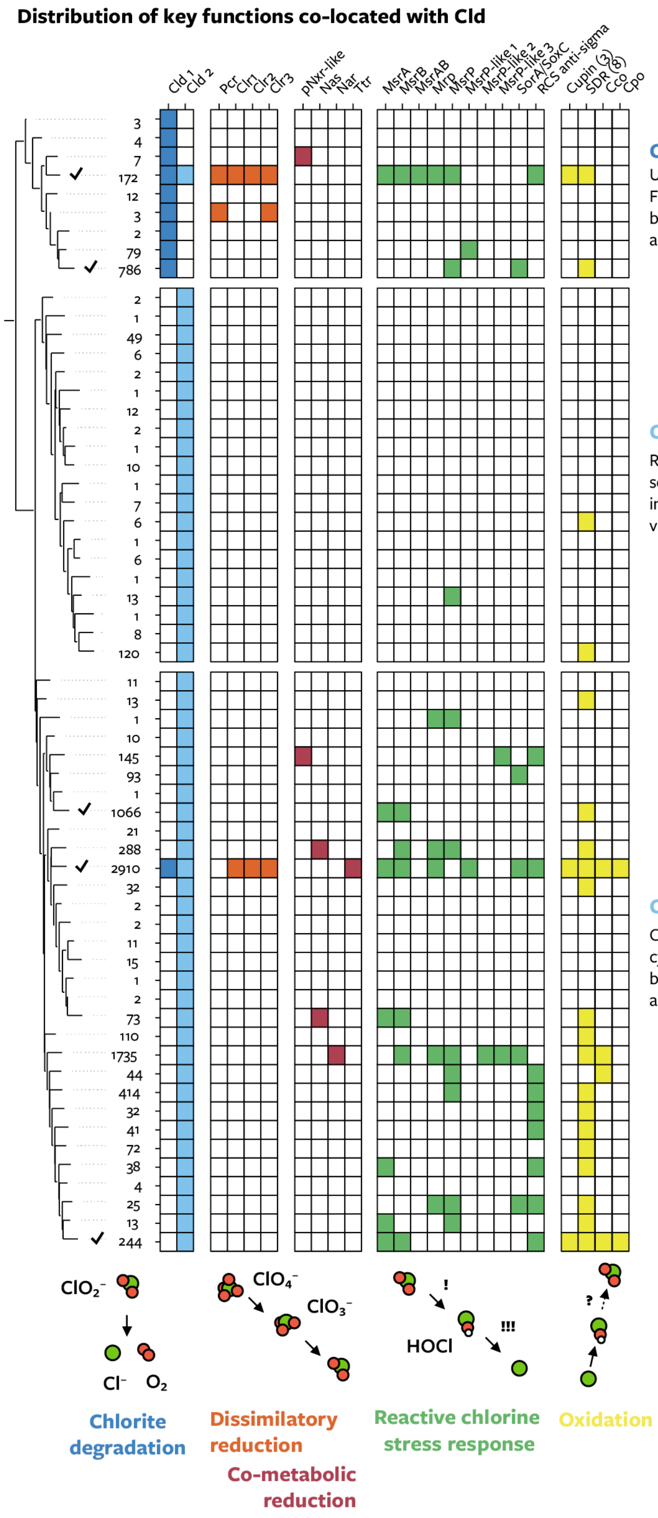
Cld was also detected near members of the SO family not yet reported to have MetO reductase activity (Figure 5-6E). Clade cd_02019, which is most closely related to MsrP but has no described enzyme activity [271], had MsrP-like proteins located near periplasmic Cld in *Nitrospira* spp. and cytoplasmic Cld and other oxidative stress response proteins (MsrA, MsrB, a peroxiredoxin, and base excision repair protein) in *Achromobacter* spp. Unlike MsrP, which is periplasmic, these MsrP-like proteins were cytoplasmic and could be found adjacent to FAD oxidoreductases that might act as electron donors. Another undefined clade contained MsrP-like proteins from *Actinobacteria* that were either fused or adjacent to a transmembrane cytochrome b subunit. The most distantly related clade of MsrP-like proteins located near cytoplasmic Cld and fused to a transmembrane domain. The one exception is the MsrP-like protein from a basaltic cave metagenome (Ga0187892_1000004275), which located near a putative periplasmic cytochrome that is also rich in methionine (8.8%). The methionine residues are concentrated between the cleaved signal peptide and the globular cytochrome domain in a disordered region where 23% of residues are Met. Potentially, this protein may act as a methionine sulfoxide substrate and as an electron donor for its partner MsrP-like protein. On the basis of their genomic association with Cld, Mrp, other reactive chlorine stress response proteins, and low-reduction potential electron donors (cytochrome b, FADH oxidoreductase), we predict that all of the uncharacterized clades above can function as methionine sulfoxide reductases.

