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Specific Inhibitors of Microbial Sulfate Reduction: Dynamics of Inhibition and Mechanisms of
Resistance across Sulfate-Reducing Microorganisms

By

Magdalena Stoeva

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

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in the

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of the

University of California, Berkeley

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Professor John D. Coates, Chair

Professor Matthew Traxler

Professor Adam P. Arkin

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Abstract

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Doctor of Philosophy in Microbiology

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Sulfate-reducing microorganisms (SRM) gain energy by coupling the oxidation of organic matter or hydrogen to dissimilatory sulfate-reduction. This use of sulfate as a terminal electron acceptor for growth results in the generation of the toxic and corrosive compound, hydrogen sulfide. SRM are ubiquitous, and their metabolism forms a critical component of the global carbon and sulfur cycles. The activity of these microbes is also of economic importance: SRM are implicated in the bio-corrosion of steel and concrete. The petroleum industry is particularly susceptible to these costs; “sour” oil, i.e. oil contaminated with SRM-produced sulfide, represents a corrosion threat to transportation infrastructure as well as a health threat for workers. The identification and characterization of specific and potent inhibitors of SRM is critical to preventing these outcomes. This thesis characterizes the specific inhibition of SRM in pure culture and in sulfate-reducing communities, both in terms of how inhibitors behave in complex systems as well as how resistance to inhibition arises.

Specific inhibitors are compounds that disrupt the metabolism of one group of organisms, with little or no effect on the rest of the community. The first chapter of this dissertation reviews the current understanding of the specific inhibition of SRM: how we define and measure specific inhibition and how we gain insight into cellular targets of inhibitors. I review mechanisms of known specific inhibitors, which target sulfate transport systems and key enzymes of the sulfate-reduction pathway. This chapter also highlights several knowledge gaps: a quantitative understanding of the “specificity” of inhibition across diverse SRM in ecological contexts, and characterizations of the activity of key sulfate transport systems. Finally, emphasis on additional targets of inhibitors will improve our basic understanding of this metabolism and our ability to control it across environmental systems.

The second chapter of this dissertation aims to quantitatively dissect SRM inhibition by the specific inhibitor, perchlorate. The inhibitory dynamics of perchlorate (i.e. rate and community effects of inhibition and rate/community structure post cessation of inhibition) are characterized in continuous-flow sulfate-reducing systems. Empirical data supports a model where perchlorate acts largely to decrease SRM growth rates, rendering planktonic SRM increasingly susceptible to wash-out. Several other key factors putatively affecting the inhibitory dynamics of perchlorate are further investigated: surface attachment, indirect effects mediated by dissimilatory perchlorate-reducing bacteria, and effects related to the donor used for growth. Inhibition in this mixed

community is compared to inhibition in a pure-culture of the dominant SRM isolated from the community, *Desulfovibrio* sp. BMSR. In contrast to community-level experiments, pure culture *D. sp.* BMSR adapts to high concentrations of perchlorate.

The third chapter of this dissertation represents work further exploring the ability of pure cultures of SRM to adapt to perchlorate stress. By passaging model SRM, *Desulfovibrio alaskensis* G20 in increasing concentrations of perchlorate we obtain a population capable of robust growth in 100 mM of the inhibitor. Whole genome-sequencing and comparison to the ancestral strain reveals a single base-pair substitution in the sulfate adenylyltransferase (*sat*) and biochemical analysis of the mutant Sat variant confirms its decreased susceptibility to perchlorate inhibition. Further adaptation experiments demonstrate the prevalence of this mutation across ten independently adapting populations of *D. alaskensis* G20. We also confirm that this mutation increases perchlorate resistance in another model SRM, *Desulfovibrio vulgaris* Hildenborough. Thus, *sat* represents a primary target of perchlorate stress and pure cultures of SRM can adapt to this stress via mutations in this critical enzyme.

Previous chapters fail to detect adaptation to perchlorate in community-embedded SRM. Thus, the fourth chapter of this dissertation explores the adaptation sulfate-reducing communities to perchlorate. Laboratory evolution experiments generate communities capable of robust sulfidogenesis in 100 mM perchlorate. Using 16s rRNA amplicon sequencing, isolation, whole genome sequencing, and molecular and analytic techniques, we describe the ecological and evolutionary mechanisms of adaptation in these communities. In contrast to pure-culture adaptations, which target *sat*, we identify anion transport systems as primary targets of adaptation in community-embedded SRM faced with perchlorate inhibition.

In an attempt to elucidate additional targets of specific inhibitors, chapter five of this dissertation again makes use of adaptive laboratory evolution in *D. alaskensis* G20. Specifically, we examine adaptations required for robust growth of *D. alaskensis* G20 under nitrate, monofluorophosphate and tungstate stress. This work highlights the importance of sulfate and other anion transport as a primary target of all sulfate analogs examined. Further targets of each inhibitor are addressed: monofluorophosphate stress necessitates overexpression of a cluster of genes likely involved in the turnover of fluorinated intermediates, and tungstate stress generates mutations in the *sat*, likely altering the specificity of this enzyme.

The final sixth chapter of this dissertation summarizes conclusions arising from the above described studies and highlights future research needed for a thorough understanding of this ubiquitous microbial metabolism, and a wider application of SRM-specific inhibitors across areas of human and environmental health.

Dedication

This dissertation is dedicated to my loving and supportive, constantly fretting, grandmothers,

Angelina Atanassova and Yordanka Stoeva

whose heartfelt wisdom has been a constant source of guidance in my life.

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Abbreviations

SRM: Dissimilatory sulfate-reducing microorganism

DPRB: Dissimilatory perchlorate-reducing bacteria

SR: sulfate reduction

Sat: Sulfate adenylyltransferase (ATP sulfurylase, *sat*)

APS: Adenosine-5'-phosphosulfate

APSR: Adenosine-5'-phosphosulfate reductase

Dsr: dissimilatory sulfite reductase

Nrf: ammonifying nitrite reductase

DvH: *Desulfovibrio vulgaris* strain Hildenborough

DvM: *Desulfovibrio vulgaris* strain Miyazaki

G20: *Desulfovibrio alaskensis* strain G20

ALE: adaptive laboratory evolution

IC50: half maximal inhibitory concentration

K_m: Michaelis constant (substrate concentration needed for $\frac{1}{2} V_{\max}$)

k_{cat}: turnover number (number of times enzyme converts substrate to product per unit time)

K_i: inhibition constant (dissociation constant for enzyme-inhibitor complex)

SI: selectivity index

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I would like to express my sincere gratitude to my advisor, John D. Coates, for his continuous support, both intellectual and financial, over the course of my Ph. D. I would also like to offer a heartfelt thanks to all members of the Coates lab, specifically Anna Engelbrektson, Hans Carlson, Misha Mehta and Israel Figueroa for their mentorship, Ouwei Wang for teaching me fundamental molecular biology techniques and for his guidance during the final years of my Ph.D., Anchal Mehra for assistance in the design and execution of laboratory experiments and general moral support, Tyler Barnum for support in analyzing, writing and presenting data, and Yi Liu for logistical support. I also owe a special thank you to the remarkable undergraduate students for their help with laboratory experiments over the years: Gilbert Nalula, Nicholas Garcia, Rowena Rushton-Green, and Livia Tran. I am also grateful to the Adam Arkin laboratory for the use of equipment, and specifically to Jennifer Kuehl, for thoughtful discussions and intellectual guidance. Of course, none of this would be possible without the continuous love and support of my partner, Geoffrey C. Maines, and my family, Eli Simova, Krassimir Stoev, Angelina Atanassova, Simo Simov, and Yordanka Stoeva, to whom I am eternally grateful. Last, I would like to thank the National Sciences and Engineering Research Council of Canada and the Energy Biosciences Institute for financial support.

Chapter 1

Specific inhibitors of respiratory sulfate reduction: towards a mechanistic understanding

Abstract

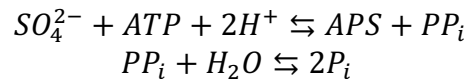
Microbial sulfate reduction (SR) by sulfate-reducing microorganisms (SRM) is a primary environmental mechanism of anaerobic organic matter mineralization, and as such influences carbon and sulfur cycling in many environments. In many industrial systems, SR results in the generation of hydrogen sulfide, a toxic, corrosive gas with adverse human health effects and significant economic and environmental consequences. Therefore, there has been considerable interest in developing strategies for mitigating hydrogen sulfide production and several specific inhibitors of SRM have been identified and characterized. Specific inhibitors are compounds that disrupt the metabolism of one group of organisms, with little or no effect on the rest of the community. Specific inhibitors of SRM have been used to control sulfidogenesis in industrial and engineered systems. Despite the value of these inhibitors, mechanistic and quantitative studies into the molecular mechanisms of their inhibition have been sparse and unsystematic. The insight garnered by such studies is essential if we are to have a more complete understanding of sulfate reduction, including the past and current selective pressures acting upon it. Furthermore, the ability to reliably control sulfidogenesis – and potentially assimilatory sulfate pathways – relies on a thorough molecular understanding of inhibition. The scope of this chapter is to summarize the current state of the field: how we measure and understand inhibition, the targets of specific SR inhibitors and how SRM acclimate and/or adapt to these stressors.

1.1 Introduction

Sulfate-reducing microorganisms (SRM) gain energy by coupling the oxidation of organic matter or hydrogen to dissimilatory sulfate reduction (SR). This use of sulfate as a terminal electron acceptor for growth results in the generation of hydrogen sulfide. SR by SRM is a critical component of the global carbon and sulfur cycles. Recent estimates suggest that up to 30% of the organic carbon flux from the ocean water column to the seafloor is oxidized by SRM [1]. The activity of these microbes is also of economic importance and can be harnessed by certain biotechnological applications. For example, SRM can be used for the immobilization and hence bioremediation of toxic and radioactive heavy metals [2, 3]. In other systems, however, SR is toxic and costly. SRM are implicated in the bio-corrosion of steel and concrete. The petroleum industry is particularly susceptible to these costs; “sour” oil, i.e. oil contaminated with SRM-produced sulfide, represents a corrosion threat to transportation infrastructure as well as a health threat for workers [4]. As a result of its critical impact on biogeochemistry, its potential applications and its corrosion/toxicity-based economic costs, microbial SR has been extensively studied.

The SR Pathway

Respiratory sulfate reducing microorganisms must overcome the chemical stability of the sulfate ion, which is not easily reduced. In order to achieve this, sulfate is initially activated, at the cost of 2 ATP equivalents per sulfate, to adenosine-5'-phosphosulfate (APS). This endergonic reaction is performed by the ATP sulfurylase (sulfate adenylyltransferase, Sat) and must be coupled to the exergonic hydrolysis of pyrophosphate by a pyrophosphatase, which pulls the sulfate activation reaction towards APS formation [5].



Because sulfate must be activated using cytoplasmic ATP, SR must occur intracellularly. Hence, sulfate needs to be transported across the cell membrane, in symport with H^+ or Na^+ [6, 7]. Post symport and activation, APS is reduced to (bi)sulfite by APS reductase (AprBA), using electrons from the quinone pool, transferred via the QmoABC complex. Finally, bisulfite is reduced to sulfide by dissimilatory sulfite reductase, DsrAB, via a DsrC-bound trisulfide intermediate, with electrons derived from DsrMK(JOP) [3, 8].

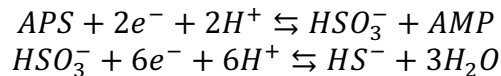


Figure 1.1 provides a summary of the SR pathway outlined above. It is important to note that this pathway functions in *dissimilatory* SRM, and is coupled to mechanisms for energy conservation. A homologous pathway exists in other prokaryotes and eukaryotes, the *assimilatory* SR pathway, whereby sulfate is reduced to sulfide and ultimately cysteine [9]. This chapter draws from research on both assimilatory and dissimilatory enzymes.

1.2 Defining Specific Inhibition

Concomitant with research on SRM and their metabolism has been a long-standing interest in controlling SR in the environment. Specifically, in oil recovery and wastewater treatment scenarios, the impetus for controlling SR is economic and has fueled research into the inhibition of SRM. The use of general inhibitors (i.e. biocides) in these contexts is suboptimal; the widespread rise of antibiotic resistance suggests that SRM subjected to biocides can develop resistance, which would only exacerbate any sulfidogenesis-related problems. Conversely, the *specific* inhibition of SRM can allow for the establishment of a stable non-sulfidogenic community that remains diverse and less likely to bounce back to SR [10].

In microbial ecology, a *specific* inhibitor is one that targets a particular group of organisms, leaving the remainder of the community unaffected. These inhibitors achieve specificity by targeting an enzyme or pathway unique to a group of organisms. As such, they are usually structural analogs of the substrate(s) for said enzyme/pathway [11]. For example, 2-bromoethanesulfonate (BES) is a structural analog of Coenzyme M (CoM), a cofactor found in all methanogens and involved in the final step of this metabolism, whereby methyl-CoM is reduced to methane. BES is thus a potent and specific inhibitor of methanogenesis, as are other structural analogs, such as 2-chloroethanesulfonate (CES) [12].

In the context of SR, specific inhibitors target one or more steps of the SR pathway, and are structural analogs of sulfate, APS or sulfite (Figure 1.1, 1.2). Molybdate is the classical specific inhibitor of sulfidogenesis, and has been used in a variety of ecological studies since the 1970s [11]. Other structural analogs of sulfate exist; in early work, Postgate tested a panel of inorganic oxyanions to identify both monovalent and divalent oxyanion inhibitors of SRM [13, 14]. Since then, several of these compounds have been characterized to varying degrees. However, their exact specificities and mechanisms have not yet been well defined.

There are several complexities in defining “specific inhibition” [11]. Inhibitors can partially or fully inhibit either the growth rate of an organism, the maximum cell yield, or both. An inhibitor can also “transiently” inhibit growth (i.e. elongate the lag phase). Adding to this complexity, inhibition can be overcome by adaptation or by rare pre-existing resistant mutants. This is well known in the emergence of microbial resistance to common antibiotics [15, 16]. Another example of this has been previously described for the SRM inhibitor, nitrate. Although 100mM nitrate is inhibitory to *Desulfovibrio vulgaris* Hildenborough (DvH), it is a “transient” inhibitor. After approx. 100h, DvH resumes normal growth. Moreover, when this culture is sub-cultured in the absence of nitrate, and then transferred back into 100mM nitrate, it retains robust growth, indicating the rise and outgrowth of spontaneous, nitrate-resistant mutant cells [17]. *Specific* inhibition entails the ability to demonstrate that other organisms are less affected. This depends both on the SRM and other organism(s) tested, and on the concentration of inhibitor used. The concentration at which “specific” inhibitors are specific remains elusive, even today. Finally, inhibition in a community context is much more relevant and still more complex. Inhibitors can be consumed or modified by organisms other than the target organism, or by the abiotic environment itself, leading to an array of *indirect* effects. For example, perchlorate and nitrate, which are both direct inhibitors of SRM in pure culture, in community contexts show increased potency as a result of turn over by perchlorate/nitrate reducing microbes [18–25]. The stability and solubility of inhibitors in natural systems are also critical factors affecting their potency.

1.3 Measuring Inhibition

Several methods for measuring inhibition have been established. Most of the quantitative measurements available come from enzymatic studies. Here, authors purify a specific protein and examine the effect of inhibitors on the turnover rate of the substrate, or else directly examine turnover of the inhibitor itself. For SR, most enzymatic studies come not from dissimilatory SRM but rather from prokaryotes and eukaryotes with homologous proteins functioning in the assimilatory pathway. Results from these can be extrapolated to dissimilatory enzymes, but with caution. Furthermore, these studies can only assess the inhibition of one specific target; if an inhibitor targets more than one enzyme, then measurements gathered from enzymatic studies are incomplete.

To capture the complete range of targets of an inhibitor, whole-cell studies are optimal. These are mostly performed in pure cultures of a handful of select SRM (most often *Desulfovibrio spp.*) [13, 14, 26]. The model SRM is exposed to one or several concentrations of inhibitor, and the resulting growth, electron donor consumption, sulfate consumption, or sulfide production is measured and compared to an uninhibited control. Specificity, when assessed, is determined by comparing inhibition of SRM to inhibition of non-SRM model organisms, e.g. *E. coli* [27].

Advances in high-throughput cultivation of SRM has enabled more fine-scale dose-response assays. For example, Carlson *et al.* devised a screen to quantitatively assess both the potency and selectivity of SRM inhibitors [10, 28, 29]. Panels of putative or established inhibitors were screened against both a model SRM, *Desulfovibrio alaskensis* G20 (G20), and a sulfidogenic marine enrichment culture. Inhibitors were also screened over a range of concentrations, in order to construct full dose-response curves. These dose-response curves allowed for the generation of an IC_{50} value: the concentration of compound necessary to inhibit 50% of growth or sulfidogenesis in 48h growth assays. For each inhibitor tested against the marine enrichment culture, one IC_{50} was calculated against total growth of the community and a second IC_{50} was calculated against sulfide production (by the native SRM fraction of the community). Thus, the inhibitory potency of a compound against the community as a whole could be compared with that against the sulfidogenic fraction specifically. If a compound inhibited sulfidogenesis at a lower concentration than the concentration required to inhibit overall growth, it was classified as a specific inhibitor of SRM. These specific inhibitors can be further ranked by their specificity, via the selectivity index (SI: growth IC_{50} / sulfide IC_{50}) (Figure 1.3). Molybdate and tungstate, the most specific inhibitors, had SIs of over 100, i.e. they are >100-fold more potent against SRM than against the community as a whole. The general biocide, tetrakis(hydroxymethyl)-phosphonium sulfate (THPS), conversely, had the same IC_{50} against both growth and sulfidogenesis, as expected for a non-specific inhibitor.

This methodology tackles several of the above-mentioned issues: it uses both a pure culture SRM as well as a complex sulfidogenic community, which tests environmentally relevant SRM in environmentally relevant conditions, while also allowing specificity to be quantified via the SI. Furthermore, as opposed to testing one or two concentrations of inhibitor, twelve concentrations were tested in order to fully define a dose-response curve. Inhibition was also validated using both qPCR and 16s rRNA amplicon sequencing. Finally, this approach was performed in high throughput: initial screens against G20 were done in 384-well plates, allowing Carlson *et al.* to screen the entirety of both the Microsource Pharmakon Collection and the ChemBridge Premium Diversity Collection (1920 and 30 000 compounds respectively). This screen should be expanded

to multiple model SRM and sulfidogenic enrichment communities, for a more thorough quantification of the diversity of potency and specificity.

Table 1.1 combines data obtained by Carlson *et al.*, across several recent studies [10, 28]. The most specific inhibitors of SR, molybdate and tungstate, are also the most potent, with IC_{50} s against sulfide production of a marine enrichment culture in the low μ M range. Interestingly, the potency of some inhibitors tested differs by up to 10-fold, depending on the SRM used: even within the *Desulfovibrio* genus, the IC_{50} for chlorate ranged from 6.3mM (95% CI: 4.9-8.0) against G20 to 58mM (95% CI 48-71) against *Desulfovibrio desulfuricans* ATCC 27774. Moreover, most inhibitors had higher potency in a community setting. At least in some cases (e.g. perchlorate) this was not due to turnover of these compounds, as no depletion was measured [28]. These observations further emphasize the need to quantify both the potency and specificity of inhibitors across a wider diversity of SRM isolates and sulfidogenic communities.

1.4 Understanding Mechanism

Nitrate and nitrite, and more recently chlorate and perchlorate (collectively (per)chlorate), are the main SR inhibitors that have been mechanistically characterized. Transcriptomic and proteomic techniques have been helpful in establishing the *stress-response* of *Desulfovibrio spp.* upon exposure to these inhibitors [28, 30–35]. However, bacteria may regulate gene expression *sub-optimally*, i.e. respond to indirect signals, with multiple stressors often initiating the same response. There is sometimes little correlation between the importance of a gene for fitness in a given condition and its expression pattern [36].

Fitness profiling via transposon (TN) insertion libraries is a useful tool for probing the *mechanism* of inhibition [37]. This method entails the creation of thousands of barcoded TN insertion mutants, such that each mutant has one inactivated gene and the population as a whole thus contains inactivated versions of every non-essential gene in the organism's genome. Subsequent growth on a stressor is used to profile the fitness of all mutants *en masse*: mutants with TN insertions in a gene detrimental to growth under the stressor will grow more than average. Conversely, mutants with insertions that disrupt the function of a gene that is beneficial under the stress will grow poorly. By comparing the frequency of mutants, via their barcodes, pre- and post-stress, researchers can identify genes that are important or detrimental for growth under the stressor. This tool exists for three separate *Desulfovibrio spp.* (DvH, G20 and *Desulfovibrio vulgaris* Miyazaki) and, as will be discussed in subsequent sections, has been successfully used to understand the mechanism of toxicity of several stressors (nitrate, perchlorate, chlorate, and monofluorophosphate) [10, 17, 28].

One specific TN insertion mutant in G20 has been particularly useful for mechanistically distinguishing between classes of inhibitors. The *rex::TN5* mutant has an insertion in the Rex repressor and as a result, overexpresses the Rex regulon, which includes core genes for the SR pathway, such as *sat*. Regulation via Rex, and its impact on inhibition will be addressed subsequently. Relevant to this discussion is the use of *rex::TN5* for preliminary insight into the mechanism of action of inhibitors: if *rex::TN5* is more resistant than wild type, then the stressor likely competitively inhibits one or more enzymes of the central SR pathway [10, 28], and thus overexpression of this pathway confers resistance. In this way, *rex::TN5* can be used as a first-pass tool when analyzing mechanism [29].

A similar “first-pass” screen for mechanism comes from the field of toxicology and consists of evaluating interactions (synergy/antagonism) between pairs of inhibitors [10, 28]. Two

inhibitors with the same target will likely be additive or antagonistic, i.e. the combined effect of both inhibitors together will be less than the more potent inhibitor alone. Inhibitor pairs targeting distinct enzymes are likely to be synergistic; the combination will exceed the additive effect of each inhibitor [38, 39]. Determining interactions between pairs of inhibitors has several additional benefits: in natural systems, these compounds are often found in combinations and hence their activity in isolation is less relevant than their activity in a mixed cocktail of compounds. Moreover, synergistic interactions enhance the potency and can enhance the selectivity of inhibition, and hence have a practical value [40, 41].

Adaptive laboratory evolution (ALE) is a complementary approach to TN-based fitness profiling. Unlike fitness profiling, ALE has the ability to capture a wider array of mutations conferring a fitness advantage (single point mutations in essential genes, for example) and can hint at gene targets of inhibition that have been missed by fitness profiling. Both fitness and ALE approaches are currently restricted to pure cultures of SRM, but would be informative in a community context.

The remainder of this chapter will aim to summarize all research to date pertaining to the specific inhibition of SR, and where relevant, the methodologies and approaches used to gain mechanistic insight. The chapter is organized by the steps in the SR pathway (See Box 1 and Figure 1.1): from transport of sulfate into cells, through sulfate activation, to the subsequent reductions to sulfide.

1.5 Dissecting the Inhibition of SR

Sulfate Transport

Transport of the sulfate anion is the first step in both the assimilatory and dissimilatory sulfate-reduction pathways. Several classes of sulfate transporters, and a large diversity therein, exist to accomplish this critical function. The few systems that have been functionally characterized are primarily from sulfate-assimilating prokaryotes and eukaryotes [42–46]. Sulfate transport in dissimilatory SRM has been largely neglected in recent years, with the last physiological studies pertaining to transport being from the 1990s [6, 7, 47–49]. These studies highlighted the complexity and redundancy of sulfate transport in SRM – establishing the existence of a dual transport system. High-affinity transporters, which can concentrate sulfate up to 10000-fold, are expressed under sulfate limitation and repressed under conditions of excess sulfate. Low-affinity, low-accumulating systems appear to operate when sulfate concentrations exceed 1mM. These systems allow dissimilatory organisms to transport sulfate efficiently, at a relatively low cost (symport with 2 or 3 protons or sodium ions), and at different environmental concentration of sulfate. More recently, *Desulfobacterium autotrophicum* was shown to express certain sulfate transporters only at high (15mM) sulfate concentrations, others only at low (<100uM) concentrations, and yet others independently of media sulfate concentrations [50]. Moreover, some transporters were localized in operons with numerous sensory and regulatory elements, hinting at their tight regulation, whereas others were not, perhaps indicative of constitutive expression. This diversity of transport systems across SRM has been recently systematized using a comparative genomics approach [51]. Surveying 44 taxonomically diverse SRM, authors found evidence for sulfate transporters across four families: *SulP* (Sulfate Permease, Transport Classification DataBase TCDB 2.A.53), *DASS* (Divalent Anion: Na⁺ Symporter, TCDB 2.A.47), *CysP* (TCDB 2.A.20.4.1) and *CysZ* (of the TSUP family TCDB: 2.A.102) with each genome having, on average,

2, 3, 1 and 8 of each respective transporter type. The following section will aim to provide a brief review of transporters relevant to dissimilatory SRM and their specificity (Figure 1.4A). For a more broad overview of sulfate transport across the three domains of life, see several recent reviews [42, 43, 46].

Dissimilatory is Different

Most of our knowledge on sulfate transport is obtained from assimilatory organisms, where sulfate transporters of the SulT family dominate (TCDB 3.A.1.6). These ABC transporters consist of a sulfate (or thiosulfate) binding protein (Sbp), which determines the specificity of the transporter, as well as a membrane protein and an ATP-binding protein, which together couple the hydrolysis of ATP to sulfate transport [42]. SulT transporters were assumed to exist in dissimilatory organisms as well and there are examples of transporters annotated as SulT-type across SRM genomes. However, comparative genomics found no evidence of SulT-type transporters across the phylogenetic diversity of sulfate-reducers [51]. This likely reflects real differences in assimilatory vs. dissimilatory SR: assimilatory SR requires a low but specific and uni-directional influx of sulfate, for use as a S source. Coupling import to ATP hydrolysis ensures irreversible transport. Dissimilatory SR, conversely, requires a much higher influx of sulfate. Further, the energy yield from this metabolism is low: if ATP were used for transport, the net yield of SR would be 0 ATP. Indeed, inhibiting ATP-dependent transport in SRM has no effect on sulfate transport [6]. Instead, four classes of ATP-independent transport machinery exist: SulP, DASS, CysP and CysZ (Figure 1.4A).

SulP permeases function as solute:cation or solute:solute exchange transporters, in prokaryotes as well as in yeast, algae, plants (Sultr proteins) and animals, including humans (SLC26 family). For a thorough review of this family, refer to [44]. SRM genomes have on average two *sulP*; however, SulP is not essential for dissimilatory SR as it was absent from all archaeal SRM genomes examined as well as several mostly thermophilic sulfate-reducing bacteria [51]. Furthermore, in DvH, G20, and *Desulfovibrio vulgaris* Miyazaki, all SulP are non-essential and support TN insertions [52–54].

The DASS family transporters usually function as low affinity transporters (i.e. they are used at high sulfate concentrations) in prokaryotes and seem to be general oxyanion transporters. The PerO permease from *Rhodobacter capsulatus* was shown to import sulfate as well as the sulfate analogs molybdate, tungstate and vanadate [55]. Moreover, *perO* genes from 5 species (*Agrobacterium tumefaciens*, *Dinoroseobacter shibae*, *Rhodobacter sphaeroides*, and *Sinorhizobium meliloti*, and *Pseudomonas stutzeri*) can all complement sulfate uptake in sulfate-uptake deficient mutants of *R. capsulatus* [56]. In the SRM *Desulfobacterium autotrophicum*, several DASS family transporters are present and differentially regulated depending on the external sulfate concentration [50].

The CysP family contains only one characterized member. Overexpression of the *Bacillus subtilis* CysP can complement sulfate transport in transport deficient mutants of *E. coli*, confirming its function [57]. In SRM, CysP permeases seem to be of particular importance in thermophilic isolates as the majority of the thermophilic genomes analyzed lack SulP and DASS type transporters [51].

The CysZ-type permease is part of the TSUP family, and has been functionally characterized as a high affinity sulfate transporter in *Corynebacterium glutamicum* [58]. The only other characterized CysZ is from *E. coli* [59]. Interestingly, TSUP family proteins are highly

abundant across SRM genomes (8 per genome on average), though no predictions can be made as to their function. In two out of the 44 SRM genomes surveyed (*Ammonifex degensii* and *Desulfohalobium retbaense*) the only putative sulfate transporters identified were from this family, suggesting either that novel sulfate transport mechanisms are present or that CysZ can function as the primary sulfate permease [51].

The Specificity and Inhibition of Transport

Divalent oxyanions (chromate, molybdate, tungstate, selenate) are structurally analogous to sulfate. As a result, they compete with sulfate for transport machinery. Studies quantifying sulfate transport in SRM have been scarce; to our knowledge, the most recent work dates to the early 1990s (Table 1.2). In *Desulfobulbus propionicus* and *Desulfococcus multivorans*, selenate, thiosulfate and chromate were able to release accumulated ^{35}S -sulfate from cells, whereas molybdate and tungstate had insignificant effects [7]. Similarly, in *Desulfovibrio desulfuricans* strain CSN, the uptake of sulfate was unaffected by molybdate or tungstate, whereas chromate and selenate strongly inhibited this function [6].

More is known about assimilatory sulfate transport. Table 1.2 presents biochemical evidence for sulfate transport through microbial and plant sulfate transporters of different classes. The SulP family is the best characterized of the sulfate transporter families present in SRM. The *Mycobacterium tuberculosis* SulP, when heterologously expressed in *E. coli*, increases sulfate uptake. However, selenate, thiosulfate, sulfite and to a lesser degree, molybdate inhibit this uptake. Several SulP genes from yeast and plants were identified by screening for selenate or chromate resistance, highlighting the “leakiness” of these permeases [60, 61]. *Arabidopsis thaliana* mutants in SulP show decreased sulfate uptake but also decreased selenate uptake and hence increased resistance to this toxic substrate [62, 63]. The *Stylosanthes hamata* SulP homolog expressed in yeast can transport molybdate, which when present in an equal concentration to sulfate causes a 48% decrease in sulfate uptake.

Studies addressing the specificity of the other 3 transport families are rare. The *B. subtilis* CysP can transport chromate, as evidenced by the enhanced sensitivity to chromate in *E. coli* cysteine auxotrophs expressing this permease [57]. Similarly, CysZ mutants of *E. coli* are resistant to chromate (and deficient in sulfate transport) [64]. DASS family transporters appear to be more general as well: the recently characterized PerO permease from *R. capsulatus* as well as homologs from several other microorganisms can mediate molybdate, tungstate, vanadate as well as sulfate uptake [55, 56].

The research summarized above supports two general conclusions: first, sulfate-analogs can leak into SRM via the sulfate-transport machinery, and once inside the cell they can have further negative consequences. Second, import of sulfate analogs can result in decreased sulfate import, since the analogs compete with sulfate for the same transporters. Decreased transport could be inhibitory to SRM, regardless of any potential downstream effects.

An Aside: Molybdate and Tungstate Specific Transport

Molybdenum and tungsten, found most often as the soluble oxyanions molybdate and tungstate, are required trace elements for many organisms, present as cofactors in metallo-enzymes. As such, organisms have several high-affinity ABC transport systems to scavenge low concentrations of molybdate and tungstate [42]. Three such systems of varying specificity have been described: ModABC, TupABC and WtpABC [42, 65]. These transporters are composed of a periplasmic component (ModA/TupA/WtpA) which binds the specific oxyanion, a transmembrane

channel (ModB/TupB/WtpB) and a cytoplasmic ATP-binding protein (ModC/TupC/WtpC). They are all tightly regulated: ModABC is regulated by ModE, which binds molybdate and tungstate with high specificity (it does *not* bind phosphate, sulfate or vanadate). This system can uptake *n*M concentrations of molybdate and tungstate but is repressed at higher molybdate concentrations.

In SRM, tungstate and molybdate are required for many enzymes involved in energy transduction and hence are stimulatory at *n*M concentrations, but as discussed previously, are highly toxic at μ M concentrations. Homologs of high-affinity molybdate and tungstate specific transporter exist: DvH and G20 both have TupA and ModA homologs [65]. TupA from G20, which controls the specificity of transport, can bind tungstate (with higher affinity) and molybdate but does not bind sulfate, phosphate or perchlorate [66]. Moreover, both *mod* and *tup* G20 genes are downregulated at either 1 μ M molybdate or 10 μ M tungstate [67]. Hence, molybdate/tungstate import is again highly regulated. Although ModE homologs do not exist in DvH, recently a tungstate responsive regulator (TunR) was identified in many SRM. Low (0.3 μ M) concentrations of tungstate, are sufficient to stop TunR-dependent activation of ModABC [68]. Thus, these high-affinity systems likely do not play a role in inhibition; over millions of years of evolution, SRM have fine-tuned these transport systems such that they do not pose a problem at higher molybdate/tungstate concentrations. Interestingly, a 2nd group of TunR family regulators, TunR2, is hypothesized to respond to tungstate and molybdate by de-repressing TSUP and SulP sulfate transporters, perhaps in an attempt to overcome inhibition by these compounds through increased sulfate uptake [68].

