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Phylogeny and Physiology of Marine Dissimilatory Perchlorate Reducing Bacteria

By

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Abstract

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Perchlorate (ClO_4^-) is a toxic, water-soluble oxyanion of chlorine that is naturally and anthropogenically produced. Because of its extensive industrial uses and unregulated disposal prior to 1997, perchlorate contamination of water sources is widespread. In humans, perchlorate inhibits iodide uptake by the thyroid gland, which can lead to hypothyroidism. As a result, the Environmental Protection Agency decided to regulate perchlorate under the Safe Water Drinking Act of 2012.

Dissimilatory perchlorate reducing bacteria (DPRB) can grow by respiring perchlorate (ClO_4^-) or chlorate (ClO_3^-) [collectively denoted (per)chlorate] to innocuous chloride (Cl^-). Even though about 80 strains of DPRB have been isolated to date (Chapter 1), most of these isolates have been obtained from freshwater, mesophilic, neutral pH environments. As a result, most of these microorganisms do not tolerate high temperatures, salinities, or extreme pH. With few exceptions, most DPRB belong to the Alpha, Beta, Gamma and Epsilon classes of the phylum Proteobacteria. The current work aims to expand the known environmental range, metabolic strategies, and taxonomic diversity of DPRB.

Shallow sediment samples from a marina in Berkeley, CA were used to study marine perchlorate reduction. Enrichments were set up at 1, 3, 5, 7, and 10% NaCl with acetate as an electron donor and perchlorate as an electron acceptor; perchlorate was consumed in salinities of up to 7% NaCl (Chapter 2). Microbial community analysis revealed that the most active members of the community were in families Rhodocyclaceae (1% and 3% NaCl), Pseudomonadaceae (1% NaCl), Campylobacteraceae (1%, 5%, and 7% NaCl), Sedimenticolaceae (3% NaCl), Desulfuromonadaceae (5% & 7% NaCl), Pelobacteraceae (5% NaCl), Helicobacteraceae (5% & 7% NaCl), and V1B07b93 (7% NaCl; phylum Deferribacteres) (Chapter 2). DPRB in the genera *Sedimenticola*, *Azoarcus*, *Pseudomonas*, *Denitromonas*, and *Marinobacter* (Chapter 2) were isolated.

To further study the physiology and metabolic potential of marine DPRB, an *Arcobacter* sp. was isolated from marine sediment from the Berkeley Bay, and was fully characterized and genome sequenced (Chapter 3). *Arcobacter* sp. CAB is the only perchlorate reducing bacterium

(PRB) in pure culture belonging to the Epsilonproteobacteria. Interestingly, CAB lacks the *pcrC* gene previously thought to be essential for perchlorate reduction. Additionally, CAB can couple the oxidation of the aromatic compound catechol to perchlorate reduction in anaerobic conditions. However, it utilizes an aerobic pathway that requires oxygen as a co-substrate for an oxygenase. Thus, *Arcobacter* sp. CAB represents the first example of a PRB that can utilize an aerobic pathway for aromatic degradation with perchlorate as an electron acceptor by utilizing oxygen produced from chlorite dismutation in otherwise anaerobic conditions.

The PRB *Sedimenticola selenatireducens* CUZ, also isolated from Berkeley Bay sediment, and the chlorate reducing bacteria (CRB) *Dechloromarinus chlorophilus* NSS, isolated from San Diego Bay sediment, were fully characterized and represent the first case of two highly related microorganisms (99% 16S rRNA identity), one of which is a perchlorate-reducing bacterium (PRB) and one of which is a chlorate-reducing bacterium (CRB; Chapter 4). Both strains are metabolically versatile, and can oxidize the aromatic compounds benzoate and phenylacetate coupled to the reduction of oxygen, perchlorate, and nitrate (Chapter 5). Both strains encode aerobic-hybrid and anaerobic pathways of phenylacetate and benzoate degradation. While *S. selenatireducens* acts as a true anaerobe and predominantly utilizes the anaerobic pathways with perchlorate as an electron acceptor, *D. chlorophilus* NSS may utilize a mixture of aerobic and anaerobic pathways when respiring on chlorate (Chapter 5).

The marine, perchlorate-reducing microbial community and novel isolates studied in this work greatly contribute to the current knowledge in the field of microbial perchlorate reduction. With the exception of *Marinobacter*, these new isolates represent the first known perchlorate-reducers in each genus (Chapter 2), thus expanding the known phylogeny of DPRB which are dominated by the genera *Dechloromonas* and *Azospira* (Chapter 1). Further, the characterization of the PRBs *Arcobacter* sp. CAB and *S. selenatireducens* CUZ and the CRB *Dechloromarinus chlorophilus* NSS has revealed several mechanisms by which DPRB degrade aromatic compounds and the choices these microorganisms make in respect to the oxygen they produce from the dismutation of chlorite. Further characterization and genome sequencing of the other novel DPRB obtained in this study is of great importance, and will likely aid in the study of the evolution of perchlorate metabolism and in understanding the role each of these isolates in microbial communities.

For my parents

Peter Carlstrom and Mariana Carlstrom

and my beloved husband

Robert A. Rohde

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Chapter 1: Introduction

What is Perchlorate?

Perchlorate (ClO_4^-) is a water-soluble, chemically stable, oxyanion of chlorine that is extensively used in pyrotechnics, rocket fuel, flares, explosives, lubricants, and paints (1-3). Although industrially valuable, perchlorate is detrimental to human health and can lead to hypothyroidism by competitively inhibiting the uptake of iodide by the thyroid gland (4-6). Additionally, because thyroid hormones are necessary for brain development (7, 8), exposure to thyroid gland inhibitors like perchlorate in developing fetuses and infants can lead to low IQ, ADHD, reading and language deficits, behavioral problems, and cerebral palsy (9).

Although originally thought to be exclusively of anthropogenic origin, it is now known that perchlorate is also naturally produced (10-13). The detection of perchlorate in several arid pristine environments, including the Atacama Desert in Chile (14, 15), the Antarctic Dry Valleys (16), and Martian regolith (17) points towards an ancient natural geochemical origin, which is further supported by stable isotope analysis of chlorine and oxygen that allows for the distinction between natural and synthetic sources (13).

In the United States, studies of human exposure to perchlorate find that low level exposure is ubiquitous through food and water; all urine samples from 2820 adults contained detectable perchlorate levels (>0.05 μg/L) with a median concentration of 3.6 μg/L (18). Average exposure through food was estimated to be 0.08-0.39 μg/kg body mass/day (19) and the EPA has adopted a reference dose of 0.7 μg/kg/day for acceptable exposure (20). Based on this, the EPA recommended an interim health advisory level for perchlorate of 15 μg/L in drinking water (20). To date, the EPA has not adopted an official maximum contaminant level for perchlorate in drinking water, though they have begun the advisory process for adopting such a level (21). California and Massachusetts have adopted drinking water limits of 6 μg/L and 2 μg/L, respectively; and 10 other states have also developed advisory limits ranging from 1-18 μg/L (22).

After being added to the potential contaminant list, systemic testing for perchlorate in drinking water was conducted in 3870 public water systems, and levels greater than 4 μg/L were reported in 160 systems (4.1%) across 26 states (23). Additionally, detectable levels of perchlorate have been measured in soil and groundwater in at least 45 states (24). The highest density of perchlorate detections occurred in Southern California, Massachusetts, New Jersey, Long Island, and West Texas. However, this distribution may be driven by an emphasis on testing in states that regulate perchlorate more so than a true reflection of contamination patterns (25). Contamination is often associated with sites that previously manufactured perchlorate or used it as a fuel for aerospace applications. Approximately 20% of 407 Department of Defense sites sampled exceeded the 15 μg/L advisory level (24). Edwards Air Force Base in California, which includes a rocket test facility, has measured perchlorate levels in groundwater of 160,000 μg/L and the site of former perchlorate manufacturer Kerr-McGee in Henderson, Nevada has

observed levels of 350,000 μg/L (26).The Kerr-McGee contamination plume is estimated to contain approximately 20 million pounds of perchlorate (26).

Dissimilatory (Per)chlorate Reducing Bacteria

Microorganisms have evolved that can grow by respiring perchlorate (ClO_4^-) and chlorate (ClO_3^-) [collectively denoted (per)chlorate] in the absence of oxygen (1). These microorganisms, known as dissimilatory (per)chlorate reducing bacteria (DPRB), are metabolically and phylogenetically diverse and have been found in diverse environments, including pristine and hydrocarbon-contaminated soils, aquatic sediments, paper mill waste sludge, farm animal waste lagoons, underground gas storage reservoirs, and plant root homogenates (27-35) (Table 1). More than 80 strains of DPRB in 24 genera have been isolated to date (Table 1; includes DPRB isolated in this work). However, the majority of these isolates have been obtained from freshwater, mesophilic, neutral pH environments (Table 1). Exceptions include *Sporomusa* sp. An4 and the thermophilic *Moorella perchloratireducens* An10, which were isolated from an underground gas storage reservoir in Russia; *Shewanella algae* ACDC and *Dechloromarinus chlorophilus* NSS (27) (Chapter 4) which were isolated from sediment from the San Diego Bay; and *Arcobacter* sp. CAB and *Sedimenticola selenatireducens* CUZ, obtained from shallow sediment from the Berkeley Bay (Chapters 3 and 4; Table 1). Additionally, *Archaeoglobus fulgidus* VC-16, originally isolated from marine hot vents near Vulcano island in Italy (36), is the only archaeon that has been shown to grow by perchlorate reduction (37). However, unlike other DPRB, *A. fulgidus* degrades perchlorate using a combination of biotic and abiotic reactions (37). Given the inoculum sources for most DPRB enrichments, it is not surprising that most isolates cannot tolerate salinities > 2% NaCl and temperatures > 45 °C. (Table 1).

From the more than 80 DPRB isolates obtained to date, the majority belong to the Betaproteobacteria (44), Alphaproteobacteria (15), and Gammaproteobacteria (14) classes in the phylum Proteobacteria (Table 1; Fig. 1). Additionally, two DPRB belonging to the Epsilonproteobacteria (38) (Chapter 3) and the Firmicutes have been described (29, 30); and, as mentioned previously, *A. fulgidus* is the only archaeon known to reduce perchlorate (37).

DPRB are metabolically diverse and have been shown to utilize a variety of organic (acetate, lactate, propionate, valerate, butyrate, pyruvate, fumarate, succinate, methanol, ethanol, fructose, benzoate, phenylacetate, catechol, benzene, *n*-alkanes) and inorganic (hydrogen, sulfide, and ferrous iron) electron donors (1, 28, 30, 32, 33, 35, 38-45) (Chapters 3 and 4). Notably, *Dechloromonas aromatica* RCB was the first microorganism shown to be able to degrade benzene anaerobically (35). DPRB are more restricted with their use of electron acceptors. In addition to (per)chlorate, oxygen and nitrate are commonly utilized; sulfate (*Archaeoglobus fulgidus* VC-16 and *Dechloromonas denitrificans* ED-1), sulfite (*Shewanella algae* ACDC), manganese (*Azospira oryzae* GR-1 and *Dechlorobacter hydrogenophilus* LT-1)

and iron (*Shewanella algae* ACDC) are rarely consumed; and arsenate and selenate have never been shown to be utilized (1, 28, 30, 32, 33, 35, 38-46) (Chapters 3 and 4).

Mechanisms and Genetics of (Per)chlorate Reduction

DPRB are further divided into two subgroups, perchlorate reducing bacteria (PRB) and chlorate reducing bacteria (CRB). While PRB can reduce ClO_4^- and ClO_3^- to chlorite (ClO_2^-) using the perchlorate reductase (Pcr) (1, 47-49), CRB can only respire ClO_3^- using the chlorate reductase (Clr) enzyme (50-52). Pcr or Clr reduces ClO_4^-/ClO_3^- or ClO_3^- , respectively, to the toxic, reactive intermediate ClO_2^-, and chlorite dismutase (Cld) detoxifies ClO_2^- into Cl^- and molecular oxygen (1, 47, 49). The produced oxygen can then be respired or used as a co-substrate for oxygenases (1, 43) (Chapter 3). Pcr and Clr are distinct enzymes that belong to the functionally diverse DMSO reductase family (49, 50).

Genes encoding the key enzymes Pcr (*pcrABCD*) and Cld (*cld*) in PRB have been shown to be part of a 10-25 kb perchlorate reduction island (PRI) that is horizontally transferred across diverse phylogenetic boundaries (53). In the model PRB *Azospira suillum* PS, the PRI is encoded by 17 genes encoding Cld, Pcr, and other genes predicted to be involved in regulation, electron transport, and cofactor biosynthesis (53) (Fig. 2). Further work in *A. suillum* PS demonstrated that 8 of 17 genes in its PRI were essential for perchlorate reduction (54). As expected, these genes included *pcrABCD* and *cld*, but genes encoding a response regulator (*pcrR*), a histidine kinase sensor (*pcrS*), and a PAS domain-containing protein (*pcrP*), were also found to be essential (54). Together, *pcrP*, *pcrR*, and *pcrS* encode a histidine kinase signal transduction system (54). Additionally, three other genes outside the PRI resulted in a perchlorate null phenotype (54) and one of these genes encoded the sigma-54 type sigma factor *rpoN*, which is thought to play a key role in the transcription of genes in the PRI and is predicted to interact with PcrR and with the promoter region upstream of *pcrA* (54). In CRB, genes encoding the machinery needed for chlorate reduction are found in composite transposons, which like the PRI, are thought to be horizontally transferred (50).

Unlike other PRB described, the recently studied archaeon *Archaeoglobus fulgidus* VC-16 does not rely on Cld for ClO_2^- dismutation and utilizes a mixture of biotic and abiotic reactions to respire ClO_4^- (37). *A. fulgidus* VC-16 is thought to rely on a NarG-type nitrate reductase to reduce ClO_4^- to ClO_2^-, which is abiotically detoxified by reacting with sulfide (HS^-) (37). *A. fulgidus* VC-16 can reduce ClO_4^- and sulfate (SO_4^{2-}) simultaneously, and therefore, the HS^- needed to detoxify ClO_2^- is produced under perchlorate-reducing conditions (37). However, in an environmental setting, it is possible that the HS^- needed for detoxification of ClO_2^- may originate from another sulfur or sulfate-reducing microorganism, thus creating a symbiotic relationship based on sulfur cycling. This type of metabolism, which relies on a DMSO family

type reductase for ClO_4^- reduction and on HS^- scavenging for detoxification of ClO_2^- is referred to as "(per)chlorate reduction *sensu lato*" (55).

Bioremediation of Perchlorate

Due to the detrimental effects of perchlorate on human health, several methods for perchlorate remediation have been developed. Ion-exchange (IX) technology is widely used in the *ex situ* removal of perchlorate from drinking water, during which a concentrated brine containing perchlorate is produced (56, 57). Using PRB to remove perchlorate from brine is appealing, as it is costly to dispose of it (56, 57). The biggest obstacle, however, is the inability of most PRB to grow at high salinity (3-6 % NaCl; Table 1) (1, 39, 57). In fact, most PRB (excluding those described in this work) are unable to function at salinities > 2% NaCl (1, 39, 57) (Table 1). Exceptions include *Moorella perchloratireducens* An10, *Citrobacter* sp. IsoCock1, and *Marinobacter vinifirmus* P4B1, which albeit slowly, could reduce perchlorate in salinities of up to 4, 10 and 10.5%, respectively (58, 59) (Table 1). As such, the isolation of novel halophilic isolates capable of reducing perchlorate effectively at high salinity is of great need.

The following work describes an in-depth study of a marine, perchlorate-degrading microbial community and isolates obtained from the Berkeley Marina (Berkeley, CA). Enrichments with varying salt concentrations (1, 3, 5, and 7% NaCl) were shown to reduce perchlorate and novel PRB in the genera *Arcobacter* (Chapter 3), *Sedimenticola* (Chapters 4 and 5), *Azoarcus*, *Pseudomonas*, *Denitromonas*, and *Marinobacter* (Chapter 2) were isolated. With the exception of *Marinobacter* (58), these isolates represent the first known perchlorate-reducers in each genus, thus greatly expanding the known phylogeny of DPRB which are dominated by the genera *Dechloromonas* and *Azospira* (Table 1; Fig. 1). Of these isolates, *Arcobacter* sp. CAB (Chapter 3) and *Sedimenticola selenatireducens* CUZ (Chapters 4 and 5) were fully characterized. *Arcobacter* sp. CAB represents the only DPRB in pure culture belonging to the Epsilonproteobacteria and notably lacks the *pcrC* gene previously thought to be essential for perchlorate reduction (54) (Chapter 3). The *pcrC* gene encodes a periplasmic multiheme c-type cytochrome that putatively mediates electron transport from the cytoplasmic membrane to the periplasmic functional PcrAB protein (49). Instead, the PRI of strain CAB contained an *Arcobacter*-like *c*-type monoheme cytochrome that may be functionally replacing *pcrC*. Additionally, characterization of *Arcobacter* sp. CAB led to the discovery that DPRB can, in the absence of exogenous oxygen, oxidize aromatic compounds via aerobic pathways by recycling the oxygen produced from dismutation of chlorite (Chapter 3). Subsequent studies in the DPRB *S. selenatireducens* CUZ, on the other hand, showed that DPRB can also act as true anaerobes and utilize completely anaerobic pathways in the presence of perchlorate, even when both aerobic and anaerobic pathways are available (Chapters 4 and 5). As such, this study of marine perchlorate reduction has significantly contributed to the current understanding of the phylogeny, habitats, and metabolic capacity of DPRB.

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Tables and Figures

Table 1. List of all perchlorate-reducing bacteria (PRB) and chlorate-reducing bacteria (CRB) isolated to date.

Organism	Isolation Source	Donor and Acceptor	PRB or CRB?	Temp (°C)	% NaCl	pH	Comments	References	Accession Number
Phylum Proteobacteria									
Class Alphaproteobacteria									
Azospirillum sp. ABL1	Perchlorate contaminated aquifer material/ groundwater from Ballistics Laboratory	Acetate & Perchlorate	PRB					(59)	AY265875
Azospirillum sp. AJ2	Perchlorate contaminated aquifer material/ groundwater from Aerojet NPL site (subsite 2)	Acetate & Perchlorate	PRB					(59)	AY265865
Azospirillum sp. cl-19	Sarno River basin, Italy	Acetate & Perchlorate	PRB					(60)	GU294120
Azospirillum sp. PMS1	Industrial site (source area; perchlorate contaminated)	Acetate & Perchlorate	PRB					(59)	AY265868
Azospirillum sp. SN1A	Perchlorate contaminated aquifer material/ groundwater from Navy site (subsite 1)	Acetate & Perchlorate	PRB					(59)	AY265874
Azospirillum sp. SN1B	Perchlorate contaminated aquifer material/ groundwater from Navy site (subsite 1)	Acetate & Perchlorate	PRB					(59)	AY265869
Azospirillum sp. SN2	Perchlorate contaminated aquifer material/ groundwater from Navy site (subsite 2)	Acetate & Perchlorate	PRB					(59)	AY265870
Azospirillum sp. TTI		Acetate & Chlorate	PRB					(1, 27, 34)	AF170353

Azospirillum sp. PMS2	Industrial site (source area; perchlorate contaminated)	Acetate & Perchlorate	PRB					(59)	AY265873
Dechlorospirillum anomalous JB116	Sewage treatment facility in Inchon, South Korea	Acetate & Perchlorate	PRB	22-35; 25-35	0-2; 0	7-7.8		(61)	Not in Genbank
Dechlorospirillum anomalous WD	Swine waste lagoon	Acetate & Chlorate	PRB	35	≤ 1; 0	7.2		(27, 34, 62)	AF170352
Dechlorospirillum sp. cl-31	Sarno River basin, Italy	Acetate & Perchlorate	PRB					(60)	GU294121
Dechlorospirillum sp. DB	Perchlorate contaminated site in Los Alamos		PRB					(63)	AY530551
Magnetospirillum bellicus VDY	Water from Strawberry Creek at UC Berkeley, Berkeley, CA	Electrode & Perchlorate	PRB	10-42; 42	≤ 1.5; 0	6.0-7.5; 6.8	Enriched in cathodic chamber of BER	(32)	EF405824

Class Betaproteobacteria

Alicycliphilus denitrificans BC	Wastewater/ soil mixture contaminated with benzene	Benzene & Chlorate	CRB				Degrades benzene and catechol with chlorate or oxygen	(43)	NR_074585
Azoarcus marinus PHD	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137430
Azospira oryzae GR-1	Activated sludge	Acetate & Perchlorate	PRB					(44)	AY277622
Azospira sp. AH			PRB					(1)	AY171616
Azospira sp. cl-6	Sarno River basin, Italy	Acetate & Perchlorate	PRB					(60)	GU294119
Azospira sp. Iso1		Acetate & Chlorate	PRB					(1, 27)	AF170350
Azospira sp. Iso2		Acetate & Chlorate	PRB					(1, 27)	AF170351
Azospira sp. KJ	Sludge/ effluent from an acetate-fed, perchlorate degrading packed bed bioreactor	Acetate & Perchlorate	PRB					(51)	AF323491

Azospira sp. PCC	Activated sludge	Hydrogen & Perchlorate	PRB				Also known as Dechlorosoma sp. HCAP-C and Dechlorosoma sp. PCC	(64)	AY126453
Azospira sp. PDX	Sludge/ effluent from an acetate-fed, perchlorate degrading packed bed bioreactor	Lactate & Chlorate	PRB					(51)	AF323490
Azospira sp. SDGM		Acetate & Chlorate	PRB					(1, 27, 34)	AF170349
Azospira suillum PS	Swine waste lagoon	Acetate & Chlorate	PRB	25-42; 37	≤ 1; 0	6.5-7.5		(27, 34, 62)	AF170348
Dechlorobacter hydrogenophilus LT-1	Soil from Longhorn Army Ammunition Plant, TX		PRB	4-37; 37	≤ 1; 0	6.0-7.2; 6.5	Enriched in slightly acidic medium (pH 6.6)	(33)	AY124797
Dechloromonas agitata CKB	Paper mill waste	Acetate & Chlorate	PRB	25-40; 35	0-2; 1	6.5-8.5; 7.5		(28)	NR_024884
Dechloromonas aromatica RCB	Sediment from the Potomac River, Maryland, USA	4-Chlorobenzoate & Chlorate	PRB				Degrades benzene with (per)chlorate, nitrate, oxygen	(35, 42)	AY032610
Dechloromonas denitrificans ED-1	Earthworm gut	Tryptic soy broth (no dextrose), nitrate, and glucose	PRB	5-36; 30		6.1-8.3; 7	Can reduce sulfate	(46)	AJ318917
Dechloromonas hortensis MA-1	Garden soil	Acetate & Chlorate	PRB	30		7.2		(65)	AY277621
Dechloromonas sp. ABL2	Perchlorate contaminated aquifer material/ groundwater from Ballistics Laboratory	Acetate & Perchlorate	PRB					(59)	AY265867
Dechloromonas sp. CCO		Acetate & Chlorate	PRB					(1, 34)	AF288776
Dechloromonas sp. CL		Acetate & Chlorate	PRB					(1, 27)	AF170354
Dechloromonas sp. CL24		Acetate & Chlorate	PRB					(1, 34)	AF288775

Dechloromonas sp. CL24+		Acetate & Chlorate	PRB					(1, 34)	AF288774
Dechloromonas sp. EAB1	Perchlorate contaminated aquifer material/ groundwater from Edwards Air Force Base	Acetate & Perchlorate	PRB					(59)	AY265877
Dechloromonas sp. EAB2	Perchlorate contaminated aquifer material/ groundwater from Edwards Air Force Base	Acetate & Perchlorate	PRB					(59)	AY265879
Dechloromonas sp. EAB3	Perchlorate contaminated aquifer material/ groundwater from Edwards Air Force Base	Acetate & Perchlorate	PRB					(59)	AY265871
Dechloromonas sp. FL2		Acetate & Chlorate	PRB					(1, 34)	AF288771
Dechloromonas sp. FL8		Acetate & Chlorate	PRB					(1, 34)	AF288772
Dechloromonas sp. FL9		Acetate & Chlorate	PRB					(1, 34)	AF288773
Dechloromonas sp. HZ	Gas-phase anaerobic packed-bed biofilm reactor treating perchlorate contaminated wastewater	Hydrogen & Perchlorate	PRB				Can fix CO_2	(66)	AF479766
Dechloromonas sp. INS	Industrial site, CA (perchlorate contaminated)	Acetate & Perchlorate	PRB					(59)	AY265880
Dechloromonas sp. JDS5	Perchlorate contaminated groundwater and soil from Longhorn Army Ammunition Plant	Hydrogen & Perchlorate	PRB				Can fix CO_2; microaerophilic	(67)	AY084086
Dechloromonas sp. JDS6	Perchlorate contaminated groundwater and soil from Longhorn Army Ammunition Plant	Hydrogen & Perchlorate	PRB				Can fix CO_2; microaerophilic	(67)	AY084087
Dechloromonas sp. JM	Activated sludge	Hydrogen & Perchlorate	PRB				Cannot fix CO_2	(1, 68)	AF323489

Dechloromonas sp. MissR		Acetate & Chlorate	PRB					(1, 34)	AF170357
Dechloromonas sp. MLC33			PRB					(1)	AF444791
Dechloromonas sp. NM		Acetate & Chlorate	PRB					(1, 27, 34)	AF170355
Dechloromonas sp. PC1	Hydrogen-based, autotrophic hollow-fiber membrane biofilm reactor reducing nitrate/perchlorate	Hydrogen & Perchlorate	PRB					(69)	AY126452
Dechloromonas sp. PMC	Industrial site (plume core; perchlorate contaminated)	Acetate & Perchlorate	PRB					(59)	AY265878
Dechloromonas sp. PR	Pristine site, Ontario	Acetate & Perchlorate	PRB					(59)	AY265866
Dechloromonas sp. RC1	Perchlorate contaminated aquifer material/ groundwater from rocket test site (subsite 1)	Acetate & Perchlorate	PRB					(59)	AY265876
Dechloromonas sp. RC2	Perchlorate contaminated aquifer material/ groundwater from rocket test site (subsite 2)	Acetate & Perchlorate	PRB					(59)	AY265872
Dechloromonas sp. SIUL		Acetate & Chlorate	PRB					(1, 27)	AF170356
Denitromonas halophilus SFB-1	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137426
Denitromonas halophilus SFB-2	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137427
Denitromonas halophilus SFB-3	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137428
Ideonella dechloratans	Activated sludge		CRB					(70)	X72724
perclace	Biosolids from water quality control plant in Riverside, California	Acetate & Perchlorate	PRB	25-30	0-2.5; 0	7-7.2		(58, 71, 72)	Not in Genbank

Propionivibrio militaris CR	Soil from Los Alamos National Laboratory		PRB	30	< 1; 0	7		(33)	AY530552
Propionivibrio militaris MP	Water from Strawberry Creek at UC Berkeley, Berkeley, CA	AQDS & Perchlorate	PRB	10-37; 30	≤ 1; 0	6.0-7.5; 6.8	Enriched in the cathodic chamber of BER	(33)	NR_125528

Class Gammaproteobacteria

Acinetobacter sp. NIIST	*Pistia* root homogenate	Acetate & Perchlorate	PRB					(31)	JX467695
Citrobacter amalonaticus JB101	Sewage treatment facility in Inchon, South Korea	Acetate & Perchlorate	PRB					(73)	Not in Genbank
Citrobacter farmeri JB109	Sewage treatment facility in Inchon, South Korea	Acetate & Perchlorate	PRB					(73)	Not in Genbank
Citrobacter sp. IsoCock1	High density hydrocarbon oxidizing bacterial cocktail	Acetate, Yeast extract & Perchlorate	PRB	20-40; 30	0-10; 0	6-9.5; 7.5		(58)	Not in Genbank
Dechloromarinus chlorophilus NSS	Hydrocarbon contaminated sediment from San Diego Bay, CA	Acetate & Chlorate	CRB	30-45; 37-42	0-4.5; 1.5-2.5	6-9; 7.5-8	Enriched in medium containing 4% NaCl	(27); Chapter 4	AF170359
Marinobacter vinifirmus P4B1	Marine sediment	Acetate & Perchlorate	PRB		1.5-10.5; 1.5			(57)	JN861074
Marinobacter vinifirmus UCB	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137425
Pseudomonas chloritidismutans ASK-1	Sludge from bioreactor treating bromate/chlorate polluted wastewater	Acetate & Chlorate	CRB					(65)	AY125329
Pseudomonas chloritidismutans AW-1	Anaerobic bioreactor treating chlorate and bromate polluted wastewater	Acetate & Chlorate	CRB	10-37; 30	0.1-4; 2-4	8-9; 8.5		(50)	AY017341
Pseudomonas sp. CAL	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP137429
Pseudomonas sp. CFPBD		Acetate & Chlorate	CRB					(1, 27)	AF288777

Pseudomonas sp. PDA	Sludge/effluent from an acetate-fed, perchlorate degrading packed bed bioreactor	Lactate & Chlorate	CRB					(51)	AF323492
Pseudomonas sp. PDB	Sludge/effluent from an acetate-fed, perchlorate degrading packed bed bioreactor	Lactate & Chlorate	CRB					(51)	AF323493
Pseudomonas sp. PK		Acetate & Chlorate	CRB					(1, 27, 34)	AF170358
Sedimenticola selenatireducens BK-1	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP122946
Sedimenticola selenatireducens CUZ	Marine sediment from Berkeley Bay, CA	Hydrogen & Perchlorate	PRB	15-30; 25-30	0.5-6; 4	6-7.5; 7	Enriched in medium containing 3% NaCl	Chapter 4	KM192219
Sedimenticola selenatireducens BK-2	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate						Chapter 2	KP122947
Shewanella algae ACDC	Hydrocarbon contaminated sediment from San Diego Bay, CA	Acetate & Chlorate	CRB	4-40; 20-30	0-8; 2	6.5-9; 8	Isolated in medium containing 2% NaCl; accession number for IMG	(45)	ACDC_00040 000 (IMG)
Vibrio dechloraticans Cuznesove B-1168			PRB					(74)	Not in Genbank

Class Epsilonproteobacteria

Arcobacter sp. CAB	Marine sediment from Berkeley Bay, CA	Acetate & Perchlorate	PRB	15-33; 25-30	0.3-6; 3	6-7.5; 7	Enriched in medium containing 3% NaCl; accession number for IMG	(75); Chapter 3	KP137431
Wolinella succinogenes HAP-1	Anaerobic digestor	Yeast extract, peptone & Perchlorate	PRB				Culture has been lost; microaerophilic; accession number for ATCC 29543 not HAP-1	(38)	NR_025942

Phylum Firmicutes

Moorella perchloratireducens An10	Underground gas storage in Russia	Methanol & Perchlorate	PRB	40-70; 55-60	0-4; 1	6.5-7	Enriched at 55 °C	(29)	NR_125518
Sporomusa sp. An4	Underground gas storage in Russia	Methanol & Perchlorate	PRB	20-40; 37		5.5-8; 7	Enrichment was mesophilic (37 °C) even though original sample was thermophilic (65 °C)	(30)	EF060193

Phylum Euryarchaeota

Archaeoglobus fulgidus VC-16	Marine hot vents close to Vulcano island in Italy	Yeast extract & Sulfate	PRB	64-92; 83			Hyperthermophilic archaeon; does not utilize Pcr or Cld; chlorite is removed abiotically	(36, 37)	X05567

Figure 1. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic position of known perchlorate-reducing and chlorate-reducing bacteria. Bootstrap values are based on 1,000 replications and branches with values >70 are marked with a circle. The scale bar represents 0.2 expected change per site. iTOL was used for tree visualization (76, 77).

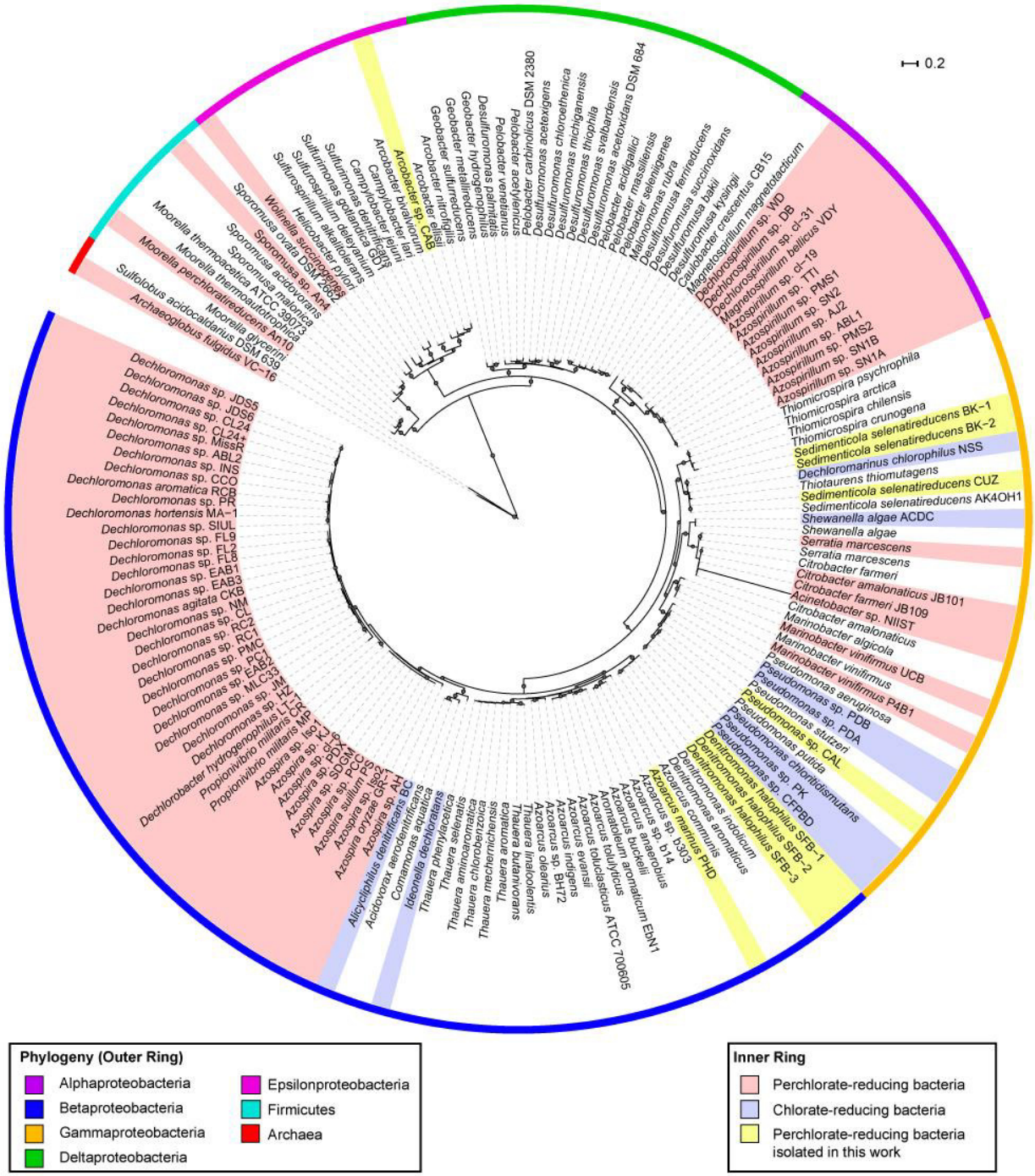

Figure 2. Perchlorate Reduction Island in the model DPRB *Azospira suillum* PS. Modified from Melnyk et al. 2014 (53). Green: perchlorate reductase and chlorite dismutase; blue: histidine kinase system; red: genes involved in electron transport; orange: putative oxidoreductase system; teal: putative sigma factor/antisigma system; yellow: molybdenum cofactor biosynthesis protein.

Chapter 2: Characterization of a Marine Microbial Community in Response to Perchlorate and Salinity

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Abstract

The study of dissimilatory perchlorate reducing bacteria (DPRB) has traditionally focused on freshwater environments; thus information on marine isolates and microbial communities is scarce. Enrichments were set up at 1, 3, 5, 7, and 10% NaCl using marine sediment with acetate and perchlorate as the electron donor and electron acceptor. The most rapid consumption of perchlorate was at 1% NaCl and decreased as NaCl concentrations increased, with enrichments at 10% NaCl showing no activity. Salinity impacted the microbial community, and the most active members were in families Rhodocyclaceae (1% and 3% NaCl), Pseudomonadaceae (1% NaCl), Campylobacteraceae (1%, 5%, and 7% NaCl), Sedimenticolaceae (3% NaCl), Desulfuromonadaceae (5% and 7% NaCl), Pelobacteraceae (5% NaCl), Helicobacteraceae (5% and 7% NaCl), and V1B07b93 (7%). Sulfur reducing families, which made up a large fraction of the microbial community at 5 and 7% NaCl, may be growing by reducing sulfur and not perchlorate, as DPRB have been shown to oxidize sulfide to sulfur. Additionally, this is the first time that an archaeon has been shown to be enriched in a community actively consuming perchlorate (Parvarchaeota, 5% NaCl). This phylum has only been described in metagenomics experiments of acid mine drainage, and is unexpected in a marine community. Novel bacteria belonging to the genera *Sedimenticola*, *Marinobacter*, *Denitromonas*, *Azoarcus*, and *Pseudomonas* were isolated and expand the known phylogeny of DPRB.

Introduction

Perchlorate (ClO_4^-), is a stable, water-soluble chemical used in explosives and rocket fuel (1-3). ClO_4^- can lead to hypothyroidism in humans; thus it is a proposed EPA regulated compound (1, 4-7). Although considered to be primarily of anthropogenic origin, naturally occurring ClO_4^- has been detected in pristine environments (8-11) and Martian soil (12).

Dissimilatory perchlorate reducing bacteria (DPRB) couple energy metabolism to ClO_4^- reduction (1). DPRB can reduce perchlorate or chlorate (ClO_3^-) [collectively known as (per)chlorate] (1, 13) and produce the toxic intermediate chlorite (ClO_2^-), which is dismutated into chloride and oxygen (14). The oxygen produced is respired or used as a co-substrate for oxygenases (1, 15). The genes encoding perchlorate reduction are part of a perchlorate reduction island (PRI) (16) that can be horizontally transferred. The archaeon *Archaeoglobus fulgidus* does not contain a PRI and it reduces ClO_4^- via biotic and abiotic reactions involving sulfur compounds (17).

Most DPRB have been obtained from freshwater, mesophilic, neutral-pH environments (1, 18). Exceptions are *Moorella perchloratireducens* and *Sporomusa* strain An4, isolated from an underground gas storage facility (19, 20), *Archaeoglobus fulgidus*, isolated from marine hot vents (17, 21), and the marine *Arcobacter* sp. CAB (15) and *Sedimenticola selenatireducens* CUZ (Carlstrom et al submitted). In addition, two marine chlorate (ClO_3^-) reducing bacteria (CRB) have also been isolated (22, 23).

Ion-exchange technology is widely used for the removal of perchlorate from drinking water; however, this process leads to the accumulation of perchlorate in brine which is costly to dispose of (24, 25). Using DPRB to remove perchlorate from brines is possible, but most DPRB in pure culture cannot grow at salinities >2% (1, 18). Thus, several groups have developed and characterized bioreactor communities that are capable of reducing perchlorate (or perchlorate and nitrate) at higher salinities (3-10%) (26-30). Nitrate was often included because it is also concentrated during ion-exchange treatments and can inhibit perchlorate reduction (1, 31).