Prokaryotic sulfite oxidases (SoxC, SorA) from this protein family were also found with Cld. Sulfite oxidases and dehydrogenases (clades cd_02114 and cd_02115), oxidize sulfite to sulfate either for energy, as in lithotrophic sulfur oxidation, or for detoxification of sulfite, as in organotrophic sulfur catabolism [271, 274]. Seven sulfite oxidases were found with either periplasmic or cytoplasmic Cld. Why? HOCl oxidizes sulfhydryls (R-SH) to sulfonates ($R-SO3^-$). In order to recycle the carbon and sulfur, the sulfonate is enzymatically cleaved to release sulfite, then either reduced to sulfide for assimilation or, to limit its toxicity, exported and oxidized to sulfate [275]. Enzymes for those reactions were also found in Cld gene neighborhoods, as were various other enzymes for sulfur homeostasis (Figure 5-6F). The production of both methionine sulfoxide and sulfonates from RCS raises the interesting possibility that MsrP originated from an ancestral sulfite oxidase responding to RCS. The association of Cld with proteins across the sulfite oxidase family distinguishes it as an important family for understanding sulfur homeostasis during reactive chlorine species response.

Cld was also found with genes for responding to targets of reactive chlorine species other than methionine and cysteine (Figure 5-6E). Formaldehyde response enzymes, such as formylglutathione hydrolase, were encoded near Cld in *Acinetobacter baumannii* OIFC074. HOCl reacts readily with amines such as amino acids, and the chloramines that are produced spontaneously dissociate into aldehydes [60, 80, 98]. Glyoxalase I, which is part of a glutathione-based system to detoxify glyoxals, was found in Cld gene neighborhoods with Msr proteins and sulfite oxidases (Figure 6E). Glyoxals can be produced by the oxidation of glucose by HOCl [276]. Reflecting the reactivity of HOCl and the large number enzymes used to repair oxidative stress, genes for responding to reactive chlorine species could be found in nearly every type of Cld gene neighborhood (Figure 6F). Genomic regions with Cld are therefore a rich, untapped resource for identifying new genes that sense or respond to reactive chlorine species.

Cld and chlorine oxyanion biology

Chlorite degradation by Cld is important for the fitness of organisms other than dissimilatory perchlorate- and chlorate-reducing bacteria. Across its phylogeny, Cld has conserved active site residues for activity (Figure 5-2) and is found with various enzymes that produce chlorite or respond to reactive chlorine stress (Figure 5-7). Organisms use Cld to degrade chlorite produced by the inadvertent reduction of perchlorate and chlorate (Figure 5-4) and possibly chlorite produced by the oxidation of lower oxidation states of chlorine (Figure 5-5). Some data suggested that Cld might even be used to produce oxygen from chlorite in oxygen-limiting environments. Thus, the roles of Cld in biology are more varied than dissimilatory perchlorate and chlorate reduction (Figure 5-7). The presence of Cld in diverse taxa and various oxic environments shows that these roles are broadly important in nature (Figure 5-1).



Cld 1

Usually longer and periplasmic. Found in (per)chlorate-reducing bacteria, nitrite-oxidizing bacteria, and halophilic archaea.

Cld 2B

Rare, intermediate size, and sometimes periplasmic. Found in nitrite-oxidizing bacteria and various slow-growing bacteria.

Cld 2A

Common, usually smaller and cytoplasmic. Found diverse bacteria and occassionally in archaea and eukarya.