Monovalent oxyanions: The Nitrate Example

The above section detailed the non-specific uptake of divalent oxyanions by systems intended for sulfate transport. However, monovalent oxyanions such as nitrate, perchlorate, and chlorate are also toxic to SRM. How do these compounds leak into cells? It is possible, and likely, that several of these compounds enter through sulfate-transport machinery.

Nitrate is the most extensively studied monovalent oxyanion inhibitor of SRM, and nitrate stress has been characterized in pure cultures of DvH and G20 [17, 28, 69]. Although initially, nitrate inhibition was attributed to low-level nitrite production, with nitrite being the causal stressor, it has since been shown that nitrate and nitrite act separately. Transcriptomic and proteomic approaches have shown few similarities in the response to nitrate vs. nitrite stress and DvH was recently shown to be incapable of nitrite production from 100mM nitrate [31, 33, 69]. Fitness profiling has further defined the mechanism of inhibition: a cluster of genes referred to as the “nitrate cluster” comes up across fitness pools in DvH, G20 and *Desulfovibrio vulgaris* Miyazaki [17, 28, 54]. Mutants with insertions in DVU0246 (Dde_0604, pyruvate-phosphate dikinase), DVU0247 (Dde_0603, CheY-like response regulator, receiver domain), DVU0250 (Dde_0600, hypothetical protein) and DVU0251 (Dde_0598, DUF81/CysZ-type permease) all grow with identical kinetics with and without 100mM nitrate, a concentration completely inhibitory to the control strain [17]. The huge fitness advantage portrayed by these TN mutants is conserved across the three SRM and, strikingly, is unique to nitrate stress. The CysZ permease (DVU0251/Dde_0598), and the surrounding genes (potentially regulatory to its function) likely cause nitrate to leak into cells; inactivation of this operon therefore prevents nitrate entry and alleviates nitrate inhibition. The native function of this operon is unknown (neither DvH nor G20 can use nitrate as an electron acceptor for growth). This CysZ may be a thiosulfate transport system; mutants in this gene cluster show a slight growth defect with thiosulfate as a terminal

electron acceptor. Because this family of transporters is poorly characterized, predictions pertaining to function are difficult. This emphasizes the need to further investigate sulfate/oxyanion transport in SRM and highlights transport as a primary target of inhibition.

Sulfate Activation

The sulfate adenylyltransferase (Sat, ATP sulfurylase) catalyzes the activation of the inert sulfate molecule. ATP is used to generate adenosine-5'-phosphosulfate (APS) and pyrophosphate (PP_i). A pyrophosphatase then rapidly cleaves pyrophosphate, to pull the overall reaction forward. Sulfate adenylyltransferases from a variety of organisms have been characterized, as Sat performs critical metabolic functions across several pathways (see Table 1.3). Assimilatory prokaryotic and eukaryotic Sat functions as part of the pathway for sulfur recruitment; in dissimilatory prokaryotes, Sat functions in sulfate respiration; in sulfide-oxidizing bacteria, Sat functions in the reverse direction as part of the sulfide oxidation pathway. The structural and sequence diversity of Sat is vast, depending on the organism in which it is found [70, 71]. In the dissimilatory SRM *Desulfovibrio desulfuricans* ATCC 27774 and *Desulfovibrio gigas*, Sat exists as a homotrimeric metalloprotein [72]. Key residues in the substrate binding site of Sat are surprisingly conserved, given that the enzyme likely originated prior to the divergence of prokaryotes and eukaryotes [71].

Alternative Substrates: APS Analog Half-life Dictates Toxicity

Sat is also conserved in its promiscuity towards other inorganic divalent oxyanions (Figure 1.4B). Sulfate, monofluorophosphate, selenate, molybdate, tungstate, and chromate are all substrates for Sat from *Penicillium chrysogenum*. Sat turnover of tungstate and molybdate is fastest, followed by chromate, selenate, and monofluorophosphate (slow turnover). All yield APS analogs of varying degrees of stability [73]. The stability (i.e. half-life) of APS analogs can serve to classify these alternative substrates: molybdate, tungstate and chromate yield APMo, APW, and APCr respectively, all of which are unstable and degrade immediately. Because formation of the APS analog requires input of ATP, this instability results in the constant hydrolysis of ATP since each molecule of molybdate, tungstate or chromate can cycle through the Sat-catalyzed reaction many times, thus depleting intracellular ATP pools. In two *Desulfovibrio* spp. tested, chromate caused a 93-95% depletion of intracellular ATP (compare to an uninhibited control), whereas molybdate and tungstate depleted between 45-79% of intracellular ATP. ATP depletion was specific to active SR, with other metabolisms being unaffected or even slightly stimulated by molybdate [27]. This “futile cycling” is the central mechanism of inhibition of these alternative substrates in SRM, and likely why they are potent inhibitors even at low μ M concentrations.

Selenate, on the other hand, forms a semi-stable APS analog, APSe, with a half-life on the order of minutes [73, 74]. Taylor and Oremland quantified ATP pools under selenate stress to be 54-83% of controls, rendering its ATP-depleting effect most minor of the oxyanions tested [27]. This is likely a combination of a slower selenate turnover by Sat compared with other oxyanions, as well as APSe being more stable, enough so to be detected [75].

Apart from sulfate, monofluorophosphate is the only alternative substrate known to form a stable analog (ADP β F), and hence the mechanism of inhibition here is different [76]. The downstream reactions and effects of ADP β F remain unknown, but it is hypothesized that intracellular release of the toxic fluoride ion is a main mechanism of inhibition. This is supported by the fact that a fluoride efflux pump TN mutant (*Dde_2107::TN5*) displays increased a specific

sensitivity to both fluoride (at 30mM) and monofluorophosphate (1mM) as compared to wild type [10].

Table 1.3 details alternative substrates across various Sat proteins that have been biochemically characterized.

Monovalent Oxyanions: Dead-end Inhibitors

In contrast to divalent oxyanions, monovalent oxyanions (nitrate, chlorate, and perchlorate) do not serve as substrates for Sat (Figure 1.4B). Although they can fit in the sulfate-binding pocket, they do not have a fourth oxygen (perchlorate does not have a sufficiently electronegative fourth oxygen) for catalysis to occur [73]. Thus, these inhibitors bind the enzyme-ATP complex but are not turned over, and hence they are termed “dead-end” inhibitors. These compounds inhibit the activity of Sat by preventing sulfate binding and turnover; hence, SRM grown fermentatively or with sulfite (growth that bypasses Sat) are more resistant to these inhibitors [10]. They are often referred to in the literature as being competitive with sulfate [74, 77–79]. More recently, the yeast Sat was crystallized with chlorate, bound at a different site (not the sulfate-binding pocket), and instead of acting as a competitive inhibitor, prevented movement of the enzyme back to the “open” state/release of the products [80]. Regardless of the mechanism, monovalent oxyanions have been shown to be inhibitory to Sat function across a diversity of organisms, with perchlorate/fluorosulfonate/chlorate being consistently more potent inhibitors compared to nitrate (For a summary, see Table 1.3). This mirrors whole-cell patterns: perchlorate and chlorate are more potent inhibitors of *Desulfovibrio spp.* in pure culture as compared to nitrate (IC₅₀ values range from 14-50mM, 4-58mM, and 32-104mM respectively) [28]. The higher IC₅₀ values as compared to K_i values (Table 1.3) likely result from minimal entry of dead-end inhibitors into the cytoplasm.

APS and sulfite reduction

The activated APS molecule is reduced to sulfite and AMP via APS reductase (*aprBA*). Although dissimilatory APS reductases from several SRM have been purified [81] and two have been crystalized (from *Archaeoglobus fulgidus* and *Desulfovibrio gigas*), very little information exists on APS analogs or on the inhibition of this enzyme. What is known, again, comes from the assimilatory APS reductase (APSR). APSR from *M. tuberculosis* is required by the latent phase of TB infection. As such, it is a potential drug target, and has received attention from the field of medicinal chemistry [82]. Cosconati *et al.* used a 3-D crystal structure of the *P. aeruginosa* APSR with APS substrate to virtually screen the National Cancer Institute Diversity Set for ligands that would be inhibitory to this enzyme [83]. The screen yielded 39 compounds, which were biochemically tested and 5 of which showed inhibition of APSR activity of >50%. One hit, “348401”, caused a 90% reduction in APSR activity. A second round of screening, yielded another set of compounds, 40 of which were biochemically characterized; five of these had dissociation constants (K_d) of <50μM, with one APS analog, the most potent inhibitor, having a K_d of <10μM. Hong *et al.* used a different approach: they defined ligand binding determinants critical for enzyme-APS interaction by synthesizing and testing APS analogs with key modifications [84]. Thus, they were able to identify a critical set of features for the rational design of APSR inhibitors. More of this research is expected, as a recent review by Campanini *et al.* highlighted the value of targeting key metabolic functions of pathogens, such as cysteine biosynthesis/sulfate assimilation,

to increase the efficiency of antibiotic treatment [85]. However, whether these APS reductase inhibitors will have similar inhibitory effects in SRM, and whether they will show specificity to sulfidogenesis in environmental systems remains completely unaddressed.

The last step of the SR pathway is the reduction of sulfite to sulfide by *dsrAB*. Nitrite is the only sulfite analog studied extensively in SRM and considered for practical application. Enzymatic characterization of desulfoviridin, the dissimilatory sulfite reductase (Dsr) from DvH, shows that it can bind and *slowly* reduce nitrite, with a K_m of 0.028mM and a k_{cat} of 0.038mol $\text{NO}_2^-/\text{s}\cdot\text{mol}$ heme. In contrast, the K_m and k_{cat} for sulfite are 0.06mM and 0.31 mol $\text{NO}_2^-/\text{s}\cdot\text{mol}$ heme[86]. DvH can also use *dsrAB* to reduce low levels of nitrite for use as a sole nitrogen source supporting growth [69]. Dsr being the primary target of nitrite inhibition is further evidenced by the *intrinsic* nitrite resistance of DvH – the concentration of nitrite inhibitory to growth of single cells into colonies on plates – being 0.04mM. This is markedly similar to the K_m of Dsr for nitrite [32]. DvH inhibition by 1mM nitrite can be completely alleviated by the addition of 5mM sulfite, implying that nitrite is competitive with sulfite [69]. Thus, the evidence suggests that nitrite is an alternative substrate of Dsr, competing with and preventing sulfite reduction to sulfide.

Importantly, many SRM can grow by nitrate or nitrite reduction in addition to sulfate reduction [87]. Moreover, SRM can use nitrite as a sole nitrogen source [69]. This reduction is accomplished via the periplasmic ammonifying NrfHA nitrite reductase. DvH can use sub-inhibitory concentrations of nitrite both as a sole nitrogen source and as a terminal electron acceptor supporting growth [69]. A *nrfA* mutant, however, cannot use nitrite as an electron acceptor, indicating that Nrf is the reductase responsible for this growth [69]. The native function of Nrf, however, is likely to protect SRM from inhibition by nitrite. Wild type DvH can grow in 5mM nitrite after a 1-2h lag and completely reduce the nitrite; a *nrfA* mutant, meanwhile, cannot [32]. Nrf likely functions in the mM range of nitrite: the Nrf from *D. desulfuricans* 27774 has a K_m for nitrite of 1.4mM [88]. Thus, in SRM, Nrf likely protects the cells from mM amounts of nitrite via rapid nitrite reduction to ammonium. Depending on the duration of exposure, this strategy may fail as it diverts electrons from sulfate reduction to periplasmic nitrite reduction. Further, nitrite reduction, whether by Dsr or Nrf, may result small quantities of nitric oxide (NO) formation. NO can react with metal ions and nitrosylate 4Fe-4S clusters of proteins. SRM *D. desulfuricans* 27774 has a two-fold approach for dealing with nitrosative stress: upregulation of genes for scavenging NO, such as *hcp*, and upregulation of systems for repairing proteins, such as YtfE ortholog Ddes_1165 [89].

Regulation via rex and Inhibition

SRM are metabolically flexible and diverse, performing sulfidogenic, fermentative, and syntrophic metabolisms [90]. This flexibility necessitates regulation of sulfate reduction genes, a task performed by the redox-sensitive Rex repressor [91, 92]. Rex responds to intracellular $\text{NADH}:\text{NAD}^+$ ratios. When $\text{NADH}:\text{NAD}^+$ is high (limiting sulfate/excess reducing equivalents), Rex binds NADH, causing it to lose the ability to bind DNA and hence de-repress its regulon, which consists of several genes essential for sulfate respiration, including *sat*, *qmoABC*, and pyrophosphatase [91, 92].

Using fitness profiling in both DvH and G20, *rex* has been identified as a key gene detrimental to nitrate and (per)chlorate stress. It is the only detrimental gene in common across all three inhibitors. TN5 insertion mutants of this gene have a fitness advantage over other mutants in the library when grown on (per)chlorate/nitrate. The TN insertion deactivates Rex, leading to de-

repression of the rex regulon and hence increased expression of Sat and other key respiratory enzymes. Increasing the number of Sat enzymes and sulfate symporters relieves the competitive inhibition imposed by perchlorate/nitrate. Thus, *rex::TN5* mutants display increased resistance to these compounds. In contrast, *rex::TN5* shows no resistance or increased sensitivity to alternative substrates of Sat, resulting from increased turnover/entry of these compounds into cells [28].

Further evidence for the importance of Rex regulation comes from wild type G20 subjected to sub-lethal doses of these inhibitors. Mid-log phase cultures of cells exposed to (per)chlorate and nitrate show increased NADH:NAD⁺ ratios when compared to un-inhibited controls [28]. Moreover, the presence of these inhibitors induces upregulation of the Rex regulon, as quantified on a transcript and protein level [28]. Likely, these compounds inhibit Sat and respiration, leading to an accumulation of NADH and subsequent de-repression of the Rex regulon. This, in turn, increases protein levels of Sat, sulfate transporters, and other core enzymes, and partially alleviates the effects of inhibition. As a result, G20 cells pre-grown on (per)chlorate or nitrate (i.e. de-repressed Rex) show increased resistance to all three of these inhibitors in subsequent transfers [28].

Other targets

Molybdate is a known specific inhibitor of SR that targets sulfate import and Sat, which causes a depletion of intracellular ATP pools, as discussed in the above sections. However, recent work by Zane and colleagues has shown that molybdate may have additional targets in the model SRM, DvH [93]. A Δsat strain of DvH was constructed and phenotyped in fermentative media with molybdate. This strain should be resistant to molybdate under these conditions as 1) there is no sulfate and hence molybdate cannot interfere with sulfate transport, and 2) there is no Sat, and hence no turn-over of molybdate and subsequent depletion of ATP pools. However, the Δsat strain retained sensitivity to molybdate. Current efforts are underway to characterize targets of molybdate in this strain, using adaptive laboratory evolution combined with whole-genome sequencing of the molybdate adapted, Δsat strains (Zane, personal communication).

Other potentially specific inhibitors of SR with unknown targets have been identified via a recent high-throughput screen of >30,000 small-molecules [29]. Several of the compounds discovered were antibiotics selective against gram-negative bacteria (ciprofloxacin, clinafloxacin, and nalidixic acid). The specificity seen here may result from the screen performed: specificity was assayed as the ratio of IC₅₀ for overall community growth of a marine enrichment to the IC₅₀ for sulfidogenesis of said enrichment (see “*Measuring Inhibition*” section of Introduction). The dominant SRMs in this community are *Desulfovibrio spp.* (gram-negative), while the dominant fermentative (non-SRM) organisms are gram-positives (Firmicutes); hence gram-negative selective antibiotics may appear “specific” against SRM. The most specific inhibitor identified was lincomycin, though the basis for this specificity was unresolved. Some less specific but very potent inhibitors of sulfidogenesis included nitroimidazoles, which have known selectivity against anaerobes [94, 95]. The most potent inhibitor of sulfidogenesis identified was Zinc pyrithione. The mechanism of action here was linked to both zinc/copper toxicity and pyrithione, which can act as a chelator, transporting these toxic metals into the cytoplasm. For all compounds identified in this screen, the precise mechanism, targets, and actual specificity remain to be validated across the diversity of SRM and sulfidogenic communities.

1.6 Conclusions and Future Perspective

Understanding the specific inhibition of SRM is critical across applied and theoretical pursuits, in both natural and engineered systems. Specific inhibitors have industrial applications for treating costly sulfidogenesis, and are also tools for probing the ecological and physiological functioning of SRM. The success of these applications depends on a thorough understanding of specific inhibition. This chapter has aimed to highlight our current understanding of SR inhibitors: how we define and measure inhibition, and how we gain mechanistic insight into its targets.

Sulfate analogs compete with sulfate for entry into cells (via sulfate transport machinery) and activation to APS (via Sat). If activated into an unstable APS analog, toxicity is mainly attributed to ATP depletion (e.g. molybdate and tungstate). Monofluorophosphate, conversely, forms a stable analog and toxicity may be at least partially attributed to intracellular fluoride release. If Sat cannot turn over a sulfate analog, then inhibition is of a competitive nature, and increasing transcription and translation of the Rex regulon can increase resistance (e.g. the case for nitrate and (per)chlorate). Other specific inhibitors, such as selenate, tellurate, and vanadate, remain mechanistically uncharacterized. APS and sulfite analogs are rare across the literature, and their identity and effects remain largely unknown. Nitrite is the exception here, with nitrite toxicity to Dsr being easily overcome in certain SRM, via periplasmic nitrite reductases. Finally, completely new to the field is the idea that SR-specific inhibitors may have ‘alternative’ targets, outside of the SR pathway.

Although SRM have been extensively studied, their specific inhibition leaves many unanswered questions. There is an obvious lack of quantification in regards to the concentration of inhibitor needed to maintain “specificity”. Indeed, without this critical information, it is impossible to use SR-specific inhibitors as either ecological tools or to control sulfidogenesis. Investigating specificity necessitates understanding inhibition in an ecological context, which again has practical benefits, and may shed light onto why SRM seem more resistant in pure culture as compared to in sulfidogenic communities. The determinants of potency also remain unknown: why are certain SRM more resistant to certain inhibitors? Differences in sulfate transport may explain some of the differences in sensitivity. Sulfate transport in SRM is the least studied component of the SR pathway, and perhaps the one that differs most significantly among SRM. Whether or not this results in differences in resistance remains to be investigated. Future research should aim to experimentally describe sulfate transport systems in SRM, their affinity towards sulfate vs. sulfate analogs, and their regulation, all of which can impact the potency of inhibition. Moreover, insights into sulfate transport will improve our ability to harness SRM for remediation efforts. Finally, continuing to probe the mechanism of inhibition (with emphasis on ‘alternative’ targets), using fitness-profiling, ALE, and other approaches will increase our basic understanding of this metabolism and its importance. With a greater understanding of SR inhibition will hopefully come an improved and wider application of SR inhibitors across areas of human and environmental health.

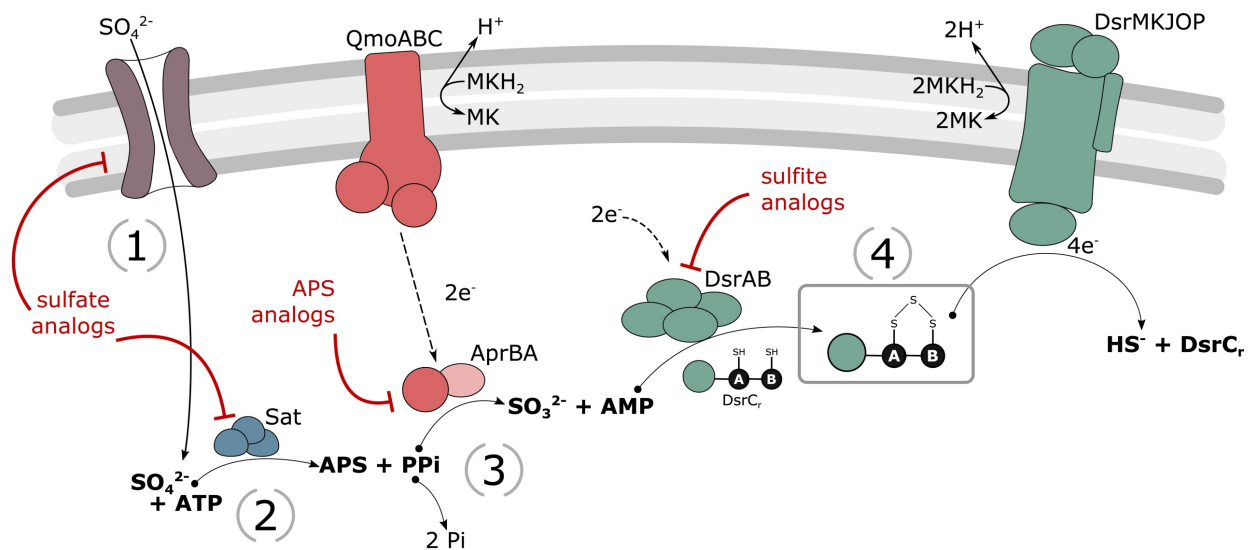


Figure 1.1: Sulfate reduction pathway, adapted from [8]. (1) Import of sulfate, (2) activation of sulfate to APS, via Sat, (3) APS reduction by AprBA, (4) reduction of sulfite by DsrAB – through a DsrC trisulfide – to sulfide. Specific inhibition can occur at (1) and (2) by sulfate analogs, at (3) by APS analogs and at (4) by sulfite analogs.

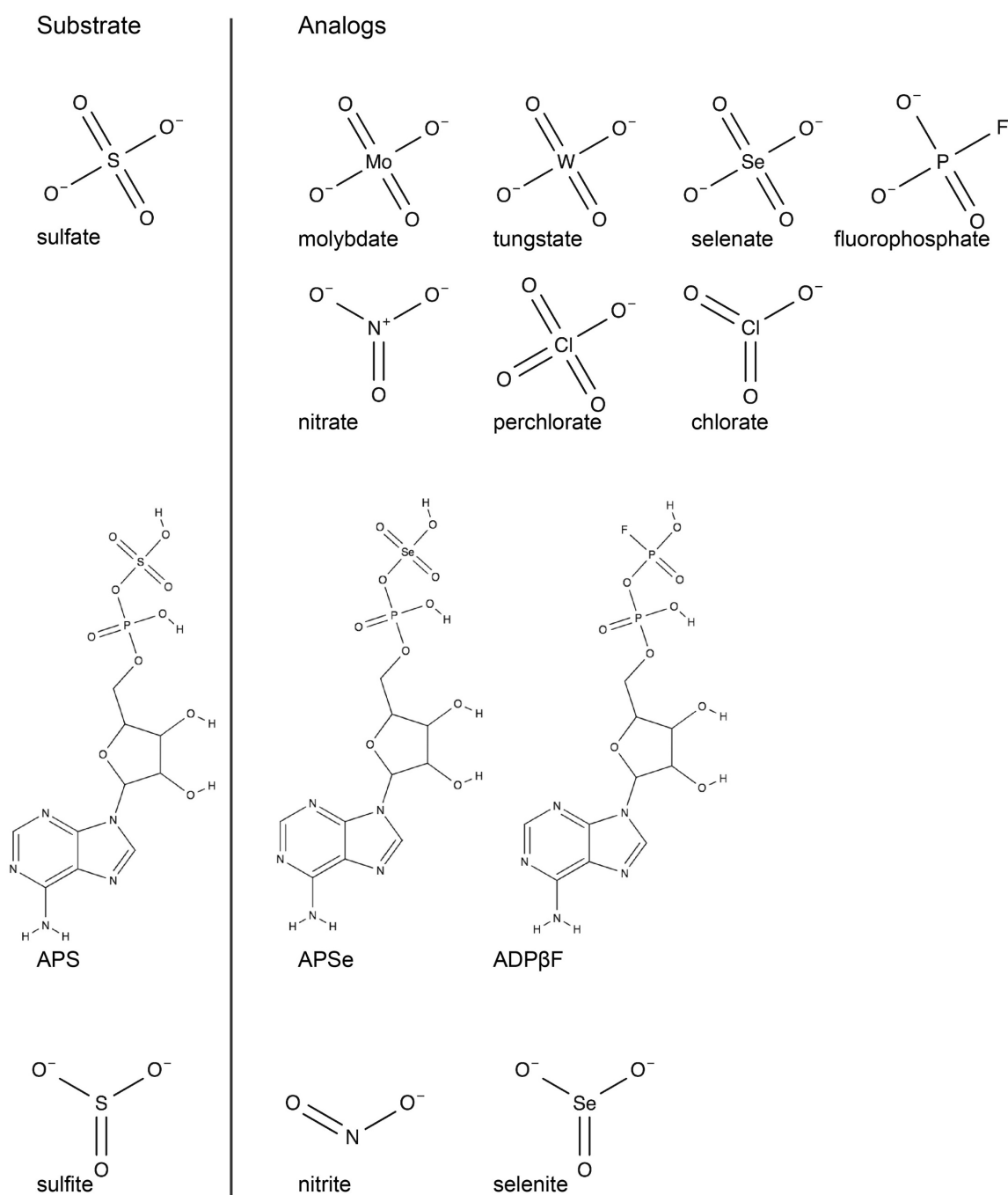


Figure 1.2: Structural analogs as specific inhibitors. Molybdate, tungstate, selenate and monofluorophosphate are divalent oxyanion analogs of sulfate; nitrate, perchlorate and chlorate are the monovalent equivalent. Sulfate analogs compete and inhibit sulfate transport and activation. APS analogs and sulfite analogs inhibit downstream APS and sulfite reduction.

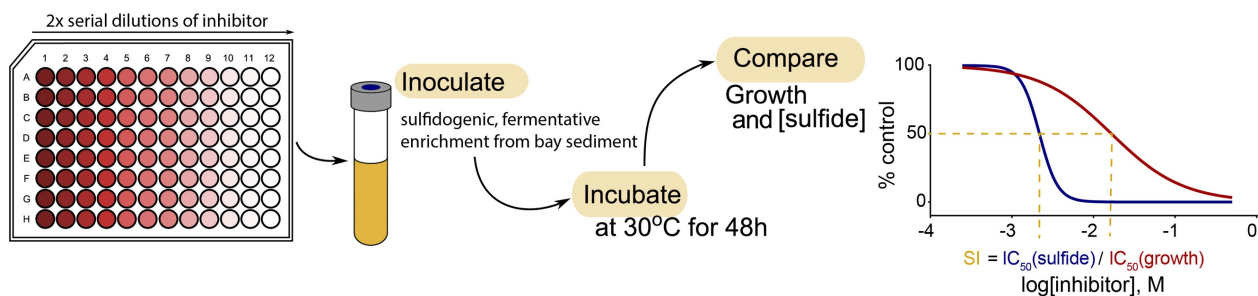


Figure 1.3: A screen for identifying specific inhibitors of SR and quantifying a selectivity index (SI) [10, 29]. Sulfidogenic enrichment cultures and subjected to serial dilutions of inhibitors, and allowed to grow for 48h. Totally community growth and sulfide production are measured across the gradient of inhibitor concentrations and used to generate dose-response curves. The SI is defined as the ratio of the IC_{50} against sulfide to the IC_{50} against growth.

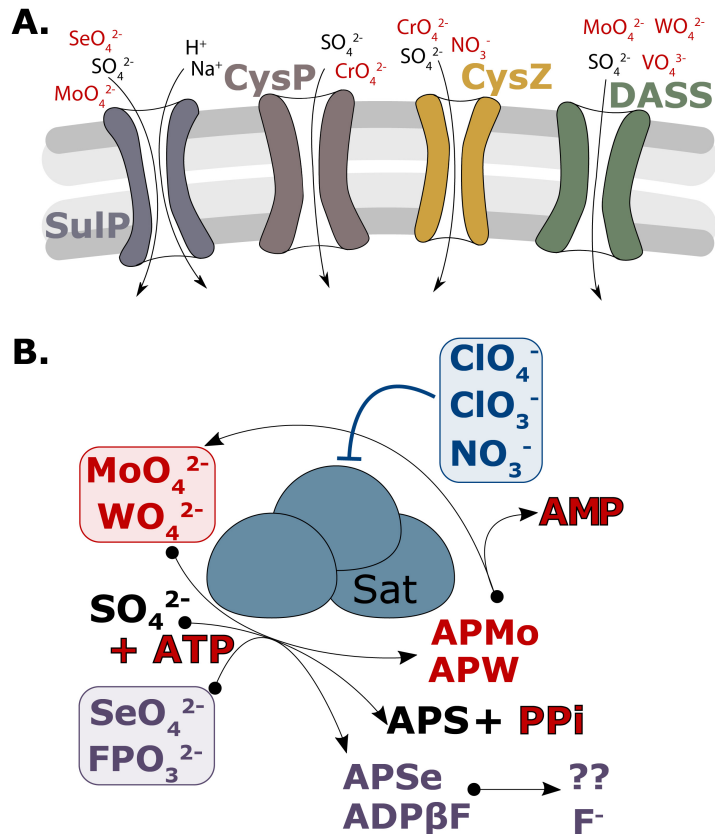


Figure 1.4: Sulfate analogs compete with sulfate for entry into the cell and activation via Sat. A. The four classes of ATP-independent sulfate transporters present in SRM, and sulfate analogs known to leak into cell through these systems (adapted from [42]). B. Classes of Sat inhibitors. Specific inhibitors MoO_4^{2-} or WO_4^{2-} are alternative substrates of Sat and lead to unstable APMo and APW analogs, which quickly degrade and lead to futile cycling, exhausting ATP stores (red box). SeO_4^{2-} and FPO_3^{2-} form stable analogs with uncharacterized downstream toxic effects; FPO_3^{2-} toxicity may be partially attributed to intracellular F^- release (purple box). Monovalent oxyanions ClO_4^- , ClO_3^- and NO_3^- are dead-end inhibitors, not turned over by Sat. They inhibit sulfate binding and activation (blue box).

Table 1.1: IC₅₀ values and selectivity indices (SI) of specific inhibitors of SRM. Table adapted from [10, 28].

Compound ¹ (name, chemical formula)	IC ₅₀ (95%CI) (mM) ^{2,3}					Selectivity Index ⁴
	ME growth	ME sulfide	G20	Other		
Selenate, SeO ₄ ²⁻	>10	0.38 (0.21-0.72)	0.022 (0.018-0.028)			>26.3
Selenite, SeO ₃ ²⁻	>10	0.57 (0.56-0.59)	0.45 (0.32-0.63)			>17.5
Tellurate, TeO ₄ ²⁻	>1	0.012 (0.020-0.013)	0.11 (0.028-0.4)			>83
Tellurite, TeO ₃ ²⁻	>1	0.053 (0.040-0.071)	0.022 (0.023-0.13)			>18.9
Perchlorate, ClO ₄ ²⁻	21 (14-31)	2.3 (2.0-2.6)	24 (20-32)	DvH DvMF 27774	14 (13-15) 28 (27-29) 50 (45-57)	>9.1
Chlorate, ClO ₃ ⁻	44 (29-60)	1.6 (1.4-1.8)	6.4 (4.9-8)	DvH DvMF 27774	14 (13-16) 4 (3-5) 58 (48-71)	27.5
Nitrate, NO ₃ ⁻	46 (34-62)	8.0 (7.0-9.0)	51 (40-65)	DvH DvMF 27774	38 (32-45) 32 (30-34) 104 (74-145)	5.75
Nitrite, NO ₂ ⁻	5.5 (3.3-9.3)	0.12(0.1-0.4)	0.42 (0.32-0.56)			45.8
Monofluorophosphate, FPO ₃ ²⁻	>100	1.9(0.29-4.4)	1.2 (0.88-1.6)			53
Vanadate, VO ₄ ³⁻	2.7 (0.89-8.2)	0.17 (0.12-0.26)	>10			16
Molybdate, MoO ₄ ²⁻	0.69 (0.24-1.9)	0.031 (0.026-0.037)	0.089 (0.043-0.19)			100
Tungstate, WO ₄ ²⁻	1.3 (0.57-3.0)	0.0024 (0.00075-0.0079)	1.6 (0.71-3.4)			542

¹ *Non-specific* compounds with an SI of <2.5, such as chromate and arsenate, are omitted from this table

² IC₅₀ = concentration of compound required to inhibit 50% of growth or sulfide production over 48h, relative to un-inhibited control

³ ME growth = growth of marine enrichment, measured via optical density (OD600); ME sulfide = sulfide production of marine enrichment community (Cline assay, [96]); G20 = *Desulfovibrio alaskensis* G20; DvH = *Desulfovibrio vulgaris* Hildenborough; DvMF = *Desulfovibrio vulgaris* Miyazaki F; 27774 = *Desulfovibrio desulfuricans* 27774

⁴ SI = ME growth IC₅₀ / ME sulfide IC₅₀

Table 1.2: Summary of research pertaining to the specificity of sulfate transport/sulfate transporters.