The dominant members of the perchlorate-degrading communities varied by study, salinity, and presence of nitrate. One group found that Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were dominant under various conditions (29, 32). However, the relative abundance of Betaproteobacteria more than doubled and the abundance of Alphaproteobacteria sharply declined when nitrate was mixed with perchlorate (29). By contrast, despite including nitrate, another group found their community lacked Betaproteobacteria and was dominated by Alphaproteobacteria and Gammaproteobacteria (26, 28). The same sample also yielded appreciable numbers of Epsilonproteobacteria and Bacteroidetes at 3% salinity (26) but not at 4.5% (28). Jinwook et al. also studied a range of salinities and found mostly Betaproteobacteria, Epsilonproteobacteria, Bacteroidetes, and Firmicutes, but no Alphaproteobacteria or Gammaproteobacteria (27). Further, only Betaproteobacteria and Firmicutes were significant at salinities ≥ 3% (27). Across various studies, the classes reported to be enriched by more than one research group are the Betaproteobacteria (*Denitromonas* and *Thauera)* and the Gammaproteobacteria (*Marinobacter*, *Idiomarina*, *Halomonas*, *Dechloromarinus*, and *Alcanivorax).* Of these, only *Marinobacter*

includes a known DPRB isolate, *Marinobacter vinifirmus* P4B1 (33), although *Dechloromarinus* includes a chlorate reducer (22).

The current study expands on the known diversity of DPRB by characterizing the temporal response of a marine microbial community to enrichment with acetate and perchlorate at varying NaCl concentrations (noted as "salinity") and by identifying the most active members of the community via 16S rRNA. Additionally, this study reports the isolation of novel marine DPRB of the genera *Sedimenticola*, *Marinobacter*, *Pseudomonas*, *Azoarcus*, and *Denitromonas*.

Materials and Methods

Enrichment Setup and Sampling

Marine sediment was collected from a marina in Berkeley, CA (37.8629º N, 122.3132º W). A 1 L bottle (Corning, Tewksbury, MA), modified to be anaerobic, containing a 1:1 ratio of sediment to sterile medium, was used as the inoculum source. Five replicates were set up at 1, 3, 5, 7, and 10% NaCl in 125 mL anaerobic bottles (Bellco, Wineland, NJ) containing 90 mL of medium (15) modified to vary the % NaCl and using 10 mL inoculum slurry. Acetate (10 mM) and perchlorate (10 mM) were used as the electron donor and electron acceptor and controls for each salinity included no donor, no acceptor, and no amendment. Enrichments were maintained for 63 days at 30 ºC and tested for acetate and perchlorate consumption weekly. Biweekly (days 0, 14, 28, and 42), 20 mL of sample were filtered with sterile 0.22 μM filters (MoBio, Carlsbad, CA) and the filters were stored at -80 ºC for nucleic acid isolation.

Analytical Methods

Perchlorate was measured via ion chromatography using a Dionex ICS 2100 with a Dionex IonPac AS 16 (4 × 250 mm) column (Thermo Fisher Scientific, Sunnyvale, CA) and a 35 mM NaOH mobile phase at a flow rate of 1.0 mL/min. Background conductivity was suppressed with a Dionex ASRS operating in recycle mode. The suppressor controller was set at 100 mA for analysis and the injection volume was 25 μL.

Acetate was measured by HPLC (Dionex; model LC20), using a UV-visible detector (Dionex; AD20) at a wavelength of 210 nm and a Bio-Rad Aminex HPX-87H column with a mobile phase of 0.016 N H_2SO_4 (flow rate of 0.9 mL/min).

Nucleic Acid Extraction, Library Preparation, and Sequencing

Filters were pulverized under liquid nitrogen. Nucleic acids were isolated using the MoBio RNA PowerSoil Kit with the DNA Elution Accessory Kit (MoBio Laboratories, Inc., Carlsbad, CA). RNA samples were treated with DNAse (Turbo DNA-free, Life Technologies) and tested for DNA contamination using 16S universal primers. cDNA was synthesized using the

Verso cDNA kit (Thermo Fisher Scientific, Sunnyvale, CA). RNA isolation was unsuccessful in samples with low activity, so RNA analysis was carried out on samples from day 0 and the most active day from each salinity (1% = day 14; 3 and 5% = day 28; 7% = day 42).

The archaeal and bacterial primer set MiSeq 16S F

(5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCMGCCGCGGTAA 3')

and MiSeq 16S R

(5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3')

were used to amplify a 287 bp region of the 16S rRNA gene that spans HV region four. These primers were based on the S-D-Arch-0519-a-S-15 (A519F) and S-D-Bact-0785-a-A-21 (Bakt_805R) primers (34) but include the necessary Illumina adapters. PCR conditions were: 95 ºC for 3 min; 30 cycles of 95 ºC for 30 s, 64 ºC for 30 s, and 72 ºC for 30 s; and 10 min at 72 ºC.

The Nextera XT Index Kit (Illumina, Hayward, CA) was used for library preparation following the manufacturer's protocol. Quantification, validation, pooling of the samples, and sequencing was done by the DNA Technologies Core at the UC Davis Genome Center (Davis, CA) using a MiSeq reagent kit V2 (2 x 250 bp) and software version MiSeq 2.4.1.

Community Analysis

Samples were analyzed using Mothur v. 1.3.3 (35) with modifications (36). Samples were demultiplexed and FASTQ files for both forward and reverse reads were analyzed following a modified version of the MiSeq SOP on the Mothur website as of July 2014 (http://www.mothur.org/w/index.php?title=MiSeq_SOP&oldid=10004) (36). Forward and reverse FASTQ reads were merged and resulting sequences were selected for an expected length of 287 bp ± 10%, based on the region amplified. Sequences were aligned using a combined bacteria and archaea standard provided with Mothur and derived from SILVA release 102 (37). Sequences that did not overlap were discarded and sequences differing by 3 or fewer nucleotides were preclustered and any sequence that occurred only once across all samples was discarded. Chimeras were removed by applying the UCHIME algorithm (38). The remaining sequences were screened by the RDP reference taxonomy included in Mothur (version 9, type PDS) to remove eukaryotes, mitochondria, and chloroplasts and sequences were reclassified using the Greengenes bacteria and archaea reference taxonomy (39) (May 2013 release) supplemented with 34 reference sequences from known DPRB and with 17 sequences identified as Betaproteobacteria of order ASSO-13 removed. The ASSO-13 order described by Greengenes includes sequences highly similar (98% identity) to taxa of genus *Thauera* (order Rhodocyclales) and this similarity caused the classifier to exhibit high uncertainty regarding samples in the family Rhodocyclaceae. Removing the ASSO-13 sequences helped preserve the more widely used classification scheme. During taxonomic classification, a 60% confidence threshold was required and after preliminary classification, sequences were split by apparent taxonomic order and clustered into OTUs with a 3% dissimilarity threshold. The resulting OTUs and their consensus taxonomy were used for all subsequent analysis. A DNA replicate of the 1%

NaCl condition at day 28 was excluded from all analyses because of very low reads (less than 7% of the median).

The relative abundance of different phyla in each sample was plotted (Microsoft Excel). OTU data was normalized and sorted by phylum. Relative abundance for OTUs belonging to the same phylum were summed and the result plotted as relative abundance (%). Phyla contributing less than 2% of the sample were classified as "others". For other analyses, OTUs were normalized and square root transformed. Hierarchical clustering and non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity matrices assessed clustering among samples (PRIMER 6, PRIMER-E Ltd, Plymouth, UK). Similarity Profile (SimProf) was used to determine significance of the identified groupings at a 5% significance level. Low stress values (scale 0-1) indicate that the NMDS plots are a good representation of the relationships among samples. Similarity percentage (SIMPER) was used to determine which OTUs contributed to the top 30% of differences between the RNA from the original sediment (day 0) and the RNA at the most enriched day in each salinity. The relative abundance (normalized without transformation) of these SIMPER identified OTUs were summed by family, including only the OTUs that were more abundant in the enriched sample. Richness (number of distinct OTUs) was calculated using presence/absence data. Evenness and diversity were calculated using Shannon's equitability and Shannon's diversity measures.

Isolations and Phylogenetic Analysis

Agar deeps (40) and agar plates matching the % NaCl in the original enrichment were used for isolations. Isolates picked from agar deeps were subsequently serially diluted. For agar deeps, the medium was the same as the one used for enrichment; for plates, $NaHCO_3$ was replaced by HEPES (7.149 g/L) and agar (15 g/L) was added. Acetate and perchlorate (10 mM each) were used as the electron donor and electron acceptor and work was carried out in an anaerobic chamber. Plates and agar deeps were incubated anaerobically at 30 ºC. Individual colonies were picked into 5 mL of medium and assessed for a decrease in perchlorate concentration and an increase in optical density (OD_{600}).

Genomic DNA was extracted using a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene was PCR amplified using the universal primers 27F and 1492R. The Silva database and aligner (37) were used to align 16S rRNA gene sequences, and a maximum likelihood phylogenetic tree was constructed with 1,000 bootstrap values using RAxML-HPC (41). A reference tree including 13 taxa (Table S1) was used to constrain the relative placement of various phyla and classes according to previously established relationships (42). None of the DPRB, new isolates, or key OTUs were included in the reference tree. Representative sequences for key OTUs were selected using the most common sequence contributing to each OTU. iTOL was used for tree visualization (43, 44). Accession numbers for the microorganisms and isolates used in the tree are provided in Table 1.

Nucleotide Sequence Accession Numbers

The 16S sequences of the novel isolates are available on GenBank and accession numbers are provided in Table 2. MiSeq sequencing results are deposited in the Sequencing Read Archive under accession number SRP049563.

Results

Consumption of Acetate and Perchlorate

Enrichments of marine sediment at 1, 3, 5, and 7% salinity were able to oxidize acetate and reduce perchlorate (Fig. 1). No activity was detected in the 10% salinity enrichments (data not shown). All five replicates from 1, 3, and 5% salinity reduced perchlorate, while only two replicates containing 7% NaCl were able to do so (Fig.1). Time to acetate depletion increased with salinity, with samples consuming 7.7 ± 0.1 mM perchlorate and all acetate in 21 (1%), 35 (3%), 42 (5%) and 49 (7%) days. Duplicates at 7% salinity grew at very different rates, with one replicate taking 35 days and the other taking 49 days to consume equivalent amounts of acetate and perchlorate (Fig.1D). Despite taking longer to consume perchlorate, the 7% salinity samples had maximum consumption rates comparable to the 3 and 5% samples. The highest rates of consumption for each salinity, as measured by the change in perchlorate concentration in consecutive time points were 0.8 ± 0.1 mM/day (1%), 0.5 ± 0.1 mM/day (3%), 0.4 ± 0.1 mM/day (5%) and 0.5 and 0.6 mM/day (7%). Some perchlorate (2.1 ± 0.4 mM) was reduced in the no donor controls, while acetate was completely oxidized in the no acceptor controls (Fig.1), suggesting the presence of alternate electron donors and acceptors in the inoculum. The days at which replicates were most active were determined to be day 14 (1%), day 28 (3% and 5%), and day 42 (7%). This result was further supported by measures of richness, evenness, and diversity in DNA samples. Samples were the least rich, even, and diverse at the day which was selected as the most active (Fig. 2). These differences were significant ($p < 0.05$) with the exception of the day 28 and 42 samples at 3%, 5%, and 7% salinity (Table 3), which suggests that, for these samples, the communities that developed by day 28 did not substantially change during the last two weeks of the experiment.

Phyla Enriched at Different Salinities

Non-metric multidimensional scaling (NMDS) of the DNA samples showed clustering by salinity and time (Fig. 3) with some outliers. 3% and 5% NaCl samples clustered together at day 14 (Fig. 3), which is unsurprising since neither group was very enriched by this time (Fig. 1B, 1C). At 28 days, when 3 and 5% NaCl samples were most active (Fig.1B, 1C), these groups separated (Fig. 3). Two 28 day replicates from 3% NaCl did not cluster (Fig. 3), even though rates of acetate and perchlorate consumption were similar (Fig. 1B). Most samples from days 28 and 42 cluster together. Samples from 7% NaCl clustered poorly; replicates from day 14 clustered with one day 28 sample, and the other day 28 day sample clustered with a day 42 sample. The second 42 day sample formed its own group (Fig. 3). This clustering was consistent

with one replicate at 7% NaCl actively consuming acetate and perchlorate by day 28 while the second replicate was still inactive. Other outliers include a day 42 sample (5% salinity) which clustered with the 7% samples from day 14 and a 1% sample from day 42 which clustered with 5% samples from days 28 and 42 (Fig. 3).

Prior to enrichment (day 0), the community was dominated by Gammaproteobacteria (22.6 ± 1.1%), Deltaproteobacteria (19.8 ± 0.3%), Bacteroidetes (17.2 ± 0.2%), Planctomycetes (7.8 ± 0.2), and Alphaproteobacteria (5.7 ± 0.8%) (Fig. 4). Other phyla/classes included Epsilonproteobacteria, Crenarchaeota, Acidobacteria, and Verrucomicrobia (Fig. 4). Of these groups, Alphaproteobacteria, Planctomycetes, Crenarchaeota, and Verrucomicrobia decreased at all salinities and time points (Fig. 4). Acidobacteria and Bacteroidetes also decreased during perchlorate consumption; however, for Acidobacteria (1% salinity) and Bacteroidetes (1% and 3% salinity) the relative abundance recovered after perchlorate was diminished (Fig. 4).

At the most active day for each salinity, the groups showing the greatest enrichment were Betaproteobacteria (1%, 3%), Gammaproteobacteria (3%), Deltaproteobacteria (5%, 7%), Epsilonproteobacteria (1%, 5%, 7%), Firmicutes (1%, 5%, 7%), Deferribacteres (7%), and Parvarchaeota (5%) (Table 4). However, the maximum relative abundance of Firmicutes (5 and 7% salinity) occurred before the day of maximum perchlorate consumption (Fig. 4), unlike other groups discussed.

Samples from 1, 3, and 7% salinity at the most active day showed some variation, while 5% NaCl samples were more consistent (Fig. 5). Two replicates (2 and 3) from 1% salinity and three replicates (1, 3, and 4) from 3% NaCl were characterized by low Betaproteobacteria abundance (<1%) and increased Gammaproteobacteria abundance (Fig. 5A, 5B). In both cases, differences in Betaproteobacteria abundance were due to a single OTU (*Azoarcus*/*Thauera* genus) that comprised greater than 99% of the Betaproteobacteria. In the 1% salinity replicates with greater than 10% Gammaproteobacteria, a single OTU representing genus *Pseudomonas* comprised 80-90% of this class but for the 3% salinity replicates, an OTU representing genus *Sedimenticola* comprised 80-95% of the class. The samples from 7% salinity differed due to Deferribacteres (23.8% versus 0.8%) and Deltaproteobacteria (11.5% versus 45.7%) (Fig. 5D). The Deferribacteres was dominated by a single OTU (85%) assigned to family V1B07b93 while the Deltaproteobacteria was dominated by an OTU of family Desulfuromonadaceae (70%). This OTU accounted for <1% of the sample in the other replicate.

Identification of Most Active Families

RNA from the most enriched day at each salinity was used to identify the most active families. Like the DNA samples, RNA samples clustered by salinity (t0, 5%, and 7%), with the 1% and 3% salinity samples clustering together (Fig. 6).

SIMPER identified the OTUs contributing to the top 30% of the dissimilarity between unenriched RNA samples (day 0) and each salinity and these OTUs were grouped by family. In the 1% salinity samples, the largest change between days 0 and 14 was observed in members of the families Rhodocyclaceae (1 OTU, 31% ± 14), Pseudomonadaceae (1 OTU, 20% ± 9), and Campylobacteraceae (2 OTUs, 22% ± 3) (Fig. 7A). Members of the Sedimenticolaceae (1 OTU,

90-fold) and an unclassified family in the order Desulfuromonadales (1 OTU, 300-fold) also increased (Fig. 7B); however, each remained less than 5% of the total population. Because the initial relative abundance of all selected OTUs was <1%, the change in relative abundance percentage (Fig. 7A) is almost identical to the final relative abundance (Fig. 8).

In the 3% salinity samples, the Rhodocyclaceae (1 OTU, 23% ± 16) and Sedimenticolaceae (2 OTUs, 51% ± 18) increased (Fig. 7A). The relative abundance of the Pelobacteraceae (1 OTU) also increased 60-fold (Fig. 7B), but remained <1% of the total sample (Fig. 8).

In the 5% NaCl samples, the Desulfuromonadaceae (4 OTUs, 29 ± 5%), Pelobacteraceae (1 OTU, 14 ± 6%), Helicobacteraceae (1 OTU, 9 ± 3%), and Campylobacteraceae (1 OTU, 6 ± 3%) increased (Fig. 47A). The Peptococcaceae (1 OTU, 5000-fold), Deferribacteres family V1B07b93 (1 OTU, 1000-fold), Rikenellaceae (1 OTU, 800-fold), Clostridiaceae (1 OTU, 100-fold), and Parvarchaea Order YLA114 (2 OTUs, 200-fold) also increased (Fig. 7B). However, each of these constituted <2.5% of the total sample (Fig. 8).

In the 7% salinity samples, the Desulfuromonadaceae (5 OTUs, 47 ± 15%), Deferribacteres family V1B07b93 (2 OTUs, 17 ± 22%), Helicobacteraceae (1 OTU, 11.7 ± 0.3%), and Campylobacteraceae (1 OTU, 6 ± 9%) all increased (Fig. 7A). The Peptococcaceae (1 OTU) also increased by 14,000-fold (Fig. 7B), yet it remained less than 5% of the total sample (Fig. 8).

Key Taxa and Isolates

Isolates were obtained from 1, 3, and 5% salinity enrichments and tested for growth on perchlorate. A maximum likelihood phylogenetic tree was constructed using known DPRB and the key OTUs identified as enriched in Figure 7. Isolates were designated *Sedimenticola selenatireducens* strains BK-1 and BK-2, *Denitromonas halophilus* strains SFB-1, SFB-2, and SFB-3, *Azoarcus marinus* strain PHD, *Marinobacter vinifirmus* strain UCB, and *Pseudomonas* sp. strain CAL (Fig. 9, Table 2). Most of the enriched OTUs clustered within the Proteobacteria. Several OTUs were closely related to Deltaproteobacteria of genera *Malonomonas* (OTU 3), *Desulfuromusa* (OTU 5), or *Desulfuromonas* (OTUs 15 and 51). Other OTUs (2, 30, 34, and 117) were closely related to the *Pelobacter* and *Desulfuromonas* genera; however their exact placement within the Desulfuromonadaceae was unclear. OTU 219 also claded within the Deltaproteobacteria, but was most closely related to the genus *Desulfomonile*. OTU 6, the only selected OTU within the Betaproteobacteria, claded with the genus *Azoarcus* in the Rhodocyclaceae. Three Gammaproteobacteria OTUs (OTUs 4, 22, and 49) were enriched in response to perchlorate. OTUs 4 and 49 claded with the DPRB *S. selenatireducens* CUZ (Carlstrom et al., submitted), the chlorate-reducing bacterium *D. chlorophilus* NSS (22, 45) and the selenate-reducing bacterium *S. selenatireducens* AK4OH (46). OTU 22, which was SIMPER selected only in the 1% salinity samples, clustered within the genus *Pseudomonas*. Of the four enriched Epsilonproteobacteria, two OTUs (OTU 23 and 14) claded within the genus *Arcobacter*. OTU 23 had 100% 16S sequence identity to the previously isolated perchlorate-reducer *Arcobacter* sp. CAB (15). The other two OTUs clustered with the genera *Sulforospirillum* (OTU 28) and *Sulfurimonas* (OTU 1).

Some OTUs claded outside the Proteobacteria, including Firmicutes (11, 128, 325, 371), Bacteroidetes (OTUs 121 and 25) and Deferribacteres (OTUs 52 and 83). OTUs 98 and 282 clustered within the Archaea phylum Parvarchaeota.

Discussion

More than 80 DPRB have been isolated to date; however, the majority of these isolates have been obtained from freshwater sediments (1, 15, 18, 22, 33, 40, 47-50). We examined the effect of varying salinity on a marine microbial community growing on perchlorate and isolated novel DPRB. This work expands the current understanding of the phylogeny of DPRB, their place in microbial communities, and identifies new taxa that may operate efficiently at high salinities.

Both perchlorate and salinity strongly impacted community structure, leading to significant declines in taxonomic richness, evenness, and diversity (Fig. S12, Table 3). Similar to previous reports (33, 51), we observed that increasing salinity led to slower consumption of perchlorate (Fig. 1). However, our results also showed that the dominant taxa in an enrichment vary substantially as a function of salinity (Fig. 7). Of the OTUs contributing most to the dissimilarity between the unenriched sample and each salinity at its most enriched day, only two Desulfuromonadaceae (OTUs 2 and 30) substantially increased in abundance in all salinities (Fig. 7). The Campylobacteraceae were the only other family to show substantial increases at all salinities, but different OTUs were present at high salinity (5% and 7%; OTU 14) than low salinity (1% and 3%; OTUs 23 and 28). In addition, Helicobacteraceae (OTU 1) was enriched in three out of four conditions (1, 5, and 7%). No other family was enriched in more than two different salinities. In particular, the Rhodocyclaceae and Sedimenticolaceae were dominant community members in 1% and 3% salinity but not in 5% or 7% salinity (Fig. 7).

Since this study focused on communities, it cannot be concluded that every microbe that was enriched in the presence of perchlorate is itself capable of reducing it. Of the enriched families, DPRB are known to exist in the Rhodocyclaceae (40, 49, 52), Sedimenticolaceae (Carlstrom et al., submitted), Pseudomonadaceae (53, 54), Campylobacteraceae (15), and Helicobacteraceae (55) and we obtained isolates from three of these families (Rhodocyclaceae, Sedimenticolaceae, & Pseudomonadaceae). DPRB are known to reduce sulfide (H_2S) to elemental sulfur (S^0) in the presence of perchlorate (56) and previous flow-through column experiments have shown enrichment of potential sulfur-reducing bacteria in the presence of perchlorate (57). Among the families that were enriched but from which no DPRB are known, several are known to contain sulfur-reducing bacteria, including Desulfuromonadaceae, Pelobacteraceae, and Peptococcaceae (58-65). Since Deferribacteres contains several sulfur reducers (66-68), it is possible that family V1B07b9 also contains them; however, information on this family is scarce. Helicobacteraceae and Campylobacteraceae contain both sulfur-reducing microorganisms and known DPRB (15, 55, 58), but the significant OTU in the Helicobacteraceae (OTU 1) claded with the sulfur-reducing bacteria. Key OTUs in the Campylobacteraceae clustered with a known DPRB of the genus *Arcobacter* (15) and a sulfur-reducing bacterium (69) (Fig. 5).

The elemental sulfur produced by DPRB may provide an electron acceptor for sulfur-reducing bacteria and stimulate sulfur cycling. Hence it is possible that the enrichment of sulfur-reducing bacteria is a consequence of sulfur cycling in the presence of perchlorate and not an indicator of DPRB. Oxygen is produced as an intermediate of perchlorate respiration (1), so this hypothesis is further supported by the strictly anaerobic lifestyle of bacteria in these families (Desulfuromonadaceae, Pelobacteraceae, Peptococcaceae) (59-64, 70). The inability to isolate Desulfuromonodaceae on perchlorate, despite them dominating the enriched community (at 5% and 7% salinities, Fig. 8), supports the hypothesis that their growth may be secondary. Alternatively, sulfur-reducing bacteria may reduce perchlorate to chlorite using an unspecified reductase and the produced chlorite could then be detoxified by an abiotic reaction with sulfide, as has been shown for the anaerobic archaeon *Archaeoglobus fulgidus* (17). Although Peptococcaceae have been shown to reduce both sulfate and sulfur (65, 71), Desulfuromonadaceae and Pelobacteraceae have only been shown to reduce sulfur (59-64), and it remains to be elucidated whether non-sulfate reducing bacteria can reduce perchlorate using a metabolism analogous to the one used by *A. fulgidus*.

If the growth of bacteria in sulfur-reducing groups is secondary, then potential DPRB communities appear to consist primarily of the *Azoarcus*/*Thauera*/*Denitromonas* branch of the Rhodocyclaceae (1% and 3% salinity), Pseudomonadaceae genus *Pseudomonas* (1% salinity), Campylobacteraceae genus *Arcobacter* (1% and 3% salinity), and Sedimenticolaceae genus *Sedimenticola* (3% salinity). At both 5% and 7% salinity the only potential DPRB is in Campylobacteraceae (genus *Arcobacter*; different OTU than in 1% salinity).

All isolates obtained belong to Rhodocyclaceae, Pseudomonadaceae, or Sedimenticolaceae, with the exception of the *Marinobacter* sp. (Alteromonadaceae) isolated from 5% salinity. *Marinobacter* sp. was not a SIMPER selected OTU and was found to occur only at 1.5 ± 1.1% relative abundance at 5% salinity on day 28 (data not shown). Among the isolates, the *Denitromonas*, *Azoarcus* (family Rhodocyclaceae), and *Pseudomonas* strains come from genera not previously containing perchlorate-reducing isolates. *Denitromonas* and *Azoarcus* were enriched in previous community studies, but perchlorate-reducing strains were not isolated (25, 29, 32). Chlorate-reducing *Pseudomonas* strains have been isolated but they cannot reduce perchlorate (54, 72).

Although several studies have investigated the microbial community of enrichments in response to perchlorate (25, 26, 28, 29, 32), this is the first report of an archaeon (phylum Parvarchaeota) being enriched in a community actively consuming perchlorate (Figs. 4 and 7). The obscure Order YLA114 of the Parvarchaeota were enriched at 5% NaCl (Fig. 4 and 7), but it has only been previously described in metagenomics experiments of acid mine drainage (73-75). Interestingly, *Candidatus* Parvarchaeum acidiphilum and *Candidatus* Parvarchaeum acidophilus are likely to be aerobic microorganisms (73), which strengthens the hypothesis that the Parvarchaeota seen in our study may be a true DPRB.

These findings present challenges and opportunities for perchlorate bioremediation via ion-exchange technology. Microbial communities were sensitive to salinity, showing reduced efficiency at high % NaCl (Fig. 1). However, our marine community could reduce perchlorate in brines of up to 7% NaCl (Fig. 1). Because salinity influenced the rate of perchlorate removal, the brine would need to be carefully monitored to maintain efficient perchlorate reduction. Furthermore, though diverse taxa were capable of perchlorate reduction at lower salinity (1-3%

NaCl), the prominent DPRB at high salinity (5-7% NaCl) consisted of only one *Arcobacter* species. Further acclimation of the enrichment to 7% NaCl and isolations of the halophilic *Arcobacter* species from this sample may expand the opportunities for bioremediation. Perchlorate also enriched for DPRB and bacteria in sulfur-reducing families. Although no known DPRB can grow by sulfide oxidation (56), it remains to be seen whether sulfur cycling and the presence of a sulfur reducing partner is beneficial or detrimental to DPRB and the rate of perchlorate reduction in a microbial community.

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Tables and Figures

Table 1. Accession numbers for microorganisms used in the 16S rRNA tree. Bolded species were used to create a reference tree.

Accession Number (Genbank unless otherwise specified)	Species
AJ457191	*Acidovorax aerodenitrificans*
AM084230	***Actinomyces europaeus***
CP002449	*Alicycliphilus denitrificans BC*
AF467248	*Alkaliphilus crotonatoxidans*
EF382660	*Alkaliphilus peptidifermentans*
AB037677	*Alkaliphilus transvaalensis*
AF091150	***Anabaena cylindrica***
X05567	*Archaeoglobus fulgidus VC-16*
FJ573217	*Arcobacter bivalviorum*
FR717550	*Arcobacter ellisii*
EU106661	*Arcobacter nitrofigilis*
KP137431	*Arcobacter sp. CAB*
CR555306	*Aromatoleum aromaticum EbN1*
Y14701	*Azoarcus anaerobius*
AJ315676	*Azoarcus buckelii*
AF011343	*Azoarcus communis*
X77679	*Azoarcus evansii*
AF011345	*Azoarcus indigens*
EF158388	*Azoarcus olearius*
EU434484	*Azoarcus sp. b303*
EU434556	*Azoarcus sp. b14*
NR_074801	*Azoarcus sp. BH72*
ARJX01000004	*Azoarcus toluclasticus ATCC 700605*
AF229861	*Azoarcus tolulyticus*
NR_024852	*Azospira oryzae*
AF323491	*Azospira sp. KJ*
AY126453	*Azospira sp. PCC*
AF170348	*Azospira suillum PS*
AF170353	*Azospirillum sp. TTI*
ABVO01000045	*Bacteroides eggerthii DSM 20697*
L16487	***Bacteroides heparinolyticus ATCC 35895***

AB626629	*Bacteroides zoogleoformans*
AF521195	*Brumimicrobium glaciale*
DQ660382	*Brumimicrobium mesophilum*
AB250968	*Calditerricola satsumensis*
AB308475	*Calditerricola yamamurae*
AF550626	*Campylobacter jejuni*
AB066098	*Campylobacter lari*
AE005673	***Caulobacter crescentus CB15***
AB054671	***Chlorobium limicola***
AB680436	***Citrobacter amalonaticus***
Bardiya and Bae, 2003	*Citrobacter amalonaticus JB101*
AB504755	*Citrobacter farmeri*
Bardiya and Bae, 2003	*Citrobacter farmeri JB109*
X79862	*Clostridium acetireducens*
AE001437	*Clostridium acetobutylicum ATCC 824*
HQ328061	*Clostridium botulinum*
AB680704	***Comamonas aquatica***
AY124797	*Dechlorobacter hydrogenophilus LT-1*
AF170359	*Dechloromarinus chlorophilus NSS*
NR_024884	*Dechloromonas agitata CKB*
AY032610	*Dechloromonas aromatica RCB*
AY277621	*Dechloromonas hortensis MA-1*
AF170357	*Dechloromonas sp. MissR*
AY126452	*Dechloromonas sp. PC1*
AY530551	*Dechlorospirillum sp. DB*
AF170352	*Dechlorospiriullum sp. WD*
AJ515881	*Deferribacter abyssi*
EU407777	*Deferribacter autotrophicus*
AB086060	*Deferribacter desulfuricans*
U75602	*Deferribacter thermophilus*
AB049763	*Denitromonas aromaticus*
AY972852	*Denitromonas indolicum*
CP001968	*Denitrovibrio acetiphilus DSM 12809*
AF282177	*Desulfomonile limimaris*
CP003360	*Desulfomonile tiedjei DSM 6799*
CP001720	*Desulfotomaculum acetoxidans DSM 771*
CP002736	*Desulfotomaculum carboxydivorans CO-1-SRB*
CP003273	*Desulfotomaculum gibsoniae DSM 7213*
AB778017	*Desulfotomaculum intricatum*

CP002770	*Desulfotomaculum kuznetsovii DSM 6115*
HE582755	*Desulfotomaculum luciae*
DQ208688	*Desulfotomaculum thermosubterraneum*
U23140	*Desulfuromonas acetexigens*
AAEW02000008	***Desulfuromonas acetoxidans DSM 684***
U49748	*Desulfuromonas chloroethenica*
AF357914	*Desulfuromonas michiganensis*
U28172	*Desulfuromonas palmitatis*
AY835388	*Desulfuromonas svalbardensis*
Y11560	*Desulfuromonas thiophila*
X79412	*Desulfuromusa bakii*
AY835392	*Desulfuromusa ferrireducens*
X79414	*Desulfuromusa kysingii*
X79415	*Desulfuromusa succinoxidans*
U46860	*Geobacter hydrogenophilus*
L07834	*Geobacter metallireducens*
FW306010	*Geobacter sulfurreducens*
X95744	*Geovibrio ferrireducens*
AJ299402	*Geovibrio thiophilus*
CP001804	*Haliangium ochraceum DSM 14365*
AB062751	*Haliangium tepidum*
CP991217	***Helicobacter pylori***
NR_026108	*Ideonella dechloratans*
AB176674	*Lishizhenia caseinilytica*
EU183317	*Lishizhenia tianjinensis*
EF405824	*Magnetospirillum bellicus VDY*
Y10110	*Magnetospirillum magnetotacticum*
Y17712	*Malonomonas rubra*
AJ294359	*Marinobacter algicola*
EU440994	*Marinobacter alkaliphilus*
DQ235263	*Marinobacter vinifirmus*
JN861074	*Marinobacter vinifirmus P4B1*
U82327	***Moorella glycerini***
EF060194	*Moorella perchloratireducens*
CP000232	*Moorella thermoacetica ATCC 39073*
L09168	*Moorella thermoautotrophica*
GQ863487	*Natranaerovirga hydrolytica*
GQ922846	*Natranaerovirga pectinivora*
AM711529	*Nostoc calcicola*
CP000140	*Parabacteroides distasonis ATCC 8503*

AGZQ01000007	*Parabacteroides merdae CL03T12C32*
AB681144	*Pedobacter africanus*
KF528723	*Pedobacter alluvionis*
AQGK01000001	*Pedobacter heparinus DSM 2366*
X70955	*Pelobacter acetylenicus*
X77216	*Pelobacter acidigallici*
CP000142	*Pelobacter carbinolicus DSM 2380*
FR749901	*Pelobacter massiliensis*
DQ991964	*Pelobacter seleniigenes*
U41562	*Pelobacter venetianus*
ABCE01000003	***Planctomyces maris DSM 8797***
AY530552	*Propionivibrio militaris CR*
EU849004	*Propionivibrio militaris MP*
AB037546	*Pseudomonas aeruginosa*
AY017341	*Pseudomonas chloritidismutans AW-1*
AY277620	*Pseudomonas putida strain ASK-1*
AF323492	*Pseudomonas sp. PDA*
AF323493	*Pseudomonas sp. PDB*
AF170358	*Pseudomonas sp. PK*
AB109011	*Pseudomonas stutzeri*
AB176954	*Pseudomonas xanthomarina*
AB517714	*Salinirepens amamiensis*
AF432145	*Sedimenticola selenatireducens*
KM192219	*Sedimenticola selenatireducens CUZ*
AB061685	*Serratia marcescens*
JQ807993	*Serratia marcescens*
AB681980	*Shewanella algae*
ACDC_00040000 (IMG)	*Shewanella algae ACDC*
AJ279798	*Sporomusa acidovorans*
AJ279799	*Sporomusa malonica*
ASXP01000001	*Sporomusa ovata DSM 2662*
EF060193	*Sporomusa sp. An4*
AB184196	*Streptomyces coelicolor*
CP000077	***Sulfolobus acidocaldarius DSM 639***
CP000153	*Sulfurimonas denitrificans*
ABXD01000012	*Sulfurimonas gotlandica GD1*
GQ863490	*Sulfurospirillum alkalitolerans*
CP001816	*Sulfurospirillum deleyanium*
AB757831	*Thauera aminoaromatica*

AF229881	*Thauera aromatica*
AB021377	*Thauera butanivorans*
AF229887	*Thauera chlorobenzoica*
AJ005816	*Thauera linaloolentis*
Y17590	*Thauera mechernichensis*
AJ315678	*Thauera phenylacetica*
Y17591	*Thauera selenatis*
AJ401017	*Thermotoga maritima*
JF754948	*Thermotoga neapolitana*
HG917903	***Thermotoga subterranea***
AJ404731	*Thiomicrospira arctica*
AF013975	*Thiomicrospira chilensis*
AF064545	*Thiomicrospira crunogena*
AJ404732	*Thiomicrospira psychrophila*
JN882289	*Thiotaurens thiomutagens*
FJ424814	*Wandonia haliotis*
NR_025942	*Wolinella succinogenes*
KP137426	*Denitromonas halophilus* SFB-1
KP137427	*Denitromonas halophilus* SFB-2
KP137428	*Denitromonas halophilus* SFB-3
KP137425	*Marinobacter vinifirmus* UCB
KP137429	*Pseudomonas sp.* CAL
KP122946	*Sedimenticola selenatireducens* BK-1
KP122947	*Sedimenticola selenatireducens* BK-2
KP137430	*Azoarcus marinus sp.* PHD

Table 2. Isolate information. Underlined species are known DPRB (Carlstrom et al submitted, 33).

Isolate Name	Accession Number	% NaCl	Closest BLAST match in NCBI nt database and percent identity
Sedimenticola selenatireducens BK-1	KP122946	1	*Sedimenticola selenatireducens* AK4OH1 (98%); *Sedimenticola selenatireducens* CUZ (98%)
Sedimenticola selenatireducens BK-2	KP122947	3	*Sedimenticola selenatireducens* AK4OH1 (98%); *Sedimenticola selenatireducens* CUZ (98%)
Denitromonas halophilus SFB-1	KP137426	1	*Denitromonas indolicum* (98%); *Denitromonas aromaticus* (98%)
Denitromonas halophilus SFB-2	KP137427	1	*Denitromonas indolicum* (98%); *Denitromonas aromaticus* (98%)
Denitromonas halophilus SFB-3	KP137428	3	*Denitromonas indolicum* (98%); *Denitromonas aromaticus* (98%)
Marinobacter vinifirmus UCB	KP137425	5	*Marinobacter vinifirmus* (98%); *Marinobacter vinifirmus P4B1* (98%)
Pseudomonas sp. CAL	KP137429	1	*Pseudomonas putida* (99%); *Pseudomonas chloritidismutans* (99%)
Azoarcus marinus PHD	KP137430	1	*Azoarcus* sp. b303 (99%); *Azoarcus* sp. b14 (99%)

Table 3. p-values for selected comparisons with respect to richness, Shannon's diversity Index, and Shannon's Equitability (EH).