Figure 5-7. The distribution of genes predicted to be involved with key functions across the phylogeny of Cld. Check marks indicate clades that include biochemically verified Cld. The number of proteins in each clade is indicated at the leaf of each branch. Enzyme abbreviations are as follows: Cld, chlorite dismutase; Pcr, dissimilatory perchlorate reductase; Clr, dissimilatory chlorate reductase; pNxr, periplasmic nitrite oxidoreductase; Nas, assimilatory nitrate reductase; Nar, dissimilatory nitrate reductase (cytoplasmic); Ttr, tetrathionate reductase; Fdh, formate dehydrogenase; Msr, methionine sulfoxide reductase; Mrp, methionine rich peptide; SorA, sulfite dehydrogenase; SoxC, sulfite oxidase; RCS anti-sigma, reactive chlorine species stress-sensing anti-sigma factor (subfamily 37); Cupin, uncharacterized cupin domain protein (subfamily 3); SDR, uncharacterized SDR family oxidoreductase (subfamily 8); Cco, cytochrome c oxidase; and Cpo, chloride peroxidase.

Anthropogenic chlorite, too, now shapes the roles and evolution of Cld. Bacterial isolates and metagenome-assembled genome (UBA7901, UBA7900, UBA5190, UBA5191) from drinking water systems were detected with Cld, and Cld-containing organisms could be a majority of microbial communities (Figure 5-5F). Cld was readily detected in metagenomes from water treatment plants, including clades of Cld most closely related to perchlorate- and chlorate-reducing bacteria. *Piscinibacter defluvii* was isolated from a wastewater treatment plant and had complete set of genes for dissimilatory chlorate reduction [277]. *Nitrosomonas mobilis* Mb-1 encoding periplasmic Cld was isolated from a wastewater treatment plant that used chlorine-based disinfectants (personal communication). Cld has never before been observed in aerobic ammonia-oxidizing microorganisms, and a fragment of PcrD that indicates the genes were a recent acquisition from perchlorate- or chlorate-reducing bacteria [87]. The simplest explanation is that the use of chlorite-producing disinfectants in wastewater treatment processes has selected for organisms with Cld and for the horizontal transfer of Cld to new organisms. Thus, with the use of oxidized chlorine by humans, microorganisms today must use Cld against chlorite from both natural and unnatural sources.

Conclusions

The use of Cld to degrade chlorite, once thought unique to anaerobic perchlorate- and chlorate-reducing bacteria, is common in various types of microorganisms and in many oxic environments. Cld is rarely conserved within a phylogenetic lineage and has been subjected to horizontal gene transfer between distantly related organisms multiple times, indicating intermittent selection. Comparative genomics indicated that Cld has conserved activity across its phylogeny but participates in several different processes. In addition to respiratory perchlorate and chlorate reduction, the co-metabolic reduction of perchlorate or chlorate selects for Cld in natural populations. Some organisms have Cld but do not have any known perchlorate or chlorate reductases; the provenance of those organisms, the content of gene neighborhoods, and the broad distribution of Cld strongly suggest that chlorite can be produced by oxidative chemistry. Because the reduction of chlorite to hypochlorous acid produces substantial oxidative stress, Cld is co-localized with various genes for sensing and responding to reactive chlorine species. While a source of stress, chlorite may also be a benefit for microorganisms in oxygen-limited conditions. This research identifies the quantification of metabolic and co-metabolic (per)chlorate reduction in nature and the production of chlorite by oxidative (photo)chemistry as major research avenues and provides a set of gene targets that are highly likely to be involved in chlorine metabolism.

Tables

Table 5-1. Genetic linkage of protein subfamilies to Cld. All subfamilies with a network clustering coefficient of less than 0.1 are shown. Columns: SID, subfamily ID; CC, clustering coefficient; Gene Function, the predicted role for the gene in chlorine oxyanion biology; Length (aa), mean protein length; Count, total number of genes in the in the subfamily found in Cld gene neighborhoods; and Examples, RefSeq accession for proteins in the subfamily from different classes of organisms.