Organism	Oxyanion(s) tested	Main Results	Reference
Whole cell experiments in SRM			
<i>Desulfohalobus propionicus</i> and <i>Desulfococcus multivorans</i>	100uM of analogs and 2.5uM ³⁵ S-sulfate (i.e. 40-fold excess analog)	Accumulated ³⁵ S-sulfate was quickly released from cells within 1min, with addition of selenate (65% in <i>Desulfohalobus propionicus</i> /75% in <i>Desulfococcus multivorans</i>), thiosulfate (53%/80%) and chromate (89%/84%). Molybdate (14%/44%), tungstate (13%/31%) and selenite (34/-1%) had no significant influence.	[7]
<i>Desulfovibrio desulfuricans</i> , strain CSN	Analog added at 100uM; 2.5 uM of labelled ³⁵ S-sulfate	Accumulation of sulfate was not affected by molybdate, tungstate or selenite (result in 105%, 107% and 107% of control sulfate uptake, respectively). Chromate caused complete inhibition of sulfate uptake (2% of control); and selenate and thiosulfate caused partial inhibition (50% and 39%).	[6]
SulP			
<i>Mycobacterium tuberculosis</i> SulP in <i>E. coli</i>	5mM Selenate, sulfite, and thiosulfate; 100uM ³⁵ S-sulfate	Induction of SulP expression in <i>E. coli</i> increased sulfate uptake. Sulfate uptake was inhibited by 5mM sulfite, selenate, and thiosulfate, and to a lesser degree by molybdate.	[97]
<i>Saccharomyces cerevisiae</i>	Selenate/chromate	Selenate-resistant mutants allow the identification of two sulfate permeases; sulfate uptake deficiency and selenate resistance in double mutants.	[60]
<i>Arabidopsis thaliana</i>	Selenate	A lesion in SulP (SULTR1;2) causes an 8-fold increase in selenate resistance (LS50 increases from 1.5mM for wild type to 12.7mM in mutant plant). This mutant has also reduced ³⁵ S-sulfate uptake, and allows less selenate to leak into cells (i.e. has a higher sulfate:selenate ratio compared).	[63]
<i>Arabidopsis thaliana</i>	Selenate (20uM) and chromate (40uM)	Resistance to selenate caused by lesions in SulP. Mutants are deficient in sulfate transport.	[62]
<i>Arabidopsis thaliana</i>	Selenate	Sultr1;1 and Sultr1;2 double mutant shows a drastic decrease in sulfate uptake and significant increase in selenate resistance	[98]
<i>Stylosanthes hamata</i> SulP (SHST1) in yeast	Molybdate	Expression of SHST1 in sulfate-uptake deficient yeast (YSD1) increases sulfate and molybdate uptake. Molybdate at 25uM reduces influx of sulfate (also at 25uM) by 48%. Molybdate transport (80nM) is not affected by an equal or 10x concentration of sulfate, tungstate or nitrate.	[99]
CysP			
<i>B. subtilis</i> CysP in <i>E. coli</i>	chromate	<i>B. subtilis</i> CysP confers sensitivity to chromate in sulfate-transport deficient <i>E. coli</i> strains. Results indicate that CysP can transport chromate.	[57]
CysZ			
<i>E. coli</i>	chromate	CysZ mutants are resistant to chromate	[64]
DASS			
<i>Rhodobacter capsulatus</i>	10mM molybdate 5mM tungstate, or 25uM vanadate	Identification and characterization of PerO, a general oxyanion uptake permease. A perO mutant displayed resistance to concentrations of molybdate, tungstate and vanadate that are toxic to wild type cells.	[55]
Various PerO in <i>R. capsulatus</i>	Molybdate, tungstate, vanadate	PerO permeases from the <i>Agrobacterium tumefaciens</i> , <i>Dinoroseobacter shibae</i> , <i>Rhodobacter sphaeroides</i> , <i>Sinorhizobium meliloti</i> , and <i>Pseudomonas stutzeri</i> can all complement the sulfate-uptake function of a sulfate-uptake deficient strain of <i>R. capsulatus</i> . Disruption of <i>perO</i> in <i>A. tumefaciens</i> , <i>R. sphaeroides</i> , and <i>S. meliloti</i> lead to increased tungstate or molybdate resistance	[56]

Table 1.3: Alternative substrates and inhibitors of Sat enzymes from various organisms.

Organism	Biochemical properties	Reference
Alternative substrates (divalent oxyanions)		
<i>Aquifex aeolicus</i>	Sat can turn over tungstate, molybdate, arsenate, selenate, and chromate (relative V _{max} values in the order listed); V _{max} of forward reaction with molybdate (molybdolysis) is 3.6 U/mg protein vs. with sulfate is 1.5 U/mg protein.	[100]
<i>Penicillium chrysogenum</i>	V _{max} for molybdolysis reaction is 100 times greater than for sulfate (18.5 vs 0.13 uM/min mg protein, respectively)	[101]
	k _{cat} values (s ⁻¹): tungstate (26.6) > molybdate (23.9) > sulfate (10.2) > chromate (3.4) > monofluorophosphate (2.3) > selenate (2.1/1.0 depending on assay) V _{max} (U/mg protein): APS synthesis (10.2); molybdolysis (22.8)	[73]
	V _{max} (U/mg protein): tungstate (30) > molybdate (24) > sulfate (8.7) > chromate (4.4) > selenate (1.8)	[102]
<i>Penicillium chrysogenum</i> <i>Saccharomyces cerevisiae</i>	V _{max} (U/mg protein) <i>P. chrysogenum</i> : molybdate (21) > sulfate (9) > monofluorophosphate (2.6) > selenate (1.8) <i>S. cerevisiae</i> : molybdate (22) > sulfate (5.1) > monofluorophosphate (1.8) > selenate (1.5)	[103]
Rat liver	V _{max} (U/mg protein): tungstate (11.6) > molybdate (10) > chromate (2.4) > sulfate (1.0) > selenate (0.2)	[78]
Spinach leaf (cytosol and chloroplast)	V _{max} (U/mg protein): Cytosol form: molybdate (120) > (sulfate (14.6) > selenate (3.66) Chloroplast form: molybdate (229) > sulfate (16.1) > monofluorophosphate (11.1) > selenate (4.1)	[74]
Dead-End Inhibitors (monovalent oxyanions)		
<i>Aquifex aeolicus</i>	Relative potencies: FSO ₃ ⁻ ≈ ClO ₄ ⁻ > ClO ₃ ⁻ > NO ₃ ⁻	[100]
<i>Penicillium chrysogenum</i>	Nitrate and chlorate inhibit 81 and 91% of forward (APS synthesis) reaction	[101]
	K _i (uM): FSO ₃ ⁻ (7.4) ≈ ClO ₄ ⁻ (7.9) > ClO ₃ ⁻ (21) > NO ₃ ⁻ (69)	[73]
	Sat inhibited by FSO ₃ ⁻ , ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ ; FSO ₃ ⁻ most potent	[104]
	K _i (uM): FSO ₃ ⁻ (18) > ClO ₄ ⁻ (18) > ClO ₃ ⁻ (68) > NO ₃ ⁻ (192)	[103]
<i>S. cerevisiae</i>	K _i (uM): FSO ₃ ⁻ (25) > ClO ₄ ⁻ (29) > ClO ₃ ⁻ (44) > NO ₃ ⁻ (116)	[103]
Rat liver	K _i (mM): ClO ₃ ⁻ (0.033 ± 0.013) > ClO ₄ ⁻ (0.28 ± 0.14) > FSO ₃ ⁻ (0.5 ± 0.08) > NO ₃ ⁻ (1.13 ± 0.23)	[78]
Rat chondrosarcoma	K _i is 1.97 ± 0.4mM for ClO ₄ ⁻	[79]
Soybean	Sat displays 35%, 66% or 80% of APS synthase activity with 100uM chlorate, nitrate and carbonate respectively; K _i for ClO ₃ ⁻ is 103 ± 19uM;	[105]
<i>Brassica capitata</i>	Chlorate and nitrate are dead-end inhibitors of Sat; competitive with molybdate	[106]
Spinach leaf (cytosol and chloroplast form)	K _i (uM) for cytosol form: ClO ₃ ⁻ (10) > ClO ₄ ⁻ (50) > FSO ₃ ⁻ (210) > NO ₃ ⁻ (300) K _i (uM) for chloroplast form: ClO ₃ ⁻ (4.9) > ClO ₄ ⁻ (24) > FSO ₃ ⁻ (100) > NO ₃ ⁻ (135)	[74]

Chapter 2

Dissecting the inhibition of sulfidogenic communities by the specific inhibitor perchlorate

Abstract

The sulfate analog perchlorate is emerging as a promising inhibitor of SRM across sulfidogenic systems. The aim of this work is to quantitatively dissect the inhibitory dynamics of perchlorate. Sulfidogenic mixed continuous-flow systems were treated with perchlorate. SRM number, sulfide production and community structure were monitored pre-, during and post-treatment. The data generated was compared to a simple mathematical model, where SRM growth slows as a result of inhibition. The experimental data supports the model that perchlorate acts largely to decrease SRM growth rates, rendering planktonic SRM increasingly susceptible to wash-out. Surface-attachment is identified as an important parameter preventing SRM wash-out and thus governing inhibitory dynamics. A subsequent experiment confirmed the lesser depletion of surface-attached SRM as compared to planktonic SRM during perchlorate treatment. Indirect effects of perchlorate (bio-competitive exclusion of SRM by dissimilatory perchlorate-reducing bacteria, DPRB) were also assayed by amending reactors with DPRB. Indeed, low concentrations of perchlorate coupled with DRPB amendment can drive sulfide concentrations to zero. Further, inhibition in a complex community is compared to that in a pure culture, highlighting similarities and differences between the two scenarios. Finally, we quantify susceptibility to perchlorate across SRM in various culture conditions, showing that prediction of complex behavior in continuous systems from batch results is possible. This study thus provides an overview of the sensitivity of sulfidogenic communities to perchlorate, as well as mechanisms underlying these patterns.

2.1 Introduction

Microbial sulfate reduction (SR) by sulfate-reducing microorganisms (SRM) is one of the main pathways of anaerobic terminal organic matter mineralization, and as such is a determinant of carbon and sulfur cycling in many environments [3, 90, 107, 108]. SR results in the generation of hydrogen sulfide, a toxic gas with adverse human health effects [109]. Sulfide production also has significant economic and environmental consequences owing to its corrosive nature [107]: Roberts et al. quantified sulfide-induced concrete corrosion rates of up to 5mm/year in sewer systems [110]. Further, reservoir souring – sulfide production in oil reservoirs – causes a decrease in oil quality, infrastructure corrosion, and reservoir plugging (Gieg et al., 2011). As a result of this economic and industrial importance, SR has been studied with a multiplicity of different tools, and in various systems [3, 90, 111, 112].

As outlined in Chapter 1, central to studies on the ecology of SR has been the identification and characterization of specific inhibitors of SRM. Specific inhibitors are compounds that disrupt the metabolism of one group of organisms, with little or no effect on the rest of the community [11]. Such compounds have been used in microbial ecology to uncover electron flow through certain metabolic processes in natural systems [11]. Additionally, specific inhibitors are of potential value to industrial systems. Postgate (1952) was the first to screen a panel of sulfate analogs in search of specific inhibitors of the sulfate reducer *Desulfovibrio desulfuricans* strain Hildenborough, and identified perchlorate, among other inhibitors [14]. Perchlorate has since been characterized as a competitive inhibitor of the SR pathway [28]. Furthermore, since perchlorate can also serve as an electron acceptor for microbial respiration, it has several indirect mechanisms of inhibition: (1) bio-competitive exclusion, whereby dissimilatory perchlorate-reducing bacteria (DPRB) perform a more energetically favorable metabolism than SR and thus outcompete SRM [21, 23]; (2) production of toxic intermediates by DPRB: chlorite or oxygen; and (3) sulfide re-oxidation to insoluble sulfur by DPRB [22, 113]. Due to its potency and selectivity, perchlorate has been proposed as a promising compound for the inhibition of sulfide production in oil reservoirs [21, 24, 28, 114]

Much of the current insight on specific inhibition of SR has been gained via pure culture studies [13, 17, 28, 69, 115]. Although useful in interrogating mechanistic aspects, this pure culture, model organism view is challenging to extrapolate to complex natural communities. On the other hand, several studies have examined the effects of perchlorate in complex systems [10, 21, 24, 28, 114, 116]. These systems, though highly relevant, are confounded by complexity and variability. Here, we use a simple planktonic, continuous-flow, sulfidogenic community to characterize the relevant biology pertaining to inhibition of SR by perchlorate. This set-up allows for the generation of simple mathematical models and the dissection of inhibitory dynamics, yet retains biological complexity and relevance.

We characterize the effects of perchlorate in terms of the rate and level of inhibition of SR, as well as the rate and level of post-treatment rebound of SR. Empirical data is compared to a simple model, elucidating two factors key to inhibitory dynamics, namely: (1) the dose-response/inhibition curve of SRM against perchlorate and (2) surface-attachment of microbial cells. Surface-attachment, as well as the indirect effects of perchlorate (mediated by DPRB) are explored further, via direct manipulation. Finally, inhibition in a complex community is compared to that in a pure culture, highlighting similarities and differences between the two scenarios. Together, the experiments performed provide a comprehensive illustration of the inhibitory dynamics of perchlorate, and factors that affect it, both in pure culture and in a community setting.

2.2 Methods

Chemostat Setup and Operation

Chemostats were custom made from glass bottles equipped with influent and effluent lines and a liquid sampling port (Adams & Chittenden, Berkeley, Ca) (Figure 2.1A). Inflow media consisted of 20 g/L NaCl, 0.67 g/L KCl, 6.8 g/L TES, vitamins, and minerals [117], as well as salts prepared separately in a concentrated stock and added post-autoclaving (20x: 21.2 g/100 mL $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 3.04 g/100 mL $\text{CaCl}_2 \times 2\text{H}_2\text{O}$; 50x: 4 g/100 mL NaCl, 5 g/100 mL NH_4Cl , 0.5 g/100 mL KCl, 0.5 g/100 mL KH_2PO_4 , 1 g/100 mL $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.1 g/100 mL $\text{CaCl}_2 \times 2\text{H}_2\text{O}$). Yeast extract (2 g/L) or sodium lactate (60 mM) served as the electron donor and carbon source and 20mM sodium sulfate served as the electron acceptor. Media was flushed with N_2 to render it anaerobic, and was pumped in from a reservoir bottle (also kept under N_2) at a set flow rate of ~ 2.6 mL/h, resulting in a residence time of approximately 48 h. This flow rate was chosen to accommodate slow-growing microorganisms and maintain diversity within the system. Chemostats were kept on a stir plate at 30°C and effluent drained passively such that a constant volume of $V = 125$ ml was maintained. Replicate chemostats were sterilized, rendered anaerobic (by degassing with N_2) and inoculated at 10% with a marine sulfidogenic community collected from San Francisco Bay sediment and pre-enriched on yeast extract and sulfate. Post inoculation, chemostats were allowed to reach steady-state (defined as stable sulfide production over several days) prior to treatment with perchlorate. Control chemostats were left untreated. Once inhibition stabilized, treatment was ceased and rebound of sulfide was monitored (Figure 2.1B). Sodium perchlorate was added at 20, 50 or 80 mM depending on the experiment. Perchlorate and sulfate concentrations were quantified via ion chromatography on a Dionex ICS-1500 using Dionex IonPac AS25 4X250 mm anion-exchange column, with an AG25 4X50 mm guard column and a 36mM NaOH flow rate of 1ml/min. Sulfide was measured using a modified Cline assay [96] and read at 660 nm on a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Optical density was measured at 600 nm on the same instrument.

An initial chemostat experiment was performed using 50 mM perchlorate, where community structure was monitored via 16s rRNA amplicon sequencing. A second experiment with 20 mM, 50 mM or 80 mM perchlorate was subsequently run to explore the effect of different concentrations of perchlorate. A third experiment assessed increased surface-attachment at 50 mM perchlorate by filling chemostats with glass beads (4.5mm, Zymo Research, Irvine, CA). A fourth experiment using 20 mM perchlorate with and without dissimilatory perchlorate-reducing bacteria (DPRB) amendment teased apart direct and indirect mechanisms of perchlorate. DPRB were added as a mixture of 4 strains: *Arcobacter* sp. CAB, *Sedimenticola selenatireduces* CUZ, *Marinobacter vinifirmus* UCB, and *Denitromonas halophilus* SFB-3 [118–120].

To compare perchlorate inhibitory dynamics in pure culture vs. in a mixed community, the dominant SRM species was isolated from the community, using methods previously described for SRM isolation [111]. Briefly, serial dilutions were performed in agar plugs with the media described above, supplemented with a trace amount of ferrous iron ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$) as ferrous iron reacts with sulfide and renders SRM colonies black. Single colonies were picked and cultured in APM. The purity of the isolate, *Desulfovibrio* sp. BMSR (IMG ID 2687453694), was confirmed via 16s rRNA sequencing, using primers 27F and 1525R [121]. This organism was then used to inoculate a final (fifth) round of chemostats. Sodium lactate (60 mM), supplemented with casamino acids (5 g/L, Bacto, technical grade), replaced yeast extract as the donor for this round

and perchlorate was added at 20 mM or 50 mM. OD600, as well as sulfide, sulfate and perchlorate concentrations were once again monitored. Table 2.1 provides a summary of the five chemostat experiments.

Community Analyses

Chemostats were sampled for planktonic and surface-attached communities: 1ml of culture was spun down (5 min at 13 000 rpm) and immediately stored at -80°C. DNA was extracted using a DNeasy Powersoil HTP 96 kit (Qiagen), following manufacturer's instructions. For surface-attached communities, 15-20 glass beads were collected and DNA was extracted immediately using a DNeasy PowerBiofilm Kit (Qiagen). DNA concentrations were quantified via a Quant-iT HS Kit (ThermoFisher Scientific, Waltham, MA), on an Infinite M1000 Pro microplate reader (Tecan), following manufacturer's instructions.

Amplicon sequencing of the 16s rRNA HV region V4 was conducted using primer pair Miseq 16s F and Miseq 16S R (A519F and Bakt_805R, modified to include Illumina adapters) [120, 122]. Libraries were prepared as per the Illumina 16s Metagenomic Sequencing Library Preparation protocol. For analysis where >96 samples were multiplexed, the Nextera XT Index Kit v2, sets A-D (Illumina) was used. Samples were quantified (Quant-iT), normalized using a Biomek XFP liquid handling robot, and pooled. Sequencing was performed at the UC Davis Genome Center (Davis, CA) on a Miseq (Illumina Miseq Reagent Kit V2). Demultiplexed samples were analyzed according to the Mothur Miseq SOP [120, 123]. Briefly, chimeras were removed using UCHIME [124] and remaining sequences were screened based on an expected length of approx. 287bp. Sequences were then clustered at the 97% similarity level and classified using the RDP and SILVA databases. The resulting OTUs were used to generate an OTU table across samples. Abundances were normalized to total amplicon counts per sample. The proportion of SRM was calculated as the combined relative abundance of the *Desulfovibrio*, *Desulforhopalus*, *Desulfocurvus*, and *Desulfomicrobium* genera. No other known SRM were identified. Primer 7 [125] was used to square-root transform normalized abundance data and generate a Bray Curtis similarity matrix, which was in turn used to create non-metric multidimensional scaling (nMDS) plots. SimProf and SIMPER were used to identify significant groupings and to determine OTUs contributing to differences between groupings [126]. Significance of SIMPER-identified OTUs was confirmed using ANOVA and Sidak's multiple comparison test.

SRM were also quantified using qPCR on the *dsrA* gene (a specific marker of dissimilatory SRM). The *dsrA* gene was PCR amplified with primers DSR1-F+ and DSR-R [127] and cloned into a TOPO cloning vector (TOPO TA Kit, Invitrogen), according to manufacturer's instructions. The vector was quantified (Quant-iT) and used to generate standard curves (300-3000000 copies). Both SYBR and Taqman workflows were used, as previously described [10, 128, 129]. All samples and controls were run in triplicate on a StepOnePlus RealTime PRC System (Applied Biosystems). Negative controls and the size of the PCR amplicon were confirmed via gel-electrophoresis. *dsrA* copy number was normalized to total ng gDNA.

Mathematical Model Construction

A simple mathematical model was developed according to basic chemostat theory [130], which posits that in a well-mixed system, the resident population increases proportional to a maximum growth rate constant and decreases proportional to the washout (flow rate). The

population is limited by an undetermined factor, represented in this case by the carrying capacity, K , of the system. Hence, at steady-state, growth of SRM can be modeled as

$$\frac{dN}{dt} = \mu N \left(1 - \frac{N}{K}\right) - \frac{N}{V} F \quad (1)$$

Where N is the SRM population, μ is the growth rate, K is the carrying capacity of the ‘environment’ towards SRM, V is the volume of the chemostat and F is the flow rate.

Perchlorate inhibition is assumed to affect the growth rate of SRM, which is consistent with previously published work suggesting that the main mechanism by which SRM overcome perchlorate inhibition is by upregulating the SR pathway [28]. Perchlorate concentration in the chemostat (P) is modeled based on the following differential equation:

$$\frac{dP}{dt} = F_{Pin} - \frac{P}{V} F \quad (2)$$

where F_{Pin} is the rate of perchlorate entering the system (equal to the perchlorate concentration in the media reservoir multiplied by the flow rate, F). Perchlorate leaves the chemostat proportional to its concentration in the chemostat ($\frac{P}{V}$) at time t multiplied by the flow, F . Solving this equation then allows for the determination of P through time.

Dose-response curves connect the concentration of perchlorate in the chemostat at a given time, to μ , via the parameter y . The equation governing perchlorate inhibition is:

$$y = \frac{1}{1 + 10^{(\text{LogIC50} - \log[P])(\text{HillSlope})}} \quad (3)$$

Where $IC50$ denotes the concentration inhibiting 50% of SRM, and $HillSlope$ describes the steepness of the slope of the dose-response/inhibition curve (refer to the Measuring Inhibition section of the Methods for the empirical determination of these parameters). Thus, y is a fraction representing the effect of a specific concentration of P . As the model assumes that the only effect of perchlorate is a change in the growth rate, y multiplies by μ_{max} , to give the actual growth rate as a function of perchlorate concentration and time. See Model Parameterization and Validation below for parameter values for equation (3).

Finally, growth was modelled additively as a combination of planktonic SRM (N_P) and surface-attached SRM (N_A), with each population being subject to its own carrying capacity, K_P and K_A respectively:

$$N_t = N_P + N_A \quad (4a)$$

$$\frac{dN_P}{dt} = \mu N_P \left(1 - \frac{N_P}{K_P}\right) - \frac{N_P}{V} F \quad (4b)$$

$$\frac{dN_A}{dt} = \mu N_A \left(1 - \frac{N_A}{K_A}\right) \quad (4c)$$

and from equation (3), $\mu = \mu_{max} \times y$.

The initial model output, N_t , was converted to sulfide concentration, $[sulfide]$, for easier comparison to experimental data, using the regression of measured sulfide and measured N_t .

Measuring Inhibition

Our current understanding of perchlorate inhibition implies a non-biocidal effect of perchlorate on SRM. To confirm this, triplicate *Desulfovibrio sp.* BMSR cultures were incubated in APM media with 20 mM sulfate and with either: no lactate, no lactate and 50 mM perchlorate or lactate (60 mM) and perchlorate (50 mM). Samples were taken at 24h and mixed with LMT agarose (1%), SYTO 9 (1x, Thermo Fisher) and propidium iodide (8 ug/mL, Thermo Fisher). Cells were imaged using a Zeiss AxioImager M2 at the Berkeley Imaging Facility. ImageJ was used to

merge images from different filters and cells were counted manually. Statistical analysis was performed in GraphPad Prism.

Perchlorate inhibition of SR was quantified via dose-response/inhibition curves, generated as previously described [10]. Briefly, 96 or 384-well plates (Costar, ThermoFisher Scientific) containing anaerobic media and a horizontal gradient of perchlorate concentrations, from 0-500mM (in a series of 2-fold dilutions) were made. These gradient plates were inoculated in an anaerobic glove bag (Coy) with culture (pure or mixed community) at an OD of 0.02, sealed, and incubated anaerobically at 30°C for 48-72 h. Growth (OD600) and sulfide (cline assay) were quantified on an Infinite M1000 Pro microplate reader (Tecan). Cline assay was performed in HT using a Biomek XFP liquid handling robot. Data generated was normalized to a control and used to construct dose-response curves in GraphPad Prism 7 (GraphPad Prism Software, Inc., La Jolla, CA) using the “Dose-Response – Inhibition, log(inhibitor) vs. normalized response – variable slope” function. Various donors for growth were used: sodium lactate (40 mM), sodium pyruvate (20 mM), sodium fumarate (20 mM), sodium succinate (20 mM), glucose (30 mM) and yeast extract (2 g/L). Dose-response/inhibition curves were also generated from growth-curve data. Briefly, triplicate anaerobic tubes were inoculated under various perchlorate concentrations and either/and OD600 (measured on a Spectronic 20D+) or sulfide (measured via cline assay) were monitored. Data generated was used to construct growth curves and calculate growth rates. Growth rates were normalized to the control growth rate and used to construct dose-response/inhibition curves, as described above. IC50 and Hillslope values determined were used in the model described above.

2.3 Results

Inhibitory dynamics of perchlorate in a sulfidogenic chemostat community

To test the inhibitory dynamics of sulfidogenesis in the presence of perchlorate, a mixed sulfidogenic community was inoculated into triplicate chemostats, treated with 50 mM perchlorate and compared to untreated control chemostats. Upon treatment, sulfide decreased rapidly from 2.0 ± 0.57 mM and stabilized at 0.17 ± 0.076 mM (Figure 2.2A, Figure 2.3A). This decrease in sulfide corresponded to a decline in the SRM population, as evidenced by qPCR of the *dsrA* gene as well as by the percentage of 16s rRNA amplicons assigned to known SRM (Figure 2.2B-C). SRM represented $33 \pm 8.2\%$ of the steady-state community, and fell to $1.3 \pm 0.33\%$ during treatment. Rebound post-treatment was immediate, and pre-treatment levels of sulfide were reached.

The 16s rRNA amplicon data generated allowed us to dissect community-wide effects of perchlorate. An nMDS plot showed that changes in communities clustered by treatment as well as by time (groups I-IV), with treated communities undergoing the same temporal effect as control communities, and rebounding to the same end-point. Control and treated communities grouped together before and after the treatment phase (at >70% similarity), and separated, at 35% dissimilarity, during treatment (Figure 2.4A). SIMPER analysis revealed that 6 groups contributed over 50% to the total difference between treated and control communities during treatment: *Desulfovibrio*, *Spirochaeta*, *Acetoanaerobium*, *Petrimonas*, *Fusobacterium*, and a group of unclassified *Bacteroidetes* and Bacteria. *Desulfovibrio* declined from $19 \pm 6.7\%$ to <1% of the community, representing the largest contribution (11%) to the difference between treated and control communities during treatment. Several non-SR organisms also decreased significantly with treatment: *Spirochaeta*, *Acetoanaerobium*, and *Petrimonas* (Kruskall-Wallis test, $p < 0.001$).

Conversely, there was an increase in relative abundance of other, likely fermentative organisms including *Fusobacterium*, the unclassified *Bacteroidetes* and other unclassified *Bacteria*. Interestingly, *Desulfocurvus*, the only other SRM present in any significant proportion, as well as *Dethiosulfovibrio* were unaffected by perchlorate treatment (Figure 2.4B).

A subsequent chemostat experiment was performed using 20 mM, 50 mM and 80 mM perchlorate (Figure 2.5, Figure 2.3B). At 50 mM perchlorate, very similar behavior to the initial chemostat experiment was observed, with sulfide dropping from 2.2 ± 0.64 mM to 0.16 ± 0.010 mM. At 80 mM perchlorate, inhibition was most pronounced (drops to 0.067 ± 0.022 mM). Once again, rapid rebound was observed upon cessation of treatment (Figure 2.6). The qPCR data validated these trends. The inhibitory effect of 20 mM perchlorate was less clear. At 20 mM, the system seemed significantly inhibited initially, with sulfide falling to 0.64 ± 0.060 mM ($p=0.0012$, t-test for 20 mM 660 h-852 h vs all control at all time-points), but then rebounding to control levels within 250 h ($p=0.2$ for 20 mM at 899-1114 h vs control at all time-points). qPCR of *dsrA* showed a trend of slight inhibition of SRM throughout treatment, although this trend was non-significant ($p=0.4$ for 20 mM 539 h-1114 h vs control at all time-points). In subsequent runs, there was no significant effect of 20 mM perchlorate on sulfide production.

Model parameterization and validation

A theoretical model of inhibition was constructed based on the assumption that perchlorate affects the growth rate of SRM. In support of this, it has been shown that SRM can overcome perchlorate inhibition via increased de-repression of the SR pathway (i.e. perchlorate is not biocidal but rather competes with sulfate for enzyme active sites), and additionally that pyruvate-fermenting *D. alaskensis* G20 cells are uninhibited by >100 mM perchlorate [10, 28]. To confirm this non-biocidal effect, triplicate populations of *Desulfovibrio* sp. BMSR were exposed to 50 mM perchlorate for 24 h, with and without lactate (donor). The proportion of dead:live cells was compared to that in control cultures with no lactate and no perchlorate, via live-dead staining. All cultures exhibited a similar proportion of dead cells post-incubation ($21 \pm 6.0\%$, $p>0.2$ for all pairwise comparisons via Kruskal-Wallis test).

The max growth rate ($\mu_{max} = 0.13h^{-1}$) for the model was determined from batch growth curves of community sulfide production. The dose-response/inhibition curve of perchlorate against SRM was also determined empirically. The final function for quantifying the perchlorate effect (y) on μ is based on growth rates (Figure 2.7A-B), and is as follows:

$$y = \frac{1}{1 + 10^{(-1.914 - \log[P])(-1.813)}} \quad (5)$$

From here, y is multiplied by μ_{max} ($0.13h^{-1}$) to give the actual growth rate, μ . In the final model, the population of SRM then follows equations 4a – 4c.

The model was calibrated using data from the initial chemostat run. N_t was set to 40% based on the average % SRM in control chemostats over time ($\sim$ steady state SRM population). Several surface-attached fractions were simulated before settling on $N_A = 0.5\%$. The model output was converted from % SRM to [sulfide], according to the regression of $\log(\text{sulfide})$ against $\log(\% \text{reads})$ ($R^2=0.79$):

$$[\text{sulfide}] = 10^{(0.6786 \times \log(N_t) - 0.7375)} \quad (6)$$

The model was then validated against the sulfide data generated in the subsequent chemostat run at 20, 50 and 80 mM perchlorate (Figure 2.5C-E). Predictions agreed well with empirical results, supporting the model that perchlorate acts largely to decrease μ , rendering planktonic SRM increasingly susceptible to wash-out. Simulation results highlighted the

importance of several parameters: (1) The max growth rate, μ_{max} was critical in establishing rebound: if it was slower than the residence time, SRM did not rebound (Figure 2.8A). (2) As the dose-response/inhibition curve was responsible for setting the actual μ , it also defined the shape of the inhibition curve (Figure 2.8B). (3) Finally, surface attachment played a role (Figure 2.8C): in the absence of surface-attached SRM, sulfide decreased continuously, asymptotically towards 0. This is in contrast to empirical observations, where a steady “inhibited” sulfide level and SRM population were reached.

The role of surface-attachment

Surface-attachment may alter inhibitory dynamics by preventing SRM washout. If surface-attached SRM constrain inhibition, then increasing their proportion relative to planktonic SRM should decrease the effectiveness of perchlorate and result in higher “inhibited” sulfide levels. To achieve this effect, we added glass beads to chemostats, increasing surface area for attachment, and treated with 50 mM perchlorate. This chemostat run produced higher levels of sulfide than previous runs ($7.5 \text{ mM} \pm 2.2 \text{ mM}$). However, inhibition followed a similar pattern to these previous runs when 50 mM perchlorate was applied, and qPCR validated this trend (Figure 2.9, Figure 2.3C). Normalized to the total sulfide production, the rate of inhibition (decline in sulfide upon treatment) was not significantly different between planktonic and biofilm runs (Graphpad Prism linear regression analysis, $p=0.3$). However, rebound in these chemostats was not as obvious as previously observed (Figure 2.6).

Amplicon sequencing of planktonic and surface-attached populations confirmed the inhibition of SRM. SIMPER analysis identified *Desulfovibrio* as, again, contributing 11% to the difference between control and treated planktonic communities, and 8% for biofilm communities. A nMDS plot showed control samples and pre-treatment treated samples grouped together, and that with treatment, treated samples separated out. The same trend is true for biofilm communities. However, planktonic communities showed a greater separation than biofilm communities upon treatment (36% vs 32% dissimilarity between control/pre-treatment and treatment groups) (Figure 2.10A). Examining the SRM population specifically confirmed that planktonic SRM were significantly more inhibited than surface-attached SRM ($p=0.011$, Mann-Whitney test), with surface-attached SRM dropping to $12 \pm 6.5\%$ and planktonic SRM dropping to $3.9 \pm 1.4\%$ of the community (Figure 2.10B). The proportion of SRM in planktonic and surface-attached control communities, on the other hand, was not significantly different ($p=0.45$, Mann-Whitney test). Interestingly, *Desulfobacterium* and *Desulforhopalus* were enriched in the surface attached SRM fraction as compared to the planktonic (Figure 2.10C). *Desulfobacterium* was indeed inhibited during treatment, but *Desulforhopalus* was unaffected.