		Distinct OTUs (Richness)				Shannon's Diversity Index				Shannon's Equitability (EH)			
Salinity	Comparison	T Statistic	P value	Significant at 0.05	Highest Richness In	T Statistic	P value	Significant at 0.05	Higher Diversity In	T Statistic	P value	Significant at 0.05	Higher in (more even)
1%	DNA vs RNA Day 14	-1.65	0.15	N		3.85	0.006	Y	DNA	3.82	0.007	Y	DNA
3%	DNA vs RNA Day 28	2.25	0.065	N		2.45	0.044	Y	DNA	2.41	0.047	Y	DNA
5%	DNA vs RNA Day 28	-0.91	0.404	N		2.99	0.024	Y	DNA	4.06	0.005	Y	DNA
7%	DNA vs RNA Day 42	10.65	0.06	N		1.48	0.378	N		0.73	0.599	N	
1%	Initial vs Day 14	7.12	0.019	Y	Initial	16.33	0	Y	Initial	11.74	0	Y	Initial
1%	Initial vs Day 28	3.74	0.02	Y	Initial	7.97	0.004	Y	Initial	7.59	0.005	Y	Initial
1%	Initial vs Day 42	3.23	0.032	Y	Initial	5.18	0.007	Y	Initial	4.86	0.008	Y	Initial
3%	Initial vs Day 14	0.62	0.562	N		5.18	0.006	Y	Initial	7.23	0.002	Y	Initial
3%	Initial vs Day 28	3.79	0.013	Y	Initial	8.12	0.001	Y	Initial	7.83	0.001	Y	Initial
3%	Initial vs Day 42	1.18	0.289	N		6.55	0.003	Y	Initial	7.45	0.002	Y	Initial
5%	Initial vs Day 14	1.17	0.328	N		26.43	0	Y	Initial	29.05	0	Y	Initial
5%	Initial vs Day 28	5.51	0.031	Y	Initial	23.19	0	Y	Initial	17.76	0	Y	Initial
5%	Initial vs Day 42	4.48	0.007	Y	Initial	8.77	0.001	Y	Initial	9.12	0.001	Y	Initial
7%	Initial vs Day 14	0.65	0.58	N		15.5	0.041	Y	Initial	10.33	0.061	Y	Initial
7%	Initial vs Day 28	0.34	0.793	N		3.68	0.169	N		4.54	0.138	N	
7%	Initial vs Day 42	6.06	0.026	Y	Initial	42.12	0.015	Y	Initial	36.61	0.017	Y	Initial
1%	Day 14 vs Day 28	-6.37	0.001	Y	Day 28	-6.37	0.001	Y	Day 28	-6.37	0.001	Y	Day 28
1%	Day 14 vs Day 42	-5.1	0.001	Y	Day 42	-5.1	0.001	Y	Day 42	-5.1	0.001	Y	Day 42
3%	Day 28 vs Day 14	-4.06	0.007	Y	Day 14	-4.06	0.007	Y	Day 14	-4.06	0.007	Y	Day 14
3%	Day 28 vs Day 42	-2.25	0.059	N	Day 42	-2.25	0.059	N		-2.25	0.059	N	Day 42
5%	Day 28 vs Day 14	-6.03	0.001	Y	Day 14	-6.03	0.001	Y	Day 14	-6.03	0.001	Y	Day 14
5%	Day 28 vs Day 42	-0.34	0.75	N		-0.34	0.75	N		-0.34	0.75	N	
7%	Day 42 vs Day 14	-21.84	0.029	Y	Day 14	-21.84	0.029	Y	Day 14	-21.84	0.029	Y	Day 14
7%	Day 42 vs Day 28	-0.47	0.719	N		-0.47	0.719	N		-0.47	0.719	N	

Table 4. Relative abundance (%) and fold-change of SIMPER selected phyla/classes at the most active day for each salinity. Values are averages of 5 replicates, except for the 7% NaCl condition in which duplicates were used. Uncertainty represents standard error. Cells were left empty if fold change < 1 or relative abundance of sample was < 4%.

	NaCl Concentration and Time			
Phylum/Class	1% (day 14)	3% (day 28)	5% (day 28)	7% (day 42)
Betaproteobacteria	17 ± 8 53-fold	16 ± 11 47-fold		
Gammaproteobacteria		43 ± 13 1.8 fold		
Deltaproteobacteria			28 ± 3 1.4-fold	30 ± 20 1.4-fold
Epsilonproteobacteria	29 ± 4 7-fold	4.9 ± 0.4 1.2-fold	23 ± 2 7-fold	29 ± 8 7-fold
Deferribacteres				12 ± 16 8000-fold
Firmicutes	4.3 ± 0.4 7-fold		8.7 ± 0.6 13-fold	5.9 ± 0.7 9-fold
Parvarchaeota			6 ± 1 5.5-fold	

Figure 1. Acetate and perchlorate concentrations in the enrichment samples at A) 1%, B) 3%, C) 5%, and D) 7% NaCl. Closed triangles: acetate concentration; closed circles: perchlorate concentration; open diamonds: acetate in the no perchlorate control; open squares: perchlorate in the no donor control. Solid lines represent the averages of five replicate samples except in (D) where the solid line represents one replicate and a dashed line represents a second replicate. Error bars represent standard error.

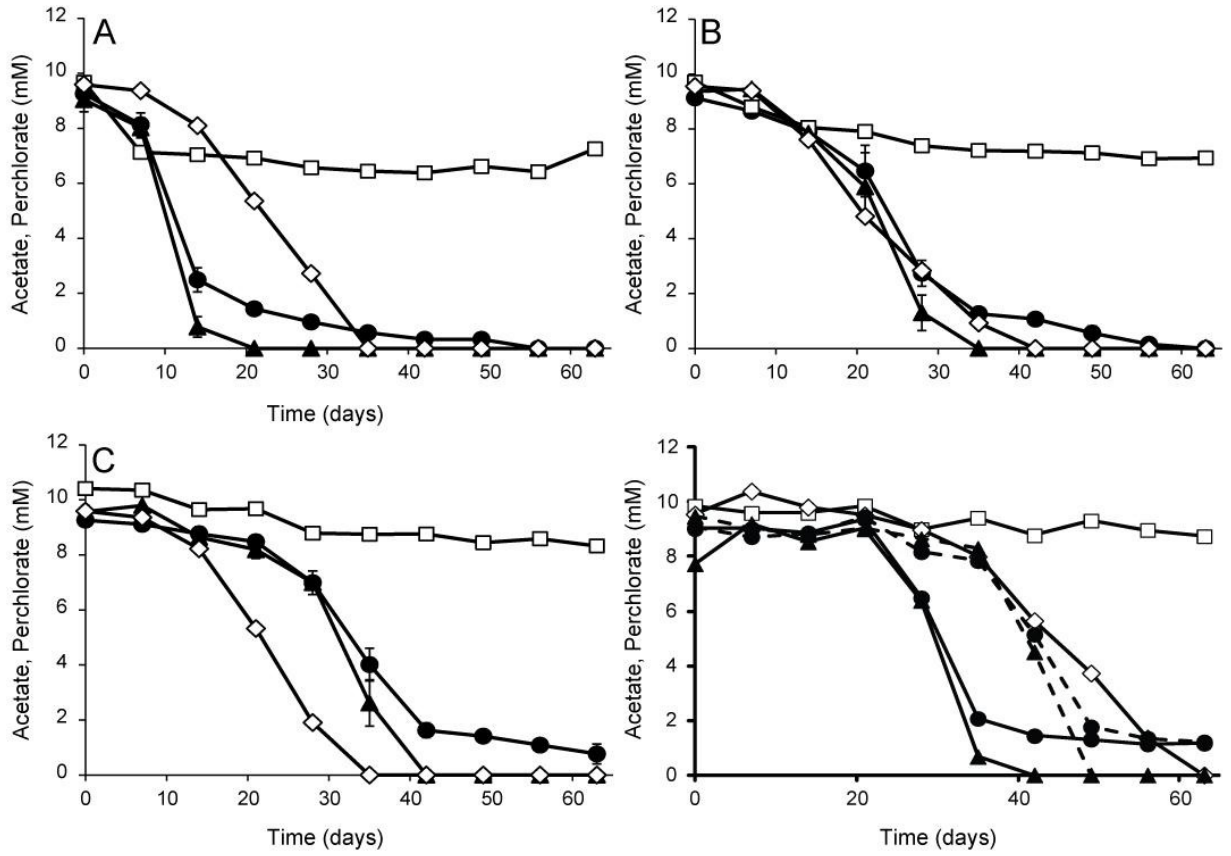


Figure 2. Richness, evenness, and diversity in DNA samples over time. A) Number of distinct OTUs (richness), B) Shannon's Equitability (evenness), and C) Shannon's Diversity. Red: 1% NaCl, blue: 3% NaCl, green: 5% NaCl, purple: 7% NaCl, black: initial samples. X-axis represents time (days) for DNA samples and the RNA at the most active day of each salinity (1% = day 14, 3 and 5% = day 28, and 7% = day 42). Error bars represent the standard error of 3-5 replicate samples except in the 7% NaCl condition in which duplicates were used.

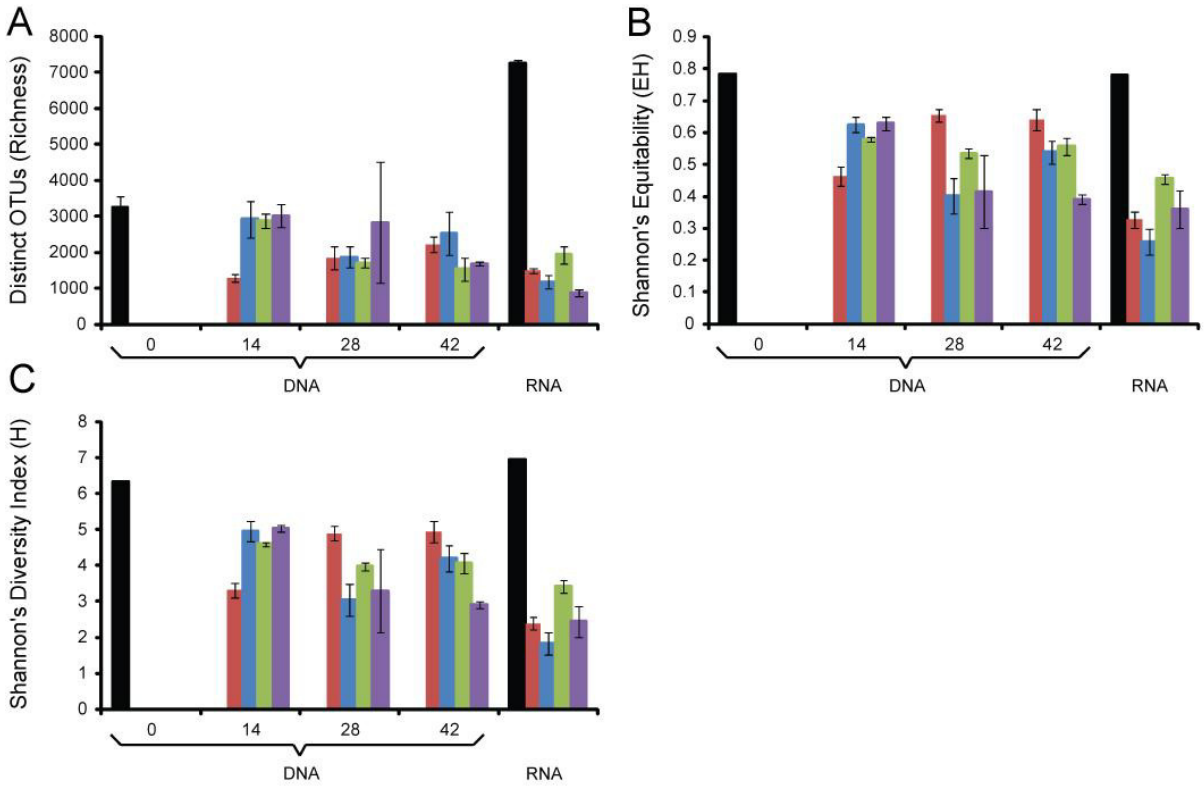


Figure 3. Non-metric multidimensional scaling (NMDS) plot of DNA microbial community data at the OTU level based on a Bray-Curtis similarity matrix. Circles represent percent similarity based on hierarchical clustering. Colored symbols represent salinity (1, 3, 5, and 7%) and the original sample (t0). Number labels represent time (day) of sampling and red, bold font marks samples from the most active day at each salinity.

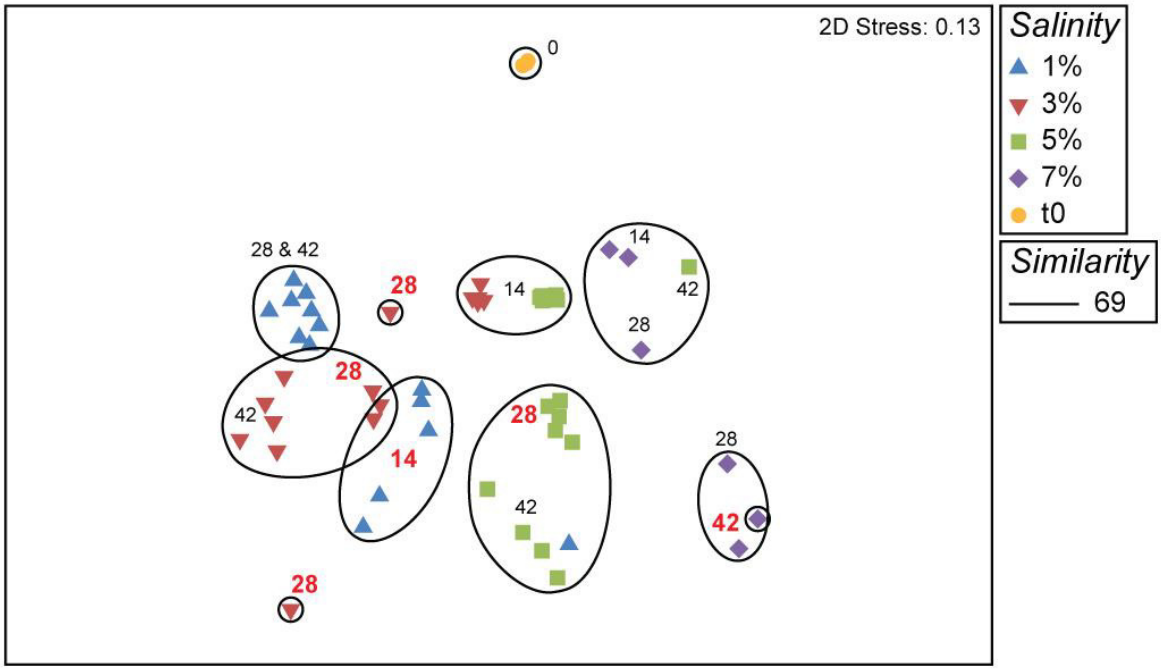


Figure 4. Relative abundance of bacterial and archaeal phyla (classes for Proteobacteria) in DNA samples over time. A) 1% NaCl, B) 3% NaCl, C) 5% NaCl and D) 7% NaCl. Columns represent the average of 3-5 replicate samples, except for days 14-42 at 7% NaCl which is the average of duplicate samples. Red rectangles mark the most active day (based on Fig. 1) at each salinity.

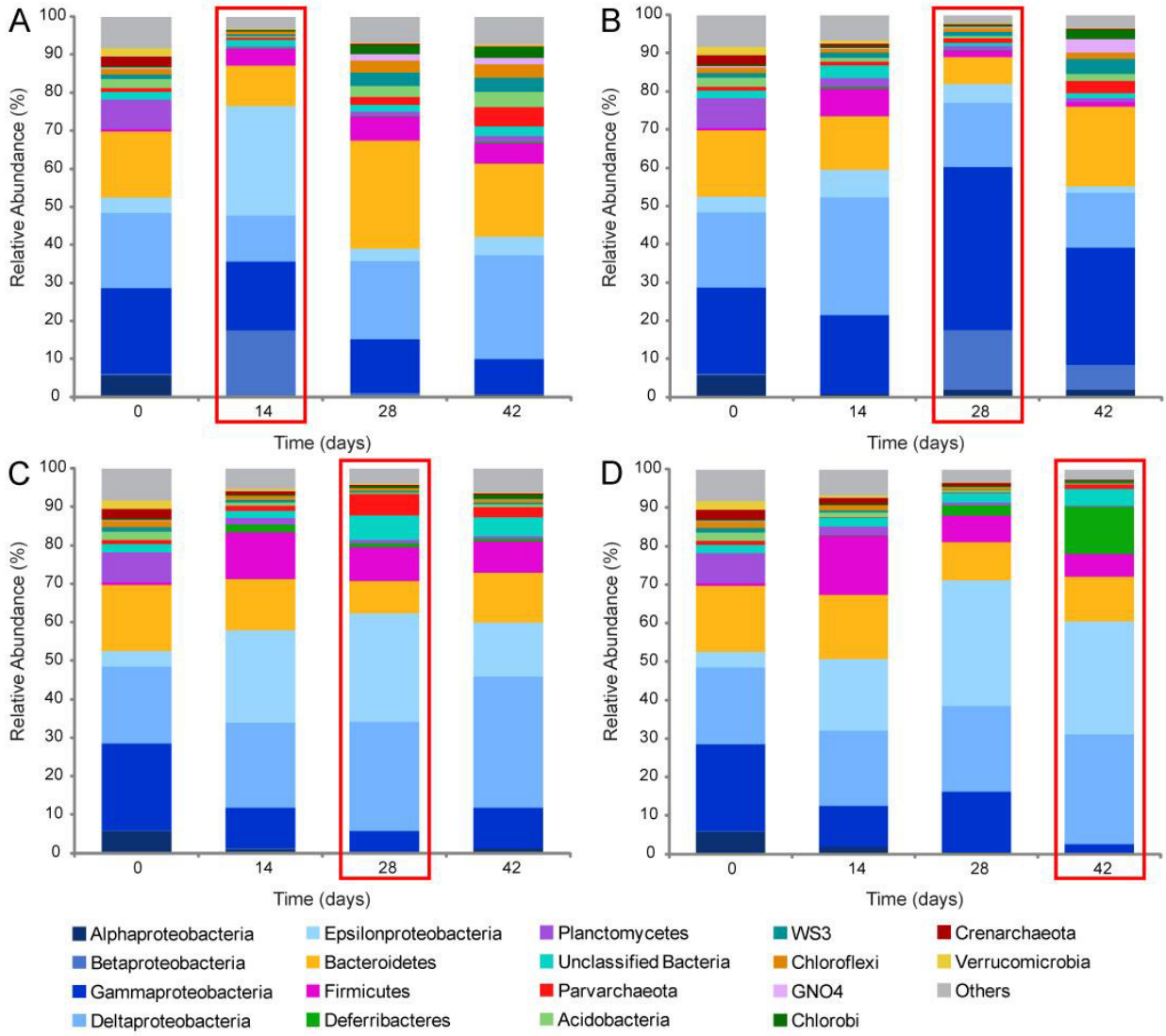


Figure 5. Relative abundance of bacterial and archaeal phyla/classes in DNA samples at A) 1% NaCl (day 14), B) 3% NaCl (day 28), C) 5% NaCl (day 28), and D) 7% NaCl (day 42) at their most enriched day. Each column represents a single replicate (1-5) and the last column in each panel represents their average. Only two of five replicates at 7% NaCl consumed perchlorate, thus only two replicates are shown in panel D.

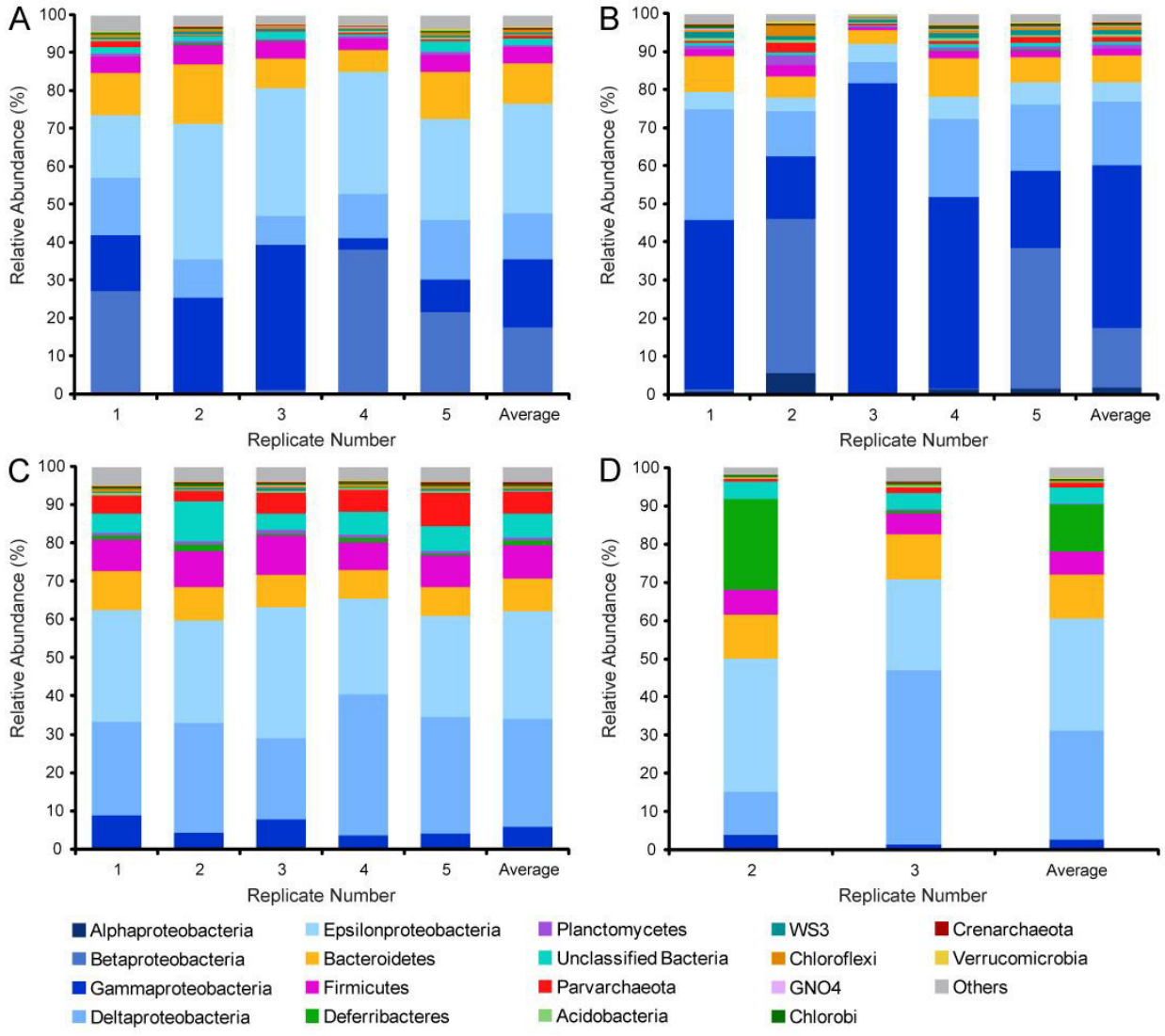


Figure 6. Non-metric multidimensional scaling (NMDS) plot of RNA microbial community data at the OTU level based on a Bray-Curtis similarity matrix. Circles represent % similarity based on hierarchical clustering. Colored symbols represent salinity (1, 3, 5, and 7%) and the original sample (t0). Number labels represent time (day) of sampling. The same plot in three dimensions is inset in the upper right corner.

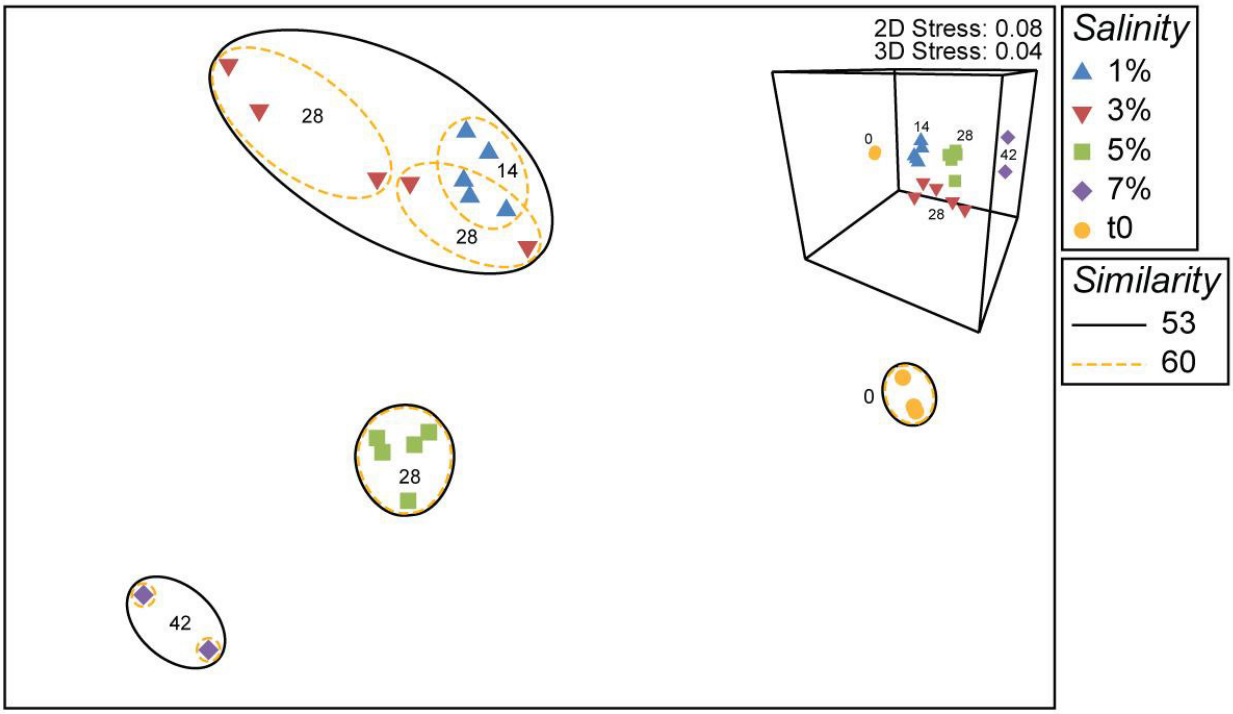


Figure 7. A) Change in relative abundance and B) log2 fold change of key OTUs grouped by family in RNA samples. Key OTUs contributing to the top 30% of the dissimilarity between the unenriched RNA and the RNA at the most active day at each salinity were identified by SIMPER and normalized data were used to calculate change in relative abundance and log2 fold change. Colored bars represent different salinities (% NaCl) and families are color coded by phylum/class. Error bars represent the standard error of 3-5 replicate samples except in the 7% NaCl condition in which duplicates were used. * indicates OTUs were not detectable in the initial sample and thus a log2 fold could not be calculated.

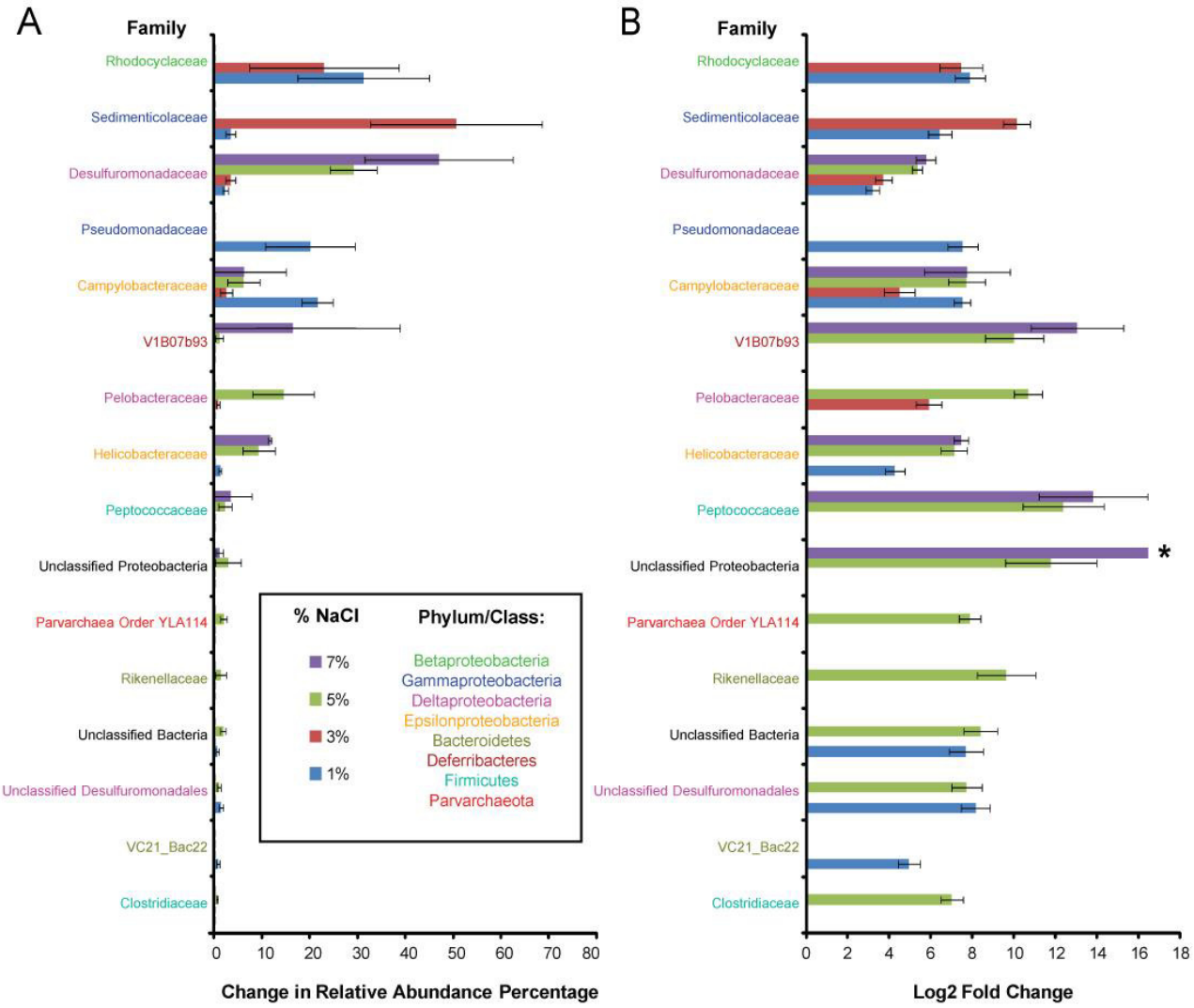


Figure 8. Relative abundance of key OTUs summed by family in RNA samples at their most enriched day. Colored bars represent different salinities (1,3,5, and 7% NaCl) and families are color coded by phylum/class. Error bars represent the standard error of 3-5 replicate samples except in the 7% NaCl condition in which duplicates were used.

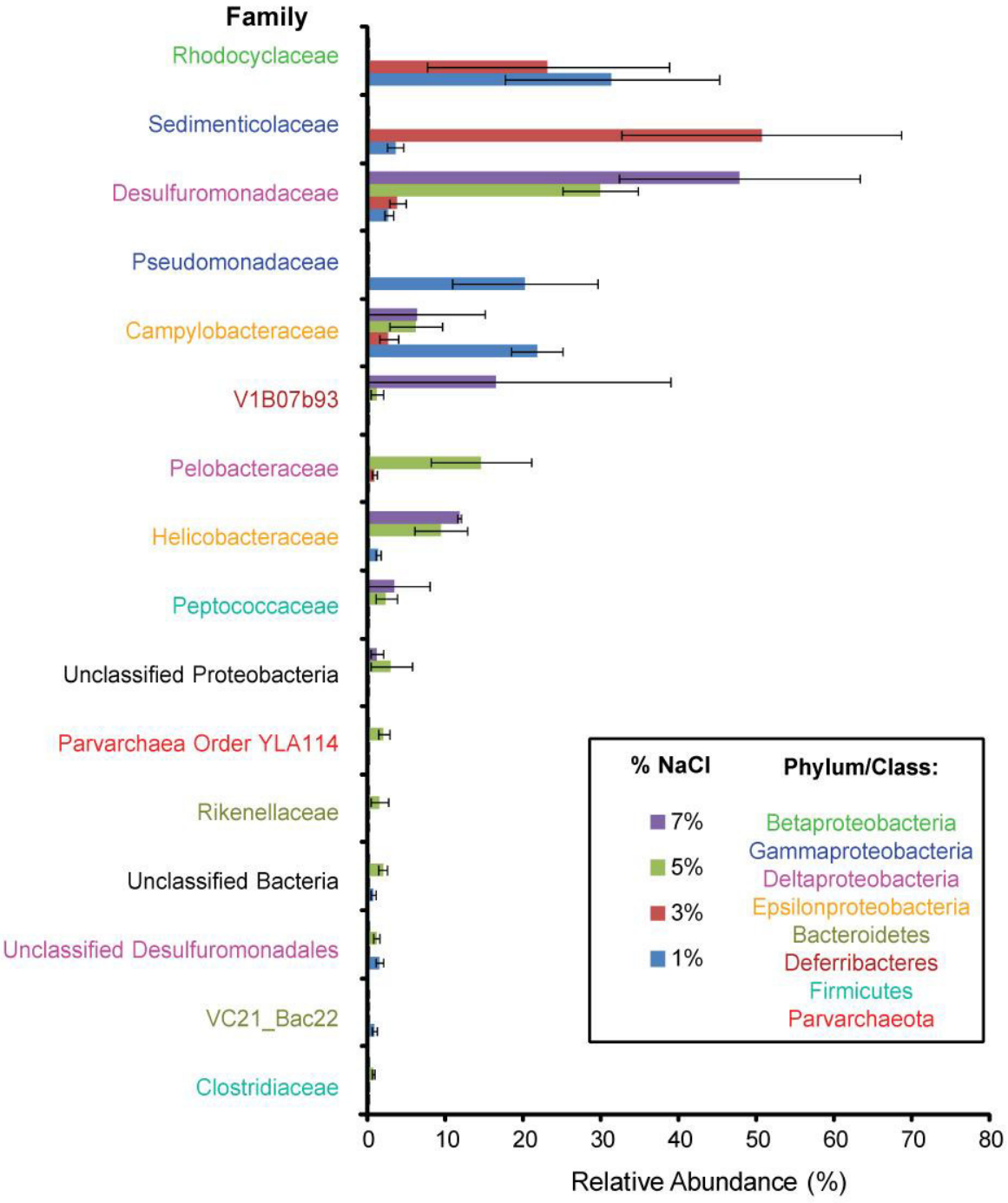
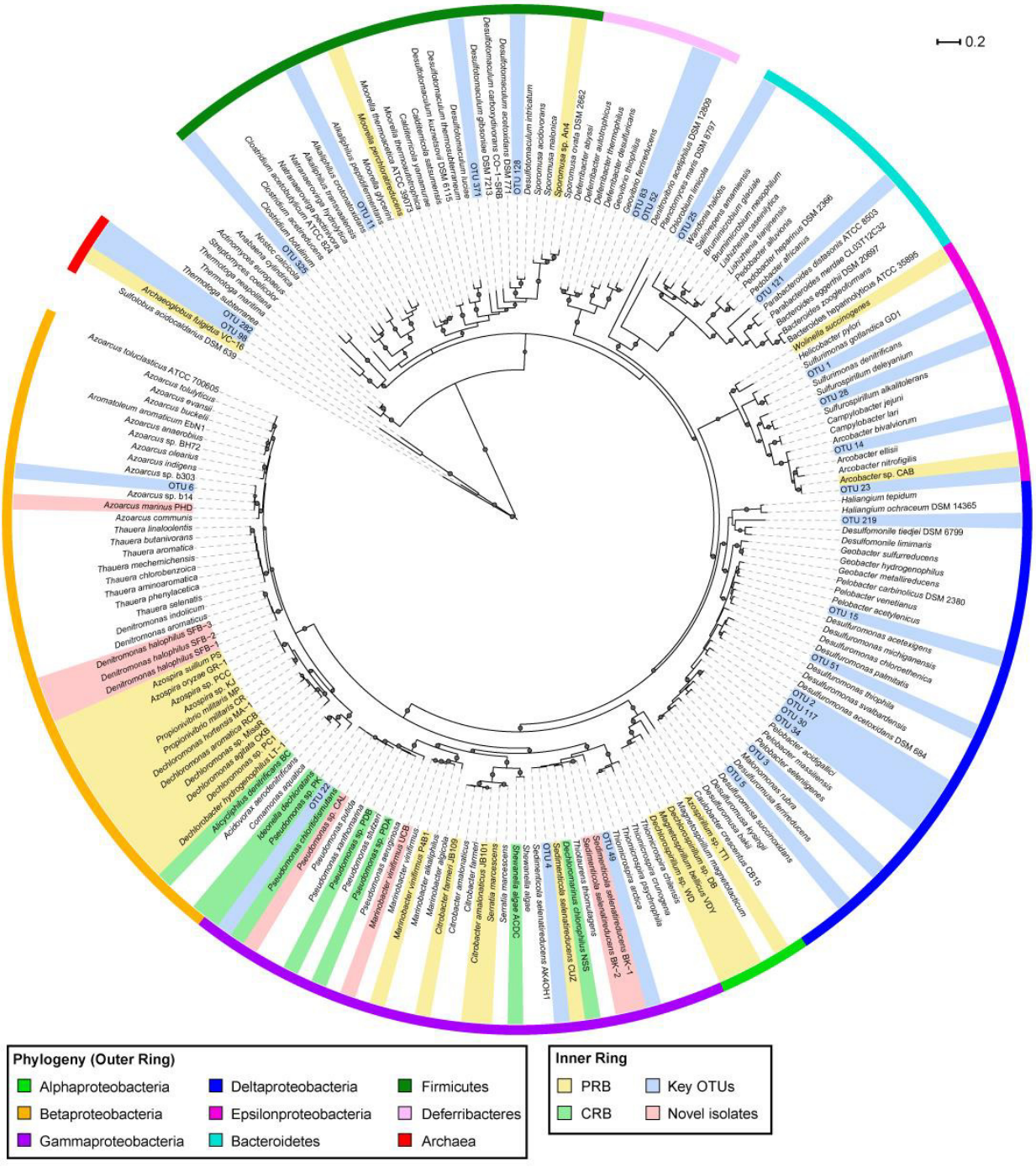


Figure 9. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic position of novel isolates and key OTUs enriched with perchlorate among known perchlorate reducing bacteria (PRB) and chlorate reducing bacteria (CRB). Bootstrap values are based on 1,000 replications and branches with values >70 are marked with a circle. The scale bar represents 0.2 expected change per site.



Chapter 3:
Physiological and Genetic Description of Dissimilatory Perchlorate Reduction by the Novel Marine Bacterium *Arcobacter* sp. strain CAB

This chapter has been previously published as an article under the following reference:

Physiological and Genetic Description of Dissimilatory Perchlorate Reduction by the Novel Marine Bacterium *Arcobacter* sp. Strain CAB. Charlotte I Carlstrom, Ouwei Wang, Ryan A. Melnyk, Stefan Bauer, Joyce Lee, Anna L. Engelbrektson, and John D. Coates. *mBio*. 2013, 4: e00217-13.

Abstract

A novel dissimilatory perchlorate-reducing bacterium (DPRB), *Arcobacter* sp. strain CAB, was isolated from a marina in Berkeley, CA. Phylogenetically, this halophile was most closely related to *Arcobacter defluvii* strain SW30-2 and *Arcobacter ellisii*. With acetate as the electron donor, strain CAB completely reduced perchlorate (ClO_4^-) or chlorate (ClO_3^-) [collectively designated (per)chlorate] to innocuous chloride (Cl^-), likely using the perchlorate reductase (Pcr) and chlorite dismutase (Cld) enzymes. When grown with perchlorate, optimum growth was observed at 25 to 30°C, pH 7, and 3% NaCl. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) preparations were dominated by free-swimming straight rods with 1 to 2 polar flagella per cell. Strain CAB utilized a variety of organic acids, fructose, and hydrogen as electron donors coupled to (per)chlorate reduction. Further, under anoxic growth conditions strain CAB utilized the biogenic oxygen produced as a result of chlorite dismutation to oxidize catechol via the *meta*-cleavage pathway of aerobic catechol degradation and the catechol 2,3-dioxygenase enzyme. In addition to (per)chlorate, oxygen and nitrate were alternatively used as electron acceptors. The 3.48-Mb draft genome encoded a distinct perchlorate reduction island (PRI) containing several transposases. The genome lacks the *pcrC* gene, which was previously thought to be essential for (per)chlorate reduction, and appears to use an unrelated *Arcobacter c*-type cytochrome to perform the same function.