SID	CC	Gene Product	Gene Function	Length (aa)	Count	Examples
cld_2	0.002	Chlorite dismutase, group 2	Chlorite degradation	188	7721	NP_773991.1, NP_924112.1
cld_1	0.019	Chlorite dismutase, group 1	Chlorite degradation	264	1069	WP_011288310.1, WP_013247962.1
3	0.026	Cupin domain-containing protein	Unknown	131	1591	WP_083761619.1, WP_011914407.1
19	0.027	ABC transporter ATP-binding protein	Transport	285	215	WP_008060994.1, WP_083842800.1
16	0.028	tRNA	Genetic mobility		260	
8	0.029	SDR family NAD(P)-dependent oxidoreductase	Unknown	258	409	NP_773983.1, WP_012078779.1
23	0.042	Site-specific DNA recombinase	Genetic mobility	193	201	WP_012435588.1, WP_001556711.1
80	0.050	Sigma-70 factor (ECF subfamily)	Regulation	217	70	NP_924104.1, WP_026605404.1
67	0.060	Signal transduction histidine kinase	Regulation	440	79	WP_003032875.1, WP_085107402.1
4	0.063	Transposase (IS66 family)	Genetic mobility	157	1202	WP_001515734.1
66	0.067	DNA-binding transcriptional regulator (LysR family)	Regulation	298	81	WP_020307717.1, WP_012435586.1
88	0.068	DNA-binding response regulator (OmpR family)	Regulation	226	65	WP_020177235.1, WP_001572351.1
53	0.071	DNA-binding response regulator (NarL/FixJ/NrtC family)	Regulation	188	86	WP_012078773.1, WP_007803317.1
34	0.072	Site-specific recombinase (XerD family)	Genetic mobility	321	139	WP_011914410.1
85	0.072	NADPH:quinone reductase-like Zn-dependent oxidoreductase	Unknown	332	68	WP_008175571.1, WP_007535313.1
1	0.073	Thermonuclease family protein	Genetic mobility	92	2334	WP_000046891.1
119	0.073	Signal transduction histidine kinase (NrtC family)	Regulation	650	51	WP_014235261.1, WP_066325839.1
15	0.074	Transposase (IS26 family)	Genetic mobility		269	WP_031992596.1, WP_038573166.1
33	0.074	Integrase	Genetic mobility	677	144	WP_011914409.1
60	0.074	DUF4113 domain-containing DNA polymerase	Genetic mobility	321	84	WP_080695106.1, WP_043755420.1
71	0.074	Translesion error-prone DNA polymerase V, umuD	DNA repair or salvage	134	77	WP_011914412.1, WP_043760689.1
99	0.075	DNA-binding transcriptional regulator (LysR family)	Regulation	296	57	WP_012078780.1, WP_012237598.1
64	0.077	Perchlorate/chlorate reductase, subunit beta	Perchlorate or chlorate reduction	323	81	WP_011288313.1, WP_029134664.1
57	0.081	Perchlorate/chlorate reductase, subunit delta	Perchlorate or chlorate reduction	212	84	WP_049758697.1, WP_037375986.1
70	0.081	TTT family transporter, receptor subunit	Transport	294	77	WP_009515871.1, WP_082751643.1
61	0.082	Perchlorate/chlorate reductase, subunit alpha	Perchlorate or chlorate reduction	904	83	WP_011288314.1, WP_037375984.1