Indirect effects of perchlorate

While our experimental data and model confirmed the direct inhibitory effect of perchlorate on SRM, particularly at concentrations greater than or equal to media sulfate concentrations (20 mM), perchlorate also has indirect effects on sulfate-reduction. Dissimilatory perchlorate reducing bacteria (DPRB) can outcompete SRM and can also oxidize sulfide, removing it from the system altogether [21–23, 113]. Previous work has been unable to empirically distinguish between direct and indirect effects. Since the community in this study did not contain

DPRB, it presented us with an opportunity to test the magnitude of indirect effects by amending treated communities with DPRB.

Amendment with a mix of DPRB (1:10 inoculum) was initially tested in batch communities treated with 20 mM perchlorate, and led to complete sulfide removal coupled to the reduction of 7.7 ± 2.6 mM perchlorate. Indirect effects of perchlorate were subsequently tested in chemostats. Planktonic sulfidogenic communities were allowed to reach steady sulfide production and amended with either 20 mM perchlorate or 20 mM perchlorate and a spike of DPRB (phase 1). The initial amendment with DPRB had no significant effect on sulfide production (Figure 2.11A). The continuous generation of sulfide may have forced DPRB to continuously oxidize it, which they cannot couple to growth [113], thus causing them to be washed out of the system. To prevent washout, 60 mM lactate alone or with the DPRB was spiked into chemostats and flow was shut off (phase 2). In this treatment phase, sulfide production was enhanced in both the control and 20 mM treated chemostats, but remained level in 20 mM + DPRB chemostats. Thus, the DPRB did indeed inhibit sulfide production, but were not able to completely eliminate sulfide. In phase 3, flow was reinstated and perchlorate was replaced with 20 mM nitrate, in order to give perchlorate reducers, most of whom preferentially reduce nitrate, a competitive advantage [131, 132]. This completely eliminated sulfide in all chemostats containing nitrate, irrespective of the presence of DPRB. Nitrate reduction and nitrite production was observed in both amended and un-amended communities (Figure 2.3D), likely due to native organisms capable of nitrate reduction, such as *Shewanella*. Nitrate treatment was suspended and perchlorate (20 mM) or perchlorate and a large inoculum (30%) of DPRB was added (phase 4). This eliminated sulfide in the perchlorate and DPRB-amended replicates, briefly. However, subsequently 20 mM and 20 mM+DPRB treatments became indistinguishable again in two out of the three replicates, starting at hour 1700. One replicate maintained complete sulfide inhibition for the duration of perchlorate treatment, and concurrent perchlorate reduction was seen (Figure 2.3D).

An nMDS of the microbial community data confirmed that the community in this replicate was different during treatment (group I, Figure 2.11B). Comparing groups I and II revealed a significantly greater presence of *Sedimenticola* in group I, as well as a greater enrichment of *Marinobacter* (Figure 2.11C). The inhibition of SRM *Desulfovibrio* and *Desulfohalobulus* was also enhanced in group I compared to group II. Finally, the significant inhibition of a group of unclassified Bacteria in I compared to II suggested a possible negative interaction between these microbes and the DPRB.

Inhibitory dynamics in Desulfovibrio sp. BMSR

To further understand direct perchlorate inhibition of SRM in the chemostats, the dominant SRM from this community, *Desulfovibrio* sp. strain BMSR (IMG genome ID 2684623040), was isolated and tested in an additional chemostat run with 20 mM or 50 mM perchlorate. The isolate, *Desulfovibrio* sp. BMSR, was characterized in terms of max growth rate and dose-response/inhibition for perchlorate (Figure 2.7C-D).

$$\mu_{max} = 0.2$$

$$y = \frac{1}{1 + 10^{(-2.099 - \log [P])(-1.108)}}$$

The max growth rate was higher than for the community but the inhibition curve was similar. Perchlorate at 20 mM showed no significant effect on sulfide production ($p=0.8$ t-test for control vs 20mM treatments over all replicates and treated time-points). However, at 50 mM

treatment, sulfide showed a pronounced decline in two out of three replicates (Figure 2.12A). Sulfide measurements for the 3rd replicate dropped briefly, but recovered and were unaffected thereafter. In contrast to earlier community experiments, sulfide inhibition lagged, i.e. sulfide was unaffected for over 100h after treatment starts. Further, in this pure culture experiment perchlorate treatment was never stopped, yet the two inhibited replicates rebounded to control sulfide levels. The communities were tested in batch for their susceptibility to perchlorate, and indeed showed adaptation to the inhibitor, with IC50s for all treated cultures increasing 1.2 to 2.6-fold over controls (Figure 2.12B).

Several additional, as of yet unexplored, factors may affect sensitivity of SRM to perchlorate. First, the electron donor for sulfate-reduction in our community was unknown and was likely a complex mixture as the community experiments were performed on yeast extract. Hence, the effect of donor on IC50 was elucidated using *Desulfovibrio* sp. BMSR grown on a variety of carbon sources (lactate, pyruvate, fumarate, succinate, glucose) and 20 mM sulfate. Sensitivity depended on the donor, particularly in the case of pyruvate ($p < 0.0001$, Extra sum-of-squares F test) (Figure 2.13). Second, the community contained a mixed SRM population in which each individual SRM likely displayed a different sensitivity to perchlorate. The hypothesis that the sensitivity of the mixed SRM culture is determined by the sensitivity of the most resistant SRM was tested by co-culturing *D. sp. BMSR* with *D. alaskensis* G20. G20 was more resistant to perchlorate and, as expected, the co-culture (consisting of the two isolates in a 1:1 initial ratio) had the same dose-response curve against perchlorate as the pure-culture G20 (Figure 2.14A). Finally, the presence of fermentative organisms might be either antagonistic or synergistic with perchlorate inhibition. In order to address this, the fermentative fraction of the community was sub-cultured in media lacking sulfate and subsequently combined with *D. sp. BMSR* at a 1:1 ratio. Here again, sensitivity to perchlorate of the fermenter-amended co-culture was dictated by the SRM (Figure 2.14B). Thus, strikingly, the inhibitory response of a mixed community growing on a specific electron donor may be modeled based on the sensitivity of the most-resistant SRM in that community, and on that particular donor.

2.4 Discussion

The aim of this study was to elucidate the various mechanisms of perchlorate inhibition in complex sulfidogenic communities. The initial chemostat experiment confirmed that perchlorate at 50 mM is a potent direct inhibitor of SRM. SRM inhibition coincided with the relative enrichment of several presumably fermentative organisms. Members of the *Fusobacterium* genus are known fermenters, producing butyrate and acetate from various sugars, amino acids and small peptides and the enrichment of *Fusobacterium* under perchlorate treatment has been previously characterized [21, 133]. The same ecological niche likely existed for the unclassified *Bacteroidetes*/Bacteria comparatively enriched during treatment. Several non-SRM were negatively affected by treatment. Members of *Spirochaeta* are typically fermentative but some species require sulfide [134, 135]. Similarly, the growth of *Petrimonas*, a presumed fermenter, can be stimulated by the presence of sulfur [136] and a decline in sulfide may have led to a decline in abiotically produced sulfur. Interestingly, *Dethiosulfovibrio* and *Desulfocurvus* were not significantly affected by the presence of perchlorate; these genera may have persisted via thiosulfate reduction [137–141]. Patterns of inhibition and relative enrichment were similar across all chemostat runs and indicated that the largest effect was on the dominant SRM, *Desulfovibrio* sp.

By combining empirical data with mathematical modeling, we were able to further our understanding of perchlorate inhibition. Models of microbial growth often include a substrate parameter, as substrate concentration and type govern microbial growth rates [142–144]. In this study, the donor for sulfate reduction remained unknown and was likely complex. To exclude this parameter from the model, μ was empirically determined in batch, on yeast extract and sulfate, and assumed to be relevant to chemostats run on the same donor and acceptor. Further, the lack of known donor/nutrient requirements for SR necessitated an alternative mechanism to limit the SRM population. A “carrying capacity” term, K , was chosen and empirically determined. In this way, the complexity of the system was reduced to two parameters: the empirical μ of the mixture of SRM on a mixture of donors, and the empirical K , the maximum sustainable SRM population size in this system. Both parameters were assumed to stay constant over the course of inhibition. μ was a critical model parameter as it encompassed the effect of perchlorate inhibition and determined both the level of inhibition as well as the ability of sulfidogenesis to rebound post-treatment. In a system where flow was faster than μ , no rebound could occur. The second critical parameter was the dose-response/inhibition curve itself, which we empirically determined via batch growth curves. The defined function captured inhibition well in chemostats, confirming both that inhibition in batch can be used to predict inhibition in continuous-flow systems and that, in continuous-flow, the total effect of inhibition can be constrained to the effect on μ . The model was predictive at 50 mM and 80 mM perchlorate. Interestingly, 80 mM perchlorate did not seem to dramatically increase inhibition, suggesting a threshold concentration, treatment above which it is not economical. At 20 mM, there was a slight decoupling between the effect on sulfide and the effect on *dsrA* copy number. *Desulfovibrio alaskensis* G20, when exposed to competitive inhibitors, can upregulate the pathway for sulfate reduction and slightly decrease its sensitivity to perchlorate [28]. While this slight advantage would be less relevant at high perchlorate concentrations, at low concentrations it may overcome the inhibitory effect on sulfide production. Furthermore, several genera of non-SRM in this system have the capacity for low-level sulfide production upon cysteine/methionine degradation or directly from elemental sulfur (*Fusobacterium*, *Spirochaeta*) [133, 134]. At low concentrations of perchlorate, these alternative mechanisms might represent a larger confounding source of sulfide.

The model also emphasized the importance of surface attachment. SRM in biofilms were less subject to wash-out and hence could maintain a certain low level of sulfide production. Surface area was experimentally manipulated via the addition of glass beads. Although these chemostats were not stirred, diffusion did not seem to be a major issue (likely due to mixing during daily sampling events) as perchlorate concentrations followed the model well, which would not be expected in a non-uniform, diffusion-limited system (Figure 2.3C). It is also important to note that the surface-attached and planktonic populations were modeled as separate; in reality they are likely interconverting and interdependent. This is a complication that is not relevant to model predictions and functioning, as both populations are assumed to be affected by perchlorate according to the same function. It is possible that biofilm cells are innately more resistant to competitive inhibition (different inhibition function). The alignment of inhibition rates between planktonic and biofilm cultures, however, suggested otherwise. Furthermore, current evidence for overcoming perchlorate inhibition is limited to de-repression of the *sat* gene [28], which should be a similar effect in biofilm and planktonic cultures. There is no evidence currently suggesting that SRM upregulate central genes for sulfate-respiration as a specific response to growth in biofilms [145]. As noted previously, the ‘biofilm’ chemostats produced more sulfide, perhaps due to a change in the relative proportions of the dominant sulfate-reducers. In any case, the level of inhibition was more limited,

with sulfide stabilizing at a higher level, in keeping with a higher level of surface attachment. The relative persistence of surface-attached populations was verified by amplicon sequencing of the biofilm and planktonic community fraction. Previous studies in our lab also show that SRM can persist in sediment flow-through systems upon treatment [24], possibly a combination of surface-attachment and SRM performing fermentative metabolisms which are largely unaffected by perchlorate. Thiosulfate reduction may also present an alternative mechanism for persistence (i.e. via sulfide production that bypasses sulfate). Indeed, *Dethiosulfovibrio* are consistently an important part of perchlorate-treated communities.

We also explored indirect effects of perchlorate inhibition. At concentrations of perchlorate that are minimally directly inhibitory (~ 20 mM), DPRB eliminated sulfide completely. However, in our chemostats, the establishment of DPRM and elimination of sulfide was dependent on the community context. Perchlorate treatment in the field would enrich for native organisms capable of perchlorate reduction, likely more competitive in their given environment than exogenously introduced DPRB. Previous work in our lab has successfully enriched for perchlorate reduction in marine systems, concomitant with total suppression of sulfide [21, 23, 24, 114]. These were sediment systems and spatial stratification likely played a role in helping enrich for perchlorate reducers: DPRB could be spatially separated from SRM and from sulfide, whereas in our stirred chemostats DPRB were constantly encountering high levels of sulfide, which is inhibitory to their growth as it short circuits their electron transport chain preventing the generation of proton-motive force. Interestingly, in the chemostats, nitrate treatment had a drastic effect on sulfide production. Several genera (*Shewanella*, *Denitrovibrio*, *Petrimonas*) are capable of nitrate reduction and nitrite accumulation, and hence SRM inhibition via indirect effects. Nitrate is a current mechanism for reversing sulfide production in commercial oil reservoirs, and has been applied with varying degrees of success in various systems [18, 20, 146–150].

Inhibition may also be modulated by donor used for growth. In our system, *D. sp.* BMSR grown on pyruvate-sulfate was significantly more resistant than when grown on lactate-sulfate. The energetics and electron flow of pyruvate-sulfate grown *D. alaskensis* G20 and lactate-sulfate grown G20 differ [151]; how this difference potentially translates to a difference in sensitivity remains to be addressed. Further, sensitivity of BMSR did not depend on whether it was grown in pure culture or in co-culture with other fermenters. As such, at least in this system, inhibition of SRM was independent of other community members. The *D. sp.* BMSR chemostat run with 20 and 50 mM perchlorate mimicked community results, and could be modeled by adjusting μ , the interconversion factor between N_t and $[sulfide]$ and the dose-response/inhibition curve of perchlorate. Hence, remarkably, whether in a complex community or in a pure culture, the batch determination of a few parameters could be used to predict inhibition. Finally, this pure-culture chemostat run uncovered the relative ease with which pure cultures of SRM adapt to perchlorate. Adaptation of SRM in the community was not seen over the course of this study, although it was specifically assayed for after each chemostat run. Adaptation has also not been seen in other long-term community studies of perchlorate inhibition in this lab, making our observation of adaption in pure-cultures all the more interesting. Future work will aim to describe mechanisms of adaptation to perchlorate, both in pure and mixed culture.

In summary, the addition of perchlorate led to slower growth of SRM populations, which rendered them susceptible to washout. This effect could be partially mitigated by increased surface-attachment. This principal governed inhibition in a pure culture as well as in a mixed community. The data presented here highlight how simple mathematical models constrained by a few experimentally determined parameters in batch can predict the behavior of complex

continuous systems. Isolation, combined with the creation of synthetic co-cultures and mixed communities, allowed us to parse out indirect effects of perchlorate as well as to define controls on inhibition when multiple organisms are present. In this way, this work provides both a framework for testing community-relevant ecological and physiological hypotheses, as well as practical insights for controlling sulfidogenesis across environments.

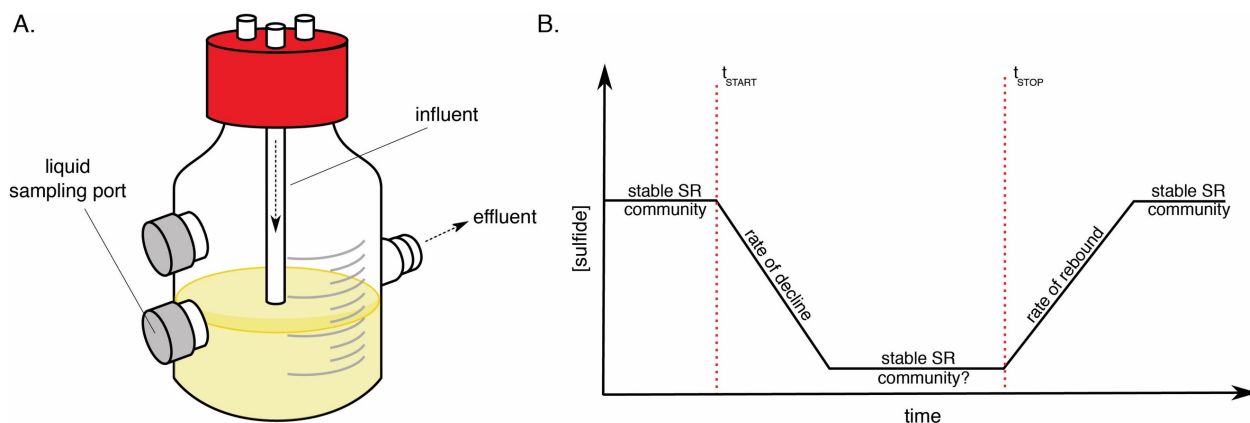


Figure 2.1: A) Chemostat design: anaerobic media (influent) is pumped in; effluent (waste culture) drains passively. A culture of approx. 125 ml is maintained and sampled through the sampling port. B) Experimental design: communities are allowed to reach stable sulfide production before treatment with perchlorate (t_{START}). Various chemical parameters are monitored during inhibition and once inhibition levels off, treatment is stopped (t_{STOP}) and rebound is monitored.

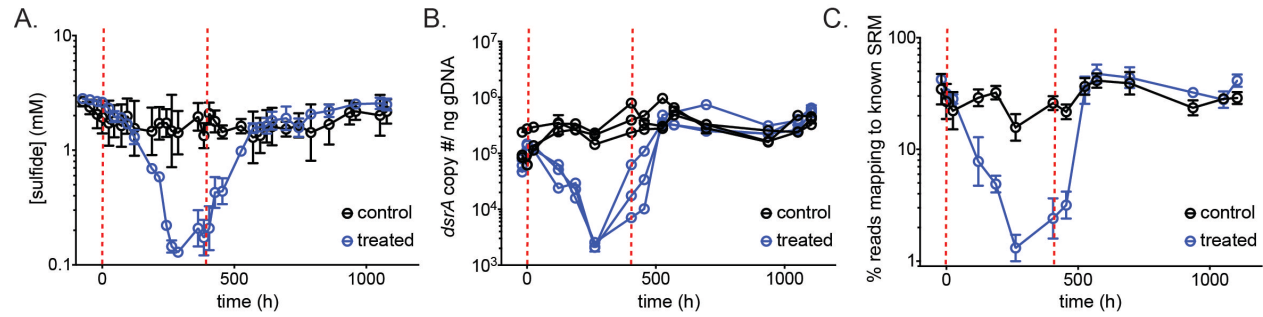


Figure 2.2: A) Average sulfide production, B) *dsrA* copy number per ng genomic DNA and C) percentage of reads mapping to known SRM, based on 16s amplicon sequencing, over time for control chemostats (black circles) and chemostats treated with 50 mM perchlorate (blue circles). Dashed red lines indicate start and stop of treatment. Error bars represent SD from 3 replicates, except in B) where replicates are graphed independently.

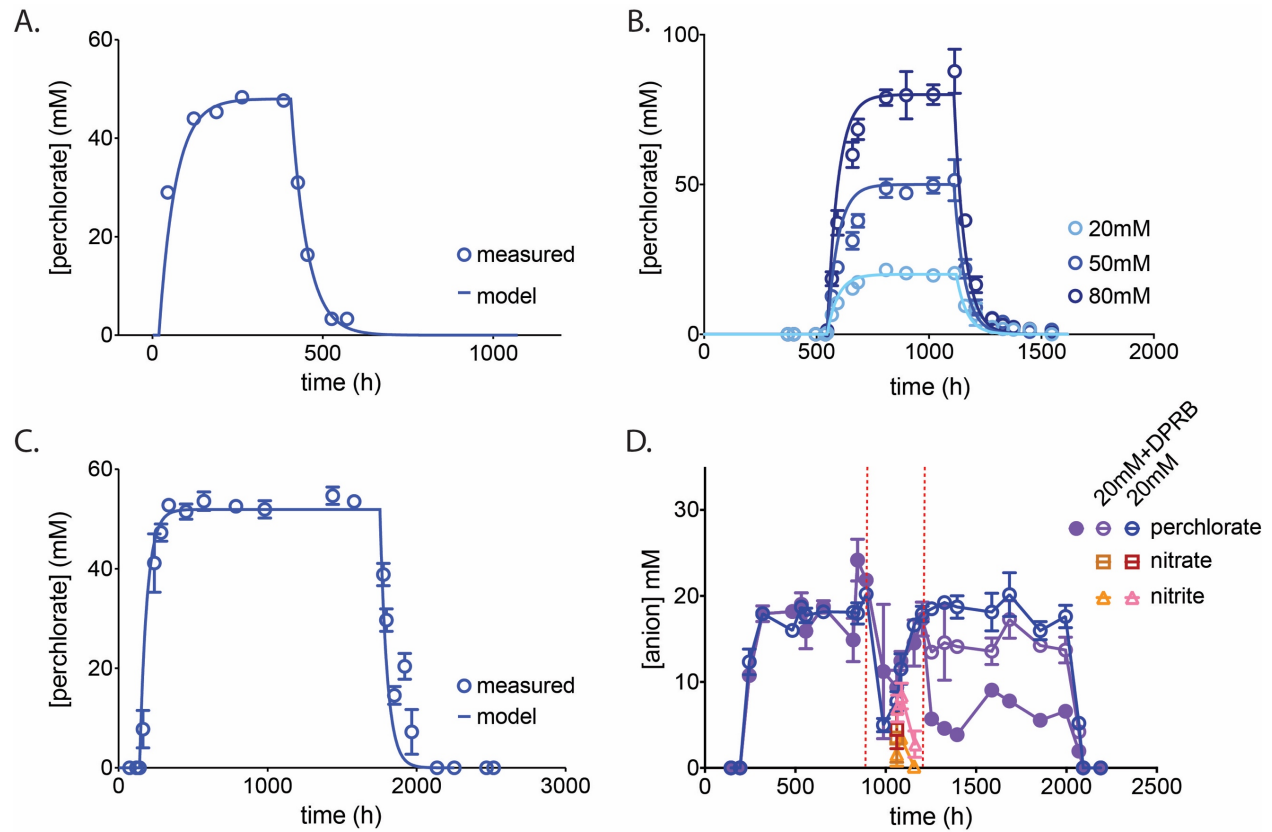


Figure 2.3: Perchlorate concentrations (circles– measured, solid line – modeled) for A) initial chemostat run at 50mM, B) subsequent run at 20, 50 and 80 mM, and C) surface-attachment run at 50 mM. D) Measured perchlorate concentrations in DPRB-amendment run, for chemostats treated with 20 mM perchlorate (blue symbols), and 20 mM perchlorate and DPRB (purple symbols). The latter treatment is split into replicates 1-2 (open symbols) and replicate 3 (closed symbols). Nitrate and nitrite concentrations are also given, for the brief period of nitrate treatment, indicated by dashed red lines. Error bars represent SD.

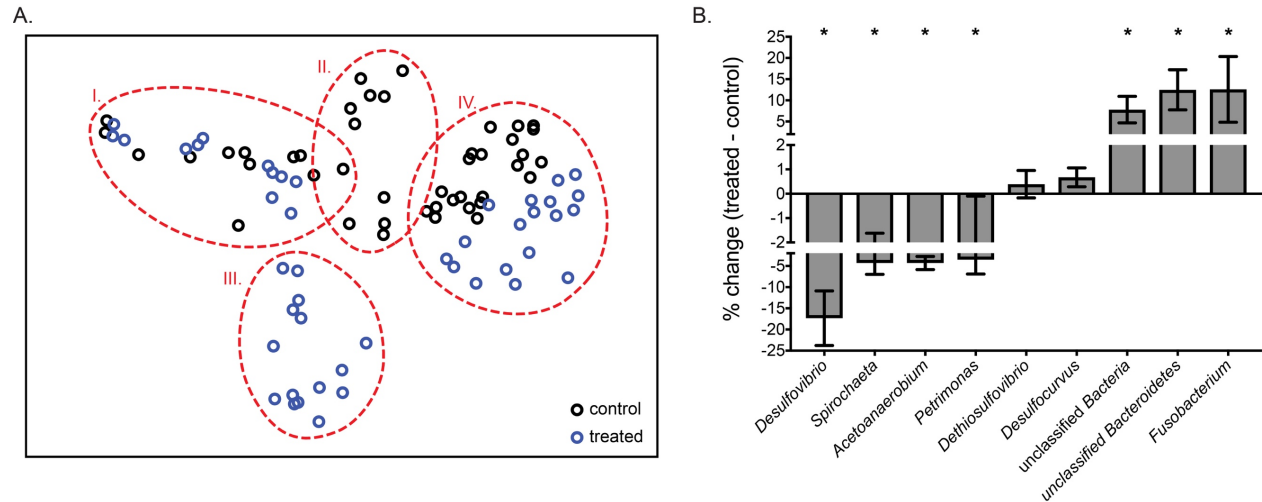


Figure 2.4: A) nMDS (non-metric multi-dimensional scaling) plot of normalized and transformed 16S amplicon community profiles for control (black) and treated (blue) communities. Dashed red lines group samples at 70% similarity. Stress value of 0.1. Group I: pre-treatment; groups II & III: treatment; group IV: post-treatment B) % change between control and treated samples during treatment (A. group II vs. III) of genera identified as important by SIMPER analysis. Error bars represent SD. * = genera which differ significantly via ANOVA ($p < 0.001$) between control and treated communities.

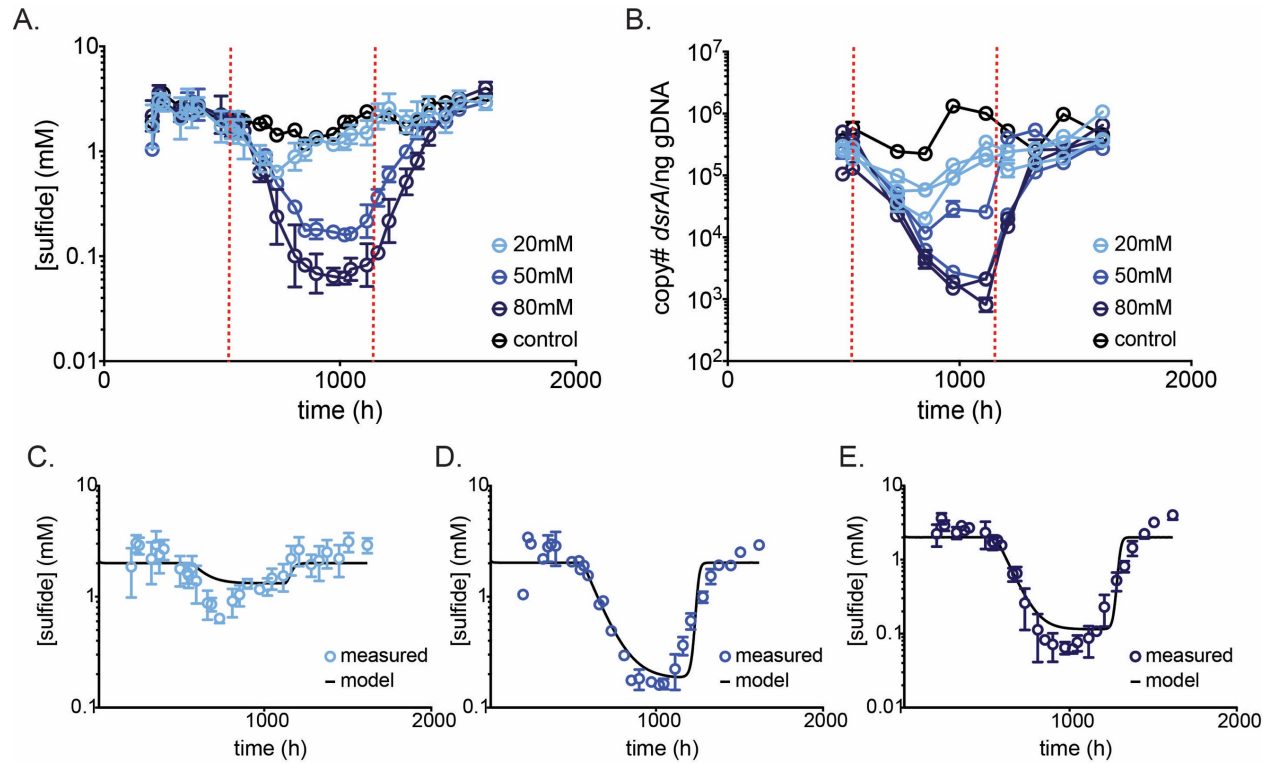


Figure 2.5: Average A) sulfide production, and B) *dsrA* copy number per ng gDNA in control (black circles) and treated (blue circles) chemostats. Dashed red lines indicate start and stop of treatment. Error bars represent SD from 3 replicates for 20 mM and 80 mM treatments and the range of 2 replicates for 50 mM treatment. In B) replicates are plotted independently. Model prediction (black line) and empirical data (blue circles) of [sulfide] over time at C) 20 mM, D) 50 mM and E) 80 mM perchlorate are compared. Model assumes a K_p of 40%, a K_A of 0.5%, and μ of 0.13.

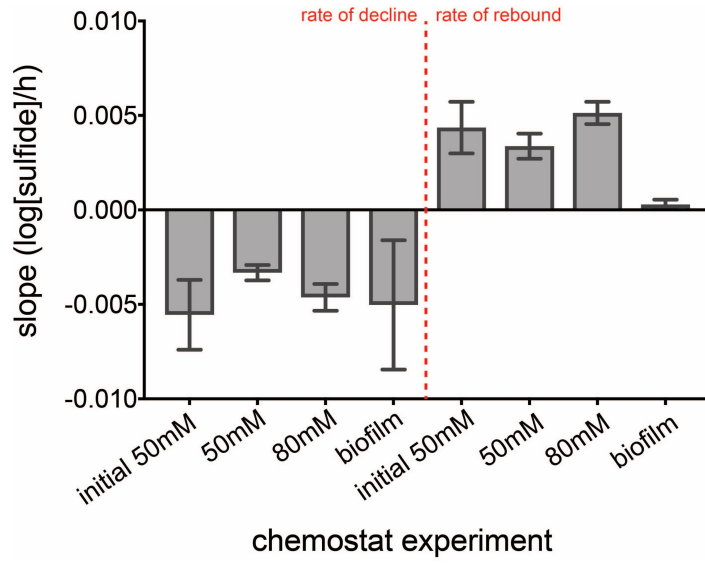


Figure 2.6: Slope of the linear regression fit to log transformed sulfide data during the period of inhibition and the period of rebound for each respective chemostat experiment. Bars represent the 95% CI.

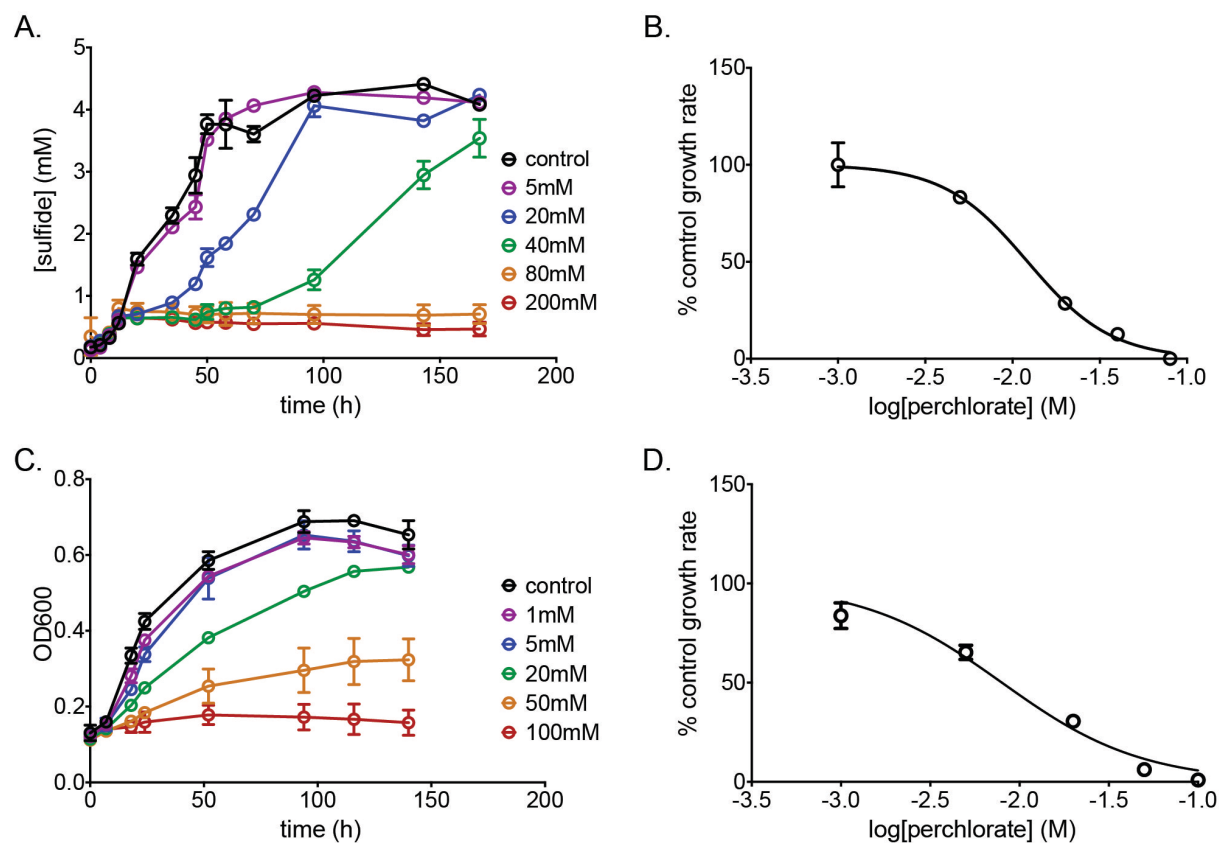


Figure 2.7: Growth curves of A) community sulfide production and C) *Desulfovibrio* sp. BMSR, at various perchlorate concentrations. Perchlorate was spiked in at 12 h and 7 h respectively. Data generated was used to construct dose-response/inhibition curves for B) community SRM and D) *D. sp.* BMSR. Error bars represent SD of three replicates.