Importance

The study of dissimilatory perchlorate-reducing bacteria (DPRB) has largely focused on freshwater, mesophilic, neutral-pH environments. This study identifies a novel marine DPRB in the genus *Arcobacter* that represents the first description of a DPRB associated with the *Campylobacteraceae*. Strain CAB is currently the only epsilonproteobacterial DPRB in pure culture. The genome of strain CAB lacks the *pcrC* gene found in all other DPRB tested, demonstrating a new variation on the (per)chlorate reduction pathway. The ability of strain CAB to oxidize catechol via the oxygenase-dependent *meta*-cleavage pathway in the absence of external oxygen by using the biogenic oxygen produced from the dismutation of chlorite provides a valuable model for understanding the anaerobic degradation of a broad diversity of xenobiotics which are recalcitrant to anaerobic metabolism but labile to oxygenase-dependent mechanisms.

Introduction

Perchlorate (ClO_4^-), a stable, water-soluble, toxic oxyanion of chlorine, has been widely produced through anthropogenic processes for use in rocket fuel, pyrotechnics, lubricants, and paints (1–3). Due to unregulated disposal of ClO_4^-before 1997, contamination is now widespread (3, 4), and it poses a significant health threat, as ClO_4^- inhibits the uptake of iodine by the thyroid gland and may lead to hypothyroidism (1, 5, 6). Recent geochemical studies indicate that extant (per)chlorate is far more prevalent than originally perceived (7–9). Although (per)chlorate has been considered to be primarily of anthropogenic origin, the widespread discovery of (per)chlorate in pristine environments (7–10), including waters and sediments from Mono Lake, CA (J. D. Coates, unpublished data); the Antarctic Dry Valleys (11); and, recently, Martian regoliths (12), has indicated a more complex and possibly ancient natural geochemical origin. This is supported by stable isotope analysis of the chlorine and oxygen isotopes of perchlorate, which allow for the distinction between anthropogenic and natural sources (13). These findings have identified the existence of an active biogeochemical redox cycle of chlorine on neoteric Earth and hint at its ancient origin.

Since the first dissimilatory perchlorate-reducing bacterium (DPRB) was isolated in 1996 (14, 15), more than 50 strains that use (per)chlorate as a metabolic electron acceptor are now known (16). All known DPRB respire perchlorate completely to chloride (Cl^-) using the key enzymes perchlorate reductase (Pcr), which can reduce both perchlorate and chlorate, and chlorite dismutase (Cld). The genes encoding Cld and Pcr, *cld* and *pcrABCD*, respectively, have recently been shown to be part of a perchlorate reduction island (PRI) (17) that is horizontally transferred across diverse phylogenetic boundaries. This genomic island also contains conserved gene families predicted to be involved in regulation, electron transport, and cofactor biosynthesis (17). In contrast, a recent study by Liebensteiner et al. (18) describes a mechanism of (per)chlorate reduction in the archaeon *Archaeoglobus fulgidus* that involves an interplay of biotic and abiotic reactions that do not involve Cld.

Thus far, the study of microbial (per)chlorate reduction has been limited mostly to isolates (e.g., *Dechloromonas*, *Azospira*, and *Ideonella*) found in freshwater, mesophilic, neutral-pH environments (1, 19–25). Exceptions are the Gram-positive species *Moorella perchloratireducens* and *Sporomusa* strain An4, both of which were isolated from an underground gas storage facility (26, 27), and the hyperthermophilic archaeon *Archaeoglobus fulgidus* (18). The current study expands the diversity of DPRB by reporting on an isolate obtained from a marine environment. The isolate, designated strain CAB, is a marine epsilonproteobacterium obtained from a marina in Berkeley, CA. It exhibits physiological and genomic features that make it unique among both existing DPRB and previously known members of the *Arcobacter* genus. Although previous studies demonstrated dissimilatory perchlorate reduction by the epsilonproteobacterium *Wolinella succinogenes* strain HAP-1 (15), cultures of that bacterium are no longer available. Therefore, strain CAB is currently the only epsilonproteobacterial DPRB available in pure culture and the first *Arcobacter* known to reduce (per)chlorate.

Materials and Methods

Culture conditions, enrichments, and isolation

Sediment samples were obtained from the Berkeley Marina in Berkeley, CA (latitude 37.8629 N; longitude 122.3132 W), using 50-ml Falcon tubes. Sample salinity was ~1.5%, and the sample contained no detectable perchlorate. The medium used for the enrichment, isolation, and culturing contained the following (g/liter): NaCl (30), KCl (0.67), $NaHCO_3$ (2.5), 10 ml vitamins, 10 ml minerals, and 20 ml RST minerals (21). The RST minerals contained the following (g/liter): NaCl (40), NH_4Cl (50), KCl (5), KH_2PO_4 (5), $MgSO_4 \cdot 7H_2O$ (10), and $CaCl_2 \cdot 2H_2O$ (1). The medium was boiled for 30 to 60 s, cooled on ice, and degassed under an N_2-CO_2 headspace (80/20). The pH was ~6.8. Each anaerobic tube (Bellco, Vineland, NJ) received 8 ml of this medium and was autoclaved. After autoclaving, 0.5 ml of $MgCl_2 \cdot 6H_2O$ (21.2 g/100 ml distilled water [dH_2O]) and 0.5 ml of $CaCl_2 \cdot 2H_2O$ (3.04 g/100 ml dH_2O) were added to each tube from sterile aqueous stock solutions.

For the enrichment, 1 g of sediment sample was mixed with 9 ml of the described medium and 10 mM (each) acetate and perchlorate were used as the electron donor and acceptor, respectively. The enrichments were maintained at 30°C for 2 weeks and then transferred into fresh medium twice prior to isolation. Agar deeps (with acetate and perchlorate) (21) and streak plates (with acetate and oxygen) were used for isolation.

Optimum pH, temperature, and salinity

Acetate and perchlorate were used to test the optimum growth temperature, pH, and salinity. The temperatures tested were 15°C, 20°C, 25°C, 30°C, 33°C, and 37°C. For salinity, the amount of NaCl added to the medium was varied from 0% to 6% in 0.5% increments. For pH, the medium described above was used, except that 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer was substituted for $NaHCO_3$. The medium was adjusted to the desired pH (6, 6.5, 7, or 7.5) using HCl or NaOH. All analyses were performed on triplicate cultures.

Alternate electron acceptors and electron donors

For the electron donor profile, perchlorate was used as the sole electron acceptor (10 mM). Electron donors were added from sterile aqueous solutions to give the following final concentrations (mM, unless otherwise specified): acetate (10), propionate (10), isobutyrate (10), butyrate (10), valerate (10), methanol (5), ethanol (5), benzoate (1 and 2.5), pyruvate (5), citrate (10), succinate (1), lactate (10), glucose (10), fructose (10), yeast extract (1 g/liter), fumarate (10), malate (10), hydrogen (~50 kPa), catechol (1), and methane (~25 kPa). The tubes containing hydrogen also contained 1 mM acetate as a carbon source. To determine if strain CAB oxidized the tested electron donor coupled to perchlorate reduction, a sample was taken at $t = 0$ and at $t = 11$ days and analyzed for a decrease in the concentration of perchlorate by ion chromatography (see below). Each donor was tested in triplicate, and negative controls included (also in triplicate) a no-donor control and a no-donor-plus-1 mM acetate control. Growth was

considered positive when perchlorate was consumed together with an increase in biomass and no loss of electron acceptor in the no-donor control.

For the electron acceptor profile, acetate (10 mM) was used as the sole electron donor and carbon source. Electron acceptors were added from sterile aqueous stocks in the following concentrations (mM unless otherwise specified): perchlorate (10), chlorate (10), nitrate (10), nitrite (2), sulfate (10), sulfite (2), thiosulfate (10), arsenate (2.5), selenate (2.5), ferric citrate (10), malate (10), and oxygen (~25 kPa). To determine if strain CAB reduced the tested acceptor coupled to the oxidation of acetate, a sample was taken at $t = 0$ and at $t = 11$ days and analyzed for a decrease in the concentration of acetate by high-performance liquid chromatography (HPLC) as described below. Each acceptor was tested in triplicate, and the experiment included a no-acceptor control (also in triplicate). Growth was considered positive when there was an acetate loss together with an increase of biomass and no loss of electron donor in the no-acceptor control.

Cell growth profiles

Cell growth was measured spectrophotometrically at 600 nm. All experimental analyses were performed in triplicate, and the results are expressed as the average of these determinations.

Analytical methods

Perchlorate concentration was measured via ion chromatography using a Dionex IonPac AS 16 (4- by 250-mm) column (Thermo Fisher Scientific, Sunnyvale, CA) with a 35 mM NaOH mobile phase at a flow rate of 1.0 ml/min. The eluting perchlorate was detected by conductivity suppressed with a Dionex ASRS operating in recycle mode. The suppressor controller was set at 100 mA for the analysis. The injection volume was 25 µl. Acetate concentrations were measured by HPLC (Dionex; model LC20), using a UV-visible (UV-Vis) detector (Dionex; AD20) at a wavelength of 210 nm and a Bio-Rad Aminex HPX-87H column with a mobile phase of 0.016 N H_2SO_4 (flow rate of 0.9 ml/min).

Catechol and 2-HMS were measured via liquid chromatography-mass spectrometry. For determination of catechol concentration, 2 µl of filtered sample was injected at 30°C onto a Rapid Resolution column (2.1 by 30 mm, 3.5 µm; Agilent Technologies, Santa Clara, CA) and analyzed on an Agilent 1100 series liquid chromatography system equipped with a degasser (G1379B), binary pump (G132B), autosampler (G1329B), and column compartment (G1316B) coupled to an Agilent 6510 quadrupole time of flight (QTOF) mass spectrometer equipped with a dual-spray electrospray ionization (ESI) source. Compounds were eluted with a flow rate of 0.5 ml/min and a mobile phase program of 1 min of isocratic flow of 98% A (0.1% formic acid) to 2% B (acetonitrile with 0.1% [vol/vol] formic acid) and then 1 min of linear flow to 10% B, 2 min of linear flow to 95% B, 2 min of isocratic flow, and an immediate return to 2% B with 3 min of equilibration. MS settings were as following: negative-mode ion polarity, 325°C gas temperature, 7-liter/min drying gas, 45-lb/in^2 nebulizer, V_{cap} (capillary voltage) of 3,500 V, scanning from 50 to 1,100 *m/z* (1.41 spectra/s), and internal mass calibration ions *m/z*112.985587 and *m/z* 1,033.988109. For analysis of 2-HMS, 10 µl of filtered sample was analyzed using the same method.

Scanning electron microscopy

Silicon wafers were washed with ethanol for 30 s and air dried. A 20-µl poly-L-lysine drop was placed on the silicon wafers for 1 min and then withdrawn. The wafers were then rinsed with ultraclean water. A drop of concentrated CAB cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was added to the silicon wafers and allowed to settle for 1 h. The silicon wafers were then processed as described in reference 24 and visualized using a Hitachi S-5000 scanning electron microscope at 20 kV.

Transmission electron microscopy

Flagella were visualized using the negative-stain technique. Copper Formvar- and carbon-coated grids (400 mesh) were glow discharged just before use to increase hydrophilicity. A suspension (5 µl) of CAB cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was deposited on the grids. This was sufficient to cover the grid. After 2 min, the suspension was removed by touching the ragged torn edges of filter paper to the sample until the grid surface was nearly dry. The grid was then washed 3 times on water droplets, and excess water was wicked off using filter paper as described above. A drop of 1% aqueous uranyl acetate was then added to the grid. After 2 min, the grid was dried with filter paper. Samples were examined the same day using a Tecnai transmission electron microscope at 120 kV.

Whole-cell fatty acid analysis

Fifty milliliters of strain CAB cells was grown aerobically using 20 mM acetate and anaerobically using 20 mM (each) acetate and perchlorate in the medium described above. The cells were then harvested via centrifugation and submitted to MIDI Labs (MIDI, Inc., Newark, DE) for whole-cell fatty acid analysis.

Genomic and phylogenetic analysis

Genomic DNA was extracted from strain CAB as described previously (50). The genomic DNA was then submitted to Eureka Genomics (Hercules, CA) for Illumina sequencing and partial assembly. The genome of *Arcobacter* sp. strain CAB is available on the Integrated Microbial Genomes (IMG) system of the Joint Genome Institute.

The Silva database and aligner (51) were used to align 16S rRNA gene sequences, and a maximum likelihood phylogenetic tree was constructed with 1,000 bootstrap values using RAxML-HPC (52). Accession numbers for the microorganisms used in the tree are provided in Text S1 in the supplemental material. *Sulfolobus acidocaldarius* was used as an outgroup.
RNA extraction and RT-PCR. Forty milliliters of triplicate cultures growing either with 1 mM catechol and 5 mM perchlorate or with 5 mM acetate and 5 mM perchlorate was centrifuged at 7,000 × *g*, 4°C, for 10 min. The pellets were resuspended in 1 ml RNAwiz reagent (Ambion, Grand Island, NY), and RNA was extracted according to the manufacturer's protocol. The Turbo DNA-free kit (Applied Biosystems, Grand Island, NY) was used to remove any contaminating DNA according to the manufacturer's protocol. cDNA was made with 200 ng RNA using the Verso cDNA kit (Thermo Fisher Scientific, Sunnyvale, CA) according to the manufacturer's

protocol. The RNA was tested for DNA contamination using the universal primers for 16S rRNA amplification 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′).

To determine if the genes in the 4-oxalocrotonate pathway of catechol oxidation were expressed when strain CAB was grown using catechol as an electron donor instead of acetate, RT-PCRs were performed using cDNA. The PCR primers used can be found in the supplemental material. Chlorite dismutase primers were used as a control. For primer pairs OEH 1F/1R, OXD 5F/5R, Cld 5F/5R, HOA 1F/1R, and CAT 1F/1R, PCR conditions were 95°C for 5 min; 30 cycles of 95°C for 45 s, 61°C for 45 s, and 72°C for 45 s; and 10 min at 72°C. For primer pair HMSD 4F/4R, the conditions were 95°C for 5 min; 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min; and 10 min at 72°C. For primer pair OXT 3F/3R, the conditions were 95°C for 5 min; 30 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 30 s; and 10 min at 72°C. For primer pair HSH 4F/4R, the conditions were 95°C for 5 min; 30 cycles of 95°C for 45 s, 65°C for 45 s, and 72°C for 30 s; and 10 min at 72°C.

Results

Enrichment, isolation, and phylogeny

Culture enrichment was performed for 2 weeks in medium containing 3% NaCl inoculated with sediment from the Berkeley Marina. The enrichment was transferred into fresh medium twice prior to isolation. Pink colonies appeared in agar plugs after 5 to 7 days, and an isolate designated CAB was obtained using acetate and perchlorate as the sole electron donor and acceptor, respectively. No other isolates were obtained. Phylogenetic analysis of the 16S rRNA gene identified strain CAB as a previously unknown epsilonproteobacterium most closely affiliated with the genus *Arcobacter* (Fig. 1). The closest relatives to *Arcobacter* strain CAB were *Arcobacter defluvii* (94% 16S rRNA sequence identity), isolated from a waste treatment plant in Spain, and *Arcobacter ellisii* (94% 16S rRNA sequence identity), isolated from mussels in the Ebro Delta of Spain (28, 29).

Morphology

Strain CAB is a Gram-negative bacterium with most cells appearing to be straight rods usually 0.3 to 0.8 µm wide by 1 to 4 µm long (Fig. 2A). Strain CAB cells are highly motile and possess one or two flagella at one of the poles (Fig. 2B). In comparison, members of the genus *Arcobacter* typically appear as motile, slightly curved, rod-shaped cells 0.2 to 0.9 µm wide and 1 to 4 µm long with one or two polar flagella (30). Although some cells of strain CAB are slightly curved, most cells lack the characteristic curvature usually associated with the *Arcobacter* genus and from which the genus derives its name. Rarely, cells appear as filaments (8 to 10 µm) or in chains (Fig. 2A).

Growth on acetate and perchlorate

Arcobacter sp. strain CAB grows by coupling the oxidation of acetate to the reduction of perchlorate (Fig. 3). Strain CAB has a doubling time of 4 to 5 h and reaches a maximum optical density at 600 nm (OD_{600}) of 0.45 when grown at 30°C with 16 mM (each) acetate and perchlorate. A 16.8 mM concentration of acetate and a 10.5 mM concentration of perchlorate were consumed over the 50-h incubation period, indicating that approximately 6.3 mM acetate (or 37%) was assimilated into biomass, which is typical for DPRB (21).

Optimum pH, temperature, and salinity

Optimum growth pH, temperature, and salinity (percent NaCl) experiments were carried out using acetate as the electron donor and perchlorate as the electron acceptor. Strain CAB grew over the tested pH range (6 to 7.5) with an optimum of pH 7 (Fig. 4). The optimum growth temperature was 25 to 30 °C, although good growth was observed between 20 and 30 °C (Fig. 5). Furthermore, growth also occurred at 15 °C, which is characteristic of the genus *Arcobacter* and differentiates this genus from the closely related but exclusively pathogenic genus *Campylobacter* (31, 32). No growth, either aerobically or anaerobically with perchlorate, was observed at 37 °C. Unlike many DPRB, which cannot tolerate levels of NaCl higher than 2% (1), strain CAB is halophilic and grew best at 3% NaCl (Fig. 6).

Fatty acid composition

The fatty acid compositions of strain CAB cells grown aerobically on acetate or anaerobically on acetate and perchlorate were analyzed and are shown in Table 1. The predominant fatty acids under both conditions were hexadecanoic acid ($C_{16:0}$), *cis*-9-hexadecanoic acid ($C_{16:1}\omega7c$), and *cis*-11-octadecanoic acid ($C_{18:1}\omega7c$) (Table 1). The presence of *cis*-11-hexadecanoic acid ($C_{16:1}\omega5c$) and *cis*-5-dodecanoic acid ($C_{12:1}\omega7c$), as well as the absence of dodecanoic acid ($C_{12:0}$), distinguishes strain CAB from other recognized members of the genus *Arcobacter* (33).

Metabolic diversity

In addition to perchlorate, strain CAB utilized both chlorate and oxygen as alternative electron acceptors, as is typical of the DPRB (Table 2). The ability of strain CAB to grow in atmospheric partial pressures of oxygen is consistent with most known DPRB and further supports its phylogenetic placement within the genus *Arcobacter*, as members of the genus *Campylobacter* are unable to grow aerobically (31, 32).

Like most members of the genus *Arcobacter*, strain CAB can utilize nitrate as an electron acceptor (29). However, strain CAB does not grow robustly by this metabolism and accumulates significant concentrations of nitrite (4.65 mM) when given 10 mM acetate and 8 mM nitrate (Fig. 7). Unexpectedly, the genome of strain CAB contains a complete set of genes encoding the reduction of nitrate and nitrite to ammonium. The genome encodes copies of the periplasmic nitrate reductase Nap (*napA-G-H-B-F-L-D*) (locus tags CAB_00029540, CAB_00029550, CAB_00029560, CAB_00029570, CAB_00029580, CAB_00029590, CAB_00029600, and

CAB_00029610), the periplasmic nitrite reductase Nrf (*nrfH-A-I-hyp-hyp*) (locus tags CAB_00023420, CAB_00023410, CAB_00023400, CAB_00023390, and CAB_00023380), the nitrous oxide reductase Nos (*nosZ-hyp-D-G-C1-C2-H-F-L*) (locus tags CAB_00024950, CAB_00024960, CAB_00024970, CAB_00024980, CAB_00024990, CAB_00025000, CAB_00025010, CAB_00025020, and CAB_00025030), and the nitric oxide reductase Nor (*nor-Q-D-B-C*) (locus tags CAB_00034190, CAB_00034200, CAB_00034210, and CAB_00034220). The presence and the arrangement of the Nap, Nrf, and Nos clusters in strain CAB are very similar to those of the ammonifying epsilonproteobacterium *Wolinella succinogenes* (34). Furthermore, these clusters contain typical signatures of nitrate reduction pathways in the Epsilonproteobacteria, such as the lack of *napC* in the Nap gene cluster and the*nos-G-C1-C2-H* gene arrangement that has, to date, been found exclusively in the Epsilonproteobacteria. Like other members of the *Arcobacter* genus (35), the genome of strain CAB also includes the Nif operon, suggesting that strain CAB can fix N_2, though this has not been empirically tested.

As shown in Table 2, strain CAB oxidizes a variety of organic acids coupled to the reduction of perchlorate. Hydrogen is also utilized as an electron donor, and the genome of strain CAB contains genes encoding several hydrogenases (CAB_00033260 to CAB_00033310, CAB_00000010 to CAB_00000060, and CAB_00000130 to CAB_00000180). However, the genome of strain CAB lacks genes necessary for CO_2 fixation, and as such, strain CAB appears to require organic carbon for biomass synthesis in the presence of hydrogen as an electron donor.

Catechol degradation

In contrast to previously described DPRB and members of the *Arcobacter* genus (28), strain CAB oxidized fructose and catechol using perchlorate as an electron acceptor (Table 2; Fig. 8). In the case of catechol, the oxidation of 1 mM catechol resulted in the reduction of 2.38 mM perchlorate (data not shown). According to the equation $C_6H_6O_2 + 3.25ClO_4^- \rightarrow 6CO_2 + 3.25Cl^- + 3H_2O$, the theoretical ratio of catechol oxidized to perchlorate reduced is 1:3.25, implying that approximately 0.27 mM (or 26.8%) catechol was assimilated into biomass. Cultures of strain CAB grown on 1 mM catechol and 5 mM perchlorate were bright yellow, which is indicative of the production of 2-hydroxymuconate semialdehyde (2-HMS), a known intermediate of the *meta*-cleavage pathway of aerobic catechol oxidation (36). Absorbance at 375 nm, which is specific to 2-HMS, was indicative of the production of this compound during growth with catechol and perchlorate (Fig. 8), but not if catechol was replaced with acetate as an electron donor (data not shown). Spectrophotometric scans (300 to 800 nm) of the filtrates show a maximum absorbance peak at 375 nm (Fig. 9), consistent with the previously published spectrum of 2-HMS (37). No maximum absorbance peak at 375 nm was seen in cultures grown with acetate instead of catechol (data not shown). The spectrum analysis could not be directly validated because of the unavailability of commercial 2-HMS. However, using the known extinction coefficient for 2-HMS of $\varepsilon_{375} = 36{,}000\ M^{-1}\ cm^{-1}$(38), the measured absorbances were converted to 2-HMS concentrations. During growth of CAB on 1 mM catechol with perchlorate, the 2-HMS peak indicated a maximum concentration of 7.0 µM at $t = 36$ h, which declined to 1.8 µM at $t = 556$ hours (Fig. 10). In order to further support the finding of 2-HMS during anaerobic oxidation of catechol with perchlorate as an electron acceptor, a separate culture of strain CAB that obtained an unusually high absorbance at 375 nm (36 µM calculated

2-HMS concentration) was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. An ion of *m/z* 141.0192 was observed, consistent with $(M\text{-}H)^-$ of 2-HMS (theoretical *m/z* 141.0193) (Fig. 11). Strain CAB did not oxidize catechol with nitrate as an electron acceptor (data not shown).

The genome of strain CAB encodes copies of all the proteins necessary for the *meta*-cleavage pathway of catechol degradation (38). These proteins include catechol 2,3-dioxygenase (CAB_00005480), 2-hydroxymuconate semialdehyde dehydrogenase (CAB_00005500), 2-hydroxymuconate semialdehyde hydrolase (CAB_00005570), 4-oxalocrotonate tautomerase (CAB_00005550), 4-oxalocrotonate decarboxylase (CAB_00005540), 2-oxopent-4-enoate hydratase (CAB_00005510), and 4-hydroxy-2-oxovalerate aldolase (CAB_00005530). Reverse transcription-PCR (RT-PCR) of cultures grown in the presence of acetate and perchlorate shows no or very little expression of genes in this pathway, whereas cultures grown with catechol and perchlorate have much higher expression of all these genes (Fig. 12). The presence and transcription of these genes under these conditions are consistent with metabolic observations of catechol consumption and transient production of 2-HMS, demonstrating the unique ability of DPRB to utilize oxygenase-dependent pathways under anoxic (per)chlorate-reducing conditions.

Draft genome and perchlorate reduction island

The draft genome of strain CAB was 3.48 Mb, which is one of the largest sequenced genomes in the Epsilonproteobacteria to date (39, 40). The genome contained 367 contigs ranging in size from 201 bp to 85,599 bp. The average contig length was 9,478 bp. The GC content was low (28.16%) but is characteristic of the family *Campylobacteraceae* and the genus *Arcobacter* (30).

The genome revealed the presence of a unique perchlorate reduction island (PRI) containing striking differences from those in other DPRB (Fig. 12). As is typical of DPRB, no genes encoding a separate chlorate reductase were found. The PRI contains copies of *cld* (locus tag CAB_00027480), *pcrABD* (locus tags CAB_00027550, CAB_00027540, and CAB_00027530), and transposases (locus tags CAB_00027490, CAB_00027500, CAB_00027510, and CAB_00027560). Of particular note was the lack of the *pcrC* gene in the genome. This gene was considered an essential component of the PRI and is present in all other sequenced DPRB (17). The *pcrC* gene encodes a periplasmic multiheme *c*-type cytochrome that putatively mediates electron transport from the cytoplasmic membrane to the periplasmic functional PcrAB protein (16). Instead, the PRI of strain CAB contained a *c*-type monoheme cytochrome (locus tag CAB_00027520) (2). BLAST results revealed that this cytochrome is similar to those known from other *Arcobacter* genomes, including *A. butzleri* RM4018, *A. butzleri* ED-1, and *A. nitrofigilis* (41). Additionally, in all previously sequenced organisms the *cld* was located either upstream or downstream of the *pcr* operon on the same strand. In contrast, in *Arcobacter* sp. strain CAB, it was located much further away from the *pcr* genes and on the opposite strand (17). Between *pcr* and *cld*, there were three transposase genes, suggesting a possible mechanism for the aberrant PRI architecture relative to those of previously sequenced DPRB.

Discussion

In this study, we describe a novel organism capable of dissimilatory (per)chlorate reduction. Although more than 50 DPRB have been isolated since 1996, *Arcobacter* sp. strain CAB is only the second DPRB belonging to the Epsilonproteobacteria to be isolated and is currently the only one in pure culture (1). Further, nearly all isolated DPRB do not tolerate NaCl concentrations higher than 2%, whereas strain CAB is a halophile and grows best at 3% NaCl (1). As such, it provides a unique opportunity to study respiratory (per)chlorate reduction in a different phylogenetic and environmental context (1). Respiration of (per)chlorate has not been reported previously in any members of the genus *Arcobacter*, and the few sequenced genomes (40, 42, 43) of this genus do not carry copies of *cld* or *pcr*. As such, the existence of strain CAB suggests a novel ecological niche among members of the genus *Arcobacter*.

Although *Arcobacter* is not exclusively a pathogenic genus, members of the *Arcobacter* genus are most often studied because of their pathogenicity. The genus *Arcobacter*, which was first described in 1991 to accommodate 2 aerotolerant *Campylobacter* species, currently comprises 13 named members (29) plus *Candidatus* "Arcobacter sulfidicus," which has not yet been fully described (44). *Arcobacter* species exhibit broad environmental diversity that can be divided between free-living species, usually found in marine environments, and pathogenic species known to infect birds and mammals, including humans (31). Strain CAB fails to grow at 37°C either aerobically or anaerobically with perchlorate, suggesting that strain CAB is unlikely to be a mammalian pathogen. Based on its environmental source and metabolism, strain CAB fits into the marine subclade of the *Arcobacter* genus. The relatively low (94%) 16S rRNA gene sequence agreement between strain CAB and its nearest relatives *A. ellisii* and *A. defluvii* indicates that strain CAB is a new species in the *Arcobacter* genus.

Strain CAB displays high metabolic versatility. In addition to the discovery that strain CAB is capable of reducing (per)chlorate, it was also found to oxidize fructose and catechol. Neither of these activities has been reported previously in members of the genus *Arcobacter*, and the inability to catabolize carbohydrates was considered diagnostic of the genus (30). These activities are also unusual among DPRB, where the majority are unable to use carbohydrates, catechol, and citrate (45).

Although (per)chlorate-reducing bacteria have been shown to degrade aromatics such as benzene in the presence of (per)chlorate, the mechanism by which this happens has not yet been completely elucidated (46–48). It has been suggested that the molecular oxygen produced by the dismutation of chlorite is internally recycled and used as a co-substrate by oxygenases to carry out aerobic processes in anoxic environments (48, 49). Strain CAB oxidized catechol coupled to perchlorate reduction. RT-PCR and genomic data point toward a dioxygenase-mediated process in which the molecular oxygen produced from chlorite dismutation is used as a co-substrate by the catechol 2,3-dioxygenase enzyme to convert catechol into 2-HMS.

The genome of strain CAB reveals the presence of an 11-kb contig containing a unique transposase-laden PRI that carries *cld* and *pcrABD* but lacks *pcrC*. This finding is surprising, as *pcrC*, which encodes a *c*-type cytochrome, was thought to be an essential part of the

perchlorate reduction machinery. Strain CAB may be replacing the shuttling function of *pcrC* with another cytochrome, possibly the monoheme *c*-type cytochrome encoded in the PRI between the *cld* and *pcr* operons. If true, this would suggest that the perchlorate reduction pathway is more flexible than previously thought.

The relatively low 16S rRNA gene sequence identity, the frequent absence of curved cells, and the presence of metabolic pathways previously unknown among members of the *Arcobacter* distinguish strain CAB from other *Arcobacter* species and expand the known diversity of the genus. Further, the existence of strain CAB significantly extends the range of known DPRB. The only previously isolated DPRB from the Epsilonproteobacteria is *Wolinella succinogenes* strain HAP-1, for which all cultures are now believed to have been lost. Though DPRB are well known among the Proteobacteria, especially within the Betaproteobacteria subclass, Epsilonproteobacteria are only distantly related to known DPRB. As such, strain CAB will contribute to our understanding of the evolutionary history and diversity of the perchlorate reduction pathway.

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Tables and Figures

Table 1. Whole-cell fatty acid composition of *Arcobacter* sp. strain CAB and other *Arcobacter* species[a]

	% of total fatty acids in organism:						
	Arcobacter sp. strain CAB						
Fatty acid	Aerobic	Anaerobic	*A. marinus*	*A. halophilus*	*A. nitrofigilis*	*A. butzleri*	*A. skirrowii*
$C_{10:0}$	ND	1	ND	ND	ND	ND	ND
$C_{12:0}$	ND	ND	6.6	8.2	7.2	6.8	9.1
$C_{14:0}$	3.92	3.03	5	5	4.8	3.2	2.4
$C_{16:0}$	19.86	25.53	26.2	19.3	32	19.6	22.2
$C_{18:0}$	ND	ND	1.6	ND	ND	ND	ND
$C_{12:1}\omega 7c$	3.23	2.79	ND	ND	ND	ND	ND
$C_{14:1}\omega 6c$	ND	ND	ND	ND	ND	4.9	1.4
$C_{14:1}\omega 7c$	1.49	ND	ND	ND	ND	ND	ND
$C_{16:1}\omega 5c$	1.56	1.29	ND	ND	ND	ND	ND
$C_{16:1}\omega 7c$	50.19	46.77	28.4	37.9	30.9	19.2	22.8
$C_{16:1}\omega 6t$	ND	ND	ND	ND	ND	13.8	7.8
$C_{18:1}\omega 9c$	ND	ND	2.1	ND	ND	ND	ND
$C_{18:1}\omega 7c$	12.39	14.51	22.3	21.7	12.8	11.6	19.7
$C_{12:0}$ 3-OH	1.58	1.25	2.9	2.7	5.5	ND	ND
Iso-$_{C16:1}$ I, $C_{14:0}$ 3-OH, and/or $C_{12:0}$ alde	1.83	2.42	2	2.1	5.3	17.4	9.2

[a] Data for all species excluding strain CAB are from reference 33. ND, not detected or <1%.

Table 2. Compounds tested as electron donors in the presence of perchlorate (10 mM) or as electron acceptors in the presence of acetate (10 mM)

Compound	Concn (mM[a])
Electron donors	
Utilized	
Acetate	10
Propionate	10
Citrate	10
Succinate	1
Lactate	10
Yeast extract	1 g/liter
Fumarate	10
Malate	10
Fructose	10
Catechol	1
Hydrogen	~50 kPa
Tested but not utilized	
Isobutyrate	10
Benzoate	1, 2.5
Butyrate	10
Valerate	10
Methanol	5
Ethanol	5
Glucose	10
Methane	~25 kPa
Electron acceptors	
Utilized	
Perchlorate	10
Chlorate	10
Oxygen	~25 kPa
Nitrate	10
Tested but not utilized	
Ferric citrate	10
Sulfate	10
Sulfite	2
Thiosulfate	10
Arsenate	2.5
Selenate	2.5
Malate	10
Nitrite	2

[a] Unless otherwise indicated.

Figure 1. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic position of *Arcobacter* sp. strain CAB (gray box) both within the genus *Arcobacter* and within known perchlorate (bold)- and chlorate (underlined)-reducing bacteria. Bootstrap values are based on 1,000 replications and are shown at the nodes of the tree. The scale bar represents 0.1 expected change per site.

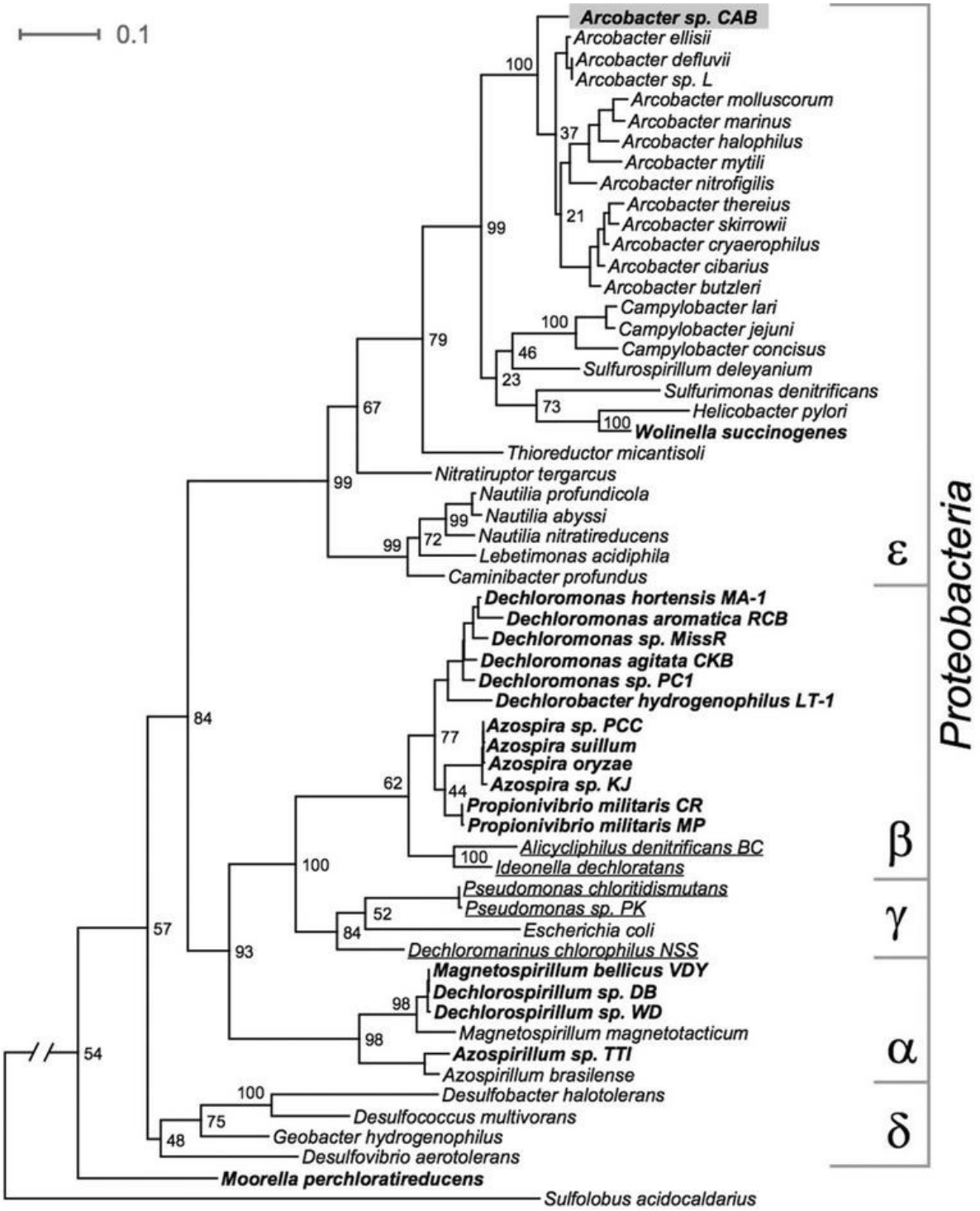


Figure 2. Scanning electron microscopy (A) and transmission electron microscopy (B) of *Arcobacter* sp. strain CAB.

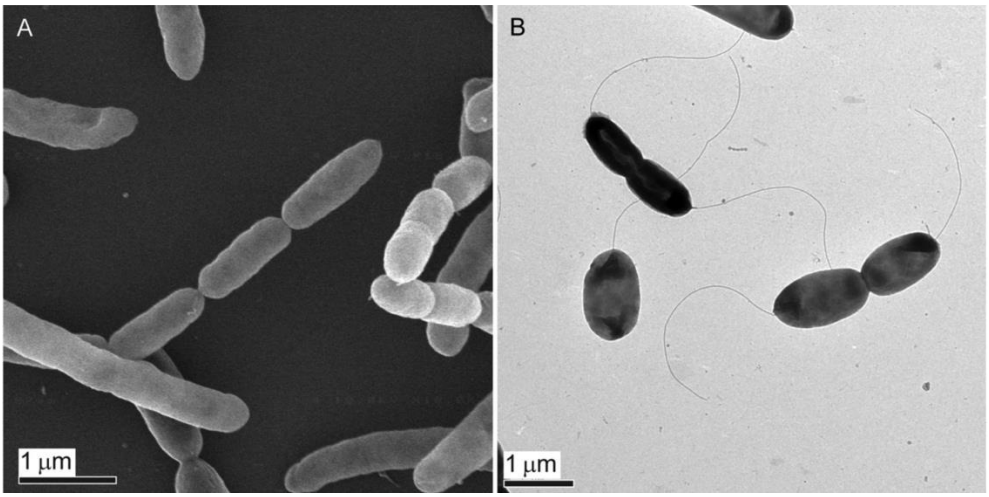


Figure 3. Growth curve of *Arcobacter* sp. strain CAB on 16 mM (each) acetate and perchlorate. Cell number increase was monitored by OD600. Diamonds, cell density (16 mM acetate-perchlorate); circles, perchlorate concentration (mM); triangles, acetate concentration (mM); squares, cell density for the negative control without perchlorate. Error bars represent standard deviations of the average of triplicate samples.