55	0.082	Alpha/beta hydrolase family protein	Unknown	279	85	WP_009734230.1, WP_026779386.1
205	0.083	Enoyl-CoA hydratase/isomerase family protein	Other	228	33	WP_019497504.1, WP_083525184.1
77	0.084	Acetate-CoA ligase	Other	508	71	WP_009734228.1, WP_060979399.1
37	0.085	RCS-sensing anti-sigma factor, DUF1109 domain-containing	Oxidative stress response	208	132	WP_011288319.1, WP_003549388.1
54	0.085	Transposase (Tn3 family)	Genetic mobility	813	85	WP_012077404.1, WP_000124025.1
6	0.085	Gamma-glutamylcyclotransferase family protein	Oxidative stress response	114	665	NP_773992.1
116	0.085	Alpha/beta hydrolase family protein	Unknown	281	52	WP_040512021.1, WP_063988050.1
240	0.086	NAD(P)-dependent oxidoreductase	Unknown	310	29	WP_017285547.1, WP_020564915.1
62	0.088	RCS-sensing sigma factor	Regulation	177	83	WP_011288320.1, WP_003549387.1
93	0.091	NAD(P)/FAD oxidoreductase, glutathione sulfide reductase-like	Oxidative stress response	488	61	WP_008567178.1, WP_003158917.1
84	0.091	Alkylhydroperoxidase, AhpD-like	Oxidative stress response	182	68	WP_007535291.1, WP_036008191.1
97	0.091	Plasmid stabilization system toxin	Genetic mobility	97	59	WP_011342942.1, WP_094538652.1
107	0.091	Nitrate/sulfonate/bicarbonate ABC transporter permease	Transport	279	54	WP_007803303.1, WP_023100455.1
123	0.092	Peroxiredoxin	Oxidative stress response	165	50	WP_020096154.1, WP_058937083.1
95	0.092	Plasmid stabilization system antitoxin	Genetic mobility	91	60	WP_011342941.1, WP_011342941.1
11	0.092	DNA primase	Genetic mobility	604	310	NP_773990.1
128	0.093	Class I SAM-dependent methyltransferase	Unknown	228	48	WP_025297811.1, WP_083129309.1
22	0.093	DNA-binding transcriptional regulator (ArsR family)	Regulation	110	205	NP_773999.1, WP_103275825.1
14	0.095	N-formylglutamate amidohydrolase	Oxidative stress response	264	292	NP_773994.1
13	0.096	Adenylosuccinate lyase	DNA repair or salvage	425	304	WP_013247961.1, WP_033925750.1
162	0.098	Peptide-methionine (S)-S-oxide reductase	Oxidative stress response	203	38	WP_081614707.1, WP_003464967.1
110	0.098	DNA-binding transcriptional regulator (HxlR family)	Regulation	137	53	WP_015215298.1, WP_036002077.1
120	0.098	Nitrate/sulfonate/bicarbonate ABC transporter substrate-binding protein	Transport	422	51	WP_007803301.1, WP_023100454.1
74	0.099	DNA polymerase V, umuC	Genetic mobility	73	75	WP_011914411.1
177	0.100	Ferredoxin of nitrite reductase or dioxygenase	Other	110	36	WP_041756587.1, WP_005004323.1
129	0.100	Glutathione S-transferase family protein	Oxidative stress response	211	48	WP_015215307.1, WP_025659664.1
159	0.100	Peptide-methionine (R)-S-oxide reductase	Oxidative stress response	167	38	WP_019497506.1, WP_003464965.1

Chapter 6
Future Directions

The above research highlights three major gaps in our understanding of the biogeochemical chlorine cycle.

Quantifying perchlorate and chlorate reduction in the environment

For some metabolisms, like nitrification, kinetics for the genes and organisms performing the metabolism has enabled a quantitative description of the metabolism in different environments [278]. The most important question is what the relative contribution of co-metabolism to perchlorate and chlorate reduction is and how might that change based on the concentrations of perchlorate, chlorate, and other substrates. If substantial, co-metabolic perchlorate and chlorate reduction may be an important selective pressure in some habitats via the production of reactive chlorine species. Another question is whether the interaction between perchlorate- and chlorate-reducing bacteria persists at naturally low concentrations of perchlorate.

The discoveries described in chapters four and five significantly inform quantitative models by identifying the interaction between perchlorate- and chlorate-reducing bacteria (chapters two and three), by describing the different enzymes involved in respiratory perchlorate and chlorate reduction in natural environments (chapters two, four, and five), and by confirming co-metabolic reduction of perchlorate and chlorate and the co-option of Cld for detoxification (chapter five). These findings emphasize the type of reductase and its localization, the presence or absence of Cld, and the composition of the microbial community as important considerations for any model.