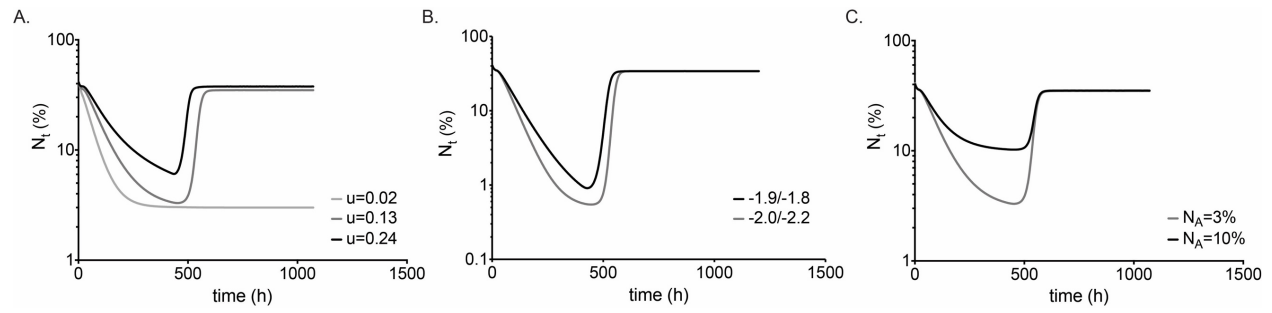


Figure 2.8: Model predictions for the effect of A) increasing μ , B) changing the dose-response/inhibition curve (IC50/Hillslope parameters) and C) increasing N_t , while keeping all other model parameters constant.

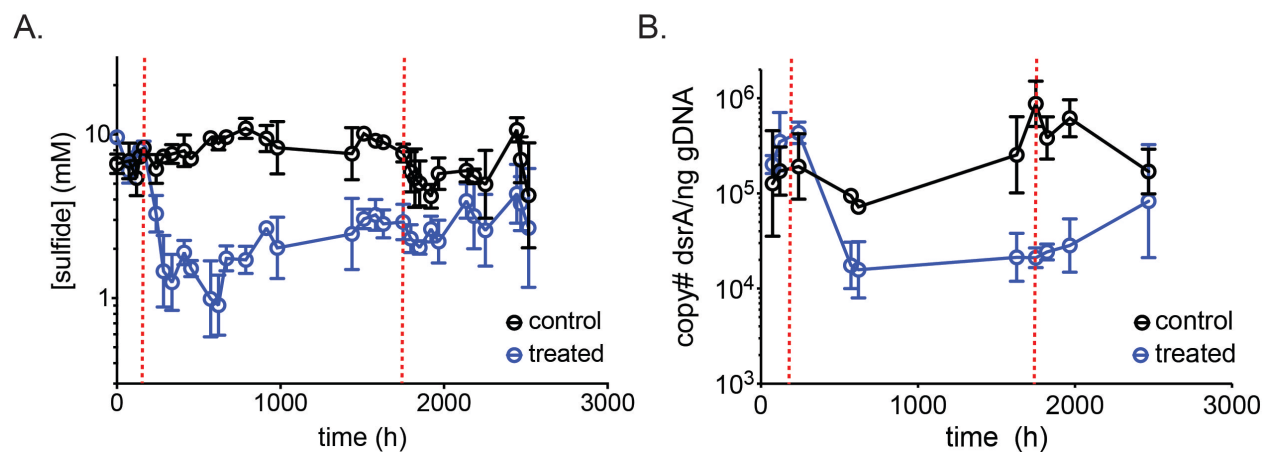


Figure 2.9: Average A) sulfide production, B) *dsrA* copy number per ng gDNA over time for control chemostats (black circles) and chemostats treated with 50 mM perchlorate (blue circles). Dashed red lines indicate start and stop of treatment. Error bars represent SD from 3 replicates.

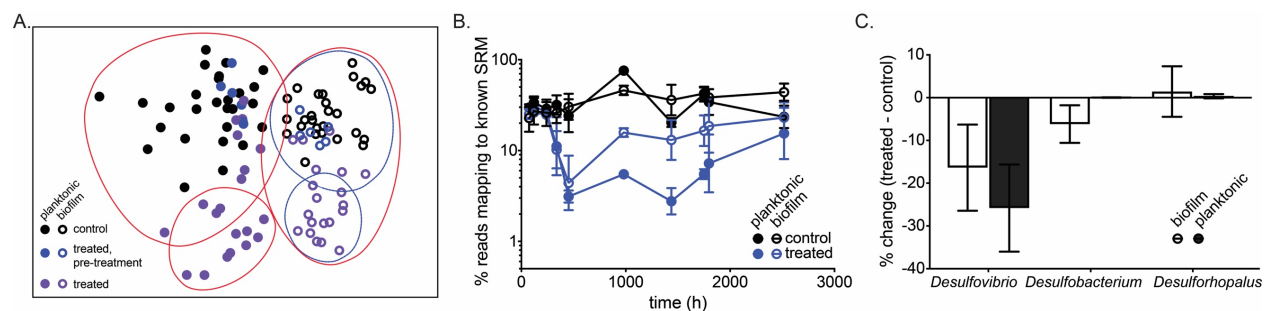


Figure 2.10: A) nMDS plot of normalized and transformed 16S amplicon community profiles for control (black) and treated (purple: pre-treatment; blue: treated) communities. Red and blue lines group samples at 64% and 68% similarity, respectively. Stress value of 0.2. B) percentage of reads mapping to known SRM, based on 16s amplicon sequencing, over time for control chemostats (black circles; open=biofilm, closed=planktonic) and chemostats treated with 50 mM perchlorate (blue circles, open=biofilm, closed=planktonic). C) % change between control and treated samples during treatment of main SRM. Error bars represent SD.

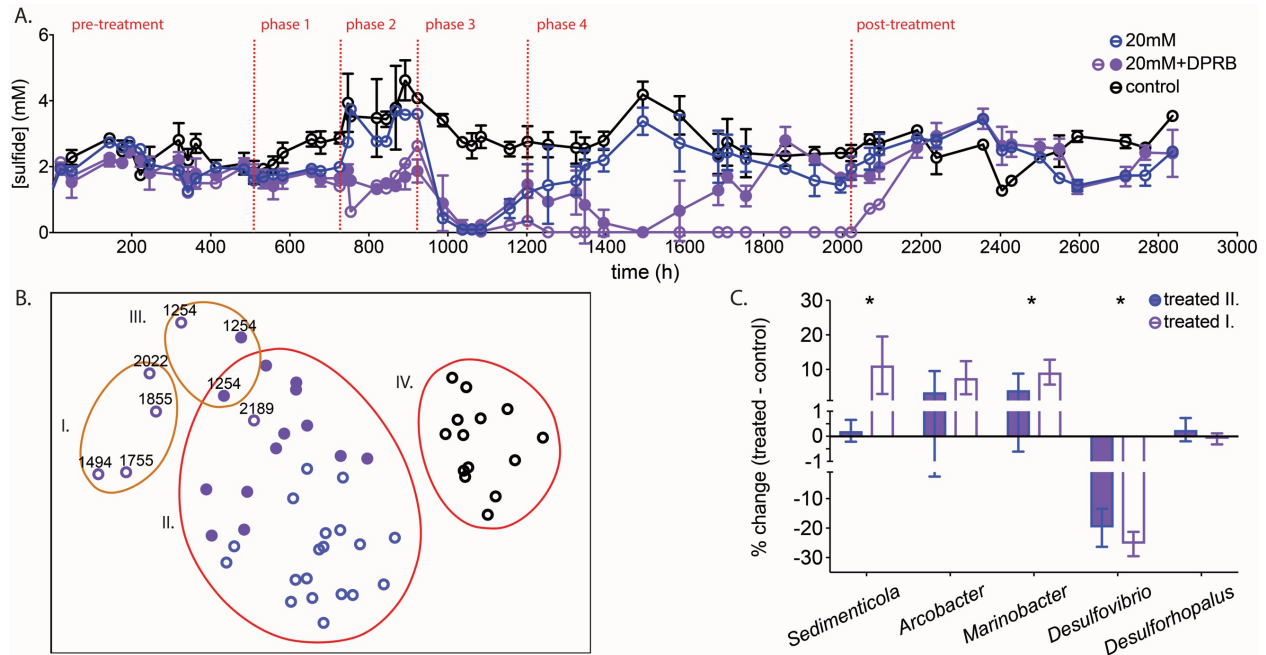


Figure 2.11: A) Average sulfide production in control chemostats (black circles), chemostats with 20 mM perchlorate (blue circles) and chemostats with 20 mM perchlorate, amended with DPRB (purple circles). Amended chemostats did not replicate each other and hence the two trajectories are shown as closed circles (2 replicates) and open circle (replicate 3). Dashed lines indicate start/stop of various treatments. Error bars represent SD for replicates. B) nMDS plot of normalized and transformed 16S amplicon community profiles for control (black circles) and treated (20 mM: blue circles, 20mM+DPRB: purple circles) communities. Orange and red lines group samples at 65% and 70% similarity, respectively. Stress value of 0.2. Numbers next to symbols represent time (h) for sample. C) % change in DPRB and SRM between groupings I. and II (B). Error bars represent SD. * = genera which differ significantly via ANOVA ($p < 0.01$) between groupings I. and II.

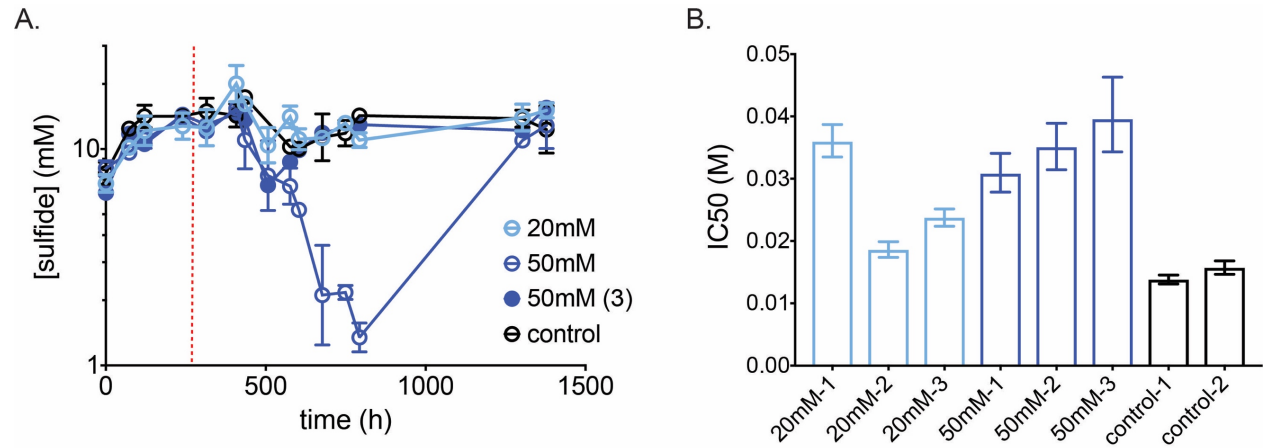


Figure 2.12: A) Average sulfide production in control (black circles) and treated (blue circles) pure-culture *D. sp.* BMSR chemostats. Chemostats treated at 50 mM did not replicate each other and hence both trajectories are shown (replicates 1-2: open circles, replicate 3: closed circle). Dashed line indicated treatment start date. Error bars represent SD of replicates. B) IC50 values for all chemostats, measured at final time-point. Error bars represent the 95% CI of the IC50 estimate.

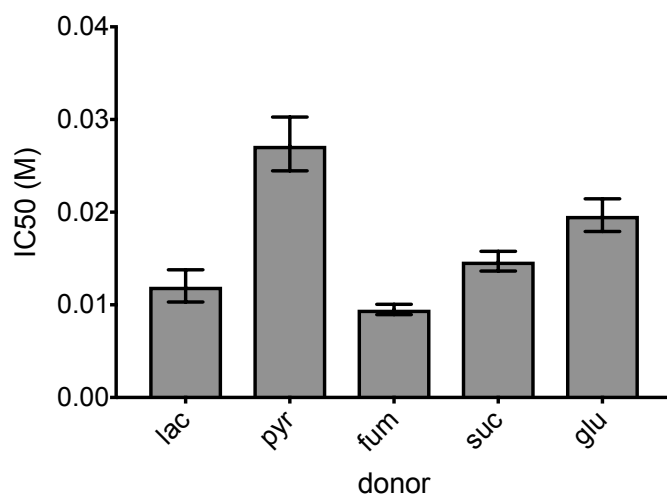


Figure 2.13: IC₅₀ measurements for *Desulfovibrio* sp. BMSR on different donors: lac - sodium lactate, pyr - sodium pyruvate, fum - sodium fumarate, suc - sodium succinate and glu - glucose. Error bars represent the 95% CI of the IC₅₀ estimate.

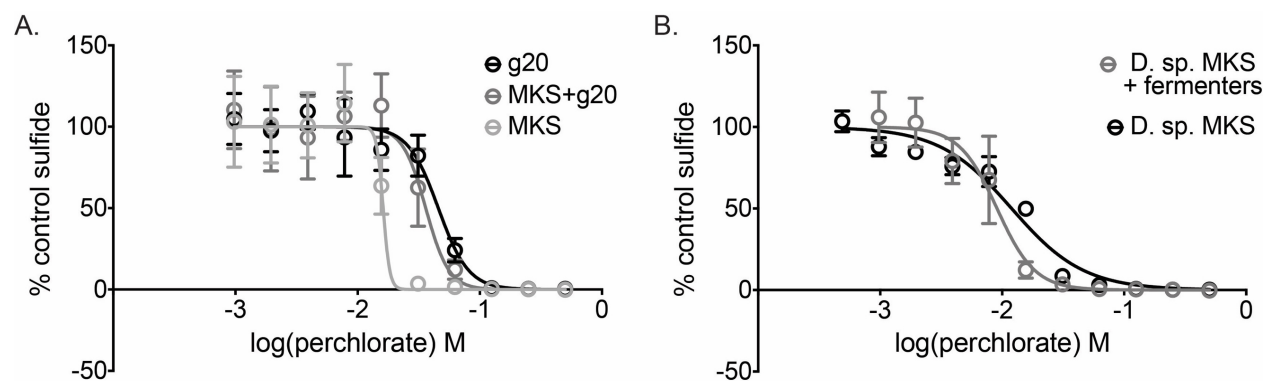


Figure 2.14: Dose-response/inhibition curves for A) *Desulfovibrio alaskensis* G20 (black circles), *Desulfovibrio* sp. BMSR (light grey circles) and a co-culture of both organisms (grey circles) and B) *Desulfovibrio* sp. BMSR in pure culture (black circles) and co-cultured with a mixed community of fermenters (grey circles). Error bars represent the SD of four replicates.

Table 2.1: Summary of chemostat experiments.

RUN	PURPOSE	TREATMENT		CULTURE
		[perchlorate] (mM)	Additional Factor(s)	
<i>1: Initial run</i>	Inhibitory dynamics and effects of community structure	50 mM		Sulfidogenic community
<i>2: Validation run</i>	Testing inhibition at various perchlorate concentrations	20,50 and 80 mM		Sulfidogenic community
<i>3: Surface-attachment run</i>	Testing the effect of increased surface attachment	50 mM	glass beads for increased surface-attachment	Sulfidogenic community
<i>4: DPRB run</i>	Testing <i>indirect</i> effects of perchlorate	20 mM	+/- DPRB to evaluate indirect effects	Sulfidogenic community
<i>5: Pure-culture run</i>	Comparing inhibition in pure culture and in a community context	20, 50 mM		<i>Desulfovibrio</i> sp. BMSR

Chapter 3

Evolving perchlorate resistance in *Desulfovibrio alaskensis* G20

Abstract

Chapter 2 defined the extent and mechanism of perchlorate inhibition in sulfate-reducing communities. However, constant addition of perchlorate to natural systems results in new selective pressures. Consequently, we investigated the ability of *Desulfovibrio alaskensis* G20, a model SRM, to evolve resistance to perchlorate. Serial transfers of G20 in increasing concentrations of perchlorate led to robust growth in the presence of 100 mM inhibitor. Adapted strains were isolated and demonstrated a three-fold increase in resistance to perchlorate compared to the wild type ancestor. Whole genome sequencing revealed a single base substitution in Dde_2265, the sulfate adenylyltransferase (*sat*). We purified and biochemically characterized the Sat from both wild type and adapted strains, and showed that the adapted Sat was approximately three-fold more resistant to perchlorate inhibition, mirroring whole cell results. The ability of this mutation to confer resistance across other inhibitors of sulfidogenesis was also assayed. The importance and generalizability of this mutation was further confirmed in multiple evolving G20 cultures as well as in another SRM, *Desulfovibrio vulgaris* Hildenborough. This study is thus not only of practical value in modeling environmental impacts of perchlorate treatment, but also extends insight into the sulfate adenylyltransferase and key residues involved in controlling the specificity of this universal enzyme.

3.1 Introduction

Sulfate-reducing microorganisms (SRM), ubiquitous across anaerobic environments, conserve energy by coupling the oxidation of organic carbon or hydrogen to the reduction of sulfate. This metabolism results in the generation of sulfide (S^{2-} , HS^- , H_2S), which is toxic and corrosive, and hence economically and environmentally costly [3, 18, 109]. The petroleum industry is particularly affected, where oil reservoir souring (production of sulfide by SRM) is a major health hazard for workers and leads to facility failure through corrosion damage. In 2011, soured oil accounted for more than 50% of all U.S. crude oil production, and the U.S. Energy Information Administration (EIA) predicts that sour oil will continue to account for greater than 20% of the total projected increase in U.S. crude oil production between 2013 and 2020 [152]. These soured oils are transported through extensive production pipelines to transportation hubs and refineries, which become at risk of catastrophic oil spills.

Identification and characterization of selective and potent inhibitors of SRM is critical to preventing these outcomes. Recent studies have characterized perchlorate amendment as a promising strategy to control souring [21, 24, 25, 28, 114]. The SRM *Desulfovibrio alaskensis* G20 (henceforth, G20) is derived from the G100A strain isolated from an oil well in Ventura, California [153, 154]. G20 has been studied as a model SRM due to its role in bio-corrosion and bioremediation. Perchlorate is a potent and specific inhibitor of G20, as well as other SRM in complex microbial communities [28]. The mechanism of perchlorate inhibition has been briefly probed by “omics” approaches (microarrays and proteomics) and fitness-profiling via a barcoded transposon-insertion library of G20 [28]. Perchlorate is presumed to enter cells via sulfate uptake systems, and competitively inhibit the central pathway of SR, specifically sulfate activation via the sulfate adenylyltransferase (*sat*). Evidence for this comes from the increased resistance of the *rex::TN5* mutant. Under normal circumstances, Rex functions as a repressor of core respiratory genes, such as *sat* [91]. Inactivation by the transposon insertion causes increased transcription of the regulon. However, both omics and fitness profiling provide only partial information. Bacteria often have suboptimal responses to stressors, and hence, data generated from omics techniques may not inform on the precise mechanism of inhibition [36]. Fitness approaches are limited to fitness effects arising from the inactivation of non-essential genes [92].

Adaptive laboratory evolution (ALE) is a powerful tool to assess the mechanism of inhibition. Microbes are grown in controlled laboratory conditions under a specific selection regime, allowing for the evolution of increased fitness under the condition tested [155]. Paired with the ability of next-generation sequencing to rapidly and economically identify variants, ALE has greatly accelerated our understanding of the underlying mechanisms of adaptation, and has allowed us to link genomic changes to specific phenotypes. Over the past two decades, there has been an explosion of ALE studies, largely pioneered by Lenski and his long-term evolution experiment in *E. coli* [156–158]. This experiment has yielded critical insight into the process of adaptation, its trajectory and replicability, and its genetic basis. Apart from basic insight into the adaptation process, ALE has allowed us to probe the mechanism of a variety of stressors and develop strains with industrially-relevant phenotypes [159–163]. Most of this work has been in *E. coli* or *S. cerevisiae*, but current efforts are expanding to other microbes [164–167].

In this study, we use ALE to determine the genetic basis of adaptation to perchlorate stress in G20. Continuous perchlorate amendment as a souring-control strategy necessitates a thorough investigation into the long-term consequences of perchlorate exposure on SRM; both the possibility and mechanism of perchlorate adaptation need to be addressed. In this study, we hope

to provide definitive evidence of the targets of perchlorate inhibition in G20 and SRM more generally, as well as practical insight into *sat* and determinants of its specificity. To our knowledge, this is the first study detailing the evolution of a microbe in the face of a *specific* inhibitor. We demonstrate that G20 develops resistance to perchlorate via a non-synonymous single base substitution in *sat*. To further evaluate the role of this mutation in perchlorate resistance, the Sat from G20 wild type and adapted strains was heterologously expressed, purified, and characterized. The ability of this mutation to confer resistance across other inhibitors of sulfidogenesis was also assayed. The frequency with which this mutation arises was subsequently quantified across ten independently adapted populations of G20. Finally, the effect of the mutation in another model SRM, *Desulfovibrio vulgaris* Hildenborough, was assessed to understand the ubiquity of perchlorate resistance in SRM.

3.2 Methods

A population of *D. alaskensis* G20 was adapted to 100 mM perchlorate. This initial adaptation was performed by Patrick Grégoire. The subsequent phenotypic and genomic characterization of isogenic adapted strains from this population was performed by Misha Mehta. This analysis revealed a critical mutation in the Sat enzyme; biochemical characterization of the mutant Sat enzyme and comparison to the wild type enzyme was performed by Matthew D. Youngblut and Steven A. Redford. In order to determine the frequency with which this mutation in the Sat arises, ten populations of wild type G20 were independently adapted to 100 mM perchlorate, by Misha Mehta and Anchal Mehra. The frequency of various *sat* alleles was quantified through time and across populations, via amplicon sequencing, by myself. In order to determine the relevance of the Sat mutation to other SRM, Grant Zane constructed a *Desulfovibrio vulgaris* Hildenborough (DvH) strain containing the mutation. Phenotyping of the control and mutant DvH was done by myself and Anchal Mehra.

Media and cultivation conditions

G20 was cultivated as previously described [28, 151, 168]. Briefly, G20 was grown anaerobically in MOLS4 basal Tris-buffered media with a N₂ headspace. The media contained 8 mM MgCl₂, 20 mM NH₄Cl, 0.6 mM CaCl₂, 2 mM KH₂PO₄, 0.06 mM FeCl₂, 30 mM Tris-HCl, 60 mM sodium lactate and 30 mM sodium sulfate. Trace elements and vitamins were added from stocks [36, 169] and the media was brought to a pH of 7.4. The incubation temperature for all whole cell experiments was 30°C. All G20 cultures were recovered from 1 mL freezer stocks in 9 mL anaerobic MOLS4 media with 1 g/L yeast extract and 1 mM sodium sulfide. All evolved strains were archived at -80°C.

Adaptation to perchlorate

The ancestral strain for the adaptive evolution experiments was taken from a freezer stock of G20, received from the Adam Arkin laboratory (University of California, Berkeley). G20 was sequentially transferred (at a 10% inoculum upon reaching early stationary phase) in MOLS4 medium with increasing concentrations of perchlorate, from 10 mM to a final concentration of 100 mM. This culture was then transferred several times (~117 generations) in 100 mM perchlorate to obtain robust growth. Subsequently, the culture was transferred three times in absence of inhibitor

and then back in presence of 100 mM perchlorate to confirm genetic adaptation. Single isogenic isolates from the adapted population (Adapted-1, Adapted-2 and Adapted-3) were obtained by plating on MOLS4 medium with 1.5% agar. Genomic DNA (gDNA) was isolated using the Qiagen DNeasy Kit (Qiagen, Redwood City, CA) following the protocol for Gram-negative bacteria, and including the RNase treatment. Whole genomes were sequenced on an Illumina MiSeq 300 PE lane (UC Davis DNA Technologies Core). Sequencing of Adapted-1 failed; for Adapted-2 and Adapted-3, genome assembly, mapping to the reference genome (NC_007519.1) and variant calling was performed using the Lasergene SeqMan Pro package (DNASTAR) or the Genome Analysis Toolkit (GATK), as outlined by Clark *et al.* [170, 171]. Mutations were further validated by PCR amplification and Sanger sequencing.

The phenotype of adapted strains was characterized with and without 100 mM perchlorate in MOLS4 medium, in triplicate, for each adapted strain. Growth was quantified via OD600. Sulfate reduction was measured in cell suspensions (in anaerobic MOLS4 medium) using late log phase cultures of wild type, Adapted-3, and Adapted-3 with 100 mM perchlorate. At each time point, 1 mL of sample was collected, centrifuged and passed through a 0.2 μ m filter. Sulfate concentrations were quantified on a Dionex IC 1500 using an AS9-HC anion exchange column with a 9mM sodium carbonate mobile phase at a flow rate of 1 mL/min.

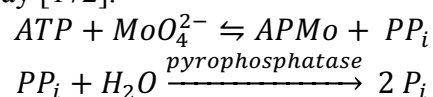
Sulfate adenylyltransferase purification

The wild type and Adapted-3 sulfate adenylyltransferase (Sat) were overexpressed in *E. coli* by first amplifying the gene from G20 gDNA with Phusion® DNA polymerase (New England Biolabs) using the primers ATPS-F (5'-GNNGGTACCGATGTCCAATTTGGTCCCCC-3') and ATPS-R (5'-GNNGAGCTCCTACATCTGGGAACCGGAAGCAG-3'). The resulting PCR product was digested using KpnI-HF and SacI (New England Biolabs) along with the expression vector pET-51b (Novagen). The digested DNA was ligated using T4 DNA ligase (New England Biolabs) and transformed into TunerTM (DE3)pLysS chemically competent *E. coli* (Novagen). Positive transformants were verified via colony PCR and Sanger sequencing. Correct constructs were plated on LB plates supplemented with 100 μ g/mL carbenicillin (LB/carb) and incubated overnight at 37°C. 5 mL LB/carb was inoculated with a single colony, incubated at 37°C and shaken at 250 RPM overnight. 1 mL of this culture was used to inoculate 1 L of Terrific Broth (Fisher) supplemented with 100 μ g/mL carbenicillin and shaken at 250 RPM at 37°C until the OD600 reached ~0.4. IPTG was then added to a final concentration of 100 μ M, and the culture was grown overnight as before. Cells were harvested via centrifugation at 10,000 x g for 10 min, resuspended in buffer A (100 mM Tris pH 8.0, 150 mM NaCl) supplemented with 1 mg/mL lysozyme (Sigma), 0.1 mg/mL DNaseI (Sigma), and frozen at -80°C until needed.

Sat was purified by first lysing cells with an EmulsiFlex-C3 cell homogenizer (Avestin). The lysate was centrifuged at 150,000 x g for 45 minutes and then applied to a 1 x 10cm StrepTactin Superflow HC column equilibrated with buffer A. The column was washed with buffer A until the absorbance of the flow-through at 280nm stabilized, and then Sat was eluted with buffer B (buffer A with 2.5mM d-desthiobiotin). Fractions showing molybdolysis activity as described below were pooled and then dialyzed against buffer C (50mM Tris pH 8.0) overnight. The dialyzed sample was applied to a 0.5 x 10cm Source Q15 column equilibrated with buffer C, and a 200 mL gradient to buffer D (buffer C with 0.5M NaCl) was run. Purified Sat typically eluted at ~200mM NaCl. Fractions containing pure Sat as shown by SDS-PAGE were pooled and concentrated with a 10 kDa MWCO centrifugal concentrator (EMD Millipore) and

subsequently diluted in buffer C. This process was repeated a total of three times to desalt the sample. The sample was then concentrated to ~100μM, snap-frozen with liquid nitrogen, and stored at -80°C until use.

The molybdolysis and ATP formation assays were performed at 25°C in 80mM Tris pH 8.0 as described previously [72, 172]. Briefly, for the forward reaction, the sulfate analog, molybdate, was used. The product of the forward (molybdolysis) reaction produced pyrophosphate. To assay molybdolysis, an inorganic pyrophosphatase (Sigma) was added, which cleaved pyrophosphate into 2 inorganic phosphate molecules. These were measured using a modified molybdenum blue assay [172].



The reverse reaction was assayed via the rate of ATP formation from APS and pyrophosphate, as previously described [173]. Substrate vs. velocity curves were obtained by measuring reaction rates at various concentrations of one substrate while holding the other at a saturating concentration. Kinetic parameters were determined by fitting substrate-velocity curves to the Michaelis-Menten model using Origin 6.0 (Microcal) and Prism 6 (Graphpad). Equilibrium plots were made by modelling the forward and reverse reactions in Mathcad 13 (PTC) and plotting the results in SigmaPlot 13.0 (Systat Software, Inc.).

qRT-PCR analysis

Wild type, *rex::TN5*, and Adapted-3 G20 cultures with and without 100 mM perchlorate were grown in triplicate to an OD600 of 0.4, as determined by a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Cells were harvested by centrifugation of 2 mL of culture at 4°C, at 4000 x g for 5 minutes, and subsequently re-suspended in Trizol (Life Technologies). RNA was extracted using a Qiagen RNeasy Midi Kit (Qiagen Inc., Valencia) and treated with DNase (Fermentas Fisher Scientific). A control PCR reaction was run with G20-specific primers (Dde_2265 5'–ATGTCCAATTTGGTCC-3' and 5'–CTACATCTGGAACCG-3') to confirm that no DNA remained post DNase treatment. RNA concentration and purity was determined via absorbance at 260/280 using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). SuperScript II reverse transcription (Life Technologies) was performed using approx. 250 ng of total RNA template and random hexamers, as per the manufacturer's instructions. *gyrB* served as the housekeeping gene based on its observed stable expression under the conditions tested. The following primers were used: *gyrB* (Dde_0003) 5'-AAAGACCCGCTTTGACAAGA-3' and 5'-GCTGACGTTCTTCTATCGCC-3', *sat* (Dde_2265), 5'-GACATCGCTGAAGAAGACCA-3' and 5'-GTCAAGGTTCTTTCCGAAGG-3', *haeI* (Dde_0401) 5'-GCGTTTCAGGTCAACATTTCAG-3' and 5'-CATAGAGACAGTGCTTGGACC-3'. The qPCR experiment was performed using a Maxima SYBR Green 2x Master Mix (Thermo Fisher Scientific), as per manufacturer's instructions, on a StepOnePlus Real-Time PCR machine (Thermo Fisher Scientific). The relative transcript abundance of Dde_2265 (*sat*) and Dde_0401 from all three cultures, in triplicate, was compared.

Proteomics analysis

Triplicate mid-log wild type, *rex::TN5*, and Adapted-3 cultures were pelleted anaerobically at 4000 x g, and re-suspended in 100mM ammonium bicarbonate at pH 7.4. Cells were sonicated

and digested with Trypsin Gold (Promega) prior to LC-MS/MS analysis as described previously [113]. For proteomic data analysis, normalized peptide counts were used as a semi-quantitative measurement of relative protein abundances. Student's *t* tests were used to compare the normalized peptide counts for the two conditions, at a significance level of $p < 0.05$ [113].

IC₅₀ measurements against growth

IC₅₀ values against growth were determined for G20 wild type, *rex::TN5*, and Adapted-3 cultures, as previously described [28]. Data analysis was carried out in GraphPad Prism and curves were fit to a dose-response/inhibition “log(inhibitor) vs. normalized response – variable slope” curve to generate IC₅₀ values. 95% confidence intervals (CI) were reported and dose-response curve fits were compared in prism among the different strains to yield *p*-values. Fits are calculated from at least three replicates.

IC₅₀ values for DvH strains were calculated based on 48 h growth curve data. Area-under-the-curve (AUC) values were obtained for each strain at each concentration of perchlorate. These values were normalized to the control AUC (no perchlorate) and used to compare dose-response/inhibition curves and IC₅₀ values, as outlined above.