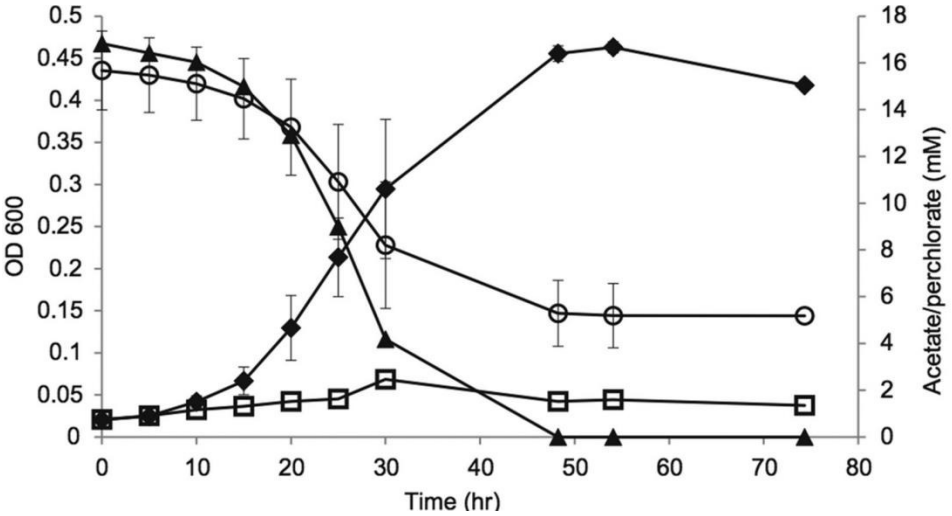


Figure 4. Effect of pH on the growth rate of strain CAB grown in medium containing 10 mM each of acetate and perchlorate. Error bars represent standard deviation of triplicate samples.

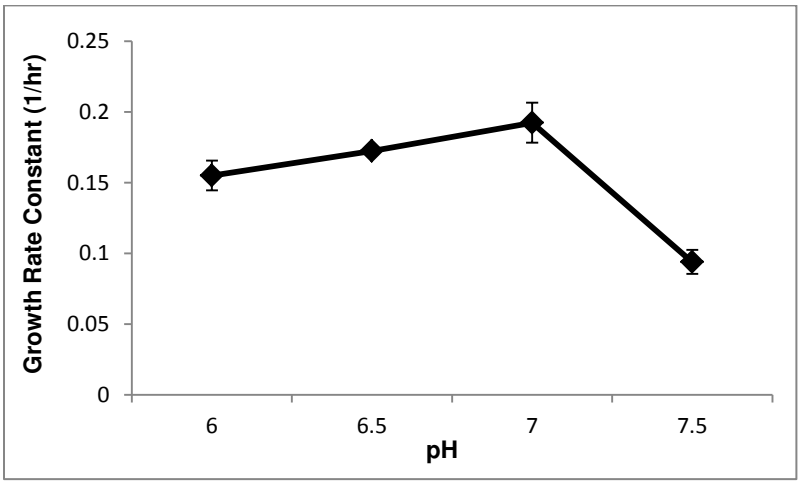


Figure 5. Effect of temperature on the growth rate of strain CAB grown in medium containing 10 mM each of acetate and perchlorate. Error bars represent standard deviation of triplicate samples.

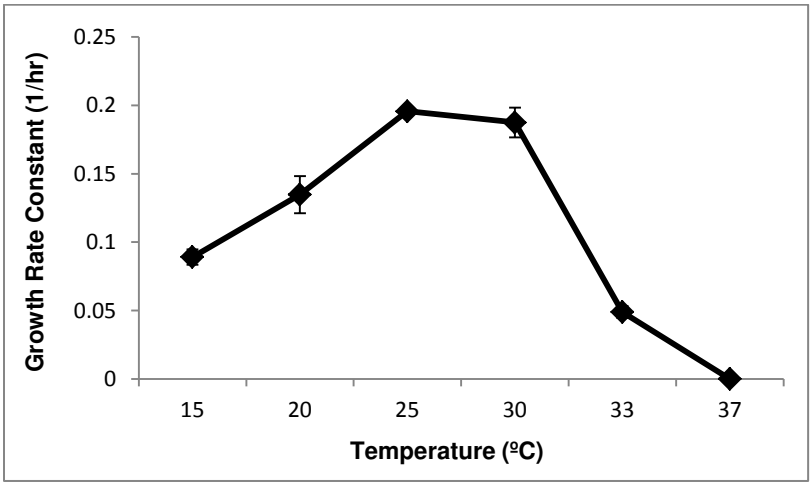


Figure 6. Effect of salinity on the growth rate of strain CAB grown in medium containing 10 mM each of acetate and perchlorate. Error bars represent standard deviation of triplicate samples.

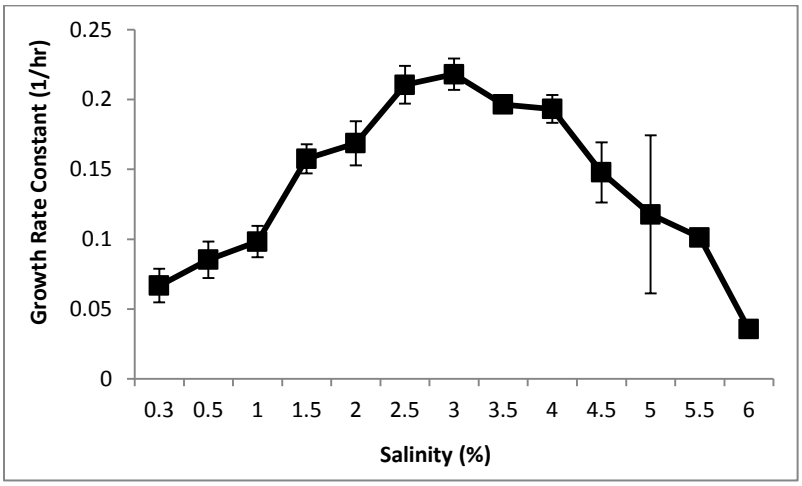


Figure 7. Growth curve of *Arcobacter* sp. strain CAB on 10 mM acetate and 8 mM nitrate. Cell number increase was monitored by optical density (OD) at 600 nm. Diamonds: cell density; squares: nitrite concentration (mM); circles: nitrate concentration (mM); triangles: cell density for no acceptor control (mM). Error bars represent standard deviation of triplicate samples.

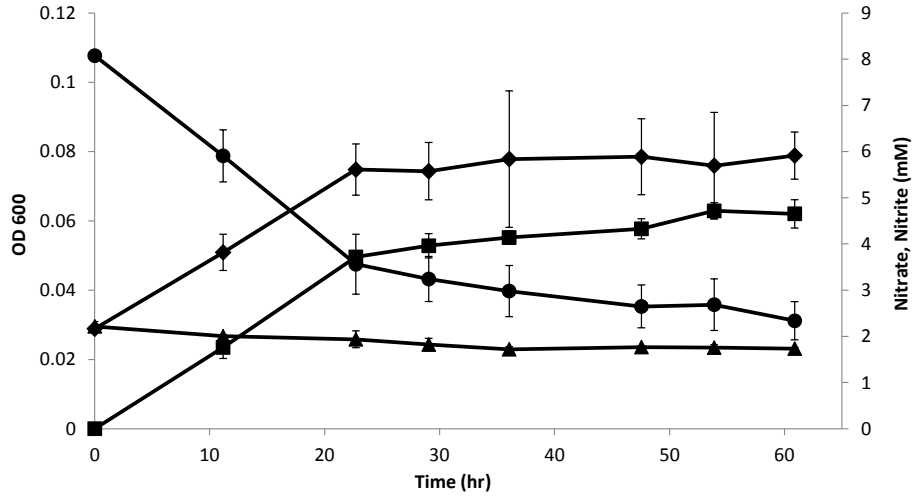


Figure 8. Growth curve of *Arcobacter* sp. strain CAB on 1 mM catechol and 5 mM perchlorate. Cell number increase was monitored by OD600. Diamonds, cell density; squares, absorbance at 375 nm; circles, catechol concentration (mM); triangles, perchlorate concentration (mM). Error bars represent standard deviations of the average of triplicate samples.

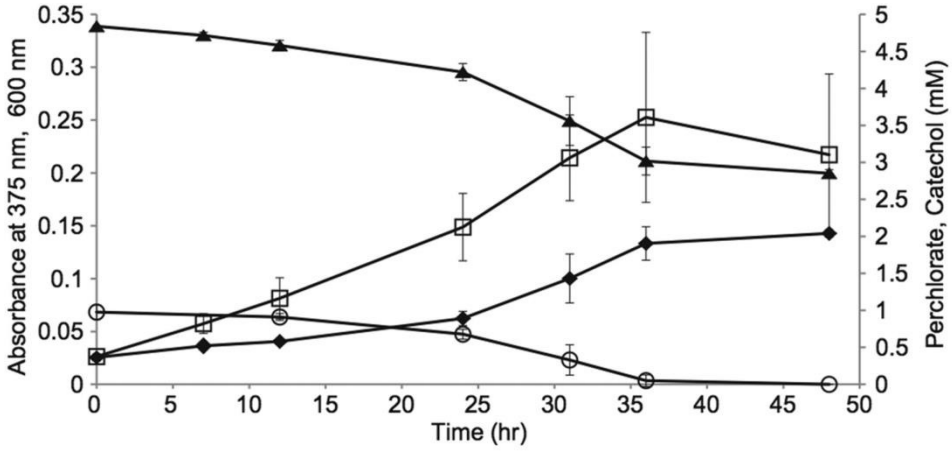


Figure 9. Spectrophotometric scans (300-800 nm) of filtrates from triplicate cultures of strain CAB grown with 1 mM catechol and 5 mM perchlorate.

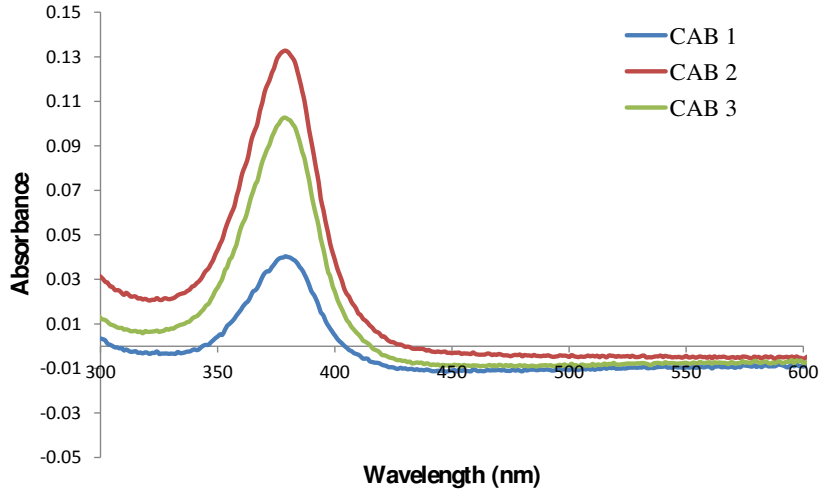


Figure 10. 2-HMS and perchlorate concentration of *Arcobacter* sp. strain CAB grown on 1 mM catechol and 5 mM perchlorate. Squares: perchlorate concentration (mM); diamonds: 2-HMS concentration (µM). Error bars represent standard deviation of triplicate samples.

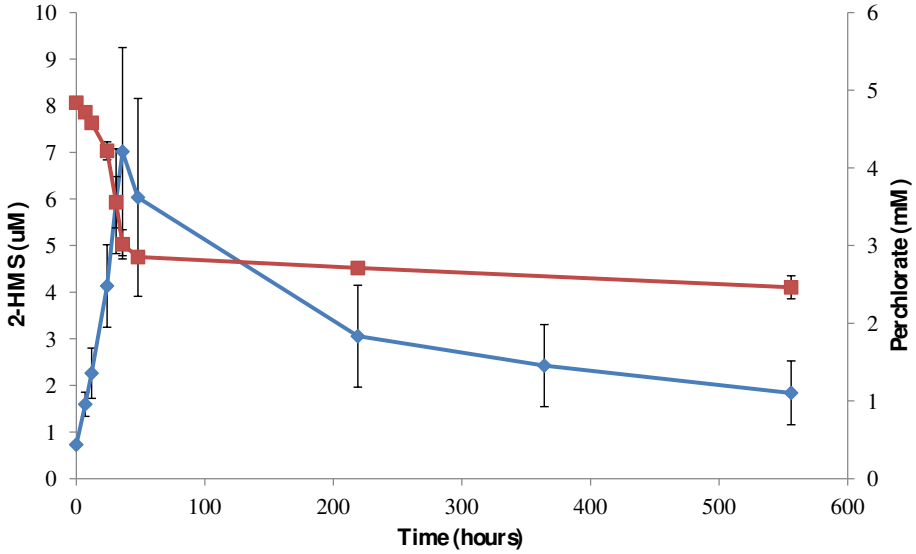
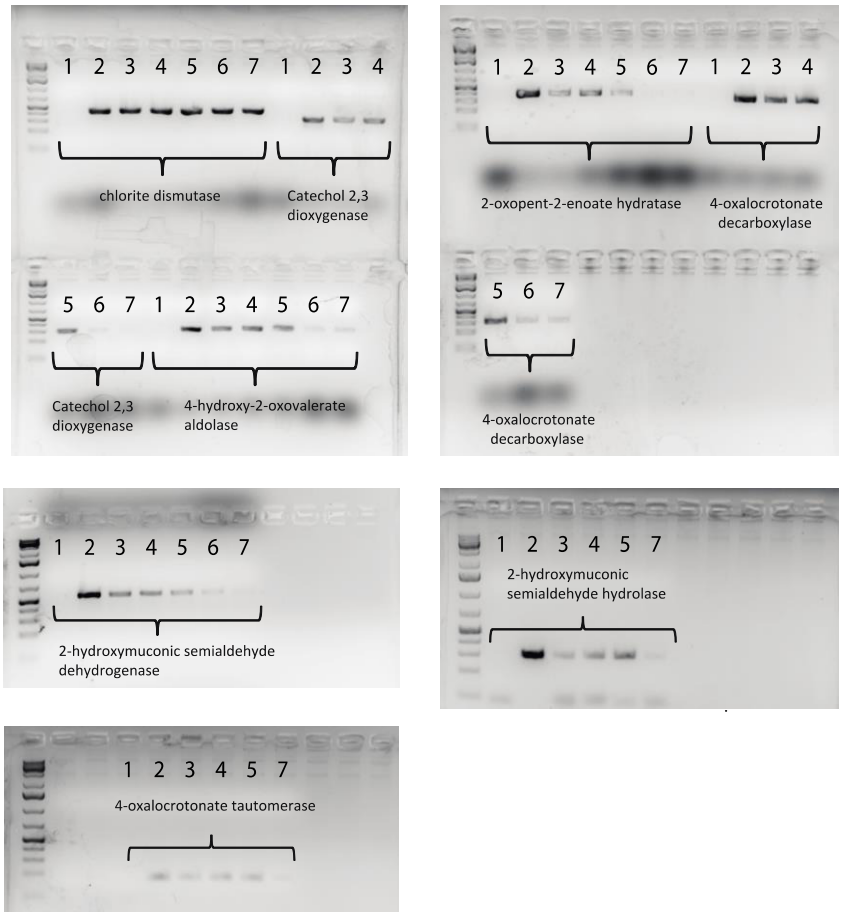


Figure 11. LC/QTOF-MS of *Arcobacter* sp. CAB culture grown on 1 mM catechol and 5 mM perchlorate. Calculated 2-HMS concentration is 35.95 µM (based on absorbance at 375 nm). An ion of m/z 141.0192 was observed, consistent (accuracy 0.71 ppm) with (M-H)- of 2-HMS (theoretical m/z 141.0193).

Figure 12. A) RT-PCR of catechol grown (3-5) and acetate grown (7) cultures of strain CAB. (1) is a PCR negative control and (2) is a genomic DNA positive control. (6) is an acetate grown culture of strain CAB that was transferred from a stock grown on catechol and perchlorate. B) Proposed pathway of catechol oxidation in strain CAB. Red text: enzymes identified in the genome; black text: intermediates of the pathway.

A



B

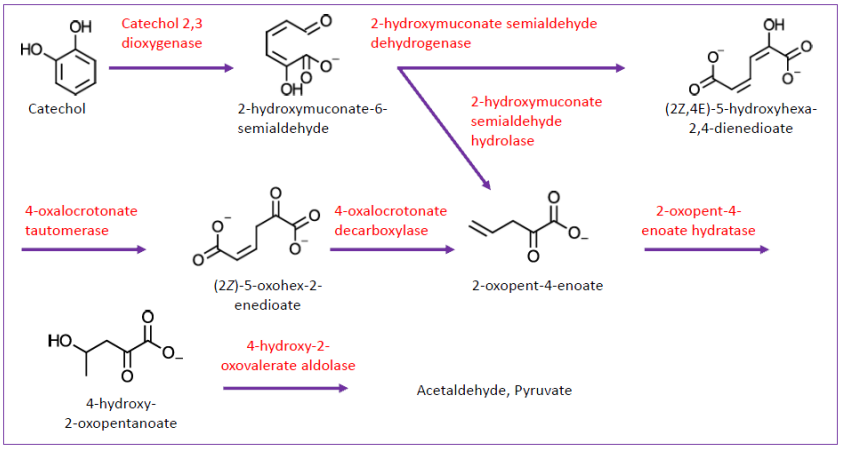
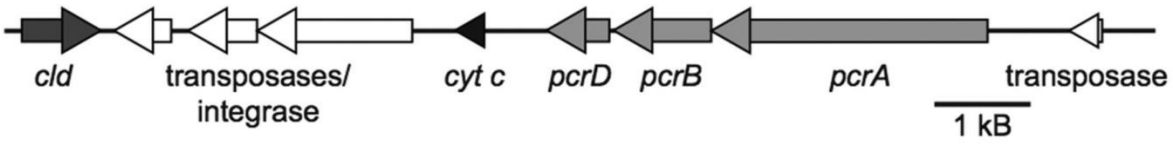


Figure 13. *Arcobacter* sp. strain CAB perchlorate reduction island. White, transposases and integrases; black, monoheme cytochrome c; dark gray, *cld*; light gray, *pcrABD*. Bar, 1 kB. Arrows indicate direction of transcription.



Chapter 4:

Sedimenticola selenatireducens CUZ and *Dechloromarinus chlorophilus* NSS: Two Novel Gammaproteobacteria Capable of (Per)chlorate Reduction

Charlotte I. Carlstrom, Dana E. Loutey, Ouwei Wang, Anna Engelbrektson, Iain Clark, Lauren N. Lucas, Pranav Y. Somasekhar, and John D. Coates

Abstract

Two novel (per)chlorate reducing bacteria, *Sedimenticola selenatireducens* strain CUZ and *Dechloromarinus chlorophilus* strain NSS were isolated from marine sediments in Berkeley and San Diego, CA, respectively. Strain CUZ reduced both perchlorate and chlorate [collectively designated (per)chlorate] and strain NSS reduced only chlorate. Phylogenetic analysis classified both strains as Gammaproteobacteria. TEM and SEM preparations of both strains showed the presence of rod-shaped, motile cells containing one polar flagellum per cell. Optimum growth for strain CUZ was observed at 25-30ºC, pH 7, and 4% NaCl, while strain NSS grew optimally at 37-42ºC, pH 7.5-8, and 1.5-2.5% NaCl. Both strains oxidized hydrogen, sulfide, various organic acids, and aromatics such as benzoate and phenylacetate as electron donors coupled to oxygen, nitrate, and (per)chlorate or chlorate as electron acceptors.

The draft genomes of CUZ and NSS encoded the requisite (per)chlorate reduction island (PRI) and chlorate reduction composite transposons responsible for (per)chlorate and chlorate metabolism, respectively. The PRI of CUZ contained a perchlorate reductase (Pcr), which reduced both perchlorate and chlorate, while the genome of NSS included a distinct chlorate reductase (Clr) that reduced only chlorate. When both (per)chlorate and nitrate were present, (per)chlorate was preferentially utilized if the inoculum was pre-grown on (per)chlorate. Historically, (per)chlorate-reducing bacteria (PRB) and chlorate-reducing bacteria (CRB) have primarily been isolated from freshwater, mesophilic environments (1-13). This study describes the isolation and characterization of two highly related marine, halophilic PRB and CRB, thus broadening the known phylogenetic and physiological diversity of this unusual metabolism.

Introduction

Perchlorate (ClO_4^-) and chlorate (ClO_3^-), collectively denoted (per)chlorate, are oxyanions of chlorine that have both natural and anthropogenic sources (6, 14-21). Perchlorate is extensively used in rocket fuel, pyrotechnics, lubricants, and paints (6, 14, 15), while chlorate is used as an herbicide, defoliant, and as a bleaching agent in the paper industry (22-24). Perchlorate contamination of groundwater due to human activity is a major environmental concern, as perchlorate inhibits the uptake of iodine by the thyroid gland and may lead to hypothyroidism (6, 22, 25, 26). Chlorate exposure has been correlated with hemolytic anemia as well as reproductive toxicity (27, 28). Thus, the ability of microorganisms to respire (per)chlorate has recently been exploited as an effective method of bioremediation (6, 29-35).

(Per)chlorate-reducing bacteria (PRB) reduce (per)chlorate with the perchlorate reductase enzyme (Pcr), while chlorate-reducing bacteria (CRB) reduce only chlorate, using a distinct chlorate reductase enzyme (Clr) (5, 6, 36-39). The product of both enzymatic reactions is chlorite (ClO_2^-), an intermediate which is detoxified in both PRB and CRB into innocuous chloride and molecular oxygen by the chlorite dismutase enzyme (Cld) (40). The molecular oxygen produced by chlorite dismutation is either respired or used as a co-substrate for oxygenases (7).

While a great number of PRB and CRB have been isolated in the last two decades, the majority of these isolates were obtained from freshwater, mesophilic, neutral pH environments (6, 9, 41-43). Exceptions are the thermophiles *Moorella perchloratireducens* (44) and *Archaeoglobus fulgidus* (45), and the halophiles *Arcobacter* sp. CAB and *Shewanella algae* ACDC (7, 13). The current study expands the diversity of PRB and CRB by characterizing two additional isolates obtained from marine environments. *Sedimenticola selenatireducens* strain CUZ was isolated from sediment in Berkeley, CA, while *Dechloromarinus chlorophilus* strain NSS was isolated from hydrocarbon-contaminated sediment in the San Diego Bay, CA. Strain NSS has previously been mentioned in studies of the diversity and evolution of chlorate reduction (6, 46, 47), but has not hitherto been characterized in detail. Strains CUZ and NSS are both halophilic Gammaproteobacteria, share 98% 16S rRNA identity, and are most closely related to the selenate-reducing bacterium *Sedimenticola selenatireducens* AK4OH1 (100% and 98% 16S rRNA identity, respectively) and poorly characterized marine endosymbionts of tubeworms and bivalves. This is the first reported case of two very closely related microorganisms, one of which is a PRB and one of which is a CRB. As such, the study of these strains will provide insight into the evolution of (per)chlorate and chlorate metabolism within a host organism as well as expand current knowledge regarding the phylogeny and physiology of PRB and CRB.

Materials and Methods

Culture Conditions, Enrichments, and Isolations

Sedimenticola selenatireducens CUZ

Sediment samples were obtained from a marina in Berkeley, CA (latitude 37.8269º N; longitude 122.3132º W) using 50 mL Falcon tubes. For enrichment, 1 g of sediment sample was mixed with 9 mL of medium in 30 mL anaerobic tubes (Bellco, Wineland, NJ). Sediment samples had a salinity of ~ 1.5%, and contained no detectable perchlorate. The medium used for enrichment and isolation is described in (7). Hydrogen gas (10 mL) and sodium perchlorate (10 mM) were used as the electron donor and electron acceptor, respectively, with 0.1 g/L yeast extract added as a carbon source. The enrichments were maintained at 30ºC for 2 weeks and transferred into fresh medium twice prior to isolation. Agar roll tubes with 10 mL hydrogen, 0.1 g/L yeast extract, and 10 mM perchlorate were used for isolation. For regular culturing post-isolation $MgCl_2 \cdot 6\ H_2O$ and $CaCl_2 \cdot 2\ H_2O$ were omitted from the medium.

Dechloromarinus chlorophilus NSS

Hydrocarbon-contaminated sediment samples (33 mg PAH per kg sediment) were obtained from the Naval Station site in the San Diego Bay, CA (32.6816º N, 117.1221º W) from a depth of 10 m and sealed under a headspace of N_2 gas using canning jars. For enrichment, 1 g of sediment sample was mixed with 9 mL of medium in 30 mL anaerobic tubes (Bellco, Wineland, NJ). The medium used for enrichment and is described in (7), except that the medium contained 40 g/L NaCl rather than 30 g/L. Sodium acetate (10 mM) and sodium chlorate (10 mM) were used as the electron donor and electron acceptor, respectively. The enrichments were maintained at 30ºC and transferred every four days for a total of seven transfers. Anaerobic agar plates amended with acetate (10 mM) and chlorate (10 mM) were used for isolation. The medium used for regular culturing of strain NSS post-isolation contained: NaCl (20 g/L), KCl (0.67 g/L), monosodium PIPES (1.6220 g/L), disodium PIPES (1.7317 g/L), vitamins (10 mL), minerals (10 mL), and RST minerals (20 mL) as described in (5).

Optimum pH, Temperature, and Salinity

Acetate and perchlorate (for strain CUZ) and lactate and chlorate (for strain NSS) were used to determine the optimum growth temperature, pH, and salinity. The temperatures tested ranged from 15 – 33ºC for strain CUZ, and 30 – 56ºC for strain NSS. The salinities tested ranged from 0 – 6% w/v NaCl in 0.5% increments for both strains. For pH, the medium described above was used, except that 10 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid] buffer was substituted for $NaHCO_3$. The medium was adjusted to the desired pH (6, 6.5, 7, or 7.5) using HCl or NaOH. All analyses were performed in triplicate cultures.

Alternate Electron Acceptors and Electron Donors

For the electron donor profile, 10 mM perchlorate (for strain CUZ) or chlorate (for strain NSS) were used as electron acceptors. The following electron donors were added from sterile aqueous solutions to give the indicated final concentrations (mM, unless otherwise specified): acetate (10), propionate (10), butyrate (10), isobutyrate (10), valerate (10), methanol (5), ethanol (5), benzoate (1), citrate (10), succinate (1), lactate (10), glucose (10), fructose (10), yeast extract (1 g/L), fumarate (10), malate (10), hydrogen (~50 kPa), catechol (1), methane (~25 kPa), protocatechuate (1), phenylacetate (1), sulfide (5). The tubes containing hydrogen and sulfide also contained 0.1 g/L yeast extract as a carbon source. To determine if strains CUZ and NSS oxidized the tested electron donors coupled to perchlorate or chlorate reduction, samples were taken at the time of inoculation and 15 days later and analyzed via ion chromatography (see below) for consumption of perchlorate or chlorate, respectively. Each donor was tested in triplicate, and negative controls included a no-donor control and a no-donor plus 0.1 g/L yeast extract control. Growth on each electron donor was considered positive when consumption of perchlorate or chlorate was observed concomitant with an increase in turbidity.

For the electron acceptor profile, acetate (10 mM) was used as the sole electron donor and carbon source. The following electron acceptors were added from sterile aqueous stocks in the indicated concentrations (mM unless otherwise specified): perchlorate (10), chlorate (10), nitrate (10), nitrite (2), sulfate (10), sulfite (2), thiosulfate (10), arsenate (2.5), selenate (2.5), malate (10), and oxygen (~25 kPa). To determine if strains CUZ and NSS reduced the tested electron acceptors, samples were taken at the time of inoculation and 15 days later and analyzed for consumption of acetate by high-performance liquid chromatography (HPLC) (see below). Each acceptor was tested in triplicate, and experiments included a no-acceptor control. Growth on each acceptor was considered positive when consumption of acetate was observed concomitant with an increase in turbidity.

To test the preferential utilization of chlorate or nitrate (strains CUZ and NSS) and (per)chlorate or nitrate (strain CUZ) in diauxic medium, cells of strains CUZ and NSS were grown with 10 mM each of acetate and chlorate or 10 mM each of acetate and nitrate. Strain CUZ was also grown on 10 mM each of acetate and perchlorate. The inocula were then transferred into tubes containing 20 mM acetate, 5 mM perchlorate or chlorate, and 5 mM nitrate. Optical density and anion concentration were measured as described below.

Cell Growth Profiles

Cell growth was measured spectrophotometrically at 600 nm (OD_{600}) using a Varian Cary 50 Bio spectrophotometer (Agilent, Santa Clara, CA). All experimental analyses were performed in triplicate.

Analytical Methods

Perchlorate concentrations were measured via ion chromatography using a Dionex ICS 2100 with a Dionex IonPac AS 16 (4 × 250 mm) column (Thermo Fisher Scientific, Sunnyvale,

CA) and a 35 mM NaOH mobile phase at a flow rate of 1.0 mL/min. Background conductivity was suppressed with a Dionex ASRS operating in recycle mode. The suppressor controller was set at 100 mA for analysis and the injection volume was 25 µL.

Chlorate and nitrate concentrations were measured via ion chromatography using a Dionex ICS 1500 equipped with a Dionex Ion Pac AS 25 column (4 × 250 mm) with a mobile phase of 36 mM NaOH at a flow rate of 1.0 mL/min. Background conductivity was suppressed with a Dionex ASRS 300 (4 mm) in recycle mode. The suppressor controller was set at 90 mA for analysis and the injection volume was 10 µL.

Acetate concentrations were measured by HPLC (Dionex; model LC20), using a UV-visible (UV-Vis) detector (Dionex; AD20) at a wavelength of 210 nm and a Bio-Rad Aminex HPX-87H column with a mobile phase of 0.016 N H_2SO_4 (flow rate of 0.9 mL/min).

Scanning Electron Microscopy

Silicon wafers were washed with ethanol for 30 s and air dried. A 20 µL poly-L-lysine drop was placed on the silicon wafers for 1 min and then withdrawn. The wafers were then rinsed with ultraclean water. A drop of concentrated cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was added to the silicon wafers and allowed to settle for 1 h. The silicon wafers were then processed as described in (24) and visualized using a Hitachi S-5000 scanning electron microscope at 20 kV.

Transmission Electron Microscopy

Flagella were visualized using the negative-stain technique. Copper Formvar- and carbon-coated grids (400 mesh) were glow discharged just before use to increase hydrophilicity. A suspension (5 µL) of cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was deposited on the grids. This was sufficient to cover the grid. After 2 min, the suspension was removed by touching the ragged torn edges of filter paper to the sample until the grid surface was nearly dry. The grid was then washed 3 times on water droplets, and excess water was wicked off using filter paper as described above. A drop of 1% aqueous uranyl acetate was then added to the grid. After 2 min, the grid was dried with filter paper. Samples were examined the same day using a Tecnai transmission electron microscope at 120 kV.

Genomic and Phylogenetic Analysis

Genomic DNA was extracted as described previously (48). The genomic DNA was submitted to Eureka Genomics (Hercules, CA) for Illumina sequencing and partial assembly. The Silva database and aligner (49) were used to align 16S rRNA gene sequences, and a maximum likelihood phylogenetic tree was constructed with 1,000 bootstrap values using RAxML-HPC (50). Accession numbers for the microorganisms used in the tree are provided in the supplementary material (Text S1). *Sulfolobus acidocaldarius* was used as an outgroup.

The genomes of *Sedimenticola selenatireducens strain CUZ* [pending; please see supplementary text for gene sequences] *and Dechloromarinus chlorophilus strain NSS are* available on the Integrated Microbial Genomes (IMG) system of the Joint Genome Institute and on GenBank (KM192219 and AF170359, respectively).

RNA-seq

Triplicate cultures with nitrate, perchlorate, and oxygen as electron acceptors (40 mL each) were harvested in mid-log phase, centrifuged at 7000g for 10 min at 4º C and resuspended in 1 mL TRIzol (Life Technologies). Anaerobic samples were processed by blowing N_2/CO_2 into 50 mL Falcon tubes prior to the centrifugation of the sample. RNA was extracted according to the manufacturer's protocol. Each sample was then treated with DNAse to remove DNA contamination (Turbo DNA-free, Life Technologies). The RNA was tested for DNA contamination using the universal primers for 16S rRNA amplification 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3'). The Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley) performed rRNA removal (Ribo-Zero rRNA removal kit, Epicentre), cDNA synthesis, library preparation (Apollo 324, WaferGen Biosystems), and 50 bp single-end sequencing using the Illumina Hiseq2000 system. Scythe (https://github.com/ucdavis-bioinformatics/scythe), seqtk (https://github.com/lh3/seqtk/), and sickle (https://github.com/ucdavis-bioinformatics/sickle) were used to remove adapter contamination, cut 5 bp on the 5' end, and quality trimm, respectively. Bowtie2 was used to map processed reads to the *S. selenatireducens* CUZ genome. The GenomicRanges package in R (Core Team, 2013) was used to count reads mapped to coding regions using summarizeOverlaps in "union" mode. The DESeq2 package in R was used to normalize counts and perform differential expression analysis. Data was log2 transformed. MeV 4.9 (http://www.tm4.org/) was used for data visualization and hierachical clustering analysis.

Results

Enrichment, Isolation, and Phylogeny

Two novel microorganisms, a PRB and a CRB, were isolated from sediment from a marina in Berkeley, CA, and from San Diego Bay, CA, respectively. Phylogenetic analysis of the 16S rRNA gene identified the (per)chlorate-reducing strain from the Berkeley sediment as a Gammaproteobacterium (Fig.1), and its 16S rRNA gene sequence was a 100% match to *Sedimenticola selenatireducens* AK4OH1, a selenate-reducer isolated from estuarine sediment from the Arthur Kill (NY-NJ) (51). Consequently, we identified this PRB as *Sedimenticola selenatireducens* strain CUZ. Despite the high 16S similarity between the two strains, strain AK4OH1 reduced selenate but not (per)chlorate (28), whereas the opposite was true for strain CUZ. Phylogenetic analysis of the chlorate-reducer from San Diego Bay sediment identified this strain as a Gammaproteobacterium (Fig. 1), and it shared 98% 16S rRNA identity with both *S. selenatireducens* AK4OH1 and *S. selenatireducens* CUZ. In previous publications, this isolate was designated *Dechloromarinus chlorophilus* strain NSS (6, 12, 43, 46, 47, 52), therefore we

uphold this designation. In addition to *S. selenatireducens* AK4OH1, strains CUZ and NSS were also both closely related to the thiotaurine-degrading autotroph *Thiotaurens thiomutagens* (52) (97% and 98% 16S rRNA similarity, respectively) (Fig. 1), and to marine endosymbionts of tubeworms and bivalves. Given the 16S rRNA sequence similarity, future research may ultimately conclude that *Sedimenticola*, *Dechloromarinus*, and *Thiotaurens* should all be consolidated under a single genus name; however, for the purposes of the present study the proposed names will be used.

Morphology

Both strains are Gram-negative bacteria with most cells appearing as straight to slightly curved rods. Strain CUZ ranged in size from 0.75-1.2 µm long and 0.3-0.45 µm wide (Fig. 2C), while strain NSS ranged from 1-2 µm long and 0.3-0.5 µm wide (Fig. 2D). Both strains were highly motile and possessed one polar flagellum at the pole (Fig. 2A and Fig. 2B). In comparison to CUZ and NSS, *S. selenatireducens* AK4OH1 was described as a non-motile rod measuring ~1.5 mm long and 0.5 mm wide (51).

Growth on Perchlorate and Chlorate

S. selenatireducens strain CUZ grows by coupling the oxidation of acetate to the reduction of perchlorate (Fig. 3A). When grown with 10 mM each of acetate and perchlorate, strain CUZ doubled every 4 hours and reached a maximum optical density (OD_{600}) of 0.52 after 32 hours. In a 47-hour incubation period, 10.6 (±0.3) mM acetate and 5.9 (±0.1) mM perchlorate were consumed, indicating that 4.8 (±0.3) mM acetate (~45%) was assimilated into biomass, which is common for PRB (2, 7). *D. chlorophilus* NSS grows by coupling the oxidation of acetate to the reduction of chlorate (Fig. 3B). Unlike strain CUZ, strain NSS cannot reduce perchlorate (data not shown). When grown with 10 mM each of acetate and chlorate, strain NSS doubled every 3.5 hours and reached a maximum optical density (OD_{600}) of 0.43 after 25 hours. In a 29-hour incubation period, 9.9 (±0.2) mM acetate and 7.4 (± 0.4) mM chlorate were consumed, indicating that 4.4 (±0.4) mM acetate (44%) was assimilated into biomass.

Optimum pH, Temperature, and Salinity

Both strains were halophilic. Strain CUZ grew optimally at 4% NaCl, pH 7, and between 25-30ºC (Fig. 4-6) while strain NSS preferred 1.5-2.5% NaCl, pH 7.5-8, and 37-42ºC (Fig. 4-6). Growth was observed at up to 6% salinity for strain CUZ and 4% salinity for strain NSS (Fig. 4). As such, both strain CUZ and NSS differ from *S. selenatireducens* AK4OH1, which only grew in salinities up to 2.3% (51). Strain CUZ exhibited optimal growth at lower temperatures than strain NSS; growth of strain CUZ was observed at 15ºC but not at temperatures higher than 30ºC, while strain NSS grew at 42ºC but not at temperatures lower than 30ºC (Fig. 5).

Metabolic Diversity

Strains CUZ and NSS utilized a wide variety of electron donors and electron acceptors (Table 1). Both strains oxidized a variety of organic acids and inorganic electron donors such as hydrogen and sulfide (Table 1). Although sulfide was oxidized, neither strain could grow by this metabolism (data not shown). Both strains respired chlorate, nitrate, and oxygen as terminal electron acceptors, but only strain CUZ respired perchlorate. In comparison, *S. selenatireducens* AK4OH1 utilized nitrate and selenate as electron acceptors, but not (per)chlorate or oxygen (51). Neither CUZ nor NSS utilized selenate. When provided with acetate (20 mM) as an electron donor, and both perchlorate and nitrate (5 mM each) or chlorate and nitrate (5 mM each) as electron acceptors, strain CUZ utilized (per)chlorate preferentially to nitrate if the inoculum was pre-grown on (per)chlorate (Fig. 7A and 7C). However, if the inoculum was pre-grown on nitrate, (per)chlorate and nitrate were concomitantly reduced (Fig. 7B, 7D). Similarly, when strain NSS was provided with acetate (20 mM) as an electron donor, and both chlorate and nitrate (5 mM each) as electron acceptors, chlorate was preferentially utilized if the inoculum was grown on chlorate (Fig. 7E). If the inoculum was grown on nitrate, simultaneous consumption of chlorate and nitrate were observed (Fig. 7F). Diauxic growth was not observed for either strain (Fig. 7).

The genomes of both strains encoded entire nitrate reduction pathways including membrane bound nitrate reductases Nar (*narGHJI*: CUZ_01713 to CUZ_01709, NSS_00031830 to NSS_00031860) and periplasmic nitrate reductases Nap (*napFDAGHBC*: CUZ_03010 to CUZ_03004, NSS_00008720 to NSS_00008660). Also present were the nitrite reductase Nir (*nirB* and *nirD*: CUZ_03993 and CUZ_03994, NSS_00004760 and NSS_00004750), the nitric oxide reductase Nor (*norE*: CUZ_03147, NSS_00018480; *norQ*: CUZ_03149, NSS_00018460; *norD*: CUZ_03151, NSS_00018440), and the nitrous oxide reductase Nos (*nosZ*: CUZ_01219, NSS_00015960; *nosDGHFL*: CUZ_01223 to CUZ_01227, NSS_00015920 to NSS_00015880).