Previous research has purified and measured the kinetics of perchlorate and chlorate reduction for respiratory perchlorate and chlorate reductases as well as a few enzymes with inadvertent perchlorate and chlorate reductase activity. Chapters four and five of this dissertation described a new type of chlorate reductase and finds that many enzymes in the DMSO reductase family of molybdopterin enzymes reduce perchlorate or chlorate in nature. Measurements of enzyme kinetics for these enzymes would provide better parameters for understanding the growth of perchlorate and chlorate reduction in the environment. The development of a system for heterologous expression and purification of DMSO reductase family enzymes would provide a substantial advance. Important considerations are (1) the need for proteins other than the catalytic subunit for enzyme activity, such as a molybdopterin chaperone (PcrD) and (2) the variation in catalytic activity even within protein subfamilies, such as that observed for Nap towards chlorate [279]. An initial description of this family by comparative genomics would be a helpful aid. Such research would ultimately help predict which proteins reduce perchlorate and chlorate and at what rates and concentrations. While enzymes that are most common or have been previously associated with Cld should be prioritized, this research could also help to describe completely unknown clades of enzymes in the DMSO reductase family (e.g. YdeP, MopB_2) and to understand the metabolism of other halogen oxyanions like iodate and bromate.

Identifying the oxidative source of chlorite (and other chlorine oxyanions)

Chlorine is oxidized to perchlorate and chlorate by atmospheric photochemistry and to hypochlorous acid by biochemical reactions in the biosphere. Therefore, unlike other elements in biology, higher oxidation states of chlorine are not known to be produced in the biosphere. Chapter five describes how the distribution of chlorite dismutase (Cld) suggests that chlorite can be produced by oxidative chemistry. Investigating the suggested chemical, photochemical, or biochemical sources of chlorite is a high-risk, high-reward research project. Reactive nitrogen species (e.g. peroxynitrite) were identified as a possible source of chlorite, perhaps via the byproducts of their reaction with biomolecules (e.g. carbonate radical). Photochemical reactions with chloride or hypochlorous acid are another possible source, despite the degradation of chlorite in pure solution by UV radiation [84], because of potential interactions with other organic or inorganic components of natural waters. Or perhaps enzymes have evolved to oxidize hypochlorous acid to chlorite to lower its reactivity. A preliminary screen for enzymatic hypochlorous acid oxidation could be performed by (1) culturing diverse bacteria, (2) exposing them to sub-inhibitory concentrations of hypochlorous acid to activate reactive chlorine responses, and (3) testing cell lysate for the oxidation of hypochlorous acid to chlorite or chlorate (chlorite is oxidized by hypochlorous acid to chlorate). The identification of chlorite produced from chloride or hypochlorous acid would be the first major transformation of chlorine discovered within the last two decades.

Describing the roles of reactive chlorine species in biology

Reactive chlorine species (RCS) contribute to oxidative stress in many natural environments and are a significant selective pressure in the built environment. However, nearly all research on RCS has studied human microbiome-associated bacteria stressed by hypochlorous acid. Chapter five identified uncharacterized genes that likely function in RCS response because they are genetically linked to Cld. These and many more genes for reactive chlorine species response would likely be discovered through simple high-throughput genetic screens of environmental bacteria. For example, one of the many screens that Price, Wetmore [252] performed identified numerous genes important for fitness under chlorite or chlorate stress in several environmental bacteria. Preferably, genes identified as important for RCS response will be subject to careful biochemical characterization. But even a genetics approach by itself would be quickly multiplied in scientific value, as the large catalogue of genomic and metagenomic sequences means that new genes can immediately be linked to different microbial habitats, helping to identify sources of RCS. Understanding the sources and roles of reactive chlorine species in different organisms is a broad topic that, like reactive oxygen or nitrogen species, will both occupy an entire field of study and be deeply intertwined with other oxidative processes. Starting by identifying genes for microbial responses to reactive chlorine species will provide a solid foundation for future research.

Chapter 7
Conclusions

Previously to this work, the biology of dissimilatory perchlorate-reducing bacteria and dissimilatory chlorate-reducing bacteria had been described from the perspective of physiology, biochemistry, genetics, genomics, and synthetic biology. In other words, scientists before me had described how these metabolisms work, how they fail, and how they vary.

Studying dissimilatory perchlorate reduction from the perspective of ecology led to unexpected insights and, ultimately, a desire to understand the evolutionary biology and biogeochemistry of chlorine as well. The general findings of this dissertation, in chronological order, are that perchlorate-reducing communities involve metabolic interactions; that chlorate-reducing bacteria interact with perchlorate-reducing bacteria by consuming the intermediate chlorate; that chlorite dismutase (Cld) is found in more metabolically diverse organisms than previously understood; that additional enzymes function as perchlorate or chlorate reductases; that chlorite may be produced from oxidizing chemistry; and that many known and unknown genes are genetically linked to chlorite degradation. A clearer view of the biogeochemical chlorine cycle was also constructed through a review of literature in several disciplines.