FREQ-Seq growth conditions, library preparation, sequencing and data analysis

To determine the frequency with which the S237A mutation arises, ten populations of wild type G20 were independently adapted to 100mM perchlorate, as described previously. For FREQ-Seq library construction, gDNA from adapted strains at all perchlorate concentrations (20-100 mM) along with G20 wild type controls (a total of 120 different cultures) was isolated as described above and quantified using a dsDNA high sensitivity Quant-iT kit (Invitrogen). Library construction was adapted from Chubiz *et al.* [174]. Briefly, the mutation-containing region of *sat* was PCR amplified using specific primers, wherein the reverse primer contained a 6-mer barcode (see Table 3.3), for multiplexing on the Illumina platform. Primers were removed post-PCR with exonuclease 1 Dpn1. A second barcode and Illumina adapters were added with a subsequent round of PCR (Table 3.3). 96-well PCR clean-up reactions were performed at the UC Berkeley DNA Sequencing Facility. Purified PCR products were then re-quantified, normalized and pooled for sequencing at the UC Davis DNA Technologies Core facility on a MiSeq 500 cycle v2, 250 PE lane.

Sequencing data were processed using Mothur v1.36.1 [123]. Samples were de-multiplexed and screened by length (350-500bp) and ambiguous base calls (maxambig=0). Identical amplicons were merged, singletons were removed and the remaining sequences were then used to generate an alignment. This alignment was used to create a table of allele frequencies across samples. Only four *sat* variants were found in any significant proportion across all samples: the wild type *sat*, the 709T>G *sat*, a 913C>T variant, and a variant with both 709T>G and 913C>T. The 913C>T substitution also occurred in control strains. A subsequent round of FREQ-Seq was performed to better quantify 709T>G abundance over the course of the adaptations. Primers were modified to create a larger amplicon size, and sequenced on a MiSeq 500 cycle v2, 250 PE run at UC Davis. Amplicons were analysed as outlined above, with one modification. Due to the larger amplicon size, forward and reverse reads overlapped only briefly. This increased the sequencing error and created artificial diversity, resulting many more ‘unique’ *sat* sequences. Consequently, the alignment generated was further trimmed to include only position 709. From here, four

versions (A,T,G,C) were found, only two of which (wild type: T, adapted: G) were observed in any significant proportion. Counts across samples were again used to create a table for 709T>G frequencies through time.

Construction of the D. vulgaris Hildenborough sat 709T>G strain

Escherichia coli cultures were grown in LC medium [175] with antibiotics (kanamycin, 50 ug/ml; spectinomycin, 100 ug/ml) as necessary.

Desulfovibrio vulgaris Hildenborough (DvH) cultures were grown in MOYL medium [175] with either 30mM sulfate or 20mM sulfite. Where noted, G418 (400 ug/ml), spectinomycin (100 ug/ml) or 5-fluorouracil (5FU, 40 ug/ml) were added.

Construction of the *sat* 709T>G point mutant strain, JW9447, was accomplished similarly to the generation of marker-less deletion strains in the uracil phosphoribosyltransferase (*upp*, pyrimidine salvage pathway enzyme) deletion mutant (JW710; resistant to 5-fluoro uracil, 5FU) of *Desulfovibrio vulgaris* Hildenborough [91, 176]. Briefly, three plasmids were constructed using the SLIC technique [177] to delete then reintroduce the wild type or 709T>G coding region. The first plasmid (pMO9444 for marker-exchange), contains the antibiotic-resistance cassette encoding the aminoglycoside-3'-phosphotransferase (*npt*, Kmr) driven by the native promoter (Pnpt) followed by the *upp* gene (Pnpt-*npt-upp*). It was designed and selected with G418 to result in a double homologous recombination event to exchange the 3' portion of DVU1295 (*sat*; bases 405 through 14 bases beyond the stop codon), with the Pnpt-*npt-upp* cassette. One key difference with this plasmid compared with previous marker-exchange plasmids is that the Pnpt-*npt-upp* cassette was oriented in the same direction as the gene being deleted. For unknown reasons, the previous approach of placing the Pnpt-*npt-upp* cassette in the opposite direction of the gene did not yield any transformants. This plasmid was transformed into JW710 for the marker-exchange deletion strain, to yield strain JW9445. The other two plasmids (pMO9462 for wild type complement and pMO9446 for 709T>G-containing complement) were used for exchanging the marker-exchange cassette (by selecting with 5FU) resulting in either the wild type *sat* (strain JW9463) or the 709T>G strain (JW9447). Following each transformation by electroporation (see [175] for protocol), putative transformants were screened for phenotypes with spectinomycin, 5FU, and G418 to determine the probability that a double homologous recombination event took place rather than a single recombination event resulting in plasmid insertion. Three of the isolates for the marker-exchange strain with the correct phenotype were confirmed by Southern blot analysis and one selected as the confirmed deletion strain. The regions of each plasmid necessary for homologous recombination and complementation were sequenced (DNA core, University of Missouri, Columbia) to determine fidelity to the published sequence or introduction of the 709T>G or wild type sequence. Furthermore, gDNA from the complemented strains was used as template for amplification and sequencing of the *sat* gene to ensure the mutation introduced was present in the JW9447 strain and lacking in the wild type complement.

Table 3.4 contains a list of strains/plasmids used and Table 3.5 lists all primers.

3.3 Results

Adaptation to perchlorate

The concentration inhibiting 50% of growth over 48 h (henceforth referred to as the IC_{50}) for perchlorate was previously reported to be 24 mM (20-32 mM 95% CI) in wild type G20 [28]. To initially investigate adaptation to perchlorate, one population of G20 was grown under standard growth conditions with gradually increasing perchlorate concentrations, to a final concentration of 100 mM. Successive serial transfers of the initially 100 mM adapted population led to robust growth in 100 mM perchlorate, with no lag. Single colonies were picked from this adapted population to yield three strains for further investigation: Adapted-1, Adapted-2 and Adapted-3. Figure 3.1 shows the growth curve of Adapted-3, as a representative. In the absence of perchlorate, Adapted-3 grew at approximately 50% of the growth rate of wild type ($0.075 \pm 0.001 \text{ h}^{-1}$ vs. $0.157 \pm 0.019 \text{ h}^{-1}$ respectively, t-test $p=0.0019$), but achieved the same final optical density (Figure 3.1A). No growth was observed for wild type G20 in 100 mM perchlorate, whereas the growth rate of Adapted-3 was essentially unaffected by perchlorate ($0.086 \pm 0.010 \text{ h}^{-1}$) (Figure 3.1A). Similarly, the sulfate reduction rate of Adapted-3 was unaffected by perchlorate (without perchlorate: $5.5 \pm 2.3 \text{ mM/h}$; with perchlorate: $4.6 \pm 2.7 \text{ mM/h}$) and was similar to that of the wild type strain ($4.3 \pm 1.6 \text{ mM/h}$, $p=0.80$ One-Way ANOVA) incubated in the absence of perchlorate (Figure 3.1B).

In order to identify the genetic basis of perchlorate resistance, the Adapted-2, Adapted-3, and G20 wild type strain, were whole genome sequenced. Sequences of these genomes covered 99% of the NCBI Reference G20 sequence with average sequencing depths of 50x for adapted-2, 64x for adapted-3 and 70x for the wild-type ancestral strain. Adapted-2 and Adapted-3 genomes revealed a mutation in Dde_2265 and a mutation in Dde_0401, both of which were absent in the ancestral strain. Strikingly, no other mutations or indels unique to the adapted strains were detected. The substitution in Dde_2265 results in a non-synonymous change in a conserved amino acid residue (Figure 3.2); the substitution in Dde_0401 results in a synonymous change in a non-conserved amino acid residue. Since both the sequenced adapted strains were genotypically identical, all further investigations were performed with Adapted-3.

Single-base pair mutation in Dde_0401, Hydrophobe/amphiphile efflux pump HAE1

Dde_0401 is an integral membrane component transporter, annotated as a hydrophobe/amphiphile multidrug efflux pump. This class of transporters allows bacteria to extrude a wide range of substrates and can be important for the detoxification of various intracellular metabolites [178]. However, the function of Dde_0401 in G20 has not been characterized. Adapted strains had a substitution in Dde_0401 changing the nucleotide position 2025 from C to T. This resulted in a *synonymous* mutation, changing the codon for proline from GGC to GGT. The effect of this mutation is likely neutral, and hence likely does not play a role in resistance to perchlorate.

Additionally, qRT-PCR analysis of the efflux pump showed no significant change in expression levels in either presence or absence of 100 mM perchlorate in the Adapted-3 strain when compared to the wild type G20 ($p=0.1$, one-way ANOVA) (Figure 3.3). Furthermore,

Dde_0401 has no fitness effect in barcoded transposon libraries of G20 grown at the IC₅₀ concentration of perchlorate [28]. Collectively, these data suggest that the mutation in Dde_0401 is not involved in resistance to perchlorate.

Single-base pair mutation in Dde_2265, the sulfate adenylyltransferase (sat)

The sulfate adenylyltransferase (Dde_2265, *sat*) is a key enzyme in all SRM. It catalyses the first step in sulfate reduction, using ATP to activate sulfate to adenosine-5'-phosphosulfate (APS) [5]. Perchlorate-adapted strains showed a substitution in Dde_2265, changing nucleotide position 709 from T to G (henceforth *sat* 709T>G, when referring to the gene). This caused a conserved serine residue to change to an alanine at position 237 in the amino acid sequence (henceforth Sat S237A, when referring to the protein) (Figure 3.2).

Transcript abundance of *sat* with or without 100 mM perchlorate was quantified. There was a 3.4-fold (± 1.6) higher expression of *sat* in the adapted strain in the absence of perchlorate, and 31.5-fold (± 1.6) higher expression in the presence of perchlorate, when compared to the wild type cells ($p < 0.001$ for all comparisons to wild type, one-way ANOVA) (Figure 3.4A). Overexpression of *sat* has previously been shown as a mechanism for overcoming perchlorate inhibition (30 mM) [28]. G20 regulates *sat* expression via the redox-sensitive *rex* repressor. The *rex::TN5* mutant thus overexpresses *sat*, and is more resistant to perchlorate [28]. Transcript abundance in the Adapted-3 strain was compared to the *rex::TN5* mutant (18.6 ± 1.6 fold), and indeed with perchlorate, *sat* expression was significantly higher than even the *rex::TN5* mutant ($p = 0.03$, Sidak's correction, t-test). Proteomics counts mirrored qPCR data (Figure 3.4B), with the *rex::TN5* mutant having higher Sat peptide counts than the wild type strain ($p = 0.06$, Dunnett's multiple comparison t-test) and the same trend being true of Adapted-3 in the presence of perchlorate ($p = 0.2$). Collectively, these data suggest overexpression of the *rex* regulon in the presence of 100 mM perchlorate in the Adapted-3 strain.

In order to define a biochemical role for the S237A mutation in perchlorate resistance, we heterologously expressed, purified and characterized Sat from the Adapted-3 and wild type strains. Table 3.1 shows all steady-state kinetic parameters for the wild type and the S237A Sat. While the kinetics of the forward reaction were almost unchanged under molybdate-limiting conditions, under ATP-limiting conditions the catalytic turnover rate decreased in the S237A Sat as compared to the wild type enzyme. Additionally, the K_M of the reaction under ATP limitation decreased 1.8-fold, suggesting that the S237A mutation decreased the efficacy of binding and turnover of substrate under ATP-limiting conditions. For the reverse reaction, while the S237A mutation did increase the turnover rate for pyrophosphate it also increased the K_M for this substrate which led to both the wild type and S237A Sat having nearly identical catalytic efficiencies (k_{cat}/K_M). In summary, in the absence of inhibitor, the kinetic parameters discussed above suggest the S237A Sat is almost as effective as the wild type enzyme under most conditions.

The benefit of the S237A mutation is obvious in the presence of perchlorate. Table 3.2 shows the inhibitory constant (K_I) for perchlorate for each enzyme. Under both molybdate and ATP limiting conditions, the K_I values were approximately three-fold greater for the S237A Sat as compared to the wild type enzyme. These data underline how the S237A mutation is a remarkable adaptation; at the cost of only a minor perturbation in basal activity it conferred a robust resistance to inhibition by perchlorate.

Evaluation of cross-resistance

Sensitivity to various inorganic oxyanions was assayed in the Adapted-3 and wild type strains to determine if the S237A mutation confers resistance more broadly against other sulfate analogs. The IC_{50} values were calculated as previously described [10, 28] for the wild type, Adapted-3 and *rex::TN5* strains. As discussed above, *rex::TN5* has higher *sat* expression and hence displays increased resistance to competitive inhibitors. Adapted-3 was compared to *rex::TN5* in an aim to understand the extent and mechanism of inhibition. Figure 3.5 represents the fold change in IC_{50} values for the *rex::TN5* strain and the Adapted-3 strain, normalized to the wild type IC_{50} . IC_{50} values across the three strains were significantly different for all inhibitors tested ($p < 0.0001$, one-way ANOVA), as well as significantly different from the wild type IC_{50} ($p \leq 0.0001$, Dunnett's multiple comparison t-test). Adapted-3 had a 3.3-fold (95% CI: 3.1 – 3.6) increase in resistance to perchlorate, while *rex::TN5* had a 1.7-fold increase in resistance (95% CI: 1.6 – 1.8). The 3.3-fold increase in resistance of the adapted strain closely mirrored the approx. 3-fold increase in resistance of the S237A Sat, quantified biochemically. As demonstrated previously, *rex::TN5* does not show increased resistance to monofluorophosphate (MFP) or molybdate [10]. However, the Adapted-3 strain had a 1.9-fold higher resistance to MFP (95% CI: 1.6 – 2.2) and a 1.4-fold higher resistance to molybdate (95% CI: 1.1 – 1.9), suggesting that the mechanism of resistance is not primarily due to the upregulation of *sat* and further supporting the biochemical role of S237A for resistance.

The frequency of sat 709T>G across independent adaptations

In an effort to evaluate the importance of the *sat* 709T>G mutation as a mechanism of adaptation to perchlorate, ten G20 populations were independently adapted to 100 mM perchlorate and compared to three independent control populations, transferred in the absence of the inhibitor. The frequency of *sat* 709T>G was monitored via FREQ-Seq, both through time and across the multiple populations (Figure 3.6A,B). At the adaptation end-point (100 mM perchlorate) all ten populations showed the 709T>G mutation. All ten populations also showed an increase in the mutation starting with the transfer into 60 mM perchlorate. Conversely, 709T>G did not come up in the control populations. In seven of the adapted populations, the mutation appeared to be sweeping, ranging from 40-70% of all variants. In the last three populations (populations 3, 5 and 9), 709T>G was at a lower abundance (<20%) but possibly on an upwards trend. Alternatively, there were other more adaptive mechanisms for resistance in these populations. Perchlorate was neither removed nor transformed during growth, and hence all cells in a population presumably had a mechanism in place to cope with perchlorate; i.e. cells could not selfishly persist by relying on detoxification by other members of the population. Current efforts are underway to characterize adapted isolates from populations 3, 5 and 9, specifically isolates that do *not* carry the 709T>G mutation, in order to understand these alternative mechanisms. Irrespective of the presence of additional adaptive mutations, *sat* 709T>G was selected for in the presence of perchlorate across all ten populations. This observation, given the biochemical characterization presented above, highlights the importance of this mutation as a mechanism for adaptation to perchlorate.

Recreating S237A in Desulfovibrio vulgaris Hildenborough

In order to assess the generalizability of S237A for perchlorate resistance, the mutation was recreated in another model SRM, *Desulfovibrio vulgaris* Hildenborough (DvH). The S237A Sat strain (JW9447) and a wild type Sat strain (JW9463) were compared in regards to their sensitivity to perchlorate. JW9447 exhibited a significantly increased IC_{50} ; 6.8 mM (95% CI 5.3-8.5mM) for JW9463 vs. 17 mM (95% CI 14-20mM) for JW9447 ($p \leq 0.0001$, Extra sum of squares F-test) (Figure 3.7).

3.4 Discussion

This study aims to elucidate possible mechanisms of adaption to perchlorate stress in model SRM, G20. An ALE experiment, transferring G20 into increasing concentrations of perchlorate, led to robust growth in the presence of 100mM of the inhibitor. Whole genome sequencing of adapted strains revealed a key mutation at position 709 in the *sat*. Recreation of the mutation acquired during adaptation is the current standard for demonstrating its importance [164]; here we went a step further and biochemically characterized the mutant protein, confirming that the S237A mutation decreases enzyme inhibition by perchlorate. The importance and generalizability of the mutation was investigated in ten independently evolving G20 cultures as well as in another SRM, *D. vulgaris* Hildenborough.

The Adapted-3 strain containing the S237A mutation displayed robust growth in 100 mM perchlorate, a concentration completely inhibitory to wild type G20. Comparing growth of wild type and Adapted-3 strains in the absence of perchlorate revealed a slight growth defect in the adapted strain. Sulfate reduction, however, was comparable, suggesting that the Adapted-3 strain, although reducing sulfate at wild type rates, could not conserve energy for growth as efficiently. A cost for the mutation is not surprising, given that the Sat has been streamlined over billions of years of evolution [71]. Biochemical characterization of the purified wild type and S237A Sat confirmed a slight cost of S237A under ATP-limiting conditions. However, in the presence of perchlorate, the mutation conferred an advantage over the wild type enzyme. Hence, we believe S237A to be sufficient to render G20 capable of growth in 100 mM perchlorate. It has been previously shown that mutations in a single gene can have large effects on phenotype, as can mutations that are not in the active site of the enzyme [179–182].

In addition to this genetic adaptation, Adapted-3 demonstrated an increase in *sat* expression. Previous work has suggested that overexpressing *sat* can relieve competitive inhibition by perchlorate [28]. Wild type G20 upregulates *sat* in response to perchlorate stress. It is likely that Adapted-3 showed the same upregulation when faced with perchlorate, which allowed it to further maintain robust growth. The *rex* repressor is sensitive to NADH:NAD⁺ ratios, with an increase in NADH causing de-repression of the regulon and increased transcription of *sat*. The slight deficiency in the S237A Sat may have caused increased NADH pools, which in turn caused upregulation of *sat* even in the absence of perchlorate. In the presence of perchlorate, this upregulation was even higher and allows the Adapted-3 strain to maintain wild type levels of sulfate reduction as well as robust growth. Hence, the S237A mutation was a primary mechanism of resistance, and functioned in combination with the overexpression of this more perchlorate-resistant Sat.

Analysis of the resistance profile of the Adapted-3 strain compared to the *rex::TN5* mutant confirmed that the primary mechanism of resistance was biochemical, underpinned by the S237A mutation. As previously shown, *rex::TN5* displays increased *resistance* to the competitive inhibitors nitrate and perchlorate and increased *sensitivity* to non-competitive inhibitors MFP and molybdate [10]. Overexpression of the *rex* regulon presumably increases the permeability and turnover of these non-competitive inhibitors: increased intracellular MFP can lead to increased intracellular fluoride release, and increased molybdate turnover can rapidly deplete ATP stores via futile cycling [10, 11]. The Adapted-3 strain, however, showed increased resistance across all four inhibitors tested, competitive and non-competitive. Hence, *sat* overexpression in the Adapted-3 strain was not sufficient to impact its susceptibility to non-competitive inhibitors. Further, this data suggested that S237A was most beneficial against perchlorate inhibition but functioned more generally against other inhibitors. Future ALE experiments, adapting G20 to a suite of sulfate analogs, will further define the significance of mutations in the *sat*. Identifying determinants of specificity has been previously accomplished for another key enzyme of the SR pathway – APS reductase – with the hope of aiding in the development of specific inhibitors of this enzyme [84]. Our study provides a complementary methodology for identifying key residues and optimizing enzyme function [179].

Strikingly, the S237A mutation came up consistently across all ten independently perchlorate-adapting populations of G20, at around the same perchlorate concentration. Although parallelism is common, it is rare to observe the *exact* same mutation across replicate ALE experiments, usually because mutations are loss-of-function, and the precise mutation is not important [183]. In this case, the precise mutation was important; parallelism may have been due to constraints imposed by *sat*, which may not be able to tolerate many mutations. Perchlorate may also represent a simpler stress with fewer targets, unlike the more complex traits of salt or oxidative stress tolerance [162, 184]. More likely, this parallelism hints at a rare pre-existing variant which came to dominate under selection. Similarly, a recent ALE experiment in *D. vulgaris* Hildenborough revealed multiple ecotypes in the ancestral population [164]. A protein alignment of Sat sequences from complete genomes on IMG shows members of the genus *Thermodesulfovibrio* have an A at position 237, which is otherwise a S, P, or M in other clades of SRM. This signals to a potential environmentally-relevant benefit to this mutation. Further to this point, recreating the mutation in DvH increased resistance approximately 2.5-fold, from 6.8 mM to 17 mM. Interestingly, in none of the ten adapted G20 populations has the S237A mutation completely swept, suggesting competition with alternative beneficial mutations (clonal interference) [185–187]. Further investigation into these alternative mechanisms, their relative fitness compared to Sat S237A and their environmental relevance is needed in order to fully define the adaptive landscape of SRM evolving to the presence of perchlorate. Finally, previous studies have suggested perchlorate addition as a technology for controlling sulfidogenesis in industrial systems [10, 21, 22, 28, 113]. In these systems, the potency of perchlorate is a combination of several independent processes involving both direct (toxicity to SRM) and indirect mechanisms (bio-competitive exclusion of SRM and enzymatic oxidation of sulfide, both mediated by dissimilatory perchlorate-reducing bacteria) [21, 23]. Hence, future ALE experiments will need to implement the ESENCE approach (embedded species evolving in a natural community experiment), in order to describe adaptation across SRM in the relevant community context [163].

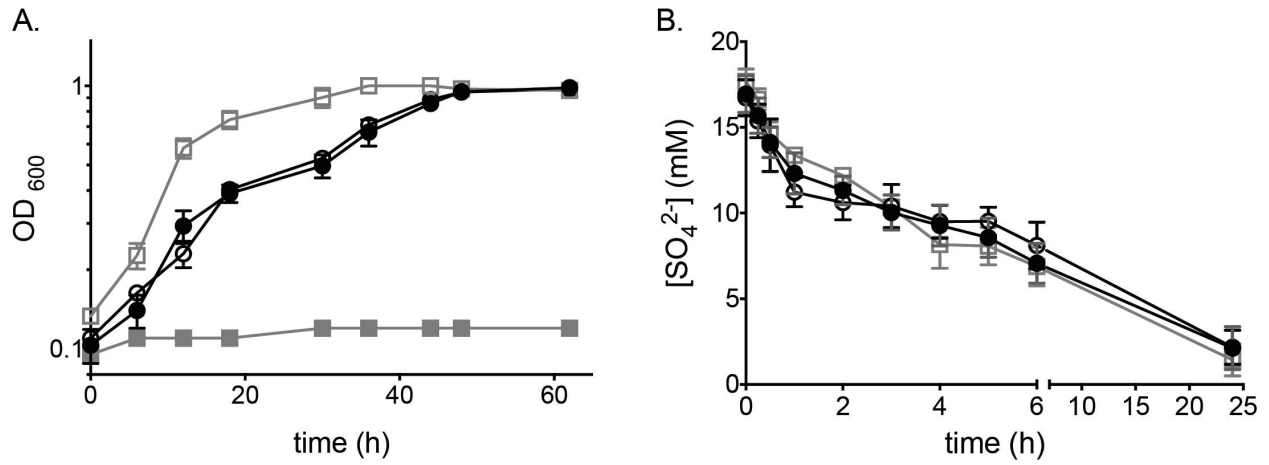


Figure 3.1: A) Growth of the wild type (grey squares) and Adapted-3 strains (black circles) in the presence (filled symbols) or absence (open symbols) of 100 mM perchlorate. B) Sulfate reduction in cell suspensions of the wild type (grey squares) and Adapted-3 strains (black circles) in the presence (filled symbols) or absence (open symbols) of 100 mM perchlorate. Error bars represent standard deviation of 3 replicates.

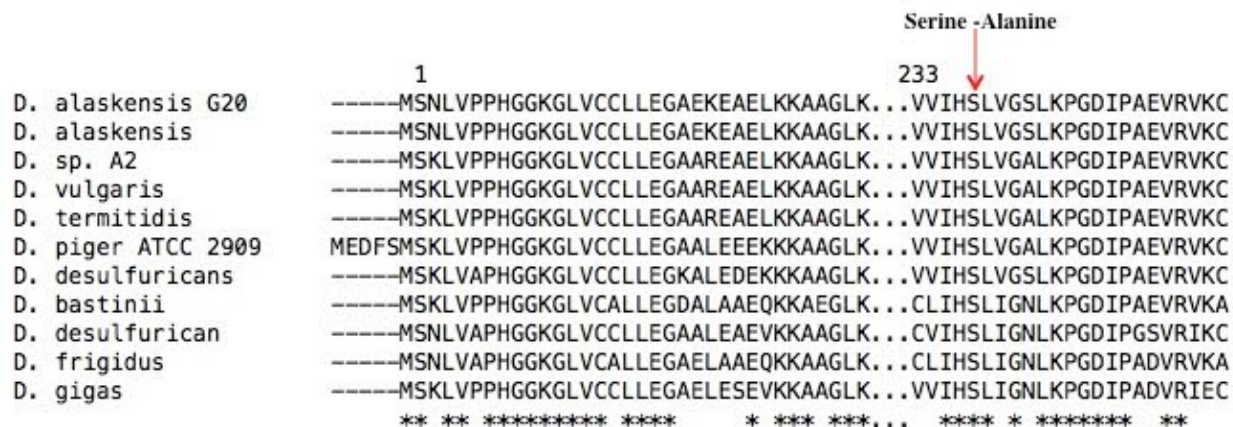


Figure 3.2: Multiple sequence alignment of the *sat* gene from various *Desulfovibrio* species, generated using Clustal Omega. Conserved positions are indicated with a star; the S237A mutation in the adapted strains is indicated by a red arrow.

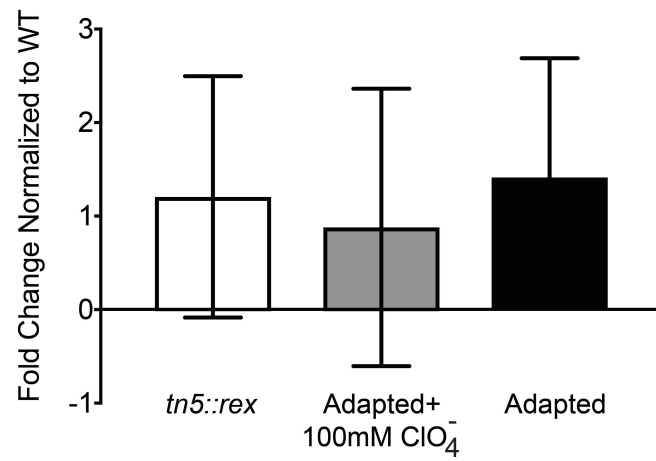


Figure 3.3: Relative fold change of Dde_0401 transcript abundance based on RT-qPCR data, normalized to wild type expression. Values represent mean of 3 biological replicates.

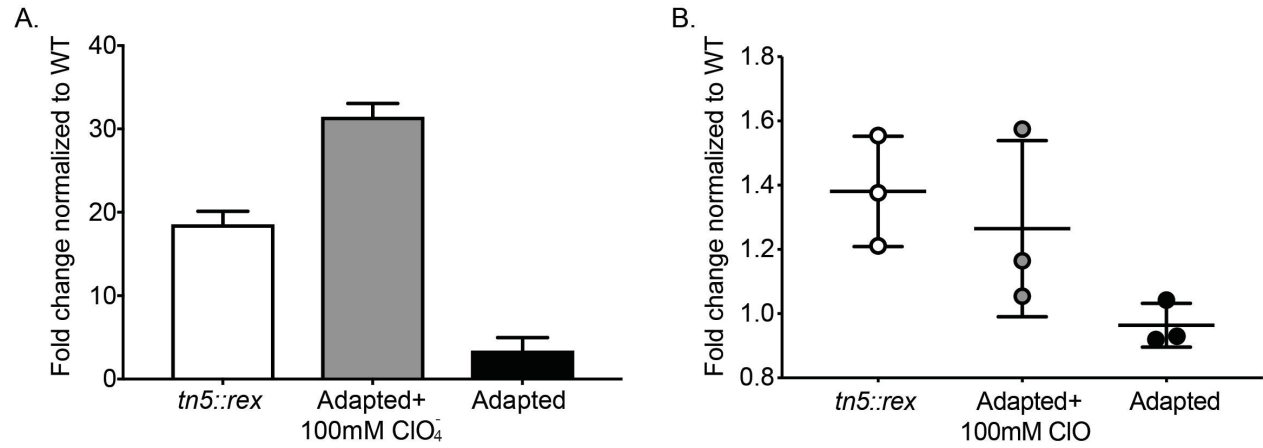


Figure 3.4: (A) Relative fold change of *sat* transcript abundance based on RT-qPCR data, normalized to wild type expression. Values represent mean of 3 biological replicates. (B) Fold change in Sat peptide counts normalized to wild type cultures. Mean and standard deviation of 3 biological replicates are plotted.

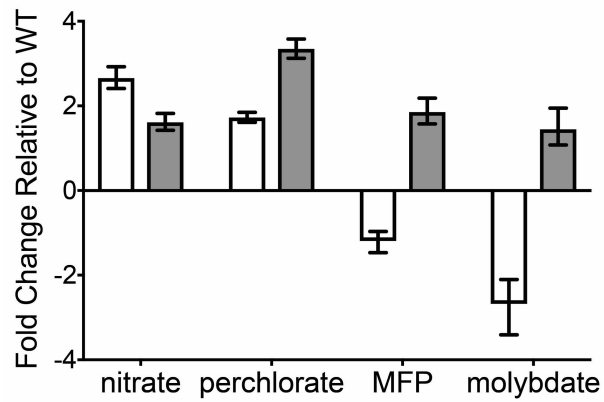


Figure 3.5: Fold change in IC_{50} values for *rex::TN5* (white bar) and adapted-3 (grey bar) strains, relative to wild type IC_{50} , for nitrate, perchlorate, monofluorophosphate (MFP) and molybdate. Error bars represent 95% CI for the IC_{50} value, fit from a dose-response inhibition curve with at least 3 replicates for 11 inhibitor concentrations (33 points).

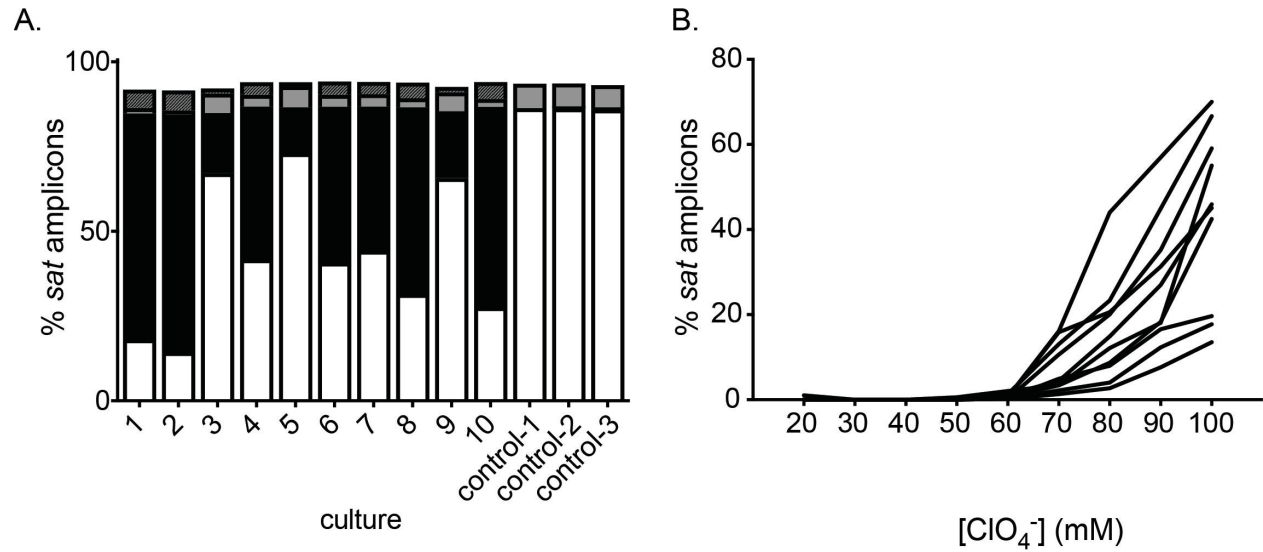


Figure 3.6: (A) % wild type *sat* (white bar), 709T>G (black bar), 913C>T (grey bar) and 709T>G+913C>T (grey/black cross-hatched bar) amplicons across ten independently adapted populations (1-10) and in the 3 control populations (control 1-3) at the end-point of the adaptation (100mM perchlorate). (B) % *sat* amplicons with a G at position 709 across all serial transfers into increasing perchlorate concentrations (20-100 mM perchlorate) and across all ten independently adapted cultures.