Both strains degraded aromatic compounds such as benzoate and phenylacetate coupled to the reduction of (per)chlorate, nitrate, and oxygen (Table 1 and data not shown). The genomes of the two strains contained genes for both an anaerobic pathway of benzoate degradation similar to that of *Thauera aromatica* (53, 54) and an aerobic-hybrid, epoxide-mediated, pathway of benzoate degradation similar to the pathway studied in *Azoarcus evansii* (55, 56). The anaerobic pathway encoded a benzoyl-CoA reductase (*bcrCBAD*: CUZ_03345 to CUZ_03342, NSS_00026600 to NSS_00026570), an enoyl-CoA hydratase (*dch*: CUZ_03346, NSS_00026610), an oxoacyl-CoA hydrolase (*oah*: CUZ_03347, NSS_00026620), and a hydroxyacyl-CoA dehydrogenase (*had*: CUZ_03348, NSS_00026630). The aerobic-hybrid pathway encoded the beta subunit of the benzoyl-CoA oxygenase (*boxB*: CUZ_03988, NSS_00004800) as well as the benzoyl-CoA dihydrodiol lyase (*boxC*: CUZ_03987, NSS_00004810). The benzoate degradation pathways appear to share a benzoate-CoA ligase (*bclA*: CUZ_01337, NSS_00014960), as previously reported in *T. aromatica* and *Magnetospirillum sp. TS-6* (53). Although BLAST(57) searches for other genes involved in the aerobic-hybrid pathway of benzoate degradation yielded several hits for *boxA*, *boxD*, and *boxE* separately from the *boxBC* cluster, it is unclear which, if any of these genes could be involved (data not shown).

In addition to benzoate, the genomes of CUZ and NSS also included genes encoding both an anaerobic pathway and an aerobic-hybrid pathway of phenylacetate oxidation (53). The

anaerobic cluster shows similarity to clusters studied in *Rhodopseudomonas palustris* and *Azoarcus* spp. (53) and included genes for the phenylacetate-CoA ligase (*padJ*: CUZ_03796, NSS_00026760), the phenylacetyl-CoA: acceptor oxidoreductase (*padBCD*: CUZ_03788 to CUZ_03790, *padBCDE*: NSS_00026680 to NSS_00026710), and the phenylglyoxylate:NAD+ oxidoreductase (*padEFGHI*: CUZ_03791 to CUZ_03795, *padFGHI*: NSS_00026720 to NSS_00026750). Downstream processing of the benzoyl-CoA intermediate produced during anaerobic phenylacetate degradation would likely proceed using the anaerobic benzoate degradation pathway (bcr) described above. In strain NSS and *S. selenatireducens* AK4OH1, the anaerobic benzoate and phenylacetate gene clusters were next to each other, and given the high genome-wide similarities between strains CUZ and AK4OH1 (95%), as well as strains CUZ and NSS (86%), this is likely also true for strain CUZ. Genes encoding proteins involved in the aerobic-hybrid pathway of phenylacetate degradation (15, 29) were also present in the genomes of both strains, and they included the oxepin-CoA hydrolase (*paaZ*), the 2,3-dehydroadipyl-CoA hydratase (*paaF*), the 1,2-epoxyphenylacetyl-CoA isomerase (*paaG*), the 3-hydroxyadipyl-CoA dehydrogenase (*paaH*), the 3-oxoadipyl-CoA thiolase (*paaJ*), and the phenylacetate-CoA ligase (*paaK*) (CUZ_04217 to CUZ_04222, NSS_00036190 to NSS_00036230 and NSS_00036090). With the exception of the *paaE* (CUZ_04215, NSS_ 00036170) gene, which is proposed to function as an iron-sulfur oxidoreductase (58), genes encoding the key oxygenase of the system, phenylacetyl-CoA epoxidase (*paaABCDE*), were missing. Nonetheless, CUZ and NSS degraded both benzoate and phenylacetate aerobically and anaerobically, and it remains to be elucidated which of these pathways or combinations of pathways are active under anaerobic, aerobic, or microaerophilic conditions.

Draft Genome and (Per)chlorate and Chlorate Reduction Genes

The draft genome of strain CUZ was 4.5 Mb and contained 86 contigs ranging in size from 204 bp to 543 kb. The average contig length was 53 kb and the GC content was 56.22 %. The genome of strain CUZ revealed the presence of a perchlorate reduction island (PRI) that resembled PRIs previously described in other PRB, though it differed from most by the presence of transposases and the lack of some of the accessory and regulatory genes that often flank the core Pcr and Cld genes (7, 59). The genes in the CUZ PRI included the previously described perchlorate reductase (*pcrABCD*: CUZ_04203 to CUZ_04206), chlorite dismutase (*cld*: CUZ_04201), two-component system composed of a response regulator (*pcrR*: CUZ_04209) and a histidine kinase sensor (*pcrS*: CUZ_04210), PAS-domain containing protein (*pcrP*: CUZ_04211), and quinol dehydrogenase tetraheme cytochrome c (CUZ_ 04202) (Fig.8A) (59, 60). However, the *pcrR*, *pcrS*, and *pcrP* and quinol dehydrogenase tetraheme cytochrome c genes of strain CUZ were only distantly related to those of the well-studied *Azospira suillum* PS, and due to their polyphyletic origin (59, 60), it is unknown whether or not they are essential for (per)chlorate reduction in strain CUZ (Fig. 8A and data not shown). Notably, the PRI of strain CUZ lacked the conserved promoter for *pcrA* that contains consensus binding sites for the sigma factor RpoN, which is essential for perchlorate reduction in *A. suillum* PS (60). Unlike most PRB, The PRI of strain CUZ contained several transposases (Fig.8A), and as such resembled the architecture of the recently published PRB *Arcobacter* sp. CAB (7) and other chlorate-reducers (47). No genes encoding a separate chlorate reductase were found.

The 3.86 Mb genome of strain NSS contained 49 contigs ranging in size from 207 bp to 715 kb, with an average length of 79 kb. GC content was 58.75 %. The genome of strain NSS encoded chlorate reduction genes flanked by insertion sequences, forming composite transposons. Chlorate reduction composite transposons were recently shown to be a common property of CRB and represent mobile genetic units by which chlorate metabolism may be transferred (47). The genome of strain NSS encoded the genes necessary for chlorate reduction: chlorate reductase Clr (*clrABCD*: NSS_00036460 to NSS_00036490) and chlorite dismutase Cld (*cld*: NSS_00036440) (Fig. 8B).

Discussion

The characterization of *S. selenatireducens* strain CUZ and *D. chlorophilus* strain NSS contributes to the known physiological diversity of PRB and CRB. To date, only two marine, halophilic bacteria have been described that can respire perchlorate or chlorate (7, 13). This study investigated the physiology and phylogeny of two additional marine, halophilic (per)chlorate-reducers belonging to the class Gammaproteobacteria. The high 16S rRNA similarity (98%) between the two isolates is the first reported case of two highly related strains, one of which is a PRB and one of which is a CRB.

S. selenatireducens CUZ reduced (per)chlorate whereas *D. chlorophilus* NSS reduced chlorate only, probably due to the specificity of the Pcr and Clr enzymes, respectively. The genome of strain CUZ did not contain a Clr, nor did that of NSS contain a Pcr. Though Clr and Pcr both belong to the dimethylsulfoxide reductase family of molybdenum-containing proteins, they belong to distinct clades. ClrA is polyphyletic (47), whereas to the best of our knowledge, PcrA is monophyletic (unpublished results).

Phylogenetically, strains CUZ and NSS are both highly related to the selenate-reducing bacterium *S. selenatireducens* AK4OH1 (100% and 98% 16S rRNA similarity, respectively), which was isolated from estuarine sediment near Staten Island, NY-NJ (51). Strains AK4OH1, CUZ, and NSS are all halophilic bacteria isolated from marine environments, but the strains differ metabolically. Strain AK4OH1 reduced selenate but not (per)chlorate. In contrast, strains CUZ and NSS reduced (per)chlorate and chlorate, respectively but neither reduced selenate (Table 1). The inability of strain AK4OH1 to reduce (per)chlorate is particularly intriguing given the presence of a PRI in its genome similar to that observed in CUZ (Supplementary Table 1; Joint Genome Institute, IMG). Overall, 10% of the *pcrA* gene differs between strains CUZ and AK4OH1, whereas the intergenic region upstream of *pcrA* differs only by one SNP, suggesting the *pcrA* gene is rapidly evolving or adapting to a selective pressure. The *pcrA* gene of strain CUZ differs from that of strain AK4OH1 by a C-terminal tail of 14 AA (Supplementary Table 1). Interestingly, this tail is unique to strain CUZ and a related PRB belonging to the Epsilon class of Proteobacteria, *Arcobacter* sp. CAB, which was isolated from the same location and is the only other marine PRB isolated to date (9). The role, if any, of the PcrA 14 AA tail modification remains to be understood. Further studies involving genetics and evolution will aid in determining what changes in the PRI are necessary to confer the ability to reduce (per)chlorate.

Other close relatives of strains CUZ and NSS include the thiotaurine degrading bacterium *Thiotaurens thiomutagens*, and unnamed, poorly studied sulfur-oxidizing marine endosymbionts (Fig. 1 and data not shown). These endosymbionts are thought to grow lithoautotrophically by utilizing sulfide (61) or thiotaurine as electron donors (52). Strains NSS and CUZ both oxidized sulfide but did not grow by this metabolism. It is unknown whether strains NSS and CUZ can utilize thiotaurine, and whether they can form symbiotic relationships with tubeworms or clams. Because there are so few isolates obtained from this poorly understood clade of the Gammaproteobacteria, the isolation and characterization of two novel strains greatly contributes to the known physiology of microorganisms in the group.

The genomes of strains CUZ and NSS included genes for (per)chlorate and chlorate reduction, respectively. In particular, the PRI of strain CUZ adds to the known diversity of the genetics of (per)chlorate reduction. The tail modification of the PcrA enzyme in strain CUZ, which is also present in *Arcobacter* sp. CAB, could indicate some adaptive advantage, given that the organisms were isolated from nearly identical environments. Previous work showed that an RpoN binding motif upstream of *pcrA* was essential for regulating (per)chlorate reduction in *Azospira suillum* PS (60), and such a motif is assumed to have a regulatory role in other (per)chlorate reducers when it is present. Notably, the PRI of strain CUZ lacks an RpoN binding motif upstream of *pcrA*, suggesting that strain CUZ utilizes a different regulatory circuit. In support of this, transcriptomics data indicates that expression of the PRI in strain CUZ is constitutive (Fig. 9). The genome of strain NSS included chlorate-reducing genes which were 99.9% identical (nucleotide identity) to those of the chlorate-reducing Gammaproteobacterium *Shewanella algae* ACDC, which was isolated from the same environment as strain NSS (47). As previously described, the presence of chlorate reduction composite transposons in NSS suggests this metabolism may be horizontally transferred (47).

Most PRB are capable of reducing nitrate, whereas this metabolism is rare for CRB (6, 9, 11, 43, 62-64). In most cases, a separate nitrate reductase exists for this metabolism, though not all strains can couple nitrate reduction to growth, nor can all strains reduce nitrate completely to nitrogen gas (2, 12). The genomes of strains CUZ and NSS encoded separate nitrate reduction pathways, and both were also able to grow by nitrate reduction. Both strains preferentially utilized (per)chlorate and chlorate, respectively, before nitrate if the inoculum was grown on (per)chlorate or chlorate. However, (per)chlorate and nitrate were concomitantly reduced if the inoculum was grown with nitrate as an electron acceptor. In contrast, other PRB preferentially utilize nitrate or utilize both electron acceptors simultaneously. This is unexpected as nitrate reduction is thermodynamically slightly less favorable than (per)chlorate reduction (E^{o}' = +746mV for NO_3^-/N_2 couple compared to E^{o}' = +797mV and +792mV for the biological couple of ClO_4^-/Cl^- and ClO_3^-/Cl^-, respectively) and to the best of our knowledge is the only example of a less favorable electron acceptor being preferentially utilized. The PRB *Azospira suillum* PS and *Dechloromonas aromatica* RCB only utilize perchlorate after complete removal of nitrate (65, 66). *Dechloromonas agitata* CKB, *Marinobacter vinifirmus* P4B1, and strain perc1ace reduced nitrate and perchlorate concomitantly regardless of whether the inoculum was grown on perchlorate or nitrate (30, 65). Further, mixed culture bed reactors have consistently demonstrated preferential reduction of nitrate over perchlorate in co-contaminated sludge (31-33). Unlike PRB, most CRB cannot grow by nitrate reduction; however, *Alicyclyphilus denitrificans* BC, the one other CRB that can reduce nitrate, differs from strain NSS in that it preferentially utilizes the electron acceptor with which the inoculum was grown (30, 62-64).

The genomes of both CUZ and NSS contain separate genes encoding the nitrate reductases Nap and Nar, as well as Pcr or Clr, respectively, thus preferential (per)chlorate or chlorate reduction cannot be attributed to competitive inhibition wherein both nitrate and (per)chlorate compete for a single binding site. However, it has been shown that Pcr enzymes reduce nitrate, and Nar has been shown to reduce (per)chlorate (5, 37, 67-70). Preferential utilization of (per)chlorate over nitrate may be genetically controlled via mechanisms which remain to be elucidated. The alternative sigma factor RpoN has been shown to be necessary for perchlorate reduction in *Azospira suillum* strain PS (60), where the promoter region upstream of the *pcrA* gene contains an RpoN-binding motif and PcrR contains an RpoN-interacting domain (60). As noted, the PRI of strain CUZ lacks the RpoN binding motif upstream of *pcrA*, and its PcrR lacks an RpoN-interacting domain, suggesting that strain CUZ possesses a distinct regulatory mechanism from that of *A. suillum* PS. Constitutive expression of the PRI of strain CUZ (Fig. 9) could explain the sequential removal of perchlorate and nitrate when the inoculum was pre-grown with perchlorate, and the simultaneous removal of perchlorate and nitrate when the inoculum was pre-grown with nitrate.

Both CUZ and NSS encode genes for aromatic degradation pathways, engendering further bioremediative applications in these strains. Aromatic compounds are the most abundant class of organic compounds after carbohydrates and are prominent environmental contaminants. Therefore, it is important to understand how these compounds are degraded in nature, especially in anoxic environments (53). Strains CUZ and NSS contain genes involved in the oxidation of benzoate and phenylacetate, both of which are key intermediates in general degradation pathways (53, 55, 71).

Strains CUZ and NSS contain known pathways for both aerobic and anaerobic benzoate and phenylacetate degradation (10, 19, 36, 41, (53, 55) as well as genes for the epoxide-mediated, aerobic-hybrid degradation pathway, (55, 56, 58) demonstrating great versatility regardless of electron accepting conditions. In anoxic environments, PRB and CRB can carry out the aerobic oxidation of aromatic compounds coupled to the reduction of (per)chlorate by utilizing oxygen derived from ClO_2^- dismutation (7, 10, 29, 72, 73), but it is unknown whether a fully anaerobic pathway that does not rely on internal oxygen generation can be coupled with (per)chlorate as an electron acceptor. Since strains CUZ and NSS appear to be capable of both anaerobic and microaerophilic oxidation of benzoate and phenylacetate, characterization of this metabolism may help elucidate the regulation of these pathways in response to various environmental conditions. Because the aerobic-hybrid pathway is hypothesized to be important in low or fluctuating oxygen concentrations (55, 74), it is conceivable that in anoxic environments, this pathway could be coupled to perchlorate reduction, utilizing the oxygen produced by ClO_2^- dismutation. However, anaerobic pathways for benzoate or phenylacetate degradation could also play an important role, as long as the concentration of oxygen produced by (per)chlorate reduction does not reach levels that are toxic to the benzoyl-CoA reductase or the phenylglyoxylate oxidoreductase, respectively (75, 76). Due to the presence of genes encoding both anaerobic and aerobic-hybrid pathways, strains CUZ and NSS may represent ideal candidates to study pathways of aromatic oxidation. (Per)chlorate reduction, and the accompanying low levels of internally generated oxygen, may help reveal how the combination of anaerobic and aerobic-hybrid pathways are regulated and whether (per)chlorate reduction can couple effectively to anaerobic pathways.

This study described two closely related marine, halophilic Gammaproteobacteria capable of metabolizing (per)chlorate. Strains CUZ and NSS share 98% 16S rRNA sequence identity, yet one is a PRB and one is a CRB, and each adds to the known physiological and phylogenetic diversity of (per)chlorate reduction. The preferential utilization of (per)chlorate or chlorate over nitrate with (per)chlorate-grown inocula is surprising, and suggests a different regulatory approach than seen in other PRB and CRB. This preference may aid in bioremediation efforts, which have historically been hindered by preferential nitrate reduction in co-contaminated environments (35, 77). Both strains encode both anaerobic and aerobic-hybrid pathways for aromatic degradation, providing a unique opportunity to study the regulation and activity of these pathways coupled to (per)chlorate reduction. This study has identified and characterized two novel marine (per)chlorate- and chlorate-reducing bacteria, each of which exhibit novel metabolic preferences and pathways coupled to (per)chlorate reduction.

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Tables and Figures

Table 1. Overview of electron donor and acceptor utilization by strains *D. chlorophilus* NSS, *S. selenatireducens* CUZ, and *S. selenatireducens* AK4OH1. Electron donors were tested with chlorate or perchlorate as electron acceptors for strains NSS and CUZ, respectively. Electron acceptors were tested using acetate as an electron donor. Data for *S. selenatireducens* AK4OH1 was adapted from (51).

Compound	**Concentration (mM)**	***D. chlorophilus* NSS**	***S. selenatireducens* CUZ**	***S. selenatireducens* AK4OH1**
Electron donors				
Acetate	10	Y	Y	Y
Benzoate	1	Y	Y	Y
Butyrate	10	Y	Y	ND
Catechol	1	N	N	ND
Citrate	10	N	N	ND
Ethanol	5	N	N	ND
Fructose	10	N	N	ND
Fumarate	10	Y	Y	ND
Glucose	10	N	N	ND
Hydrogen	50 [a, b]	Y	Y	ND
Isobutyrate	10	Y	Y	ND
Lactate	10	Y	Y	Y
Malate	10	Y	Y	ND
Methane	25 [a]	N	N	ND
Methanol	5	N	N	ND
Phenylacetate	2	Y	Y	ND
Protocatechuate	1	N	Y	ND
Propionate	10	Y	Y	ND
Succinate	1	Y	Y	ND
Sulfide [d]	5	N	N	ND
Valerate	10	Y	Y	ND
Yeast extract	1 [c]	Y	Y	ND
Electron acceptors				
Arsenate	2.5	N	N	N
Chlorate	10	Y	Y	N
Malate	10	N	N	ND
Nitrate	10	Y	Y	Y
Nitrite	2	N	Y	Y
Oxygen	25 [a]	Y	Y	N
Perchlorate	10	N	Y	N
Selenate	5	N	N	Y
Sulfate	10	N	N	N
Sulfite	2	N	N	ND
Thiosulfate	10	N	N	ND

[a] Units in kPa. [b] Samples were supplemented with 0.1 g/L yeast extract. [c] Units in g/L. [d] Although sulfide was oxidized, no growth was observed.

Figure 1. Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic position *S. selenatireducens* CUZ (*) and *D. chlorophilus* NSS (**), both Gammaproteobacteria. PRB are shown in bold and CRB are underlined. Bootstrap values are based on 1,000 replications and are shown at the nodes of the tree. The scale bar represents 0.1 expected change per site.

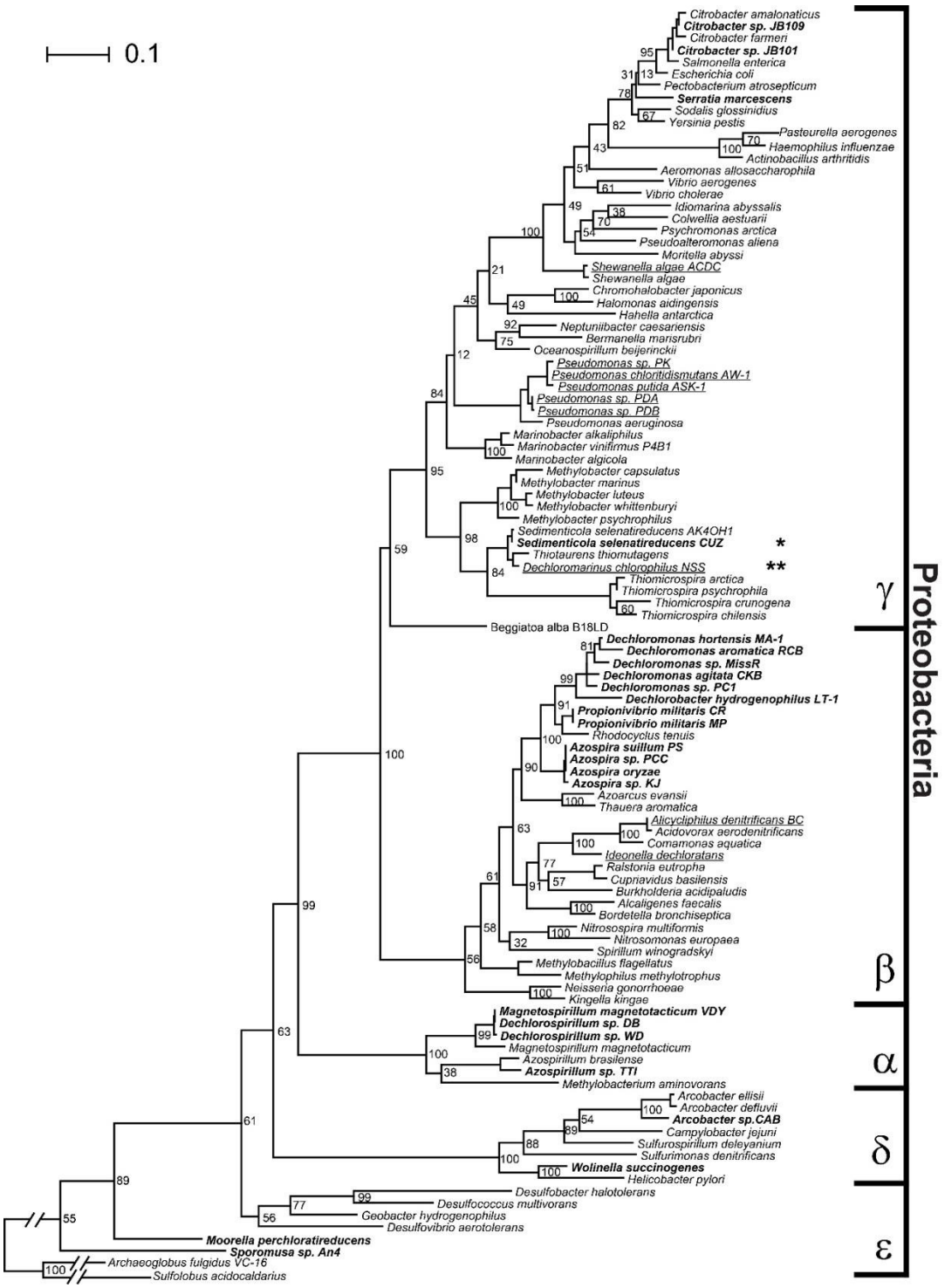


Figure 2. Transmission electron microscopy of strains (A) CUZ and (B) NSS; scanning electron microscopy of strains (C) CUZ and (D) NSS. Scale bar, 0.5 µm.

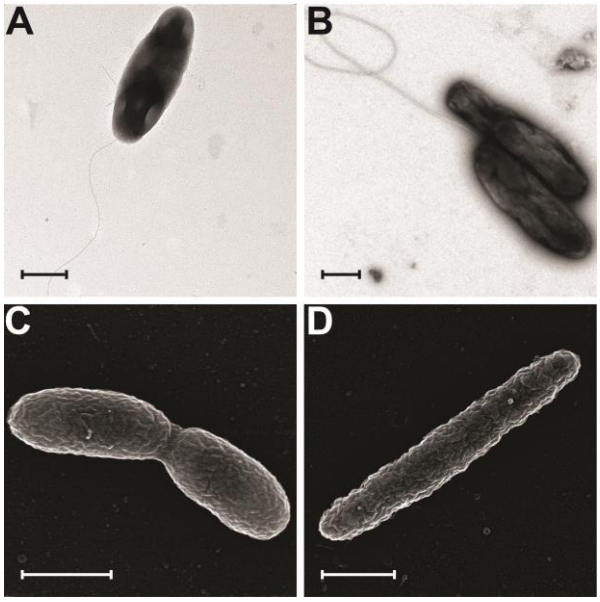


Figure 3. Growth curves of strains (A) CUZ and (B) NSS on acetate and (A) perchlorate or (B) chlorate. Cell density was monitored by OD_{600}. Circles: cell density; triangles: acetate concentration, squares: perchlorate or chlorate concentration; diamonds: cell density for the no (per)chlorate control. Error bars represent the standard deviations for the average of triplicate samples.

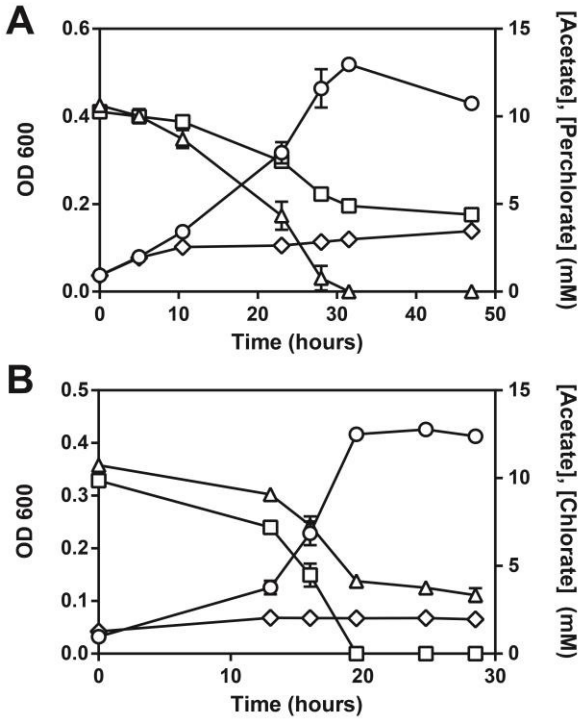


Figure 4. Salinity-dependent growth optima for strains CUZ (closed circles) and NSS (open circles). Points and error bars represent the average and standard deviations of triplicate samples.

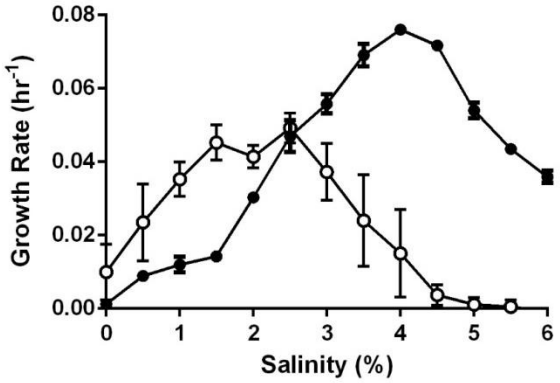


Figure 5. Temperature-dependent growth optima for strains CUZ (closed circles) and NSS (open circles). Points and error bars represent the average and standard deviations of triplicate samples.

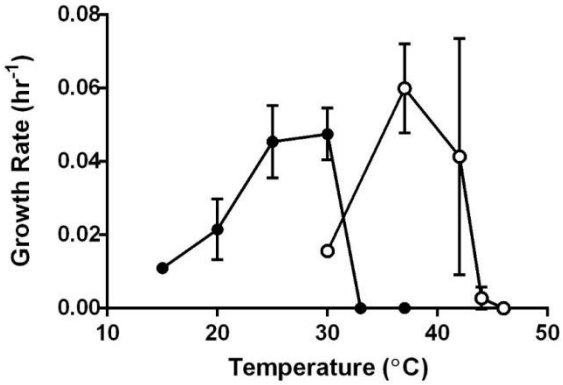


Figure 6. pH-dependent growth optima for strains CUZ (closed circles) and NSS (open circles). Points and error bars represent the average and standard deviations of triplicate samples.

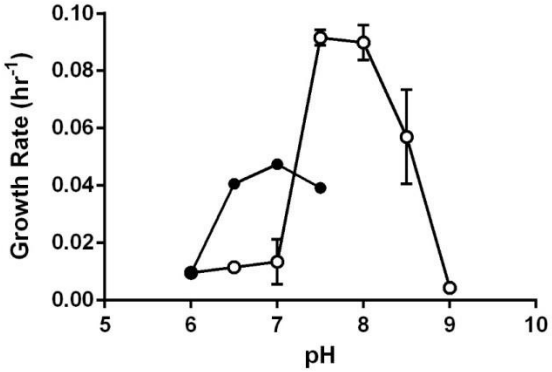


Figure 7. Growth curves of strains (A–D) CUZ and (E, F) NSS when grown in diauxic media containing both nitrate and (per)chlorate. The inoculum source was grown with either perchlorate (A), chlorate (C, E), or nitrate (B, D, F) as the sole electron acceptors. Cell density was monitored by OD_{600}. Cell density (●), perchlorate or chlorate concentration (▲), nitrate concentration (■). Error bars represent the standard deviations for the average of triplicate samples.

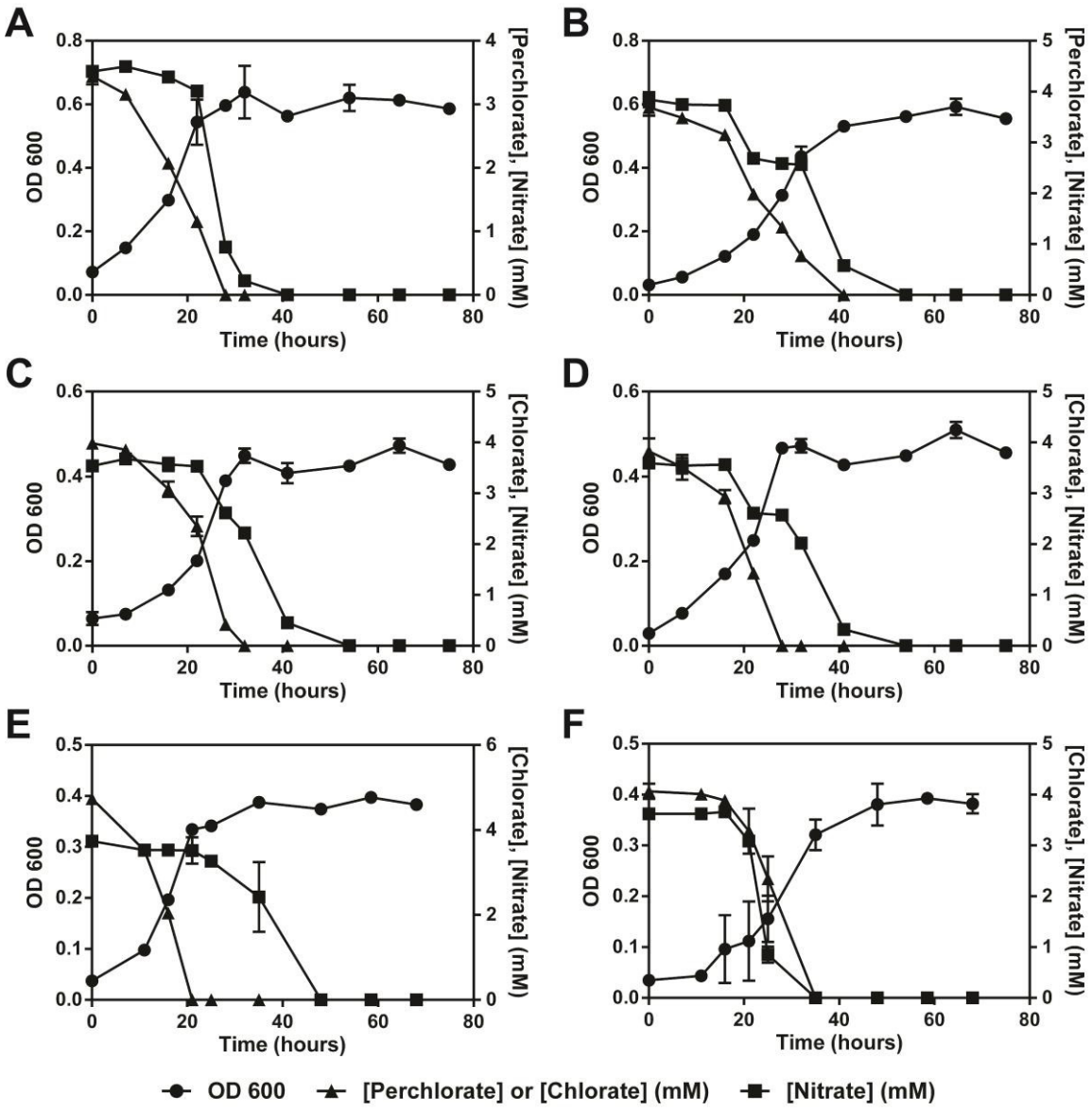


Figure 8. Comparison of genes involved in (A) (per)chlorate reduction for strain CUZ, and (B) chlorate reduction for strain NSS. Transposases and insertion sequences are indicated in light gray, and core genes required for (per)chlorate (A) and chlorate (B) reduction are indicated in dark gray.

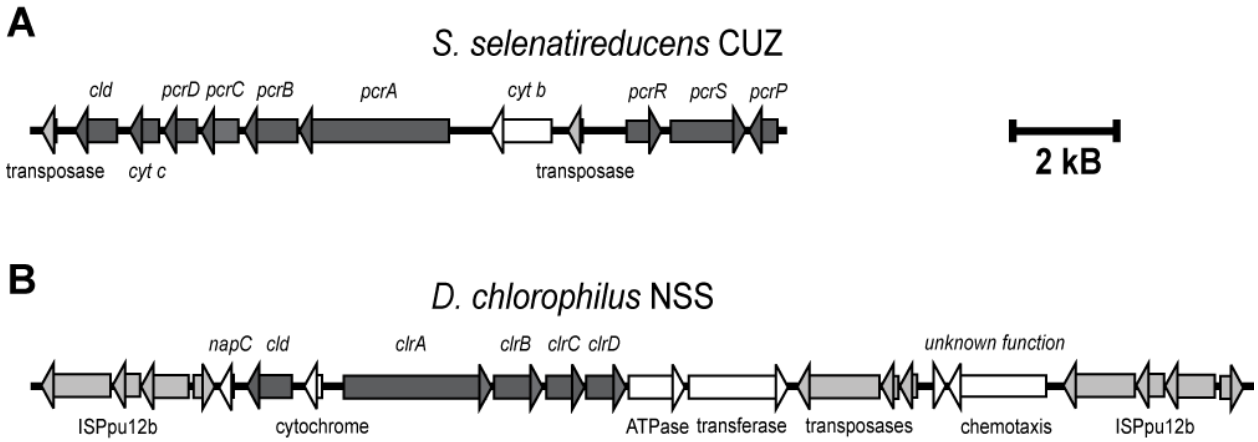
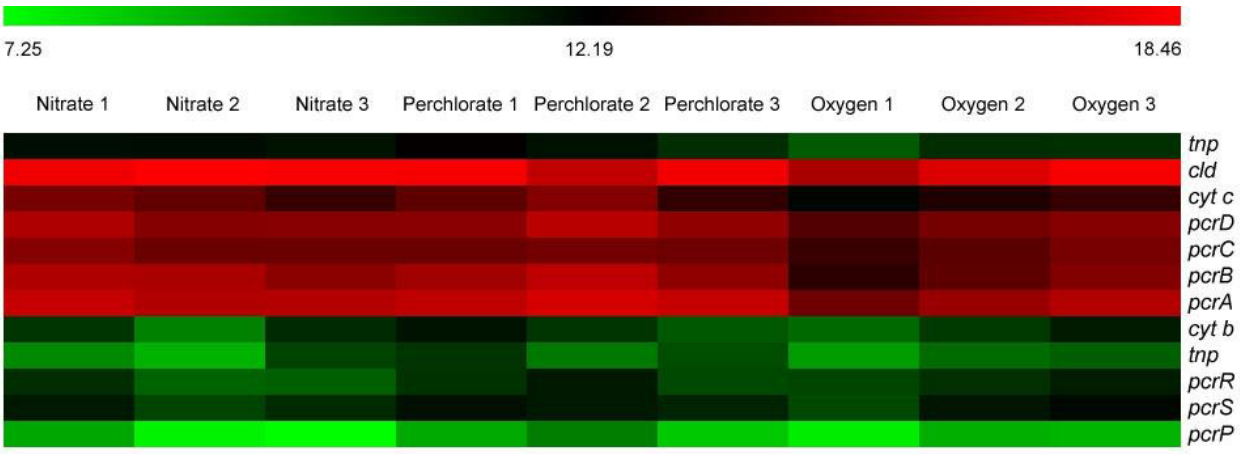


Figure 9. Heatmap of RNA-seq data from *S. selenatireducens* strain CUZ. Counts were normalized with DeSeq2 and then transformed via Log_2 (normalized counts + 1) prior to clustering and visualization. Labels above heatmap represent replicates of the electron acceptor tested; tnp = transposase.



Chapter 5: Phenylacetate and Benzoate Degradation Pathways in the (Per)chlorate Reducing Bacterium *Sedimenticola selenatireducens* CUZ and the Chlorate Reducing Bacterium *Dechloromarinus chlorophilus* NSS

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Abstract

The pathways involved in aromatic compound oxidation under perchlorate and chlorate [collectively known as (per)chlorate] reducing conditions are poorly understood. *Sedimenticola selenatireducens* CUZ and *Dechloromarinus chlorophilus* NSS oxidize phenylacetate and benzoate, two key intermediates in aromatic compound catabolism, coupled to the reduction of perchlorate or chlorate, respectively, and nitrate. While strain CUZ also oxidized benzoate and phenylacetate with oxygen as an electron acceptor, strain NSS oxidized only the latter, even at very low oxygen concentrations (1%). Strains CUZ and NSS contain similar genes for both the anaerobic and the aerobic-hybrid pathways of benzoate and phenylacetate degradation, however, the key genes (*paaABCD*) encoding the epoxidase of the aerobic-hybrid phenylacetate pathway were not found in either genome, suggesting an unknown alternative may be present. Using RNA-seq and proteomics, as well as by monitoring metabolic intermediates, we investigated the utilization of the anaerobic and aerobic-hybrid pathways on different electron acceptors. For strain CUZ, the results indicated utilization of the anaerobic pathways with perchlorate and nitrate as electron acceptors and of the aerobic-hybrid pathways in the presence of oxygen. In contrast, proteomics results suggest that strain NSS may use a combination of the anaerobic and aerobic-hybrid pathways when growing on phenylacetate with chlorate. Though microbial (per)chlorate reduction produces molecular oxygen through the dismutation of chlorite (ClO_2^-), this study demonstrates that anaerobic pathways for aromatics degradation can still be utilized.