These findings together shift several perspectives on chlorine metabolism:

When microbes respire perchlorate, this activity is not isolated but entwined in a web of ecological interactions. The reactivity of oxidized chlorine molecules alters the chemical environment around perchlorate-reducing bacteria. Sulfur or metals like iron are oxidized, providing respiratory electron acceptors for organisms that cannot respire perchlorate or chlorate. In the absence of chemical reductants, perchlorate reduction leads to the accumulation of chlorate, providing respiratory electron acceptors for chlorate-reducing bacteria. The activity of these other organisms can in turn affect the rate of perchlorate reduction and the overall structure of the microbial community.

The evolution of perchlorate and chlorate reduction is much more complicated than an ancient combination of a (per)chlorate reductase with Cld followed by co-evolution. Genomic and metagenomic data demonstrate that multiple different types of perchlorate or chlorate reductases have independently acquired Cld. Only some of those reductases have evolved for the respiration of perchlorate and chlorate. Within the genomic islands for perchlorate and chlorate respiration, different reductases or Cld have been swapped for one another, and some islands contain both perchlorate reductase and chlorate reductase; this gene transfer may be facilitated by the ecological interaction of perchlorate- and chlorate-reducing bacteria. All types of chlorate reductase have been observed at least once with both periplasmic Cld and cytoplasmic Cld, indicating intracellular chlorite stress may differ between metabolisms. Additionally, the periplasmic Cld in perchlorate- and chlorate-reducing bacteria has been recurrently exchanged with nitrite-oxidizing bacteria, and itself evolved later than some forms of

Cld, bringing into question the timeline by which different components of this metabolism evolved.

Perchlorate and chlorate reduction are not isolated metabolisms but evolutionarily linked to other processes in the biogeochemical chlorine cycle. For example, the evolution of chloromethane-producing enzymes likely increased the flux of chlorine to the stratosphere and, in turn, the flux of perchlorate and chlorate to Earth's surface. A more direct link is that perchlorate- and chlorate-reducing bacteria have acquired genes that evolved in other organisms, such as periplasmic molybdopterin methionine sulfoxide reductases and oxidative stress signaling proteins, that respond to hypochlorous acid produced by haloperoxidases and by abiotic chemistry. Even Cld is more common beyond perchlorate- and chlorate-reducing bacteria. Therefore, canonical perchlorate and chlorate reduction are metabolisms that have been layered on top of previously evolved chlorine metabolisms.

Other oxidized chlorine molecules play a more important role in biology than currently recognized. The role of perchlorate-reducing bacteria in preventing the accumulation of perchlorate is rightly emphasized, but using the number of genes in genomic databases as a heuristic, the amounts of oxidized chlorine produced in the environment follow hypochlorous acid > chlorite > perchlorate > chlorate. i.e., many more organisms are affected by hypochlorous acid and chlorite than by perchlorate or chlorate. About 5% of prokaryotic genera contained a genome with Cld, and many more genomes have genes for responding to reactive chlorine species. The use of chlorine disinfectants – molecular chlorine, hypochlorous acid, hypochlorite, and chlorine dioxide – means that hypochlorous acid and chlorite have an important impact on evolution in the built environment. Because the biology of perchlorate and chlorate has been largely settled by this study and previous studies, the biology of other hypochlorous acid and chlorite deserve substantially more scholarship.

I advise future researchers to use the trove of genomic sequences – perchlorate reductases, chlorate reductases, chlorite dismutase, and various enzymes predicted to be for reactive chlorine species response – that I provided as the starting point for future research. The comparative genomics of oxidized chlorine has yielded an abundance of information and hypotheses that remain to be tested.

The physiology *and* ecology of perchlorate and chlorate reduction are now well-defined parts of a biogeochemical chlorine cycle. The other roles for oxidized chlorine in this cycle remain to be understood.

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