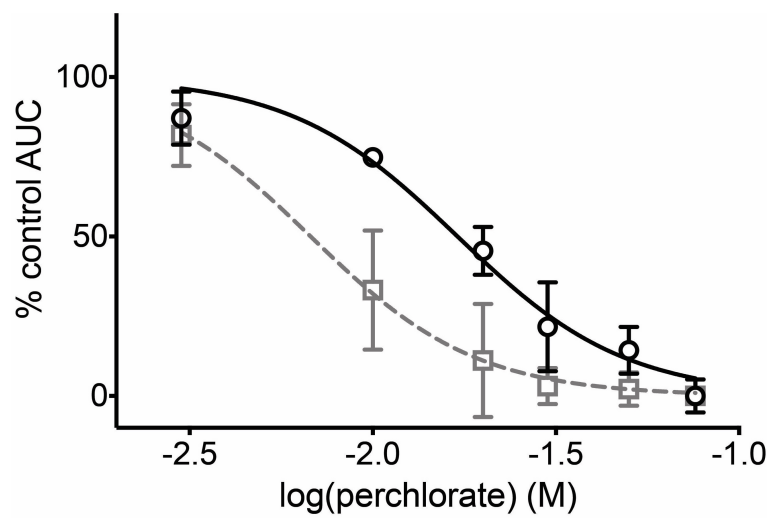


Figure 3.7: Dose-response curve for DvH strain JW9447 (*sat* 709T>G, black circles) and JW9463 (wild type *sat*, grey squares). Error bars represent SD of three replicates.

<u>Limiting Substrate</u>	<u>Steady-state parameter</u>	<u>Wild type Sat</u>	<u>S237A Sat</u>
<i>MoO₄²⁻</i> (<i>Molybdolysis</i>)	k_{cat} (s ⁻¹)	14.5 ± 1.2	14.5 ± 0.5
	K_M (mM MoO ₄ ²⁻)	3.26 ± 0.66	3.68 ± 0.31
	k_{cat}/K_M (M ⁻¹ s ⁻¹)	4.4 x 10 ³	3.9 x 10 ³
<i>ATP</i> (<i>Molybdolysis</i>)	k_{cat} (s ⁻¹)	8.68 ± 0.44	5.09 ± 0.28
	K_M (mM ATP)	0.251 ± 0.049	0.456 ± 0.084
	k_{cat}/K_M (M ⁻¹ s ⁻¹)	3.4 x 10 ⁴	1.1 x 10 ⁴
<i>PP_i</i> (<i>ATP Formation</i>)	k_{cat} (s ⁻¹)	100.4 ± 7.8	213.8 ± 23
	K_M (μM PP _i)	12.3 ± 4.6	28.2 ± 12
	k_{cat}/K_M (M ⁻¹ s ⁻¹)	8.2 x 10 ⁶	7.6 x 10 ⁶

Table 3.1: Steady-state kinetic parameters for the wild type and the S237A Sat, determined using the molybdolysis (for the forward reaction) and ATP formation (for the reverse reaction) assays. The S237A mutation has a minimal effect on the kinetic parameters of Sat under molybdate-limiting conditions but slightly impairs when ATP is limiting. For the reverse reaction, both the wild type and S237A Sat have nearly identical catalytic efficiencies (k_{cat}/K_M). Steady state parameters were calculated by fitting reaction rates under different limiting substrate conditions to a Michaelis-Menten model. Error cited represents the 95% confidence interval.

<u>Limiting Substrate</u>	<u>Wild type Sat K_I</u> (mM ClO_4^-)	<u>S237A Sat K_I</u> (mM ClO_4^-)
MoO_4^{2-} (<i>Molybdolysis</i>)	0.138 ± 0.01	0.394 ± 0.06
ATP (<i>Molybdolysis</i>)	0.915 ± 0.15	2.63 ± 0.81

Table 3.2: Perchlorate inhibition of the wild type and S237A Sat. The S237A Sat is approximately three times more resistant to perchlorate inhibition than the wild type enzyme. Substrate vs. velocity curves for the forward (molybdolysis) reaction were measured under varying concentration of perchlorate. Inhibition constants for each substrate (MoO_4^{2-} and ATP) were calculated by fitting substrate vs. velocity curves to the Michaelis-Menten model in GraphPad Prism.

Table 3.3: Primers used for amplicon sequencing of the *sat* gene.

Primer name	Sequence ¹
ABC1	AATGATACGGCGACCAC
ABC2	ACTGGCCGTCGTTTTAC
AF2	CAAGCAGAAGACGGCATAAC
Pfl_Sat_F	GTAAAACGACGGCCAGTGTGTGTGACGGCGTGGTTATC
Pfl_Sat_R_H 1	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NATCAGC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 2	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NCGATGTC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 3	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNTTAGGCCATACCGT AGAAGTCACCCACAC
Pfl_Sat_R_H 4	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NTGACCAC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 5	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NACAGTGC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 6	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NGCCAATC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 7	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NCAGATCC CATACCG TAGAAGTCACCCACAC
Pfl_Sat_R_H 8	CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NACTTGAC CATACCG TAGAAGTCACCCACAC
FREQseq_IT 001	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> AGCAAT GTAAAACGA CGGCAGT
FREQseq_IT 002	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> CCTGTT GTAAAACGA CGGCAGT
FREQseq_IT 003	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> GGGTTT GTAAAACGA CGGCAGT
FREQseq_IT 004	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> GAAGGC GTAAAACGA CGGCAGT
FREQseq_IT 005	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> ATCTCAGT AAAACGA CGGCAGT
FREQseq_IT 006	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> ATGGAT GTAAAACGA CGGCAGT
FREQseq_IT 007	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> ATGTCT GTAAAACGA CGGCAGT
FREQseq_IT 008	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> CGTGAC GTAAAACGA CGGCAGT
FREQseq_IT 009	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> TTAGGT GTAAAACGA CGGCAGT
FREQseq_IT 010	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> TGTGCAT GTAAAACGA CGGCAGT
FREQseq_IT 011	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> AACTTT GTAAAACGA CGGCAGT
FREQseq_IT 012	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> GGATCGG TAAAACGA CGGCAGT
FREQseq_IT 013	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> AGTCAAGT AAAACGA CGGCAGT
FREQseq_IT 014	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> AGTTCCG TAAAACGA CGGCAGT
FREQseq_IT 015	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> ATGTCAGT AAAACGA CGGCAGT
FREQseq_IT 016	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT</u> CCGTCCG TAAAACGA CGGCAGT

FREQseq_IT 017	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GTAGAGG TAAACGA CGGCAGT
FREQseq_IT 018	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GTCCGCG TAAACGA CGGCAGT
FREQseq_IT 019	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GTGAAAG TAAACGA CGGCAGT
FREQseq_IT 020	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GTGGCCG TAAACGA CGGCAGT
FREQseq_IT 021	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GTTTCGG TAAACGA CGGCAGT
FREQseq_IT 022	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> CGTACGG TAAACGA CGGCAGT
FREQseq_IT 023	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GAGTGGG TAAACGA CGGCAGT
FREQseq_IT 024	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> GGTAGCG TAAACGA CGGCAGT
FREQseq_IT 025	<u>AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT</u> ACTGATG TAAACGA CGGCAGT

[†]underlined region = sequencing adapter; bold = barcode

Table 3.4: Strains and plasmids used in this study.

Strain/plasmid name	Description, Genotype	Source
<i>Escherichia coli</i> strain		
α -select	<i>deoR endA1 recA1 relA1 gyrA96 hsdR17 (r_k⁻ m_k⁺) supE44 thi-1 phoA</i> $\Delta(lacZYA\ argF)U169\ \Phi80lacZ\ \Delta M15\ \lambda^- F^-$	Bioline
<i>Desulfovibrio vulgaris</i> Hildenborough strains		
JW710	parental strain for deletion strains, Δupp ; 5FU ^r	[176]
JW9445	JW710 Δsat ; G418 ^r 5FU ^s	This study
JW9447	JW710 <i>sat</i> 709T>G; 5FU ^r	This study
JW9463	JW710 <i>sat</i> (wild type) ; 5FU ^r	This study
Plasmids		
pCR8/GW/TOPO	parental plasmid, source for pUC Sp ^r	Life Technologies
pMO746	source plasmid for P _{npt} - <i>npt-upp</i> selectable/counter-selectable cassette; Amp ^r Km ^r	[188]
pMO9444	Marker-exchange plasmid for DVU1295 (<i>sat</i>); Sp ^r Km ^r 5FU ^s	This study
pMO9446	mutation-complement for DVU1295 (<i>sat</i> 709T>G); Sp ^r	This study
pMO9462	Wild type-complement for DVU1295 (<i>sat</i>); Sp ^r	This study

Table 3.5: Primers used for DvH genetics.

Name	Sequence ¹	Purpose
SpecRpUC-F	CCAGCCAGGACAGAAATGCCTCG	Amplification of Spec ^r -pUC
SpecRpUC-R	ATGTGAGCAAAAGGCCAGCAAAAGGC	Amplification of Spec ^r -pUC
Kan gene Prom Nterm	CCGGAATTGCCAGCTGGGGCGC	Amplification of kan ^r - <i>upp</i>
<i>upp</i> gene Cterm	CTTACTTGGTGCCGAATATCTTGTCGC	Amplification of kan ^r - <i>upp</i>
DVU1295-4F (pUC OH)	<u>GCCTTTTGCTGGCCTTTTGCTCACAT</u> CCAAGCTGGTTCCCCTCATG	Forward primer for amplification of 5' end of <i>sat</i> for marker-exchange plasmid, wild type complement, and 709T>G mutation complement
DVU1295-403R (KmF OH)	<u>GCGCCCCAGCTGGCAATTCCGG</u> ACCAGCTCGCATTCCTTCTC	Reverse primer for amplification of 5' end of <i>sat</i> for marker-exchange plasmid
DVU1295-dnF	<u>GCGCCCCAGCTGGCAATTCCGG</u> AAATACGAAGGAGGCACCGCAA	Forward primer for amplification of downstream region for marker-exchange plasmid
DVU1295-dnR3	<u>CGAGGCATTTCTGTCCTGGCTGG</u> GGAGTTGCTCATGCAGAGGAACCATA	Reverse primer for amplification of downstream region for marker-exchange plasmid, wild type complement, and 709T>G mutation complement
DVU1295-SNP-R	<u>GGCTTCAGCGAGCCCACGAGGGcGT</u> GGATGACCACACCGTCGCAG	Reverse primer for 709T>G mutation complement
DVU1295-SNP-F	<u>CTGCGACGGTGTGGTCATCC</u> ACgCCCTCGTGGGCTCGCTGAAGCC	Forward primer for 709T>G mutation complement
SpecR-pUC-up	GGGAAACGCCTGGTATCTTTATAGTCCT	Sequencing of cloned regions
pMO719-XbaI-dn	TGGGTTTCGTGCCTTCATCCG	Sequencing of cloned regions
DVU1295(int)-upF	CGACGGCACTTTCTGGCCT	Sequencing of cloned regions

¹underlined region represents overhang used for annealing to neighboring PCR product in SLIC reactions; lower-case letter represents location of nucleotide for 709T>G mutation in *sat*.

Chapter 4

Anion transport as a target of adaption to perchlorate in sulfate-reducing communities

Abstract

The evolution of resistance to perchlorate by SRM impacts its efficacy as a specific inhibitor. As described in Chapter 3, G20 can adapt to perchlorate stress in pure culture through mutation of the sulfate adenylyltransferase, a central enzyme in the sulfate-reduction pathway. However, evolutionary outcomes can differ in communities. Here, we explore adaptation to perchlorate across SRM in a community context. Increasing concentrations of perchlorate altered community structure by selecting for innately more resistant strains. Isolation, whole-genome sequencing, and molecular biology techniques allowed us to define the genetic mechanisms of adaptation across the dominant adapting SRM. Changes in the regulation of divalent anion:sodium symporter family transporters led to increased intracellular sulfate to perchlorate ratios, allowing SRM to escape the effects of competitive inhibition. Thus, in communities, SRM cope with perchlorate stress via changes in transport and its regulation. This highlights the need for a more thorough understanding of transport in order to manage sulfidogenesis across environmental systems.

4.1 Introduction

The use of *specific* inhibitors, i.e. inhibitors that target a particular metabolism in a diverse community, is a promising strategy for controlling the generation of harmful microbial metabolic end-products [11, 29, 189]. One such harmful byproduct is the toxic and corrosive compound sulfide (HS^-), which is primarily produced through the respiration of sulfate (SO_4^{2-}) by dissimilatory-sulfate reducing microorganisms (SRM). SRM form a crucial link between the carbon and sulfur cycles, and are nearly ubiquitous in anaerobic environments [2]. Further, they pose significant economic, environmental, and human health consequences, particularly through corrosion of infrastructure used by the oil and gas, and municipal wastewater treatment industries [112]. Efforts to develop potent and specific inhibitors of SRM have identified strong inhibition by sulfate analogs such as molybdate, nitrate, and perchlorate [10, 13, 14, 18, 28, 29].

The sulfate analog perchlorate (ClO_4^-) is a specific inhibitor of SRM which directly targets the central pathway for sulfate reduction, competitively inhibiting sulfate turnover by the sulfate adenylyltransferase (Sat) [14, 21, 23, 73, 100]. Sat catalyzes the first step in sulfate reduction, using ATP to activate sulfate to adenosine phosphosulfate (APS), which is subsequently reduced to sulfite and sulfide. In the model SRM *Desulfovibrio alaskensis* G20, the primary gene detrimental in perchlorate stress is *rex*, a redox-sensitive transcriptional regulator that represses *sat* and other genes central to sulfate respiration [28, 91, 92]. The de-repression of *sat* increases perchlorate resistance, by providing more enzymes for sulfate turnover. *D. alaskensis* G20 can adapt to 100 mM perchlorate via a single base pair substitution in *sat* (*sat*(709T>G)); *in vitro* characterization of the wild type and mutant Sat reveals that this substitution decreases perchlorate-mediated inhibition of sulfate turnover approximately three-fold [190]. While Sat is a target of inhibition by perchlorate in pure culture, previous work has failed to detect adaptation of sulfate-reducing communities to this inhibitor [25]. Thus, it remains unknown whether *sat*(709T>G) is the primary adaptive mechanism across SRM and in the community context, or whether other mechanisms prevail.

Adaptive laboratory evolution is an experimental approach that can identify mechanisms of adaptation across various contexts [155, 158, 179, 191, 192]. Recent studies highlight the importance of integrating ecology and evolution by studying adaptation in ecologically relevant contexts; accurate predictions of evolutionary outcomes must consider the diversity and composition of communities [193–196]. More recently, Lenski outlined the ESENCE method – ‘embedded species evolving in a natural community’ – as an approach for expanding adaptive evolution studies to entire communities [163]. In the case of perchlorate, understanding both targets and mechanisms of resistance in a community context is of practical value, as perchlorate is a proposed treatment for sulfide production in oil reservoirs supplied with bulk ocean water [21, 24, 28]. Moreover, understanding targets of adaptation to perchlorate stress in new organisms helps provide basic insights into dissimilatory sulfate reduction, its regulation and control over its specificity.

Here, we use adaptive evolution to probe the evolutionary trajectories of both model and non-model SRM in communities and under increasing perchlorate stress. We demonstrate the importance of ecological sorting as an initial response. Isolation, whole genome and metagenomics sequencing and classical molecular biology techniques allow us to further identify two anion transporters critical to the genetic mechanism of adaptation in the dominant adapting SRM. In contrast to previous pure culture results, which emphasize *sat* as a primary target of perchlorate, we find that in a community setting, adaptation targeted anion transport. This work emphasizes

the importance of anion transport and its regulation in response to sulfate analog stress in environmental systems

4.2 Methods

Media and growth conditions

All sulfidogenic communities and pure cultures of *Desulfovibrio bizertensis* strain MKS (MKS) were grown in modified anaerobic APM medium: 20 g/L NaCl, 0.67 g/L KCl, 6.8 g/L TES, vitamins, and minerals [117], supplemented with salt stocks prepared separately and added post-autoclaving (20x: 21.2 g/100mL MgCl₂·6H₂O, 3.04 g/100mL CaCl₂·2H₂O; 50x: 4 g/100mL NaCl, 5 g/100mL NH₄Cl, 0.5 g/100mL KCl, 0.5 g/100mL KH₂PO₄, 1 g/100mL MgSO₄·7H₂O, 0.1 g/100mL CaCl₂·2H₂O). Sodium lactate (60 mM) served as the electron donor and carbon source and 20 mM sodium sulfate served as the electron acceptor. Media was pH adjusted to 7.2 using KOH and rendered anaerobic by flushing with N₂. *D. bizertensis* pure cultures were supplemented with 2 g/L yeast extract and 1 mM sodium sulfide. *Desulfovibrio alaskensis* G20 (G20) [154] was grown in anaerobic MOLS4 medium, as described in previous chapter, with 60 mM lactate and 30 mM sulfate [175]. All cultures were grown at 30°C.

Adaptive evolution in communities

Adaptive evolution experiments were performed using a sulfate-reducing community that was enriched from the San Francisco Bay sediment in modified artificial porewater medium (APM) with 20 mM sulfate and 2 g/L yeast extract [25]. Cultures (3 replicates) were transferred into modified APM with 60 mM lactate, 20 mM sulfate, and 10 mM perchlorate and growth of SRM was monitored by quantifying sulfide production using the Cline assay [96]. Upon reaching early log phase (~ 3-5 days), cultures were transferred at a 10% inoculum to sequentially higher concentrations of perchlorate (5-10 mM increments) up to 100 mM. Control cultures were transferred in parallel in the absence of perchlorate. A second set of community adaptations was augmented with *D. alaskensis* G20. At each transfer, 1 mL was pelleted (5 min at 13,000 x g) and immediately stored at -20°C for DNA extraction, and another 1 mL was archived in 25% glycerol, at -80°C. Archived communities were recovered in the absence of perchlorate, and their resistance to the inhibitor was quantified using dose-response curves, as described below [10, 28]. DNA was extracted using a PowerBiofilm DNA Isolation Kit (Qiagen) modified to exclude bead beating, and used for 16s rRNA community profiling, and amplicon sequencing of the *sat* gene (described below).

Assessing resistance

Respective communities or isolates were used to anaerobically inoculate 384-well microplates (Costar, ThermoFisher Scientific) containing anaerobic medium and a gradient of perchlorate concentrations. These plates were incubated at 30°C for 48-96 h, depending on the growth kinetics of the isolate/community. Growth (OD₆₀₀) was quantified on an Infinite M1000 Pro microplate reader (Tecan). Sulfide was measured via Cline assay, performed using a Biomek XF^P liquid handling robot, and read at 660 nm on the same microplate reader. Growth/sulfide data were normalized to un-inhibited controls and used to construct dose-response curves in GraphPad

Prism 7 (GraphPad Prism Software, La Jolla, CA). This allowed for the determination of IC50 concentrations (concentration inhibiting 50% of growth/sulfidogenesis relative to controls). Dose-response curves were also constructed from growth-curve data: triplicate anaerobic tubes at various perchlorate concentrations were inoculated with the selected strain and growth was monitored as either/and OD600 (Spectronic 20D+) or sulfide (measured via cline assay). For each perchlorate concentration, the area under the growth curve (AUC) was normalized to the control AUC; this data was used to generate a dose-response curve and calculate IC50/Hillslope parameters as outlined previously (Section 3.2). Statistical significance was also calculated in Graphpad prism, as outlined in their Statistics Guide.

16s rRNA community profiling

Community DNA was extracted using a DNeasy Powersoil HTP 96 kit (Qiagen), following manufacturer's instructions, and quantified using a Quant-iT HS Kit (Invitrogen), on an Infinite M1000 Pro microplate reader (Tecan), following manufacturer's instructions. Primer pair Miseq 16s F and Miseq 16S R (A519F and Bakt_805R, modified to include Illumina adapters) were used to amplify the HV region V4 of the 16s rRNA gene [120, 122]. Libraries were prepared as per the Illumina 16s Metagenomic Sequencing Library Preparation protocol, quantified (Quant-it), normalized using a Biomek XFP liquid handling robot, and pooled. Sequencing was performed at the UC Davis Genome Center (Davis, CA) on a Miseq (Illumina Miseq Reagent Kit V2). Demultiplexed samples were analyzed according to the Mothur Miseq SOP [120, 123]. Briefly, chimeras were removed using UCHIME [124] and remaining sequences were screened based on an expected length of approx. 287bp. Sequences were then clustered at the 97% similarity level and classified using the RDP and SILVA databases. The resulting OTUs were used to generate an OTU table across samples. Abundances were normalized to total amplicon counts per sample.

Amplicon sequencing of the sat gene

In order to determine the importance of the *sat*(709T>G) mutation in the community-evolved G20, amplicon sequencing of a portion of the *sat* gene was performed, as described in Chapter 3 [174, 190].

Isolation, whole genome sequencing, and variant calling

Adapted and/or control isolates of *Desulfivibrio bizertensis* MKS and *Desulfovibrio alaskensis* G20 were obtained via serial dilutions of 100 mM perchlorate-adapted communities in 1.5% agar APM plugs [111], or by plating communities on solid MOLS4 medium [151, 197]. All isolates were grown in 100 mM perchlorate-amended modified liquid APM medium, prior to collecting 1 mL of culture for DNA extraction. Due to the low abundance of *D. bizertensis* MKS in the final control communities, single colony isolates could not be obtained, so community DNA was used for shotgun metagenomics. DNA was extracted as previously described and submitted to the QB3 Functional Genomics Lab and Vincent J. Coates Genomics Sequencing Lab (Berkeley, CA) for library prep and 100PE sequencing (genomic DNA) or 150PE sequencing (metagenomic DNA) on an Illumina HiSeq4000 lane.

Sequencing data was used for variant calling, as described in Chapter 3 [190]. Briefly, demultiplexed raw forward and reverse reads were mapped to the reference *Desulfovibrio alaskensis*

G20 (IMG Genome ID 637000095) or *Desulfovibrio bizertensis* MKS (IMG Genome ID 2687453693) genomes using Bowtie2 [198]. Resulting BAM files were sorted, processed to remove PCR duplicates and indexed (<http://broadinstitute.github.io/picard>) [199]. Variant calling was performed using the Genome Analysis Toolkit (GATK) HaplotypeCaller. SNPs and INDELs were filtered by quality of depth (QD <10), strand bias (FS>60) and mapping quality (MQ<40). Genome-wide coverage was assessed as previously outlined [170].

Metagenomic reads were aligned to *Desulfovibrio bizertensis* MKS using the Lasergene SeqMan Pro package (DNASTAR) and SNPs were sorted and compared among samples using the ArrayStar package (DNASTAR).

RNA extraction, cDNA synthesis and RT-qPCR

Triplicate cultures were grown to mid-log and 1 mL was pelleted via centrifugation at 4°C and stored in 0.75 mL TRIzol reagent (Invitrogen) at -80°C. RNA extractions were performed as per manufacturer's instructions (TRIzol Reagent user manual). Extracted RNA was treated with TURBO DNase (Invitrogen) twice and subsequently purified over an RNA column (Allprep DNA/RNA Mini Kit, Qiagen), according to manufacturer's instructions. RNA purity was checked via PCR using the 27F/1525R primer set [121] and first strand synthesis was performed using SuperScript (Invitrogen). The first strand synthesis reaction was used as a template for RT-qPCR. Triplicate technical replicates were set up for each biological replicate, as well as a no-RT control per sample and triplicate negatives per primer set. All primer sets used for RT-qPCR are listed in Table 4.2. qPCR analyses were run on a StepOnePlus qPCR machine (Applied Biosciences), using the SYBR qPCR Master Mix (Thermo Scientific) as per manufacturer's instructions.

RNAseq

Initially, RNAseq libraries were constructed in house, using a modified method from [200]. Triplicate cultures of Dde_0320::TN5 and wild type G20 were grown to mid-log, under no-stress conditions, and 3 X 1 mL of culture was pelleted for RNA. RNA was extracted and treated with DNase as described above, using TRIzol (Invitrogen). rRNA depletion was performed using the Ribo-Zero RNA Removal Kit (Illumina), as per manufacturer's instructions. rRNA-depleted RNA was quantified using a Qubit RNA HS Kit (Invitrogen) and used for double-stranded cDNA synthesis via the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Double stranded cDNA concentrations were quantified using a Quant-iT dsDNA High Sensitivity Kit (Invitrogen), and approx. 1 ng was used for the tagmentation. Each 20 uL tagmentation reaction containing 4 uL 5X TAPS/MgCl₂/Peg 8000 Buffer and 1 uL Tn5, as per [200], was incubated at 55°C for 7 min. The tagmentation reactions were terminated by adding 5 uL of 0.2% SDS, and incubating for 5 min at room temperature. The 25 uL tagmentation reactions were then used for a limited-cycle PCR, with the Nextera index primers (Illumina) and KAPA HiFi HotStart MasterMix (KAPA Biosystems), as per manufacturer's instructions (Nextera XT DNA Sample Preparation Kit, Illumina). A post-PCR purification was performed using AMPure XP beads and a 1:1 ratio of beads to DNA. The final elution was performed in 15 uL water, and samples were quantified using the KAPA Library Quantification kit (Kapa Biosystems) and sequenced on an Illumina Miseq with a 600bp kit (the run stopped at base 445 so we did not get the full length read). Samples were de-multiplexed post sequencing and analyzed using the RockHopper software [201].

A second round of RNAseq libraries were constructed with Dde_0320::TN5 and wild type G20, this time under “stress” conditions: triplicates of each strain were grown as previously described, but upon reaching mid-log phase, samples were spiked with 100 mM perchlorate. RNA was collected 30 min, 1.5 h and 2.5 h after the spike. RNA from each time point was extracted and treated with DNase, as above. The resulting RNA was used for cDNA synthesis and RT-qPCR and samples from the 1.5 h time point were submitted for rRNA depletion and RNAseq library preparation at the QB3 Functional Genomics Lab (QB3 Berkeley). These libraries were sequenced on a HiSeq4000 100SR lane (Vincent J. Coates Genomics Sequencing Laboratory, Berkeley), and also analyzed using Rockhopper.

Pure-culture experiments

Adaptation in the community context was compared to adaptation in pure-culture. To this end, a pure-culture laboratory evolution experiment was performed (as described above) using the ancestral *Desulfovibrio bizertensis* strain MKS (isolated as described above, prior to any adaptive evolution experiments): five adapting populations were compared to two control populations. For pure culture experiments, growth was monitored via optical density at 600 nm (Supplemental Methods).

Results from a previous pure-culture adaptation of G20 to perchlorate revealed the dominance of a mutation in the sulfate adenylyltransferase (*sat*(709T>G)). In order to assess the relative fitness of the *sat*(709T>G) mutation and the Dde_0320::TN mutation, which dominated in the community adaptations, both genotypes were compared under a variety of conditions. Specifically, the growth of Pat3 (Table 4.1) and Dde_0320::TN5 was compared in MOLS4 [175], as well as in the modified APM medium. Each strain was grown in each medium, with or without 100 mM perchlorate. Pat3 and Dde_0320::TN5 both differ from wild type G20 only in the respective gene (*sat* or Dde_0320) and hence serve as a proxy for the relative fitness of the respective mutations. However, the TN5 expresses the Kan^R gene, which may itself have a “cost” in terms of growth. Hence, the community-adapted strains carrying mutations in these same genes were also compared, both in MOLS4 and in APM: Pg11 has the *sat*(709T>G) mutation and Pg33 has the Dde_0320::TN insertion. Again, growth was compared both with and without 100 mM perchlorate. Finally, both strains were competed directly, in 100 mM perchlorate, both in the presence and in the absence of the fermentative fraction of the community. Briefly, triplicate tubes APM + 100 mM perchlorate were inoculated with Pg11 and Pg33 in a 1:1 ratio and sampled at the start of the growth curve, at the end of the growth curve (4 days later), and through two more successive transfers in APM + 100 mM perchlorate. At each sampling point, 2 mL of culture was pelleted and stored at -80°C. DNA was extracted as outlined above and used for qPCR analysis. The same competition experiment was also performed in a background of the fermentative community fraction (obtained by sub-culturing the community in the absence of sulfate), and sampled in parallel. qPCR primers were developed to quantify the G20 population as a whole (targeting the G20 *gyrB*) as well as the TN mutant specifically (TN-genome junction) (Table 4.2). The efficiency of both primer sets was tested across a dilution series of Pg33 (for which both primer sets should yield identical results assuming equal primer efficiencies). Indeed, primer sets had the same efficiencies ($p=0.25$, Wilcoxon matched-pairs signed rank test). qPCR analyses were performed in triplicate, as previously described, and ΔC_T values were calculated as the average difference in C_T values between the primer sets, and were subsequently converted to an absolute ratio of TN mutant / total G20 ($2^{\Delta C_T}$).

Construction of MKS overexpression strains

The pBMK7 plasmid used for all overexpression in MKS was linearized via PCR. Inserts were amplified from MKS gDNA, gel purified, cloned into the pBMK7 backbone using the Gibson Assembly 2x MasterMix (NEB), and transformed into chemically competent XL1 Blues cells (UCB DNA Sequencing Facility), as per manufacturer's instructions. Colonies were screened using the M13F/R primer set and mini-prepped using the QIAprep Spin Miniprep Kit (Qiagen). The constructs were then transformed into *E. coli* strain WM3064.

MKS was transformed via conjugation with WM3064, as previously described [202]. Briefly, WM3064 was grown overnight in LB with 50 ug/mL kanamycin sulfate and 0.3 mM DAP. For each conjugation, 500 uL of WM3064 was pelleted and washed in anaerobic APM (lactate, sulfate) with 1 mM sulfide and 0.3 mM DAP. The various MKS strains (wild type or adapted) were grown to mid-log, pelleted and re-suspended with the washed WM3064 in 500 uL of the anaerobic medium described above. The co-culture was re-pelleted, re-suspended in 30 uL, and left at room temperature in an anaerobic glovebag (Coy) for ~6 h. The culture was then transferred into APM media with 400 ug/mL G418. Single colony transconjugants were obtained by diluting and transferring to APM+G418 agar plugs (1.5% agar).

Construction of E. coli CysA complementation strains

The pOW107 plasmid was used for all overexpression in *E. coli* as it was assumed that constructs (consisting of the transporter Dde_0326 and surrounding genes) needed a constitutive but weak promoter to avoid toxic effects. pOW107 was linearized via PCR and gel purified. Inserts were amplified from wild type G20 gDNA, gel purified and cloned into the pOW107 as described above. Colonies were screened using the pOW_13_F/R primer set. Positive colonies were grown in 5 mL LB + 50 ug/mL carbenicillin and mini-prepped using the QIAprep Spin Miniprep Kit (Qiagen). The constructs were then transformed into the *E. coli* CysA knock-out (KO) strain JW2415. Briefly, an overnight culture of JW2415 was used to inoculate 5 mL of LB + 50 ug/mL kanamycin, which was grown at 37°C to an OD of ~0.6, and then cooled in an ice-bath. 1 mL of this culture was used per transformation: cells were washed twice in 1 mL of cold water, re-suspended in 50 uL cold water and mixed with ~100 ng of plasmid. Standard electroporation conditions for *E. coli* were used (1800 V, 200 Ω and 25 μ F). Cells were allowed to recover in 3 mL LB for a minimum of 3 h before plating on selective media (LB+kan/carb). Single colonies were confirmed via Sanger sequencing (UCB DNA Sequencing Facility) and used to test for complementation of the sulfate uptake deficiency phenotype of JW2415. Briefly, strains were inoculated in M63 minimal media, with either 250 uM sulfate or 250 uM cysteine as a sulfur source and growth was monitored as OD600 (Tecan Sunrise microplate reader). For a list of all constructs/strains and primers, see Table 4.1 and Table 4.2, respectively.

Computational reconstruction of transcriptional factor binding sites

We used the comparative genomics approach described in [203]. Briefly, close homologs of Dde_0318 and Dde_0323 proteins were identified in the NCBI GenBank database using the BLAST service [204]. Upstream regions of the operons encoding Dde_0318 and Dde_0323

homologs (Table 4.6) were used for construction of positional weight matrix representing Dde_0318 binding motif. That positional weight matrix was used for identification of putative binding sites in the G20 genome.

Intra-cellular sulfate and perchlorate measurements

Intracellular perchlorate and/or sulfate were measured and compared between *D. bizertensis* MKS strains overexpressing TF_{Pr} transporter vs. control (P24EV and P24.1), and between wild type G20 and the Dde_0320::TN5 mutant. In all cases, triplicate cultures (1 L for MKS and 100 mL for G20) of each strain were grown to OD $\approx$ 0.6, pelleted and re-suspended in 100 mM perchlorate-amended APM (10 mL, MKS strains) or MOLS4 (40 mL, G20 strains). Cultures were incubated at 30°C for 1-2 h, then pelleted and washed five times in phosphate-buffered saline, before finally re-suspending in 1 mL of DI water. OD600 was measured before lysing cells at 98°C for 30 min. The soluble fraction was filtered (0.2 μ m), diluted 1:5 in DI water and submitted to the Andrew Jackson laboratory (Texas Tech University) for sulfate and perchlorate quantification via IC-MS/MS. IC-MS/MS results were normalized to OD, for a final value in terms of concentration/OD.