Importance

Several studies have demonstrated that (per)chlorate and chlorate reducing bacteria (PRB and CRB, respectively) can use oxygen produced from chlorite dismutation to oxidize aromatic compounds using aerobic pathways in otherwise anoxic environments. However, *S. selenatireducens* CUZ and *D. chlorophilus* NSS are the first examples of PRB and CRB that degrade aromatic compounds using oxygen-independent anaerobic pathways while growing on perchlorate and chlorate, respectively. Although both strains encode genes for the aerobic-hybrid and the anaerobic pathways of phenylacetate degradation, strain CUZ preferentially utilized the anaerobic route in the presence of perchlorate, regardless of any oxygen produced from chlorite dismutation. Strain NSS, on the other hand, may be capable of carrying out both processes simultaneously. Concurrent use of anaerobic and aerobic pathways has not been previously reported for other CRB or microorganisms that encode similar pathways of phenylacetate degradation and may be advantageous in low oxygen environments.

Introduction

After carbohydrates, aromatic compounds are the most abundant class of organic compounds found in nature (1) and occur naturally in lignin, flavonoids, quinones, and some amino acids. Many aromatic compounds, including components of crude oil and fossil fuels, are considered major environmental pollutants (1, 2) and, as such, their detection and removal is of interest. Despite the high stability conferred by the resonance energy of the aromatic ring (150 kJ mol^{-1} benzene), microorganisms have evolved that can degrade most naturally occurring aromatic compounds in both oxic and anoxic environments (3). Under oxic conditions, microorganisms utilize oxygen as both a terminal electron acceptor and as a co-substrate for oxygenases to activate and cleave the aromatic ring (3, 4). In anoxic environments, aromatic degradation proceeds via coenzyme A (CoA) activation, reductive dearomatization of the ring, and hydrolytic cleavage (3, 4). A third, novel pathway, which combines aspects of both the aerobic and anaerobic catabolic routes, has been recently elucidated and its use in low or fluctuating oxygen conditions was postulated (3-5). In this pathway, known as the aerobic-hybrid pathway, intermediates are processed as CoA thioesters (similar to anaerobic pathway intermediates), but dearomatization of the aromatic ring involves an epoxidation reaction that requires molecular oxygen (5). Finally, the ring is hydrolytically cleaved (3-5).

Phenylacetate is found in the environment as a common carbon source and is a central intermediate in the degradation of many aromatic compounds such as phenylalanine, phenylacetaldehyde, 2-phenylethylamine, phenylacetyl esters, lignin-related phenylpropane units, phenylalkanoic acids with an even number of carbon atoms, and environmental contaminants like styrene and ethylbenzene (5-7). Although the anaerobic pathway of phenylacetate degradation in bacteria is well characterized (1, 4, 8, 9), the aerobic pathway has only recently been discovered (3-5). Unlike aerobic phenylacetate degradation in fungi, in which hydroxylases convert phenylacetate to homogentisate (10-12), the novel bacterial aerobic-hybrid pathway proceeds through CoA-dependent activation, epoxidation of the aromatic ring, and hydrolytic ring cleavage (4, 5). To date, this hybrid pathway is the only known aerobic pathway used by bacteria in the degradation of phenylacetate (4, 5). The production of phenylacetyl-CoA as an intermediate in both the anaerobic and aerobic-hybrid pathways is an efficient and rapid way to respond to fluctuations of oxygen in the environment, as the phenylacetyl-CoA intermediate can be routed to either pathway depending on the concentration of oxygen (4, 13). This is also true of the anaerobic and aerobic-hybrid pathways of benzoate degradation, both of which produce benzoyl-CoA as a key intermediate (4).

Perchlorate and chlorate [collectively denoted (per)chlorate] reducing bacteria (PRB and CRB, respectively) are microorganisms that can utilize perchlorate (ClO_4^-) or chlorate (ClO_3^-) as terminal electron acceptors. While PRB can reduce both perchlorate and chlorate via the perchlorate reductase enzyme (Pcr), CRB only reduce the latter with the chlorate reductase (Clr) (14). Both Pcr and Clr reduce (per)chlorate or chlorate, respectively, to chlorite (ClO_2^-), which is subsequently dismutated into molecular O_2 and Cl^- by the chlorite dismutase (Cld) enzyme present in both PRB and CRB (15). Chlorite dismutation is the only chemotrophic microbial metabolism shown to produce significant amounts of free O_2 (15). While chemotrophic nitrous oxide disproportionation could putatively produce molecular O_2 and this reaction has been invoked in anaerobic methane oxidation coupled to nitrate reduction (16), no enzyme is known that can carry out this reaction.

At present, the degradation of aromatic compounds coupled to (per)chlorate as an electron acceptor has been shown to occur through aerobic pathways that utilize the biogenic O_2 from ClO_2^- dismutation not only for respiration, but also as a co-substrate for oxygenases in anoxic environments (15, 17-19). However, the recently described PRB *Sedimenticola selenatireducens* CUZ and the closely related CRB *Dechloromarinus chlorophilus* NSS encode genes for both anaerobic and aerobic-hybrid pathways of phenylacetate and benzoate degradation (Chapter 4; Fig. 1 and 2). The anaerobic phenylacetate pathway is encoded by two gene clusters, the *pad* cluster (Fig. 1A; Fig. 2A) that converts phenylacetate to benzoyl-CoA (Fig. 1E), and the *bcr, oah, dch, had* cluster (hereafter referred to as the *bcr* cluster) (Fig. 1B; Fig. 2B) that degrades benzoyl-CoA to glutaryl-CoA and acetyl-CoA (Fig. 1E) (1, 4). The *pad* cluster (Fig. 1A) included genes for the phenylacetate-CoA ligase (*padJ*), the phenylacetyl-CoA: acceptor oxidoreductase (*padBCD*), and the phenylglyoxylate:NAD+ oxidoreductase (*padEFGHI*). The *bcr* cluster (Fig. 1B), which degrades benzoyl-CoA anaerobically (Fig. 1E), contained genes encoding a benzoyl-CoA reductase (*bcrCBAD*), an enoyl-CoA hydratase (*dch*), an oxoacyl-CoA hydrolase (*oah*), and a hydroxyacyl-CoA dehydrogenase (*had*). In addition to the *bcr* cluster, genes for the aerobic-hybrid pathway of benzoyl-CoA degradation were also found (Fig. 1D, Fig. 2D), including the beta subunit of the benzoyl-CoA oxygenase (*boxB*) and the 2,3-epoxybenzoyl-CoA dihydrolase (*boxC*). Genes involved in the aerobic-hybrid pathway of phenylacetate degradation (Fig. 1E) (1, 5) were also present in the genomes of both strains (Fig. 1C; Fig. 2C). These genes encoded an oxepin-CoA hydrolase (*paaZ*), a 2,3-dehydroadipyl-CoA hydratase (*paaF*), a 1,2-epoxyphenylacetyl-CoA isomerase (*paaG*), a 3-hydroxyadipyl-CoA dehydrogenase (*paaH*), a 3-oxoadipyl-CoA thiolase (*paaJ*), and a phenylacetate-CoA ligase (*paaK*) (Fig. 1C; Fig. 2C). However, all of the genes encoding the key oxygenase of the system, phenylacetyl-CoA epoxidase (*paaABCDE*), were missing except *paaE*, which likely functions as an iron-sulfur oxidoreductase that transfers electrons from NADPH to the active center of the oxygenase complex (5). Locus tags for genes in these clusters are provided in Table 1.

The current study demonstrates that strains CUZ and NSS degrade phenylacetate and benzoate with (per)chlorate as an electron acceptor using anaerobic pathways (either exclusively or simultaneously with the aerobic-hybrid pathway) that do not rely on O_2 produced from the dismutation of ClO_2^-.

Materials and Methods

Culture Conditions

Sedimenticola selenatireducens CUZ

The medium used for culturing contained (g/L): NaCl (30), KCl (0.67), $NaHCO_3$ (2.5), 10 mL vitamins, 10 mL minerals, and 20 mL RST minerals (31). The RST minerals contained (g/L): NaCl (40), NH_4Cl (50), KCl (5), KH_2PO_4 (5), $MgSO_4 \cdot 7H_2O$ (10), and $CaCl_2 \cdot 2H_2O$ (1). The medium was boiled, cooled on ice, and degassed under a N_2-CO_2 headspace (80/20) and the pH was ~6.8. Each anaerobic tube (Bellco®, Vineland, NJ) received 9 mL of this medium and was autoclaved. All growth experiments were carried out at 30 ºC.

Dechloromarinus chlorophilus NSS

The medium used for culturing contained (g/L): NaCl (20), KCl (0.67), monosodium PIPES (1.6220), disodium PIPES (1.7317), 10 ml vitamins, 10 ml minerals, and 20 ml RST minerals (31). The medium was boiled, cooled on ice, and degassed under a N_2 headspace and the pH was ~7.5. Each anaerobic tube received 8 mL of this medium and was autoclaved. After autoclaving, 0.5 mL of $MgCl_2 \cdot 6H_2O$ (21.2 g/ 100 mL dH_2O) and 0.5 mL of $CaCl_2 \cdot 2H_2O$ (3.04 g/ 100 mL dH_2O) were added to each tube from degassed, sterile, aqueous stock solutions. All growth experiments were carried out at 37 ºC.

Growth on Phenylacetate, Benzoate, and Acetate

To test growth on phenylacetate coupled to the reduction of perchlorate and chlorate (corresponding to strain CUZ and strain NSS, respectively) or nitrate, 2 mM of phenylacetate and 10 mM of perchlorate or chlorate or 15 mM of nitrate were added to triplicate samples. Negative controls included a no donor and a no acceptor control. Samples were taken for optical density and anion concentration measurements as described below.

For strain CUZ, growth on phenylacetate (2 mM) and oxygen (20%) was carried out in triplicate in sealed 2.8 L Fernbach flasks using a total liquid volume of 500 mL, thus allowing for a large oxic headspace. A no acceptor control was included. The Fernbach flasks were shaken at 200 rpm. The medium was made by adding all components except bicarbonate to the flasks and autoclaving it aerobically. After autoclaving, the flasks were sealed and bicarbonate was added from an aqueous, sterile stock. HCl was used to pH the medium to ~6.8. In the case of strain NSS, 160 mL sealed bottles were used with a 50 mL liquid volume. 10 mL of oxygen was injected into triplicate cultures twice, once at inoculation, and a second time in early log phase. A no acceptor control was included. Bottles were shaken horizontally at 200 rpm.

For both strains, growth on 10 mM acetate and (per)chlorate and 1 mM benzoate with 5 mM (per)chlorate or 10 mM nitrate were used as controls for RNA-seq and proteomics experiments. Growth on 1 mM benzoate and oxygen was also used as a control in strain CUZ but not strain NSS. Cultures with 1 mM benzoate and 10% oxygen in the headspace were grown in 160 mL bottles shaken horizontally at 200 rpm.

Cell Growth Profiles

Cell growth was measured spectrophotometrically at 600 nm. All experimental analyses were performed in triplicate and the results are expressed as the average of these determinations.

Analytical Methods

Perchlorate, nitrate, benzoate, and phenylacetate concentrations were measured via ion chromatography (ICS-2100, Thermo Fisher Scientific, Sunnyvale, CA). The system utilized an IonPac AG16 (4 x 50 mm) guard column, an IonPac AS16 (4 x 250 mm) analytical column, an

ASRS-300 4 mm suppressor system and a DS6 heated conductivity cell. A KOH gradient was generated using a EGC III KOH generator at an isocratic flow rate of 1.5 mL/min. The KOH gradient was 1.5 mM from 0-7 minutes, increased to 10 mM from minutes 7-13, held at 10 mM from minutes 13-16, increased to 35 mM during minutes 16-17, held at 35 mM from minutes 17-27 and decreased to 1.5 mM from minutes 27-30. The suppressor controller was set at 130 mA for the analysis. The injection volume was 25 μL.

Chlorate concentration was measured by ion chromatography using a Dionex ICS 1500 equipped with a Dionex Ion Pac AS 25 column (4 x 250 mm). The mobile phase was 36 mM NaOH at a flow rate of 1.0 mL/min. Background conductivity was suppressed with a Dionex ASRS 300 (4 mm) in recycle mode. The suppressor controller was set at 90 mA for analysis and the injection volume was 10 μL.

RNA Extraction and RNA-seq

Triplicate cultures (40 mL each) were harvested in mid-log phase, centrifuged at 7000g for 10 min at 4º C and resuspended in 1 mL TRIzol (Life Technologies). To keep samples as anaerobic as possible, N_2/CO_2 or N_2 gas (depending on the medium) was blown into 50 mL Falcon tubes prior to the centrifugation of the sample. RNA was extracted according to the manufacturer's protocol and biological triplicates were combined into 1 sample per condition at the end of the extraction. Each sample was then treated with DNAse to remove DNA contamination (Turbo DNA-free, Life Technologies). The RNA was tested for DNA contamination using the universal primers for 16S rRNA amplification 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3'). The Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley) performed rRNA removal (Ribo-Zero rRNA removal kit, Epicentre), cDNA synthesis, library preparation (Apollo 324, WaferGen Biosystems), and 50 bp single-end sequencing using the Illumina Hiseq2000 system. Scythe (https://github.com/ucdavis-bioinformatics/scythe), seqtk (https://github.com/lh3/seqtk/), and sickle (https://github.com/ucdavis-bioinformatics/sickle) were used to remove adapter contamination, cut 5 bp on the 5' end, and quality trimm, respectively. Bowtie2 (32) was used to map processed reads to the *S. selenatireducens* CUZ genome. The GenomicRanges package (33) in R (Core Team, 2013) was used to count reads mapped to coding regions using summarizeOverlaps in "union" mode. The DESeq2 package (34) in R was used to normalize counts and perform differential expression analysis. Data was log2 transformed. MeV 4.9 (http://www.tm4.org/) was used for data visualization and hierachical clustering analysis.

Protein Extraction and Liquid chromatography-tandem mass spectrometry

Triplicate cultures (40 mL) were harvested in mid-log phase and centrifuged at 7000g for 10 minutes at 4º C. To keep samples as anaerobic as possible, N_2/CO_2 or N_2 gas (depending on the medium) was blown into 50 mL Falcon tubes prior to the centrifugation of the sample. The pellet was then washed with 1 mL of 100 mM NH_4HCO_3, centrifuged for 2 minutes at 10,000g and resuspended in 0.5 mL 100 mM NH_4HCO_3. Cells were lysed at 4º C using a 550 Sonic Dismembrator (Fisher Scientific, Waltham, MA) at a power of 1.5. Each sample was subject to 3

rounds of 30 seconds each, with a 30 second rest on ice between each round. 500 ng of trypsin were then added and the samples were incubated for 3 hours at 37 ºC. Samples were then centrifuged at 14000g for 5 minutes and the supernatant was transferred to new tubes. 5 uL of 100 mM dithiothreitol and 5 uL of 100 mM iodoacetamide were then added in two sequential 30 minute incubations at room temperature. Another 500 ng of trypsin were then added in an overnight incubation at 37 ºC. 20 uL of 2.5% trifluoroacetic acid (TFA) were added followed by a 5 minute centrifugation at 14000g. The supernatant was transferred to a new tube. C18 Zip Tips (Millipore, Billerica, MA) were used to concentrate peptides and remove salts. 85% acetonitrile/0.1% TFA were used to elute the peptides and vacuum centrifugation was used to remove acetonitrile for a final volume of 30 uL.

Trypsin-digested proteins were analyzed using a Thermo Dionex UltiMate3000 RSLCnano liquid chromatograph that was connected in-line with an LTQ Orbitrap XL mass spectrometer equipped with a nanoelectrospray ionization (nanoESI) source (Thermo Fisher Scientific, Waltham, MA). The LC was equipped with a C18 analytical column (Acclaim® PepMap RSLC, 150 mm length × 0.075 mm inner diameter, 2 µm particles, 100 Å pores, Thermo) and a 1 µL sample loop. Acetonitrile (Fisher Optima grade, 99.9%), formic acid (1 mL ampules, 99+%, Thermo Pierce), and water purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA) were used to prepare mobile phase solvents for liquid chromatography-tandem mass spectrometry. Solvent A was 99.9% water/0.1% formic acid and solvent B was 99.9% acetonitrile/0.1% formic acid (v/v). Samples contained in polypropylene autosampler vials with septa caps (Wheaton, Millville, NJ) were loaded into the autosampler compartment prior to analysis. The autosampler compartment was maintained at 4 ºC. The elution program consisted of isocratic flow at 5% B for 4 min, a linear gradient to 30% B over 128 min, isocratic flow at 95% B for 6 min, and isocratic flow at 5% B for 12 min, at a flow rate of 300 nL/min.

The column exit was connected to the nanoESI emitter in the nanoESI source of the mass spectrometer using polyimide-coated, fused-silica tubing (20 µm inner diameter × 280 µm outer diameter, Thermo). Full-scan mass spectra were acquired in the positive ion mode over the range m/z = 350 to 1600 using the Orbitrap mass analyzer, in profile format, with a mass resolution setting of 60,000 (at m/z = 400, measured at full width at half-maximum peak height).

In the data-dependent mode, the eight most intense ions exceeding an intensity threshold of 30,000 counts were selected from each full-scan mass spectrum for tandem mass spectrometry (MS/MS) analysis using collision-induced dissociation (CID). MS/MS spectra were acquired using the linear ion trap, in centroid format, with the following parameters: isolation width 3 m/z units, normalized collision energy 28%, default charge state 2+, activation Q 0.25, and activation time 30 ms. Real-time charge state screening was enabled to exclude singly charged ions and unassigned charge states from MS/MS analysis. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was enabled to preclude re-selection of previously analyzed precursor ions, with the following parameters: repeat count 2, repeat duration 10 s, exclusion list size 500, exclusion duration 90 s, and exclusion mass width 20 ppm. Data acquisition was controlled using Xcalibur software (version 2.0.7 SP1, Thermo). Raw data files were searched against the *Sedimenticola selenatireducens* strain CUZ or the *Dechloromarinus chlorophilus* NSS protein database using Proteome Discoverer software (version 1.3, SEQUEST algorithm, Thermo) for tryptic peptides (i.e., peptides resulting from cleavage at the C-terminal

end of lysine and arginine residues) with up to three missed cleavages, carbamidomethylcysteine as a static post-translational modification, and methionine sulfoxide as a variable post-translational modification. A decoy database was used to characterize the false discovery rate and the target false discovery rate was 0.01 (i.e., 1%).

The data was normalized and square root transformed. The hierarchical clustering and nonmetric multidimensional scaling (nMDS) functions in Primer 6 (Primer-E Ltd, Plymouth, UK) were used to determine the clustering patterns among samples. Similarity percentage (SIMPER) analysis was used to identify genes contributing to the top 50% of differences between groups. Bray-Curtis dissimilarity matrices were used in all cases. p-values for assessing differences in protein expression were calculated using two-tailed heteroskedastic Student's t-test.

Metabolite Extraction and Liquid chromatography-tandem mass spectrometry

For growth on phenylacetate and perchlorate and phenylacetate and nitrate, 500 mL of cells were grown in triplicate in 1 L Pyrex bottles sealed anaerobically. For growth on phenylacetate and oxygen, 500 ml of cells were grown in Fernbach flasks as described above. For the nitrate and perchlorate conditions, all work except the centrifugation was performed inside an anaerobic chamber. Samples for metabolite extraction were collected at mid-log phase and centrifuged at 10,000g for 10 minutes at 4 ºC. The volume of cells collected was normalized across different conditions by total OD (OD =125). The pellet was resuspended in 500 uL of sterile, degassed, acidic acetonitrile (32 mL acetonitrile, 7.7 mL dH_2O, 314.6 µL formic acid) and incubated on ice for 15 minutes. Samples were then centrifuged at 14,000g for 3 minutes and the supernatant was filtered into LCMS vials using a 0.2 µm filter. Benzoyl-CoA and phenylacetyl-CoA standards (Sigma Aldrich) were run in concentrations ranging from 10 to 5000 nM.

Compounds were separated using a 1200 Series liquid chromatography instrument (Agilent Technologies, Santa Clara, CA). A 40 µL aliquot of each sample was injected onto an Agilent Zorbax SB-C18 (2.1 mm i.d., 30 mm length, 3.5 µm particle size) column and eluted at 30 °C and a flow rate of 0.5 mL/min with the following gradient: 2 min isocratic 99% buffer A (40 mM ammonium acetate)/1% buffer B (methanol), then in 8 min with a linear gradient to 100% B, 2 min isocratic 100% B, then 3 min equilibration with 99%A/1% B. The eluent from the column was introduced into a mass spectrometer for eleven minutes after the first minute. Water was run in between sets as a negative control.

Mass spectrometry (MS) was performed on an LTQ XL ion trap instrument (Thermo Fisher Scientific, San Jose, CA) with an ESI source operated in positive ion mode. The MS settings were capillary temperature 350 °C, ion spray voltage 3.5 kV, sheath gas flow: 60 (arbitrary units), auxiliary gas flow 10 (arbitrary units), sweep gas flow 5 (arbitrary units). For MS/MS product ion scan, the scan range was *m/z* 245-1000. The compounds at *m/z* 872.1 (benzoyl-CoA) and *m/z* 886.2 (phenylacetyl-CoA) were isolated with 2 *m/z* isolation width and fragmented with a normalized collision-induced dissociation energy setting of 35%, an activation time of 30 ms, and an activation Q of 0.250. p-values for assessing differences in metabolite concentrations were calculated using two-tailed heteroskedastic Student's t-test.

The genomes of *Sedimenticola selenatireducens CUZ and Dechloromarinus chlorophilus NSS are* available [pending] on the Integrated Microbial Genomes (IMG) system of the Joint Genome Institute and their 16S sequences are available on GenBank (KM192219 and AF170359, respectively).

Results

Growth on Phenylacetate

S. selenatireducens CUZ and *D. chlorophilus* NSS grew by the oxidation of phenylacetate coupled to the reduction of perchlorate (strain CUZ) and chlorate (strain NSS), nitrate, or oxygen (Fig. 3). Doubling time for both strains was fastest on (per)chlorate and slowest on oxygen, and the percent of phenylacetate assimilated into biomass varied from 26-41 % (Table 2). While strain CUZ grew fully aerobically (20% oxygen in the headspace) with phenylacetate as the electron donor, strain NSS could grow only with 10% oxygen in sealed bottles (Fig. 3C, F; Table 2).

Both strains also grew by benzoate oxidation coupled to the reduction of (per)chlorate and nitrate (Fig. 4). Additionally, strain CUZ could oxidize benzoate aerobically (10% oxygen in the headspace; Fig. 4) while strain NSS was unable to do so even at very low (1%) oxygen concentrations (data not shown).

Transcriptomics

RNA-seq experiments were carried out in strain CUZ to investigate which pathways of phenylacetate degradation are transcribed when perchlorate is used as the electron acceptor. Hierarchical clustering analysis of the *pad*, *bcr*, *paa*, and *box* clusters grouped samples by both electron donor and degree of aerobicity (Fig. 5A). Acetate samples grouped together as did samples containing phenylacetate and benzoate (Fig. 5A). The phenylacetate/oxygen and benzoate/oxygen samples clustered distinctly from their anaerobic counterparts according to the amount of oxygen used (see methods).

Genes in the *pad* cluster (Fig. 1A, 1E), which anaerobically convert phenylacetate into benzoyl-CoA, and genes in the *bcr* cluster (Fig. 1B, 1E), which is responsible for the downstream processing of benzoyl-CoA in anaerobic conditions, were upregulated in the presence of benzoate or phenylacetate regardless of the electron acceptor (Fig. 5B, 5C). However, genes in these clusters were more highly transcribed on nitrate and perchlorate than oxygen (Fig. 5B, 5C). Transcription of genes in the *bcr* cluster (Fig. 5C) was higher on benzoate/oxygen compared to phenylacetate/oxygen (Fig. 5C), presumably because growth on phenylacetate/oxygen does not generate benzoyl-CoA, a known inducer of anaerobic benzoate degradation pathways (1, 20).

The *paa* genes, which are involved in phenylacetate degradation in aerobic conditions (Fig. 1C, 1E) (and do not produce benzoyl-CoA as an intermediate), were found to be most highly expressed in the phenylacetate/oxygen condition, although transcription on phenylacetate/perchlorate and phenylacetate/nitrate was also observed (Fig. 5D). Expression of genes in this cluster was also seen in the benzoate conditions (Fig. 5D); however it was lower than any of the phenylacetate samples, probably because this pathway is unnecessary for the degradation of benzoate. Genes in the *box* pathway, which degrade benzoyl-CoA aerobically, were found to be transcribed in the presence of both benzoate and phenylacetate regardless of the electron acceptor utilized (Fig. 5E). Surprisingly, expression was lower in the oxygen samples than the perchlorate or nitrate samples (Fig. 5E). Overall, the phenylacetate/perchlorate condition resembled the phenylacetate/nitrate condition instead of the phenylacetate/oxygen condition, suggesting that the degradation of phenylacetate with perchlorate as an electron acceptor occurred primarily through anaerobic pathways.

Proteomics

Proteomics experiments were carried out in strains CUZ and NSS. All conditions tested were identical, except that strain NSS was tested using chlorate, rather than perchlorate, as an electron acceptor and that strain NSS did not grow with benzoate and oxygen (data not shown). Additionally, while strain CUZ was tested with 20% oxygen in the headspace with phenylacetate as an electron donor, strain NSS was only able to grow with 10% oxygen in the headspace in the presence of phenylacetate. Nonparametric Multidimensional Scaling (nMDS) analysis indicated that all triplicate samples (except one acetate and nitrate replicate for strain NSS) clustered with each other with at least 68% similarity in both strains (Fig. 6).

During growth on phenylacetate, peptide counts for gene products of the PAD and BCR clusters of strain CUZ were higher on perchlorate and nitrate compared to oxygen (Fig. 7A, 7B). With either perchlorate or nitrate, the cumulative peptide counts for the PAD cluster were 2.8 times higher than with oxygen (Fig. 7D). A two-tailed heteroskedastic Student's t-test applied to biological replicate data estimated this increase as significant at a $p<0.011$ probability for nitrate and $p<0.003$ for perchlorate. The BCR pathway was minimally expressed on phenylacetate/oxygen, probably because under these conditions the PAA pathway predominates, benzoyl-CoA is not produced, and the BCR pathway is not induced (20, 21). Cumulative peptide counts for the BCR cluster were 12.8 times higher on perchlorate than oxygen ($p<0.004$) and 6.8 times higher on nitrate than oxygen ($p<0.007$) (Fig. 7D). Peptide counts for the PAA pathway of strain CUZ were very low or absent in all conditions except for phenylacetate/oxygen (Fig. 7C), which is consistent with the *paa* genes encoding an aerobic pathway of phenylacetate degradation (Fig. 1E). The cumulative peptide counts for PAA with phenylacete/oxygen were 11 times higher than benzoate/oxygen, which was qualitatively low despite being the second most expressed condition ($p<0.002$) (Fig. 7D).

During growth on benzoate, the PAD pathway of strain CUZ was also more expressed on perchlorate and nitrate than oxygen (Fig. 7A).Cumulative peptide counts for PAD were higher with nitrate (3.6 times, $p<0.007$) and perchlorate (2.8 times, $p<0.015$) than with oxygen (Fig. 7D). Protein expression for BCR on benzoate was similar for all electron acceptors (Fig. 7B)

with only a low 1.4 times increase in expression on perchlorate relative to oxygen ($p<0.03$), and no statistically discernible difference between nitrate and either perchlorate or oxygen.

In strain CUZ, protein expression of the BOX pathway of aerobic-hybrid benzoate degradation was highest for the benzoate/oxygen samples (Fig. 7D), although a small number of peptides from BoxC and BoxB were measurable in the phenylacetate/oxygen, phenylacetate/perchlorate, and benzoate/perchlorate conditions (data not shown). Total peptide counts for the BOX pathway were 9 times higher for benzoate/oxygen than benzoate/perchlorate ($p<0.002$; Fig. 7D), while benzoate/perchlorate and benzoate/nitrate grown cells were not statistically distinguishable from each other. This reinforces the idea that benzoate/perchlorate and benzoate/nitrate rely mainly on the anaerobic BCR pathway with little or no contribution from the aerobic-hybrid BOX pathway (Fig. 7D). Although cells grown on benzoate/oxygen produce peptides for both the BCR pathway and the BOX pathway (Fig. 7D), the benzoyl-CoA reductase of the BCR pathway is highly oxygen sensitive (22), suggesting that BOX is the more likely pathway to be actually used under aerobic conditions.

Proteomics results for strain NSS were similar to those of strain CUZ with respect to the PAD and BCR clusters (Fig. 8A, 8B). Protein expression of the PAD and BCR pathways was much higher on phenylacetate/chlorate or phenylacetate/nitrate than phenylacetate/oxygen (Fig. 8A, 8B). With phenylacetate, total peptide counts for the PAD pathway were higher with nitrate (4.0 times, $p<0.004$) and chlorate (8.1 times, $p<0.002$) than with oxygen (Fig. 8D), while the BCR peptides were nearly absent on oxygen (Fig. 8B, 8D). By contrast, PAA was expressed more highly on phenylacetate/oxygen than any other condition with a statistical significance of at least $p<0.04$ for all comparisons (Fig. 8C, 8D). In contrast to strain CUZ, peptides from the PAA pathway were still detected in phenylacetate/chlorate and phenylacetate/nitrate samples of strain NSS (Fig. 8C, 8D). This creates the possibility that strain NSS may use a combination of aerobic and anaerobic pathways to degrade phenylacetate with chlorate as the electron acceptor. Finally, proteins of the BOX pathway were expressed in phenylacetate/chlorate samples at higher levels than any other condition (at least $p<0.04$ for all comparisons) (Fig. 8D). This is consistent with benzoyl-CoA (an anaerobic phenylacetate degradation intermediate) and oxygen acting as BOX inducers (20, 21). Cells grown on benzoate/nitrate or benzoate/chlorate expressed much lower levels of BOX peptides, suggesting that they rely on the BCR pathway (Fig. 8D).

Measurements of Pathway Intermediates

Samples of strain CUZ with phenylacetate as the electron donor and perchlorate, nitrate, or oxygen as electron acceptors were analyzed for concentration of benzoyl-CoA and phenylacetyl-CoA in mid-log phase. The concentration of benzoyl-CoA, a key intermediate produced only in the anaerobic pathway of phenylacetate degradation, was measured as a diagnostic for the activity of the anaerobic (PAD) pathway with perchlorate, nitrate, and oxygen as electron acceptors. As shown in Fig. 9A, the pool of benzoyl-CoA in the perchlorate and nitrate samples was very similar (1.61 (±0.3) μM and 1.8 (±0.6) μM, respectively), and 14 to 15 times larger ($p<4\times10^{-5}$) than when oxygen was used as an electron acceptor (0.119 (±0.02) μM). On the other hand, the pool of phenylacetyl-CoA, which is the first intermediate produced in both the anaerobic and the aerobic-hybrid pathways, was 87 times larger ($p<2\times10^{-9}$) in the

aerobic condition (54 (±9) μM) than it was with perchlorate (0.6 (±0.5) μM) or nitrate (0.6 (±0.6) μM) as electron acceptors (Fig. 9B).

Discussion

We investigated the mechanisms of phenylacetate and benzoate degradation under anaerobic (per)chlorate reducing conditions relative to aerobic and anaerobic nitrate reducing conditions in the PRB *S. selenatireducens* strain CUZ and the CRB *D. chlorophilus* NSS. Previous work has shown that PRB and CRB can utilize oxygen generated from (per)chlorate reduction to activate oxygenase dependent pathways (17, 18, 23). The current study demonstrated that PRB and CRB growing on (per)chlorate can also degrade aromatic compounds utilizing fully anaerobic pathways that do not rely on oxygen produced from chlorite dismutation. The PRB *S. selenatireducens* strain CUZ and the CRB *D. chlorophilus* strain NSS degraded phenylacetate both aerobically and anaerobically coupled to nitrate (Fig. 3), and their genomes encoded aerobic-hybrid and anaerobic pathways, allowing for the investigation of pathway expression on different electron acceptors. However, genes (except *paaE*) encoding the epoxidase (*paaABCDE*) of the aerobic-hybrid pathway, were not found in either genome, opening the possibility that the aerobic-hybrid pathway may differ somewhat from the previously described process.

When strain CUZ was grown on phenylacetate, the anaerobic (*pad*, *bcr*) and aerobic-hybrid (*paa*) pathways both appeared to be transcribed in the presence of nitrate, perchlorate, and oxygen (Fig. 5). However, proteomics indicated that the aerobic-hybrid pathways of phenylacetate (PAA) and benzoate (BOX) degradation were predominantly expressed in the presence of oxygen and not in the presence of perchlorate or nitrate (Fig. 7), while the PAD and BCR pathways were more highly expressed on nitrate and perchlorate. In the case of phenylacetate, this was further supported by 14 times higher concentrations of the anaerobic intermediate benzoyl-CoA during growth on perchlorate and nitrate compared to oxygen (Fig. 9A). Given these results, we conclude that with perchlorate as the sole electron acceptor, strain CUZ oxidizes phenylacetate and benzoate via anaerobic pathways.

In strain CUZ, very high levels of phenylacetyl-CoA on phenylacetate/oxygen (Fig. 9B) suggested a bottleneck in the aerobic-hybrid pathway, which may be a result of the absence of genes for the key epoxidase (*paaABCDE*) of this system that would ordinarily be expected to act on phenylacetyl-CoA. While strain CUZ grew on phenylacetate/oxygen and clearly transcribed other aerobic-hybrid pathway genes, the aerobic-hybrid pathway may rely on a yet-to-be identified oxygenase/epoxidase that could be less efficient than the canonical 1,2-phenylacetyl-CoA epoxidase. BoxB, the key oxygenase of the aerobic-hybrid benzoate degradation pathway, is the closest relative to the oxygenase of the aerobic-hybrid phenylacetate degradation pathway (PaaA) and may thus appear to be a likely candidate; however, previous studies have shown that the BoxB enzyme of *Azoarcus evansii* cannot process phenylacetyl-CoA (24). Similarity percentage analysis of proteomics samples revealed a cluster of genes (CUZ_02282-CUZ_02289; NSS_00000690-NSS_00000740) that may be important for growth in phenylacetate/oxygen. These genes are likely involved in the degradation of 4-hydroxyphenylpyruvate to acetoacetate and fumarate (25). Of particular interest is the presence of 3 genes encoding oxygenases/hydroxylases (CUZ_02283, CUZ_02285, CUZ_02288;

NSS_00000700, NSS_00000720, NSS_00000750) that may be relevant to the aerobic-hybrid phenylacetate degradation pathway in strains CUZ and NSS. Genetic studies will be necessary to fully understand this pathway and the necessary genes.

Strain NSS grew anaerobically with phenylacetate (Fig. 3) and benzoate (Fig. 4), but was unable to oxidize benzoate aerobically under the conditions tested. As with strain CUZ, proteomics demonstrated that the anaerobic phenylacetate and benzoate pathways (PAD, BCR) were upregulated in the presence of nitrate or chlorate, but not oxygen (Fig. 8). Unexpectedly, the aerobic-hybrid phenylacetate pathway (PAA) was expressed for all conditions where phenylacetate was present. The total PAA peptide count was only about 2.3 times higher in the presence of oxygen than it was on nitrate ($p<0.004$), which is much less distinctive than the more than 35-fold increase in strain CUZ when comparing PAA expression under the same conditions. Phenylacetate/chlorate in strain NSS produced intermediate PAA peptide counts that could not be statistically distinguished from phenylacetate/nitrate. Based on these data, it is possible that phenylacetate degradation coupled to chlorate reduction in strain NSS uses both the aerobic-hybrid and anaerobic pathways simultaneously. It is also possible that phenylacetate activates expression of PAA, even in the absence of oxygen. In strain NSS, the benzoate degradation pathway is also unclear. Since strain NSS cannot grow on benzoate with oxygen (even when reduced to 1%) there is no baseline level to use as a positive control for aerobic growth. The aerobic-hybrid benzoate pathway (*box*) was never activated in the presence of benzoate; hence, it appears that benzoate is degraded exclusively by the anaerobic pathway (*bcr*) in all tested conditions. However, peptides of the *box* pathway were observed in the presence of phenylacetate and chlorate. If oxygen is present, the *box* pathway could act on the benzoyl-CoA intermediate of the anaerobic phenylacetate pathway, though it is unknown if this is occurring.

These results indicate that PRB, and likely CRB, can couple (per)chlorate reduction to aromatic degradation using anaerobic pathways. This is in contrast to previous studies showing PRB and CRB using (per)chlorate to facilitate the aerobic degradation of compounds such as benzene, toluene, napthalene, phenol, and catechol (17, 18, 23, 26, 27). Even though both pathways are present, strain CUZ preferred the anaerobic pathway. This preference is somewhat unexpected, as key enzymes in the anaerobic pathways, the benzoyl-CoA reductase and the phenylglyoxylate:NAD^+ oxidoreductase, are highly oxygen sensitive (22, 28). However, because chlorite dismutase is a periplasmic enzyme (29, 30), the O_2 produced from chlorite dismutation is physically separated from the cytoplasm; thus it is likely that the cytoplasmic concentration of O_2 was low enough to prevent significant inhibition of these enzymes. One possible reason to preferentially utilize the anaerobic pathways is to conserve O_2 for other processes such as respiration. It may be more energetically favorable to respire the produced O_2 than to use it for ring cleavage. It was observed that both strains CUZ and NSS grew more rapidly with perchlorate and chlorate respectively than on nitrate, suggesting the extra oxygen does convey a metabolic advantage (Fig. 3). Thus, bacteria in microaerophilic environments may apply a mixture of anaerobic and aerobic strategies that allow them to make the best possible use of limited oxygen.

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Tables and Figures

Table 1. Locus tags for genes in the *paa*, *pad*, *bcr*, and *box* clusters of strains CUZ and NSS.

Gene	CUZ Locus Tags	NSS Locus Tags
Paa Cluster		
paaE	CUZ_04215	NSS_00036170
paaZ	CUZ_04217	NSS_00036190
paaF	CUZ_04218	NSS_00036200
paaG	CUZ_04219	NSS_00036210
paaH	CUZ_04220	NSS_00036220
paaJ	CUZ_04221	NSS_00036230
paaK	CUZ_04222	NSS_00036090
Pad Cluster		
padB	CUZ_03788	NSS_00026680
padC	CUZ_03789	NSS_00026690
padD	CUZ_03790	NSS_00026700
padE	CUZ_03791	NSS_00026710
padF	CUZ_03792	NSS_00026720
padG	CUZ_03793	NSS_00026730
padH	CUZ_03794	NSS_00026740
padI	CUZ_03795	NSS_00026750
padJ	CUZ_03796	NSS_00026760
Bcr Cluster		
bcrD	CUZ_03342	NSS_00026570
bcrA	CUZ_03343	NSS_00026580
bcrB	CUZ_03344	NSS_00026590
bcrC	CUZ_03345	NSS_00026600
dch	CUZ_03346	NSS_00026610
oah	CUZ_03347	NSS_00026620
had	CUZ_03348	NSS_00026630
Box Cluster		
boxB	CUZ_03988	NSS_00004810
boxC	CUZ_03987	NSS_00004800

Table 2. Doubling time and biomass assimilation of strains CUZ and NSS growing on phenylacetate.

	Electron Acceptor	Equation	Doubling Time (hr)	Biomass assimilation (%)
CUZ	ClO_4^-	$2\ C_8H_7O_2^- + 9\ ClO_4^- + 2\ H^+ \rightarrow 16\ CO_2 + 9\ Cl^- + 8\ H_2O$	7 ± 2	41 ± 1
	NO_3^-	$5\ C_8H_7O_2^- + 36\ NO_3^- + 41\ H^+ \rightarrow 40\ CO_2 + 18\ N_2 + 38\ H_2O$	10 ± 2	34 ± 3
	O_2	$C_8H_7O_2^- + 9\ O_2 + H^+ \rightarrow 8\ CO_2 + 4\ H_2O$	29.7 ± 0.4	ND
NSS	ClO_3^-	$C_8H_7O_2^- + 6\ ClO_3^- + H^+ \rightarrow 6\ Cl^- + 8\ CO_2 + 4\ H_2O$	4.3 ± 0.3	36
	NO_{3-}	$5\ C_8H_7O_2^- + 36\ NO_3^- + 41\ H^+ \rightarrow 40\ CO_2 + 18\ N_2 + 38\ H_2O$	7.4 ± 1.1	26
	O_2	$C_8H_7O_2^- + 9\ O_2 + H^+ \rightarrow 8\ CO_2 + 4\ H_2O$	6	ND

Figure 1. Genes and pathways of phenylacetate and benzoate degradation in *S. selenatireducens* CUZ. (A) *pad* gene cluster. (B) *bcr* gene cluster. (C) *paa* cluster gene cluster. (D) *box* gene cluster. (E) Pathways of phenylacetate and benzoate degradation in strain CUZ. Red: *paa* gene cluster involved in the aerobic-hybrid pathway of phenylacetate degradation; blue: *pad* gene cluster involved in anaerobic phenylacetate degradation; purple: *bcr* gene cluster in anaerobic benzoate and phenylacetate degradation; green: *box* gene cluster involved in the aerobic-hybrid pathway of benzoate degradation; (*): genes not found in strain CUZ. Solid arrows: anaerobic pathways; dashed arrows: aerobic-hybrid pathways. Bar, 1 kb. Arrows indicate direction of transcription.

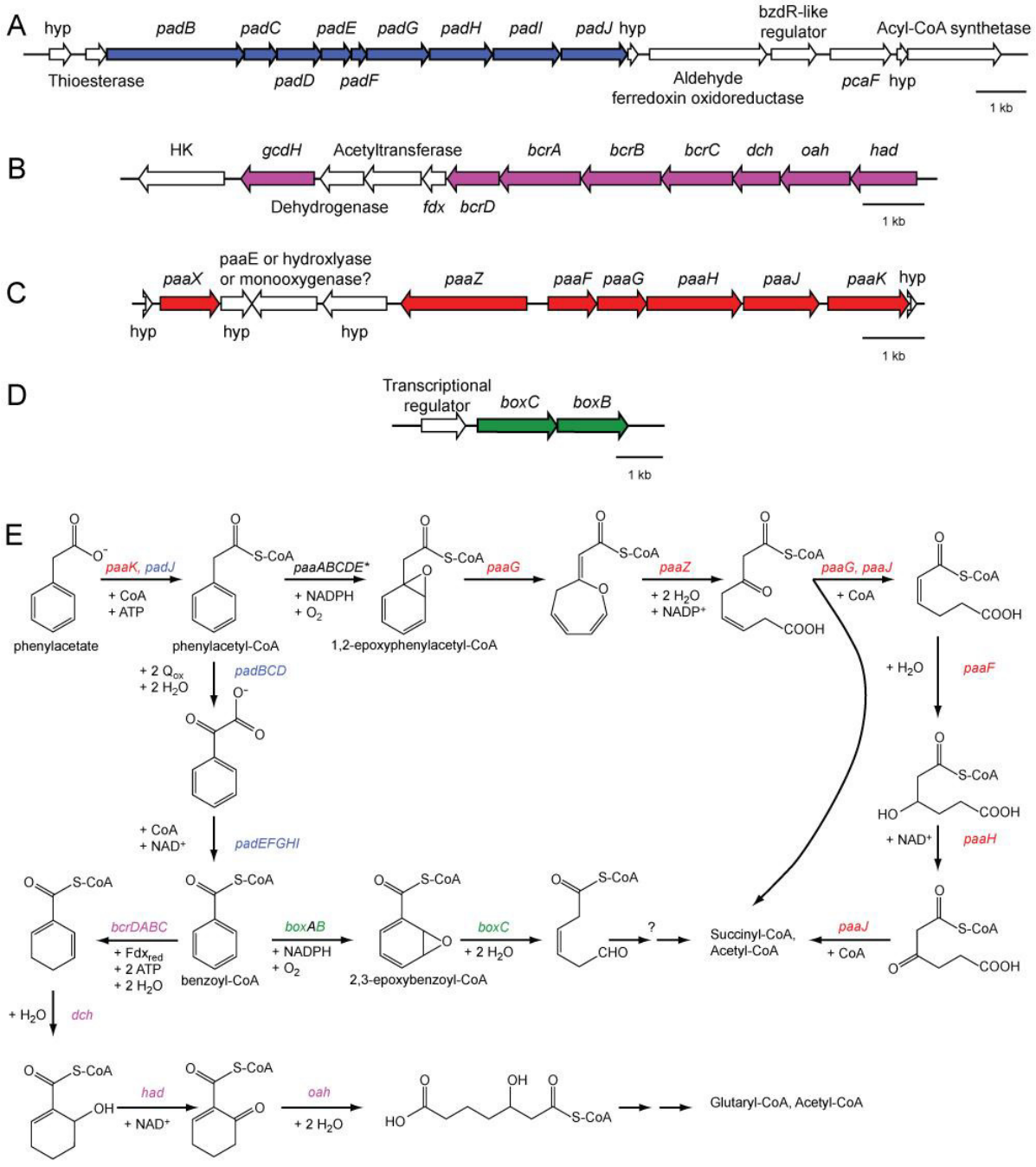


Figure 2. Genes involved in phenylacetate and benzoate degradation in *D. chlorophilus* NSS. (A) *pad* gene cluster. (B) *bcr* gene cluster. (C) *paa* cluster gene cluster. (D) *box* gene cluster. Gene colors correspond to the pathways found in Figure 2E. Red: *paa* gene cluster involved in the aerobic-hybrid pathway of phenylacetate degradation; blue: *pad* gene cluster involved in anaerobic phenylacetate degradation; purple: *bcr* gene cluster involved in anaerobic benzoate and phenylacetate degradation; green: *box* gene cluster involved in the aerobic-hybrid pathway of benzoate degradation. Bar, 1 kb. Arrows indicate direction of transcription.

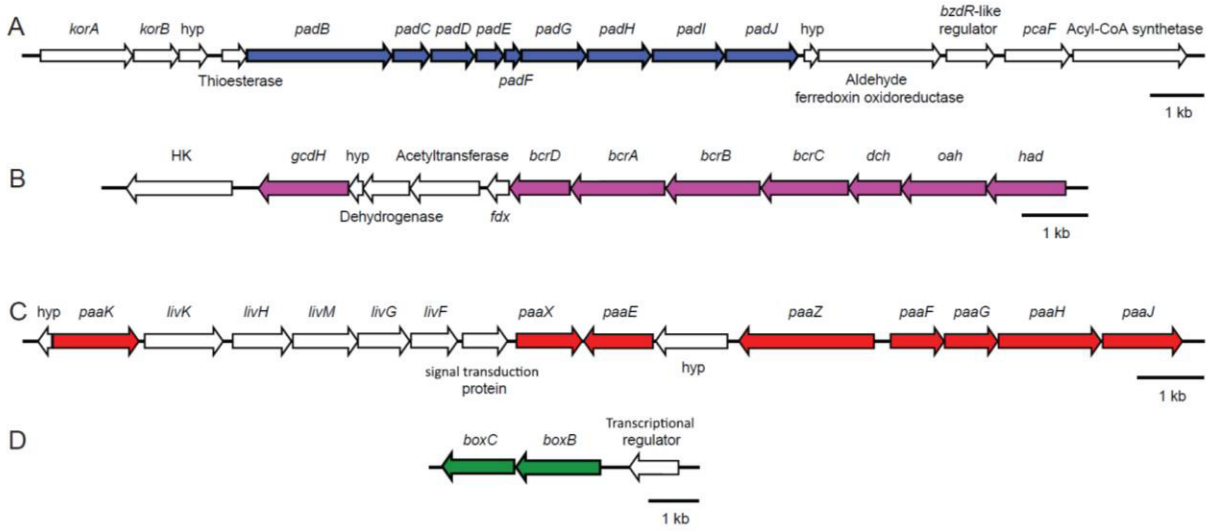


Figure 3. Growth curves of *S. selenatireducens* strain CUZ (A-C) and *D. chlorophilus* strain NSS (D-F) on 2 mM phenylacetate with perchlorate (A) or chlorate (D), nitrate (B, E) or oxygen (C, F) as electron acceptors. Blue circles: cell density; red squares: cell density for the no acceptor control; green triangles: phenylacetate concentration; black open circles: perchlorate concentration; black open triangles: chlorate concentration; orange diamonds: nitrate concentration. Points and error bars represent the average and standard deviation of triplicate samples.

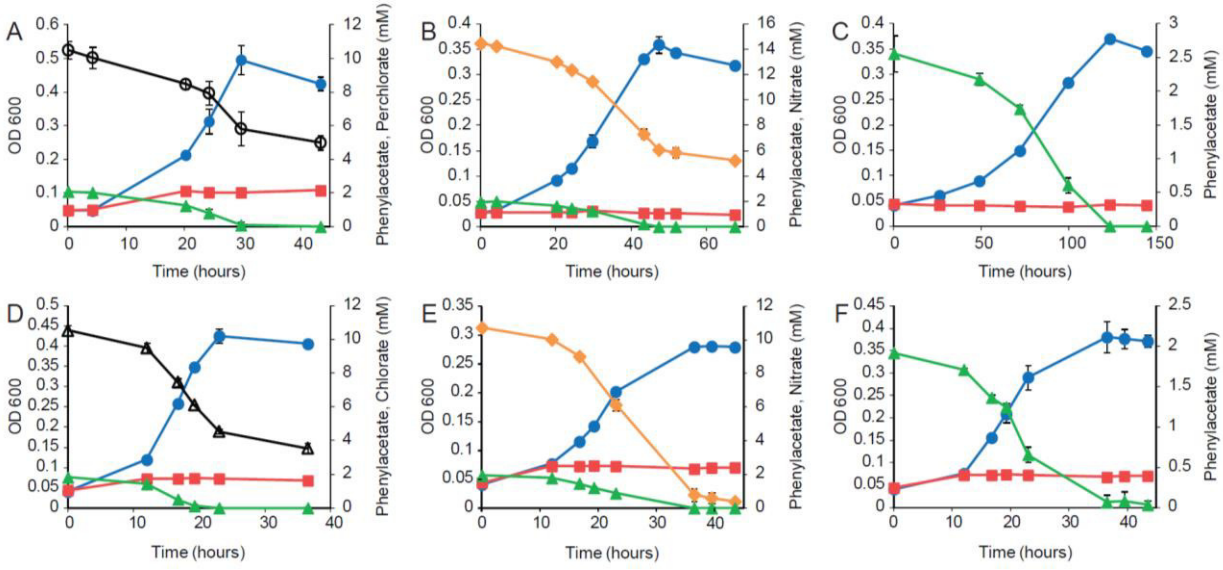


Figure 4. Growth curves of *S. selenatireducens* strain CUZ (A-C) and *D. chlorophilus* strain NSS (D-E) on 1 mM benzoate with perchlorate or chlorate (A, D), nitrate (B, E) or oxygen (C) as electron acceptors. Blue circles: cell density; red squares: cell density for the no acceptor control; green triangles: phenylacetate concentration; black open circles: perchlorate concentration; black open triangles: chlorate concentration; orange diamonds: nitrate concentration. Points and error bars represent the average and standard deviation of triplicate samples.

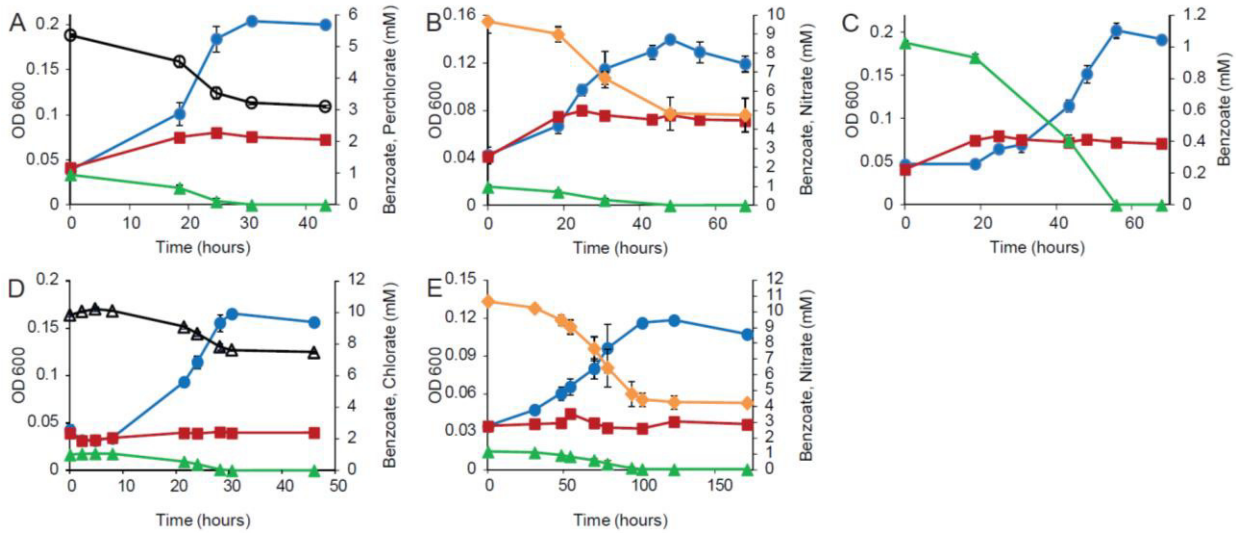


Figure 5. Hierarchical clustering (A) and heatmaps (B-E) of RNA-seq data from *S. selenatireducens* strain CUZ. Counts were normalized with DeSeq2 and then transformed via Log_2 (normalized counts + 1) prior to clustering and visualization. (B) *pad* gene cluster involved in anaerobic phenylacetate degradation. (C) *bcr* gene cluster involved in anaerobic phenylacetate and benzoate degradation. (D) *paa* gene cluster involved in the aerobic-hybrid pathway of phenylacetate degradation. (E) *box* gene cluster involved in the aerobic-hybrid pathway of benzoate degradation. Conditions: AP: acetate and perchlorate; AN: acetate and nitrate; AO: acetate and 10% oxygen; PhP: phenylacetate and perchlorate; PhN: phenylacetate and nitrate; PhO: phenylacetate and 20% oxygen; BP: benzoate and perchlorate; BN: benzoate and nitrate; BO: benzoate and 10% oxygen. Scale bar represents Euclidean distance.

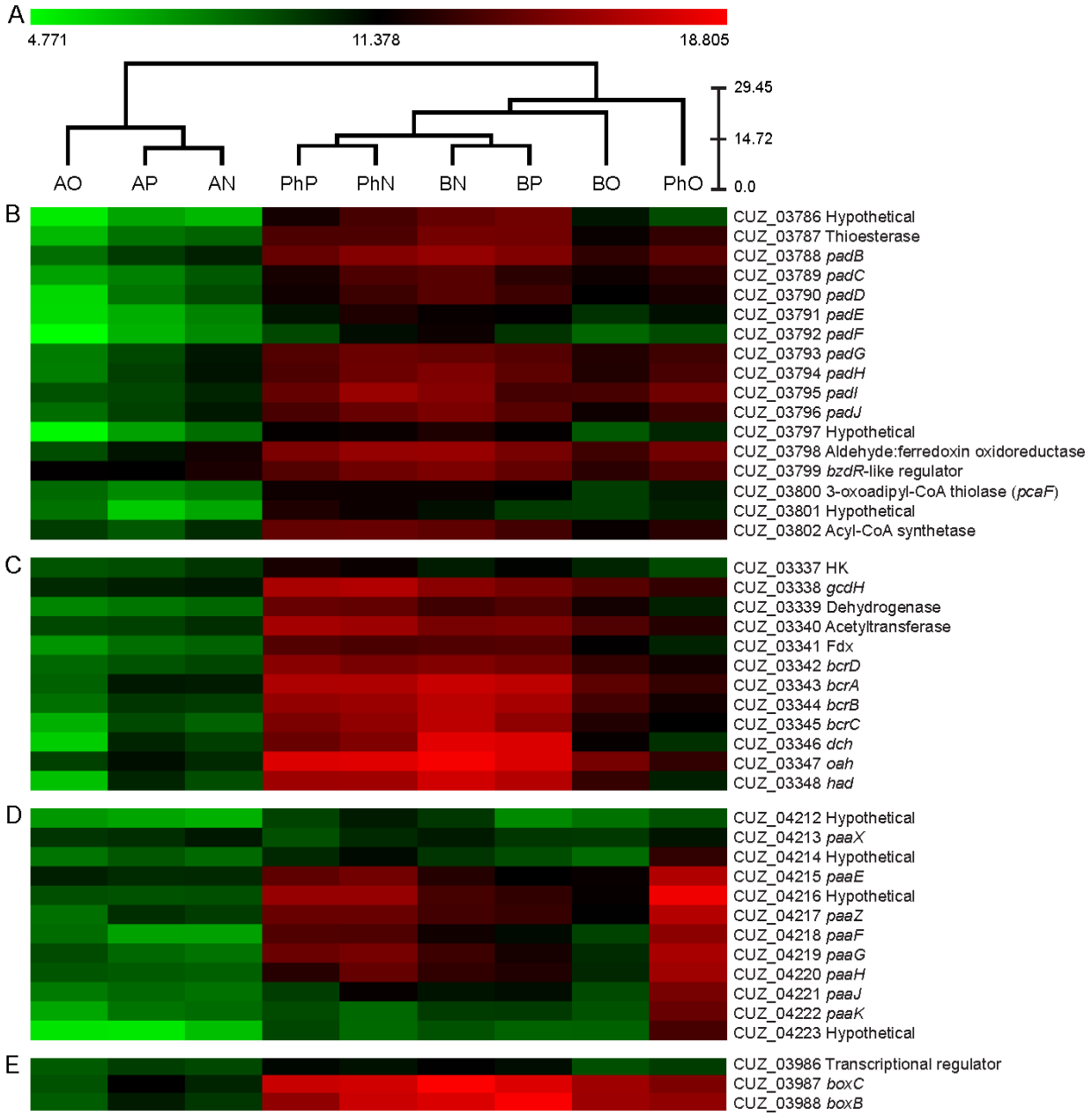


Figure 6. Non-metric Multidimensional Scaling plot of proteomics samples from (A) *S. selenatireducens* strain CUZ and (B) *D. chlorophilus* strain NSS based on a Bray-Curtis similarity matrix. Circles represent percent similarity based on hierarchical clustering. Colored symbols represent different electron donors. Letters represent different electron acceptors. N: nitrate; P: perchlorate; O: oxygen, C: chlorate.

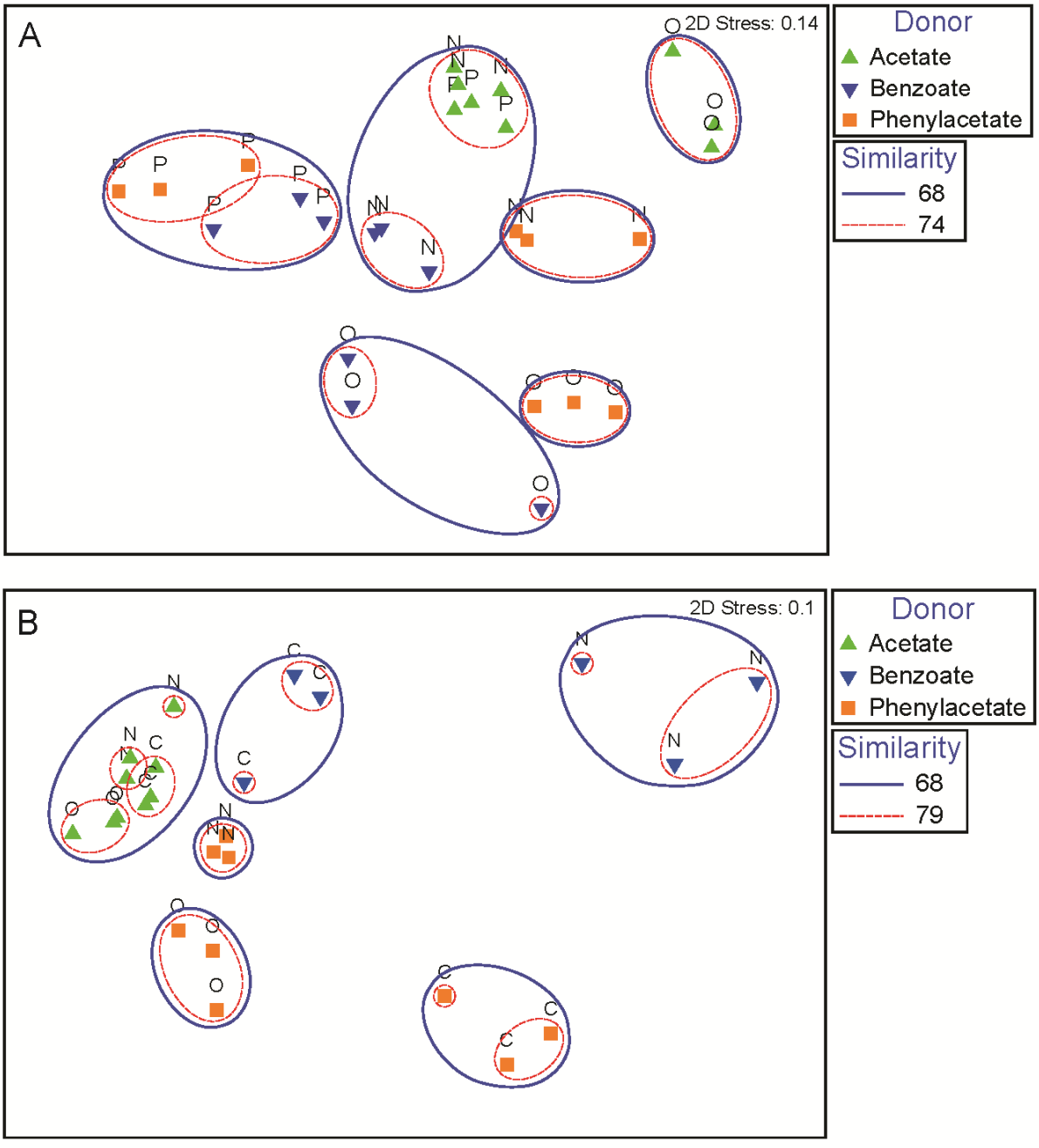


Figure 7. Normalized peptide counts of proteins from *S. selenatireducens* strain CUZ. (A) PAD cluster [anaerobic phenylacetate degradation]. (B) BCR cluster [anaerobic phenylacetate/ benzoate degradation]. (C) PAA cluster [aerobic-hybrid phenylacetate degradation]. (D) Sum of all the normalized peptide counts per pathway (PAD, BCR, PAA and BOX). Error bars represent the standard deviation of triplicate samples.

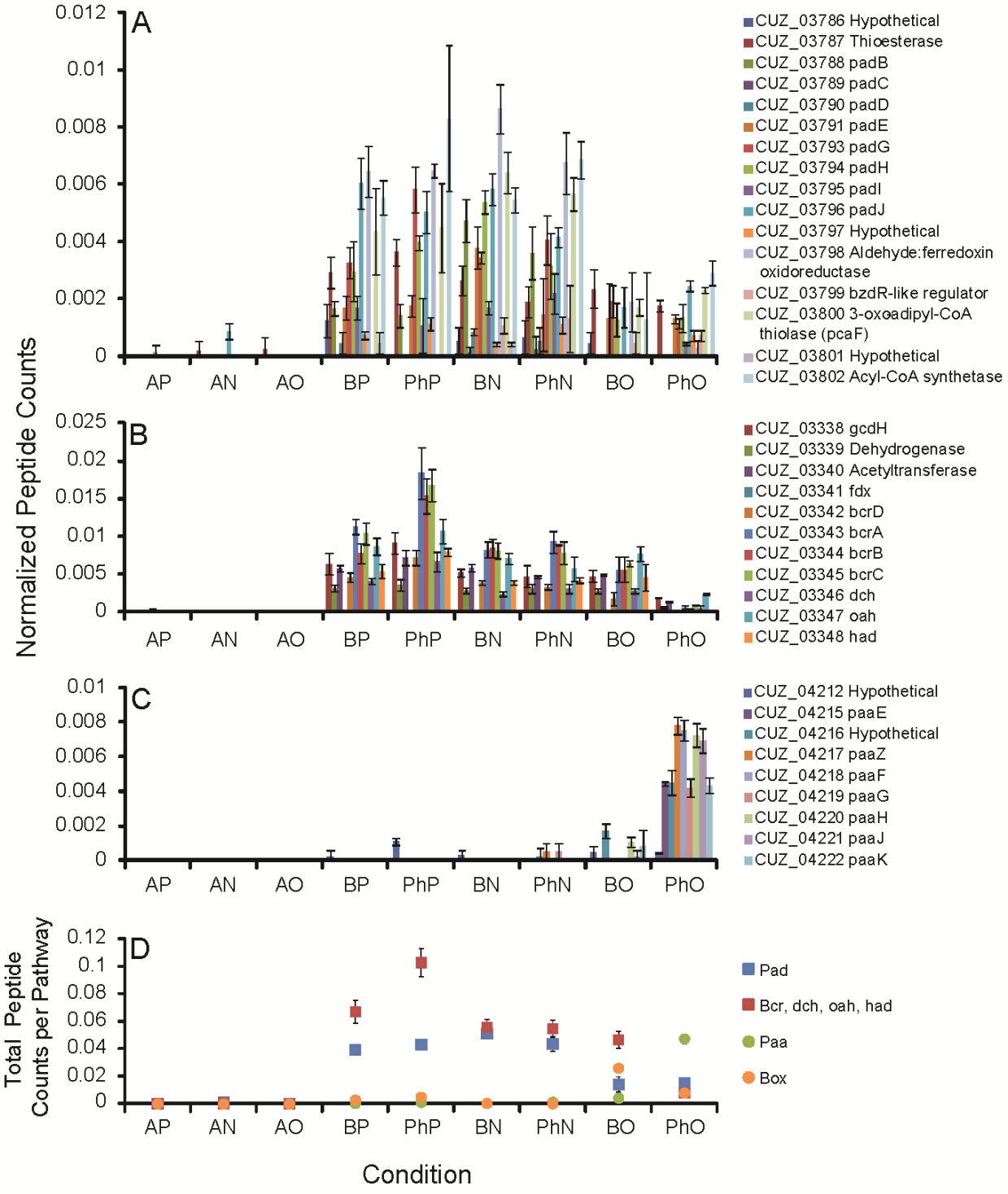


Figure 8. Normalized peptide counts of proteins from *D. chlorophilus* strain NSS. (A) PAD cluster [anaerobic phenylacetate degradation]. (B) BCR cluster [anaerobic phenylacetate/ benzoate degradation]. (C) PAA cluster [aerobic-hybrid phenylacetate degradation]. (D) Sum of all the normalized peptide counts per pathway (PAD, BCR, PAA, and BOX). Error bars represent the standard deviation of triplicate samples.

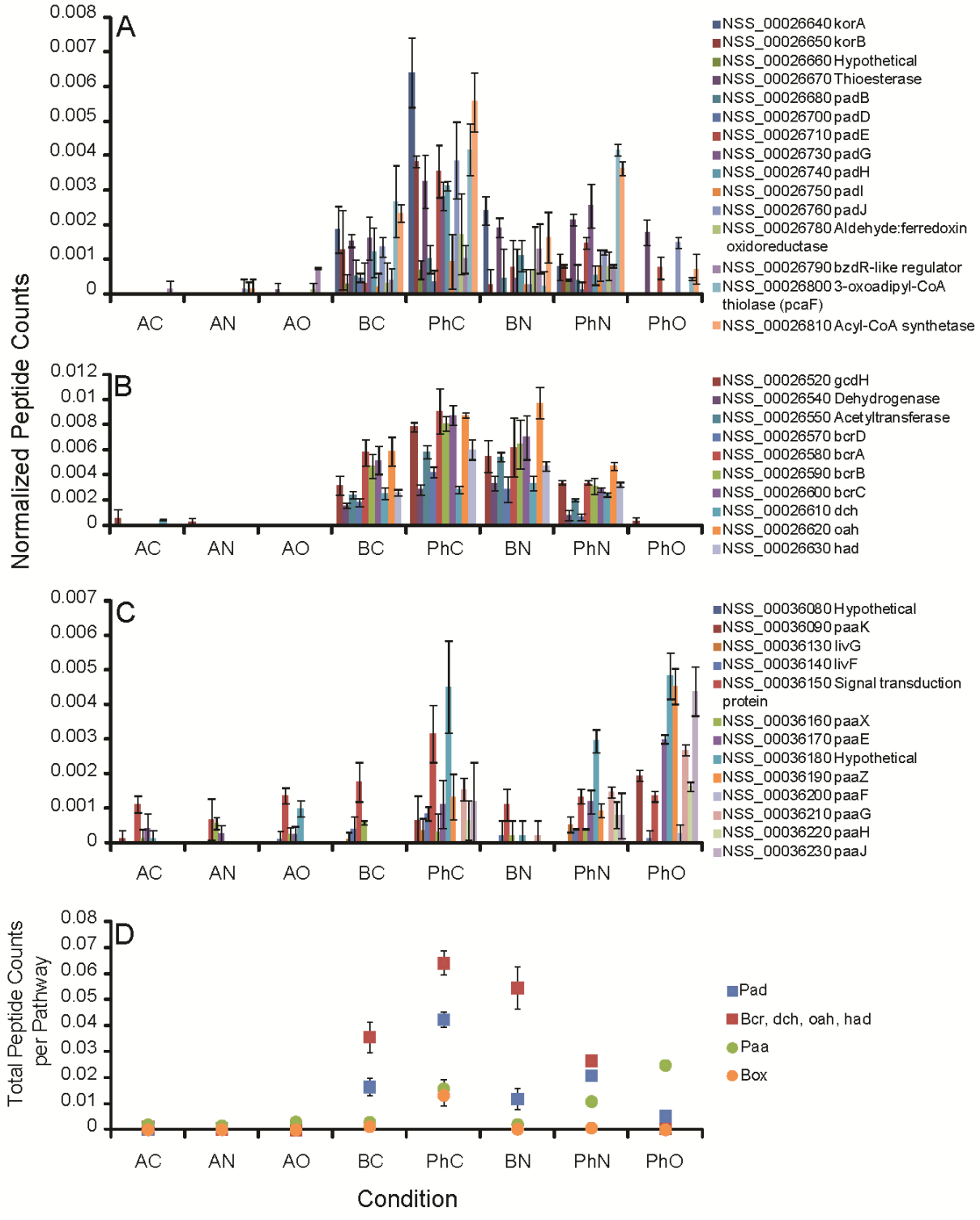
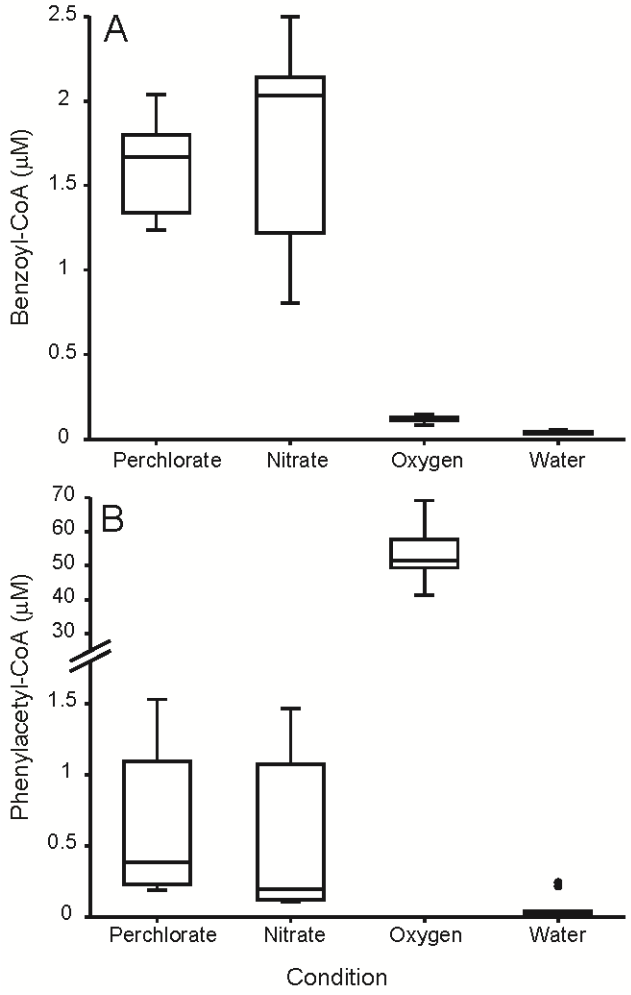


Figure 9. Metabolites extracted from mid-log phase samples of *S. selenatireducens* strain CUZ with phenylacetate as an electron donor and perchlorate, nitrate, or oxygen as electron acceptors. Water was run between sets as a negative control. (A) Concentration of benzoyl-CoA pool. (B) Concentration of phenylacetyl-CoA pool. Three biological replicates were sampled two to four times each, giving 12 perchlorate, 9 nitrate and 11 oxygen samples. Data is represented in a Tukey boxplot with the box extending to the upper and lower quartiles and the horizontal line indicating the median. Whiskers extend beyond the box to the highest and lowest measured concentration within 1.5 times the interquartile range of the edge of the box. Outlying points that exceed 1.5 times the interquartile range are indicated with open circles.



Chapter 6: Conclusions

The preceding dissertation has provided new insights into the habitats, diversity, genetics, and metabolic capabilities of dissimilatory perchlorate reducing bacteria (DPRB). In total, 10 new isolates have been cultured from 6 genera (*Arcobacter, Sedimenticola, Azoarcus, Denitromonas, Pseudomonas, and Marinobacter*), and with the exception of *Marinobacter*, they represent the first perchlorate-reducing isolate in each genus. This expanded collection of isolates includes *Arcobacter* sp. CAB, the only DPRB classified as Epsilonproteobacteria in pure culture (Chapter 3). All of these new strains originated from marine sediment and are expected to tolerate salinities greater than 3% NaCl, a property that had been absent in most existing isolates due to the tendency of prior work to focus on freshwater environments (Chapter 1).

All known DPRB except for the archaeon *A. fulgidus*, have relied on both the perchlorate reductase enzyme encoded by *pcrABCD*, and the chlorite dismutase enzyme encoded by *cld* for perchlorate reduction. However, this study showed that *Arcobacter* sp. CAB lacked the *pcrC* gene, which encodes a periplasmic multiheme *c*-type cytochrome that putatively mediates electron transport from the cytoplasmic membrane to the periplasmic functional PcrAB protein (1). Instead, the perchlorate reduction island (PRI) of strain CAB contained an *Arcobacter*-like *c*-type monoheme cytochrome which could be functionally replacing *pcrC* (Chapter 3). It is unknown what adaptive advantage this substitution may serve, but it suggests that the perchlorate reduction machinery is more flexible than previously thought. *Arcobacter* species also appear to be amongst the most salt-tolerant of currently studied DPRB, dominating cultures at 5 and 7% NaCl (Chapter 2). *Arcobacter* sp. CAB and *Sedimenticola selenatireducens* CUZ have an identical 14 AA tail on their PcrA not observed in other DPRB. The fact that this tail is present in two different classes of Proteobacteria (Epsilonproteobacteria and Gammaproteobacteria) originating from the same environment is suggestive of horizontal gene transfer, which is thought to be essential in the spread and evolution of the perchlorate reduction machinery (2). The 14 AA tail may provide some adaptive advantage in marine environments, though this has not yet been tested.

Though it has long been known that DPRB produce O_2 as a result of chlorite dismutation, less has been known about how this oxygen could be utilized. In the absence of exogenous oxygen, *Arcobacter* sp. CAB could use the O_2 produced from chlorite dismutation in the oxygenase-dependent degradation of catechol (Chapter 3). The ability to use aerobic pathways in anaerobic environments shows the metabolic versatility of DPRB growing on (per)chlorate. In contrast, despite encoding both anaerobic and aerobic-hybrid pathways, *Sedimenticola selenatireducens* CUZ preferentially utilized anaerobic pathways to degrade benzoate and phenylacetate when growing on perchlorate (Chapter 5). This study demonstrated for the first time that fully anaerobic, oxygenase-independent pathways for aromatics degradation can be utilized by DPRB growing on perchlorate. Additionally, the chlorate reducing bacterium *Dechloromarinus chlorophilus* NSS may use a combination of the anaerobic and aerobic-hybrid pathways when growing on phenylacetate and chlorate (Chapter 5), and simultaneous utilization of these pathways has not been previously reported for other DPRB. The ability of DPRB to transform (per)chlorate to O_2 allows these microorganisms to function as aerobes or anaerobes, depending on the pathways they encode. However, unlike other aerobes or anaerobes, the choice

of pathways utilized by DPRB growing on perchlorate does not depend on the electron acceptor itself, as perchlorate can be coupled to either aerobic or anaerobic pathways. As a result, DPRB display an extremely high level of metabolic flexibility.

During studies of metabolic preference, it was observed that *Sedimenticola selenatireducens* CUZ preferentially utilized (per)chlorate over nitrate when the inoculum was pre-grown on (per)chlorate (Chapter 4). Such a preference contrasts with most DPRB, which prefer to deplete nitrate before utilizing (per)chlorate. For bioremediation projects where perchlorate removal is the primary goal, identifying microbes that prefer perchlorate is important as nitrate is a common co-occurring contaminant and its presence could delay perchlorate removal. Similarly, the salt tolerance of many of the strains isolated during this project is also important for the future use of DPRB in bioremediation since the brines produced using ion-exchange technology often range from 3-6% NaCl (3). When marine sediments were exposed to perchlorate and high salinities (5% and 7% NaCl), the perchlorate-reducing community developed more slowly, but was ultimately capable of efficiently removing perchlorate (Chapter 2). Unfortunately, the high salinity microbial community appeared to be dominated by DPRB from a single genus (*Arcobacter*) which may make the community less resilient to environmental fluctuations. Additionally, microbial community analysis suggested that DPRB can form symbiotic relationships with sulfur reducers, and it remains to be elucidated whether this relationship is beneficial or detrimental to the rate of perchlorate removal.

By increasing the known diversity of DPRB, and expanding the knowledge of their habitats, genetics, and physiology, this work has provided an improved understanding of this unusual and highly flexible metabolism. Much work remains to be done to characterize the new isolates. Physiological characterization and genome sequencing of these novel isolates will not only contribute to the metabolic diversity of DPRB but will also aid in the study of horizontal gene transfer as a likely mechanism for the spread, adaptation, and evolution of the perchlorate reduction machinery. With the EPA on the cusp on enacting national perchlorate contamination limits, it is likely that interest in DRPB and their capacity to consume perchlorate will only continue to grow, and as such, basic research on DPRB is both timely and important.

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