4.3 Results

Sulfate-reducing communities initially adapted to perchlorate through shifts in community structure

We investigated the adaptation of sulfate-reducing communities to the sulfate analog perchlorate in terms of overall community structure and the physiology and genetics of the dominant taxa. Sulfate-reducing communities were adapted via serial transfers into increasing concentrations of the inhibitor (Figure 4.1A). Parallel control communities were transferred in the absence of perchlorate. The inoculum consisted of a community enriched from estuary sediment that subsequently was either unaltered (“native”) or biologically augmented with model SRM *Desulfovibrio alaskensis* G20 (“G20-augmented”). G20 adaptation to perchlorate has been previously characterized in pure culture, and thus bio-augmentation allowed for direct comparison to pure culture results [190].

All replicate, independently adapted, native and G20-augmented communities, showed significantly increased resistance to perchlorate. The half maximal inhibitory concentrations (IC50s) for native adapted communities were between 90-180 mM (95% confidence interval of 77-224 mM) and 57-98 mM (95%CI 48-121 mM) for the G20-augmented condition, compared to 22-34 mM (95%CI of 20-28mM) and 20-26 mM (95%CI 18-29 mM), for control native and augmented communities, respectively (Figure 4.1B). Further, native adapted communities, unlike controls, displayed robust sulfidogenesis during the final transfer into 100 mM perchlorate (Figure 4.2A). G20-augmented adapted communities showed varying degrees of sulfidogenesis in 100 mM perchlorate: replicate 1 demonstrated no sulfide production, replicate 2 produced sulfide robustly, and replicate 3, only slightly (Figure 4.2B).

Community structure differed between native and augmented conditions, and with increasing perchlorate concentration. Control communities, both native and G20-augmented, consisted of approximately 85% *Desulfovibrio* spp. This community structure was stable over

time, with the exception of G20 (in augmented communities), which was eventually outcompeted by native *Desulfovibrio* spp. (Figure 4.1C). G20-augmentation also increased *Desulfovibrio bizertensis* strain MKS abundance, as compared to native controls. In contrast, adapting communities started with the same structure but changed as perchlorate concentration increased. In all native adapting communities, at 20-40 mM perchlorate a population of *Desulfocurvus* increased as most *Desulfovibrio* spp. decreased, and above 40 mM a population of *Desulfovibrio bizertensis* MKS (MKS) outcompeted *Desulfocurvus*, ultimately expanding to cover the entire SRM niche across all three independent adaptations. Augmented communities also saw a decrease in various native *Desulfovibrio* spp. and a concomitant increase in G20, which came to occupy a majority of the SRM niche. However, variability among independent replicates was greater in augmented communities: in replicate 1 G20 was largely displaced by a non-sulfate reducing gammaproteobacterium *Psychromonas* (explaining the lack of sulfide production in the final 100mM transfer). In replicates 2 and 3, G20 was sustained until the final transfer. Replicate 2, where sulfidogenesis was most robust, most closely resembled native communities, showing a similar expansion of *Desulfocurvus* and ultimate rise of MKS.

Changes to community structure could be explained by variability in the innate resistance to perchlorate of different SRM. We compared the resistance of *D. bizertensis* MKS and a previously isolated *Desulfovibrio* sp. from this community (*D. sp.* BMSR) [25], both isolated prior to any adaptation on perchlorate (Figure 4.3, Figure 4.4). Although the two strains did not differ in their IC50 values, MKS had a significantly less steep hillslope ($p=0.0044$, Extra sum-of-squares F test), and was therefore less inhibited at higher concentrations of perchlorate (>20 mM), growing slowly in even 50-100 mM (Figure 4.4). *D. alaskensis* G20 was more resistant than BMSR ($p=0.04$, Extra sum-of-squares F test) and grew faster at <50 mM perchlorate than both other *Desulfovibrio* sp. tested (Figure 4.3, Figure 4.4). The *Desulfocurvus* spp. from this community was not isolated but was previously shown to persist in chemostats treated with 50 mM perchlorate [25]. These results suggested that initial adaptation of communities to perchlorate was through an ecological mechanism: the elimination of less resistant taxa. We next explored beneficial adaptations acquired by the ecologically successful taxa, *D. bizertensis* MKS and *D. alaskensis* G20, allowing them to grow robustly in 100 mM perchlorate.

Mutation of the sulfate adenylyltransferase (sat) was not a dominant mechanism of adaptation to perchlorate

The most abundant strains in each sulfate-reducing community (MKS and G20) were isolated and whole-genome sequenced to identify genetic mechanisms of adaptation. Because genomes might contain both adaptive and non-adaptive mutations, sequences from adapted and non-adapted conditions were compared to determine mutations specific to perchlorate adaptation. We hypothesized that adaptation would target the sulfate adenylyltransferase (*sat*), as had been observed previously in pure culture experiments with *D. alaskensis* G20 [190].

Single colony MKS isolates from adapted communities (8 total) were whole-genome sequenced and compared to total community sequencing reads from controls (strain MKS could not be isolated from control communities) and the reference genome. Of the small set of mutations that occurred in adapted but not control MKS (Table 4.3), no mutations were detected in *sat*.

In *D. alaskensis* G20, we initially used previously published methods to determine the frequency of the *sat*(709T>G) variant that had arisen repeatedly during previous adaptation experiments [17]. This analysis revealed that only in the first community did *sat*(709T>G) arise,

and as of the 100 mM transfer, it constituted only 0.5% of all *sat* amplicons. Isolates of G20 from control and recovered adapted communities were also whole-genome sequenced and compared. Only three of thirteen isolates, all from community 1, contained the mutation in *sat* (Table 4.4). All other G20 isolates contained a mutation in either Dde_0320 or Dde_0321, predicted signaling components.

The predominance of these mutations over the *sat*(709T>G) mutation might be explained by medium composition or by community effects. A comparison of the *sat*(709T>G) strain Pg11 to strain Pg33, with an inactivation of the Dde_0320 gene, revealed that Pg11 was less competitive in the medium used in this study (APM) than that used in previous studies (MOLS4) (Figure 4.5AB). Two additional representative strains (Pat3 and Dde_0320::TN5, Table 4.1) showed the same pattern (Figure 4.6). Direct competition of Pg11 and Pg33 in 100 mM perchlorate-amended APM revealed that Pg33 outcompeted Pg11 and its domination was stable (Figure 4.5C). In co-culture with fermentative organisms, Pg33 took longer to dominate, and did so less stably. Thus, it seems that both community and medium effects shift the cost/benefit of adaptive mechanisms. Regardless of the cause, in our community adaptations neither of the dominant SRM adapted primarily via mutations in the *sat*.

Adaptation by decreasing perchlorate import

The most common mutations in *D. bizertensis* MKS occurred in a locus containing a transcription factor, a transporter, and accessory genes (Figure 4.7A). Six of eight isolates had mutations in the DNA-binding helix-turn-helix (HTH) motif of a cAMP receptor protein-like transcription factor (TF_{Pr}, Ga0134258_11426), and all other isolates contained a nonsense mutation in the divalent anion:sodium symporter (DASS) family transporter (DASS_{Pr}, Ga0134258_11427) gene directly upstream of TF_{Pr}. A mutation in TF_{Pr} was also detected in the only sequenced MKS strain from G20-augmented community 2 (the augmented community where MKS persisted) (Table 4.3). To determine if mutations in this operon occur in pure culture adaptations, the ancestral MKS strain was independently adapted to 100 mM perchlorate. All five adapted populations showed increased resistance to perchlorate compared to the two control populations (Figure 4.8). Five single-colony adapted isolates across the populations were sequenced and all revealed mutations in either TF_{Pr} or DASS_{Pr} (Table 4.3). Therefore, in all biological conditions tested (pure culture, native community, G20-augmented community), mutations in TF_{Pr} or DASS_{Pr} were correlated with perchlorate resistance in MKS.

Comparative genomics suggested that TF_{Pr} regulates expression of the downstream operon. The operon is conserved across four other SRM: *Desulfovibrio bizertensis* DSM 18034, *Desulfofustis glycolicus* DSM 9705, *Mailhella massiliensis* Marseille-P3199, and *Desulfobulbus* sp. ORNL (Figure 4.9, Table 4.5). In three of these organisms (MKS, DSM 9705 and Marseille-P3199) there is an inverted repeat upstream of the operon, which may serve as a TF binding site. Each respective genome was scanned for the presence of additional respective TF binding sites, and none were found. Thus, TF_{Pr} likely regulates expression *only* of the downstream operon.

We hypothesized that increased resistance to perchlorate was caused by inactivation of the DNA-binding ability of TF_{Pr}, thereby changing expression of the downstream operon. In agreement with this, one of the adapted strains containing a mutation in the HTH domain of TF_{Pr}, strain P24 (Table 4.1), had significantly lower expression of DASS_{Pr} as compared to wild type MKS ($p < 0.0001$, Mann-Whitney test) (Figure 4.10). Because decreasing transcription initiation by the TF_{Pr} correlated with increased fitness in 100 mM perchlorate, we tested whether *increasing*

TF_{Pr} expression harmed the fitness of MKS in these conditions. The following strains were constructed (Table 4.1): overexpression of wild type TF_{Pr} (wt.1), overexpression of P24 mutant TF_{Pr} (wt.2), and an empty vector control (wtEV) in the wild type MKS background; and also an empty vector control in the P24 background (P24EV). Without perchlorate, all strains grew similarly (Figure 4.7B). With 100 mM perchlorate, the greatest growth occurred in the P24 control (P24EV), followed by the wild type control (wtEV) and the overexpression of the mutant TF_{Pr} (wt.2); the strain overexpressing the wild type TF_{Pr} did not grow. RT-qPCR indicated that expression of DASS_{Pr} matched the gradient of “intact” TF_{Pr} levels in each strain: from lowest to highest, P24EV, wtEV, wt.2, and wt.1 (Figure 4.7C). The degree of resistance to perchlorate was strongly negatively correlated with expression of the DASS_{Pr} transporter.

A simple explanation would be that DASS_{Pr} is permeable to perchlorate. If true, decreasing expression of the transporter would decrease perchlorate import into the cell. Direct overexpression of DASS_{Pr} in adapted strain P24 (P24.1) rendered the strain sensitive to perchlorate compared to the empty vector control (Figure 4.11A). When incubated in the presence of 100 mM perchlorate, P24.1 showed significantly more intracellular perchlorate than P24EV ($p=0.046$, Unpaired t-test) (Figure 4.11B). Additionally, overexpression of the transporter in the wild type MKS background also increased sensitivity of that strain (data not shown). These data indicate that DASS_{Pr} is detrimental to fitness in high concentrations of perchlorate because it transports perchlorate into cells. In summary, strain MKS adapted to perchlorate by inactivating this transporter, via a nonsense mutation directly in the transporter or by mutating TF_{Pr}, responsible for its expression, thereby decreasing perchlorate import into cells (Figure 4.14A).

Adaptation by increasing sulfate import

All sequenced perchlorate-adapted *D. alaskensis* G20 isolates that did not contain mutations in the *sat*, contained a mutation in the signaling proteins Dde_0320 (7 isolates) or Dde_0321 (3 isolates) (Table 4.4). Dde_0320 encodes a histidine kinase with a cytoplasmic kinase domain and an extracellular sensory domain. Dde_0321 encodes a protein with a predicted response regulator receiver domain. The resistance phenotype for the histidine kinase null was confirmed by comparing the growth of wild type G20, adapted strain Pg33 (Table 4.1) and G20 strain Dde_0320:TN5 (Table 4.1, courtesy of Adam Arkin). Disruption of the histidine kinase was sufficient for growth in 100 mM perchlorate (Figure 4.12B). We predicted that the genes regulated by the histidine kinase were responsible for adaptation to perchlorate.

The Dde_0320 histidine kinase is part of an operon, which includes genes encoding σ^{54} -dependent Fis family transcriptional regulator Dde_0318, hypothetical protein Dde_0319, protein Dde_0321 with a response-regulator receiver domain, and histidine kinase-like protein Dde_0322 (Figure 4.12A). The operon has a predicted σ^{54} -dependent promoter, as does the downstream Dde_0323-Dde_0328 operon, which contains genes encoding DASS family transporter Dde_0326, CBS domain protein Dde_0325, and four proteins again putatively involved in signal transduction [205]. Dde_0319, Dde_0320 and Dde_0321 have been previously identified as detrimental under perchlorate and monofluorophosphate stress (another specific inhibitor of SRM) [28, 53]. Thus, as this locus is consistently associated with perchlorate stress, we term genes Dde_0318-Dde_0328 the “perchlorate cluster” and henceforth refer to the transcriptional regulator, Dde_0318, as PclR, to the histidine kinase (Dde_0320) as PclS, and to the Dde_0326 transporter as PclT.

Comparative genomics suggested that PclR controls expression of both its own and the downstream operon. Orthologs of PclS were identified in *Desulfovibrio vulgaris* Hildenborough (DvH) and *Desulfovibrio vulgaris* Miyazaki (DvM) (Figure 4.12A) [205, 206]; in DvM, as in G20, the ortholog was detrimental to perchlorate stress [54]. Both DvH and DvM operons are regulated by Fis family σ^{54} -dependent transcriptional regulators DVU2956 in DvH, and DvMF_1763 in DvM [206]. G20 lacks an ortholog to DVU2956/DvMF_1763 and the entire locus contains only one gene encoding a regulatory DNA-binding protein, *pclR* (Figure 4.12A). Conserved binding sites for PclR were identified upstream of both operons in the perchlorate cluster (*Supplemental Methods* and Table 4.6). A whole-genome scan revealed three additional putative binding sites: upstream of Dde_0191, Dde_0218 and Dde_0683/Dde_0685, encoding a kinase, a methyl-accepting chemotaxis sensory transducer, and a cytochrome c family protein/ formylmethanofuran dehydrogenase subunit, respectively (Table 4.7). From this analysis, we conclude that PclR is the regulatory protein necessary for activation of both σ^{54} -dependent promoters in the perchlorate cluster, and, possibly of some other genes.

In order to determine how PclS fits into this regulatory network, we compared transcriptional profiles of wild type G20 vs. the Dde_0320::TN5 strain, both in the absence (no stress) and presence of 100 mM perchlorate (stress), using RNAseq. In both stress and no-stress conditions, the region upstream of PclR had significantly greater expression (213x and 300x, respectively) in Dde_0320::TN5 compared to wild type (Table 4.8). The downstream genes of the perchlorate cluster (Dde_0318-Dde_0319 and Dde_0323-Dde_0326) were also overexpressed (approx. two to four-fold). From the computationally identified targets of PclR described above, only Dde_0218 is differentially expressed both under stress and no-stress conditions (~9x up-regulated in Dde_0320::TN5) (Table 4.8). Additional sets of genes with differential expression (Table 4.9) are not discussed here (see: Discussion). We propose that PclS senses a signal and directly or via a regulatory cascade modulates the activity of PclR, thereby repressing transcription of the perchlorate cluster. Inactivation of PclS, hence, increases transcription of all gene targets (Fig 7B).

The regulon of PclR contains only one gene with putative activity towards sulfate or sulfate analogs: PclT, another DASS family transporter. The closest studied homolog of PclT, HRM2_40290 from *Desulfobacterium autotrophicum* HRM2, is a putative sulfate symporter expressed under high sulfate conditions and co-localized with regulatory elements [50]. We hypothesized that PclT is also a tightly regulated sulfate symporter. Direct measurements of intracellular sulfate and perchlorate in the Dde_0320::TN5 vs. wild type strains found that although intracellular perchlorate concentrations did not differ ($p=0.32$), sulfate concentrations were above detection in the Dde_0320::TN5 strain and below detection in wild type (Table 4.10). Even with a conservative estimate of sulfate in wild type, placing it at the detection limit, this difference is significant ($p=0.004$). To confirm sulfate transport by PclT, we heterologously expressed either PclT alone, or the entire operon (Dde_0323-Dde_0328) in *E. coli* strain JW2415 (deficient in sulfate uptake ($\Delta cysA$)) (Table 4.1) [207]. Both constructs complemented the sulfate uptake deficiency phenotype (Figure 4.13). Thus, PclT can transport sulfate; other genes in its operon may modulate this transport activity. In summary, G20 adapted to perchlorate by inactivating PclS, thus increasing transcription of the perchlorate cluster and sulfate import via PclT. Increased intracellular sulfate, relative to perchlorate, can overcome the competitive effects of perchlorate on Sat-mediated sulfate turnover (Figure 4.14B).

4.4 Discussion

The experiments outlined above describe community-wide and species-specific responses to increased perchlorate concentrations. Both native and G20-augmented communities initially adapted to the inhibitor via changes in community structure. Using a combination of isolation, WGS and genetics we show that across the two dominant adapting SRM, adaptation to perchlorate targets regulatory elements controlling anion transport systems, thereby modulating the intracellular concentrations of sulfate and perchlorate.

Selection initially acted on species abundances (*ecological sorting*), allowing for the expansion of innately more resistant SRM [208]. Initially dominant *Desulfovibrio* spp. made way to a *Desulfococcus* sp., which in turn made way to MKS or G20. In our community, the sulfate-reducing niche was consistently around 85%; thus, SRM had to compete for this niche during adaptation. G20 was only competitive in perchlorate-adapting conditions. In control communities G20 was outcompeted, but strikingly, caused an increase MKS; this is indicative of a possible synergy between the two, or else G20 negatively affected other *Desulfovibrio* which otherwise outcompete MKS. In contrast to the consistency observed across replicate native adapting communities, the added competition from G20 created variability in augmented communities. G20 had a slight advantage (it can grow rapidly at low perchlorate concentrations), which was tightly balanced by being innately less competitive in this medium and community context.

Genomic adaptation ultimately allowed for robust sulfate-reduction in 100 mM perchlorate. In *Desulfovibrio bizertensis* MKS, adaptation was accomplished by decreasing the transcription initiation ability of TF_{Pr} . This decreased expression of the $DASS_{Pr}$ transporter and consequently, non-specific perchlorate transport into cells. In *Desulfovibrio alaskensis* G20, adaptation targeted $PclS$, a histidine kinase, leading to increased transcription of the perchlorate cluster, including $PclT$, a sulfate symporter. Inactivation of $PclS$ led to increased intracellular sulfate, via $PclT$. Both decreased perchlorate transport and increased sulfate transport increased the intracellular sulfate:perchlorate ratio, thus overcoming the effects of competitive inhibition (Figure 4.14).

Previous work suggested that SRM adapt to perchlorate by mutating the conserved sulfate adenylyltransferase, responsible for catalyzing the first step of sulfate-reduction. In contrast, our study finds that SRM in an ecological context adapt to perchlorate via mutations in transport systems or the regulatory systems controlling them: targets that are less conserved and much more redundant. Model SRM DvH, for example, has 72 response regulators, the majority of which lack a DNA-binding domain and instead likely act via protein-protein interactions [35, 209]. The diversity of regulatory systems in SRM reflect how these organisms cope with stress [35]; unsurprisingly, they represent an easy target for adaptation.

Sulfate transport in SRM is similarly complex and redundant: G20 has 11 putative sulfate transporters, whose function and relative importance remain uncharacterized [51]. Transport systems have been previously defined as targets of various stressors: in G20, DvH and DvM, nitrate stress targets genes of the “nitrate cluster”, which include a CysZ-type permease (Dde_0598) [17, 28, 54]. Nitrate resistance is attributed to the loss of this permease and decreased nitrate entry into cells. Similarly, the TunR2 regulator in DvH and other SRM was predicted to protect against inhibition by increasing sulfate uptake via TSUP and SulP-family transporters [68].

SRM genomes contain on average three $DASS$ family transporters [51]. Few transporters of this family have been experimentally characterized. An exception is the $PerO$ anion permease of *Rhodobacter capsulatus*, which when inactivated increases resistance to molybdate, tungstate

and vanadate [55]. Moreover, sulfate transport and increased resistance was confirmed for five homologous permeases across the Proteobacteria [56]. PerO is a general, low-affinity anion transporter – i.e. promiscuous – setting a precedent for the non-specific transport observed by DASS_{Pr}. In *Desulfobacterium autotrophicum*, DASS family transporters are either expressed constitutively, or are tightly regulated [50]. PclT is homologous to HRM2_40290 from *D. autotrophicum*, which is expressed only in “high” sulfate (~15mM). Collectively, our study and others cited above define sulfate/anion transport as a critical, highly redundant and poorly understood function in SRM.

The native function of the targeted operon in MKS remains unknown. Operons regulated by CRP-like transcription factors and encoding transporters and thioredoxin/thioredoxin reductases are implicated in nitrate and oxidative stress and in U(IV)/ Cr(VI) reduction [210–212]. It is possible that in MKS this operon is involved in cytoplasmic redox balance. The native function of the perchlorate cluster is likely related to sulfate uptake via PclT. RNAseq data, however, identified several additional loci/regulons significantly up-regulated in the Dde_0320::TN5 strain (Table 4.9). The first consisted of genes involved in fumarate reduction, and critical for growth on malate or fumarate, and by pyruvate fermentation (Dde_1250-Dde_1258) [151, 168, 197]. G20 grown in pyruvate/sulfate medium was previously shown to display increased resistance against perchlorate, perhaps as a result of partial fermentation of pyruvate, consuming reducing equivalents which have built up by the perchlorate-mediated inhibition of sulfate reduction [25, 28]. It is possible that the up-regulation of fumarate reduction genes (and the downregulation of the pyruvate ferredoxin oxidoreductase) allow for a similar process in the Dde_0320::TN5 strain.

The ZraR and HcpR regulons are also upregulated in the Dde_0320::TN5 strain. In DvM and DvH, ZraR is important for chlorate and oxidative stress [213]; in G20, this regulon is induced upon exposure to molybdenum, suggesting its involvement in molybdenum tolerance [214]. The HcpR regulon consists of genes putatively involved in energy production/conversion. Across G20, DvH, DvM and *Desulfovibrio gigas*, these genes are important under nitrosative and perchlorate stress [33, 210, 215]. Whether or not there is true cross-regulation between PclS and the transcriptional regulators controlling the above regulons remains to be definitively addressed; however, cross or branched regulation are not uncommon [206, 210, 216].

Recent studies have highlighted the importance of a community context or “natural” environment in determining evolutionary outcomes, via mechanisms such as increasing costs of adaptive mutations or decreasing niche availabilities [195, 196]. Previous work in G20 has shown that the *sat*(709T>G) mutation arises across all independently adapted populations. Here, we see this mutation arise only in one community, and this community shows the lowest level of sulfidogenesis in 100 mM perchlorate and a concomitant crash of the G20 population. We have shown that in the APM medium used for community adaptations, strains with an inactivation of PclS have increased relative fitness compared to strains with *sat*(709T>G). It is possible that the community context changes the adaptive landscape in additional ways, such as supplying different donors, altering the pH, or modulating population sizes [193]. Indeed, G20, MKS and other SRM competed for the sulfate-reducing niche throughout the adaptation. This tight competition may have limited the rise of more costly mutations, and may explain why we failed to see adaptation of sulfidogenic communities in previous studies [25]. Finally, the specificity and regulation of two DASS family transporters examined herein emphasize the importance of anion transport as a conserved mechanism used by SRM in communities, when faced with sulfate analog stress. Future work will need to address the paucity of information on this subject for a predictive understanding of long-term SRM inhibition in natural environments.

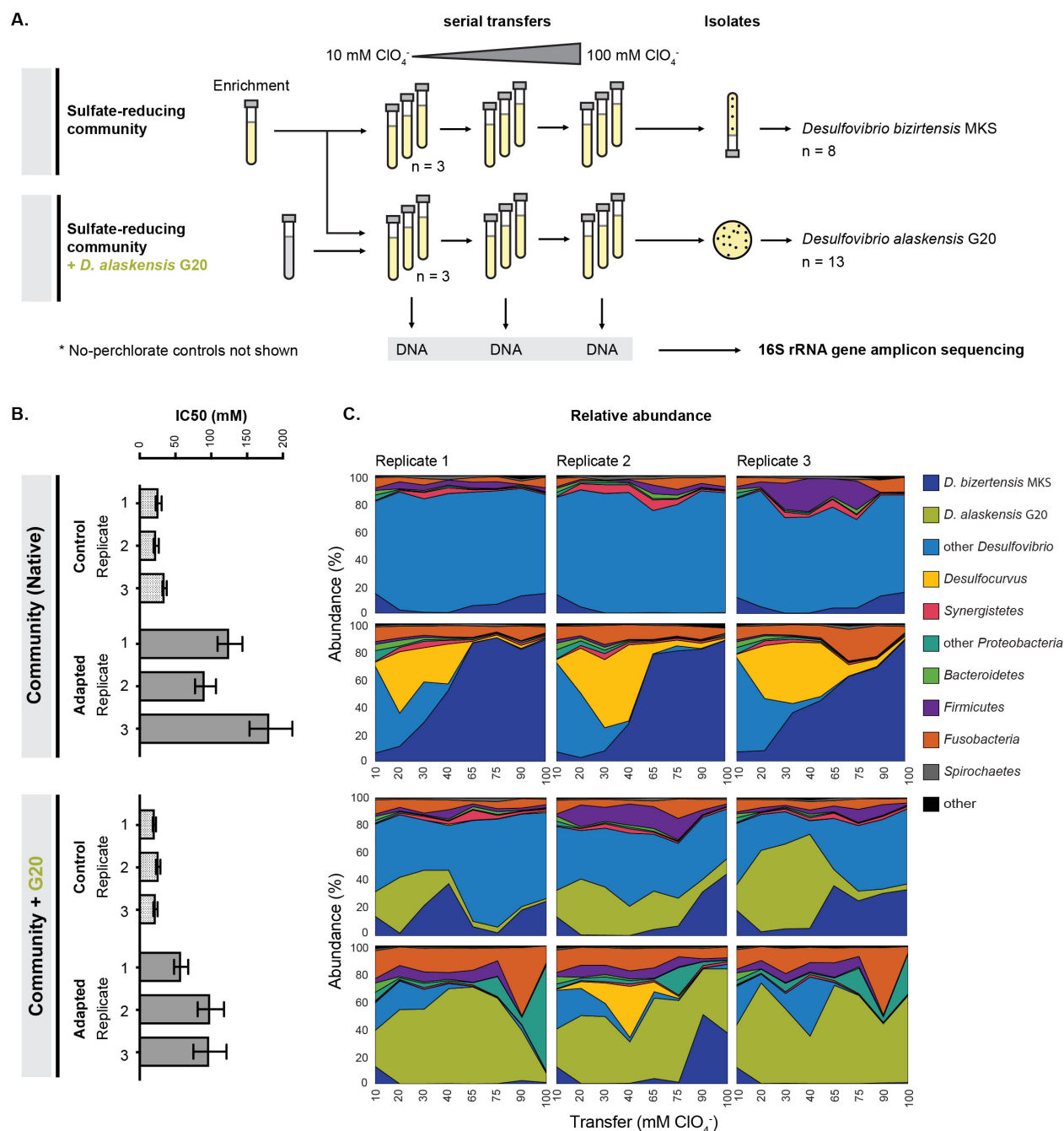


Figure 4.1: A. Experimental design: serial transfers of three native sulfate-reducing communities (top panel) and three G20-augmented communities (bottom panel) in increasing concentrations of perchlorate, up to 100 mM. DNA was sampled at each transfer and used for 16S rRNA amplicon sequencing. Post adaptation, 8 isolates of MKS and 13 isolates of G20 were obtained from adapted communities for whole-genome sequencing. B. IC50 values for control (patterned) and adapting (solid) native communities (top panel) and G20-augmented communities (bottom panel). Bars represent 95% confidence interval. For native communities, all dose-response curves generate

significantly different IC₅₀ values, except control 1 vs control 2 ($p < 0.0001$, extra sum-of-squares F test, Graphpad Prism). For G20-augmented communities, all IC₅₀s differ significantly from one another ($p < 0.0001$), except control 1 vs. control 2/3 and control 2 vs. control 3. C. Community profiles from 16S rRNA amplicon sequencing for the independently transferred control and adapting replicate communities, over the course of successive transfers in increasing perchlorate concentrations.

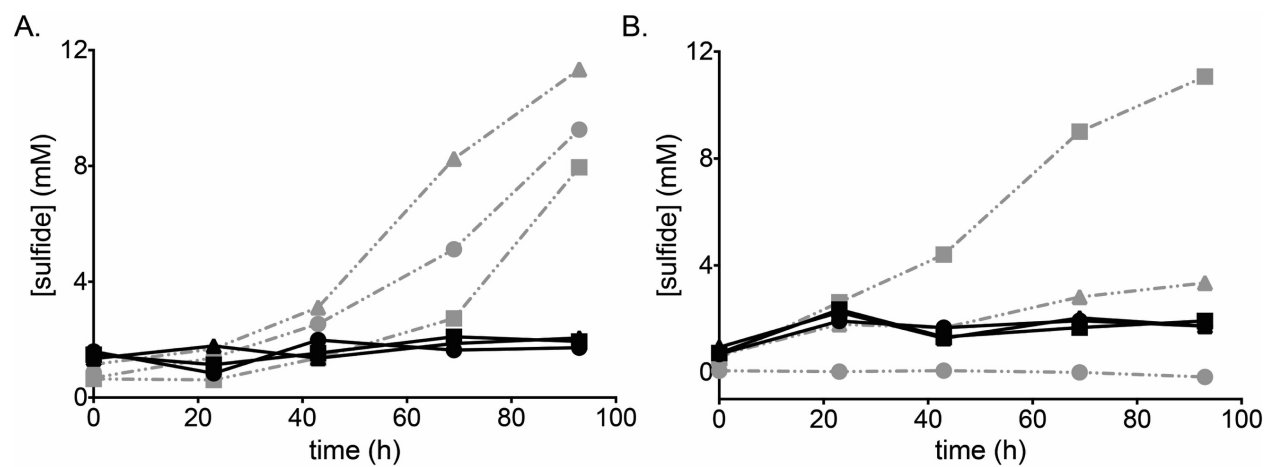


Figure 4.2: Sulfide production from control (black) and adapting (grey) A) native and B) G20-augmented communities, post adaptation, in 100 mM perchlorate.

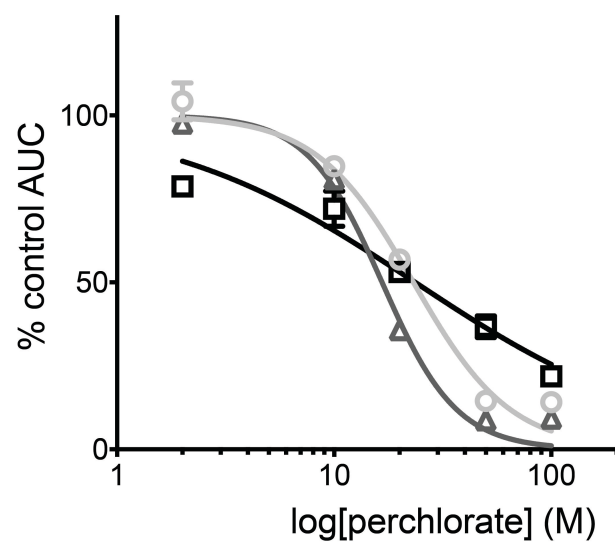


Figure 4.3: Dose-response curves for MKS (black squares), *D. sp. BMSR* (grey triangles) and G20 (light grey circles). Error bars represent SD from 4 replicates. AUC = area under the curve. IC₅₀ values do not differ significantly between *D. sp. BMSR* and MKS ($p=0.1179$, Extra sum-of-squares F test); hillslope values do ($p=0.0044$, Extra sum-of-squares F test). IC₅₀ of G20 vs. BMSR is significantly different at $p=0.04$ (Extra sum-of-squares F test).

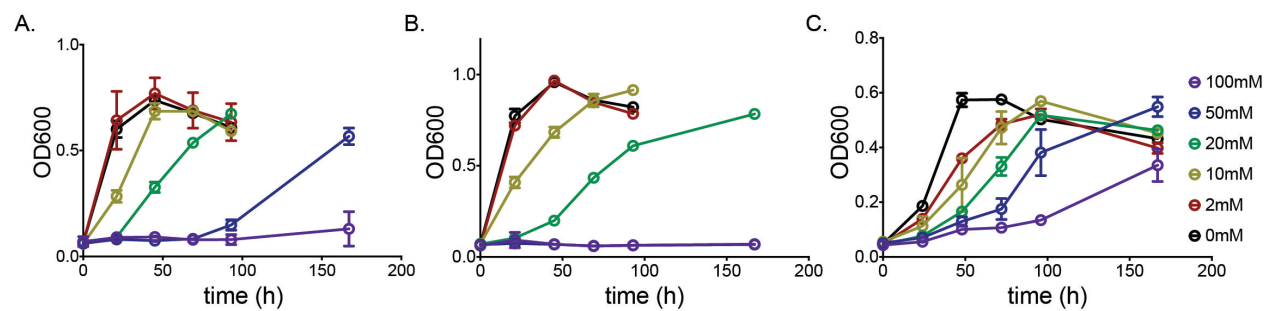


Figure 4.4: Growth curves for A) G20, B) *D. sp. BMSR*, and C) MKS at various concentrations of perchlorate. Bars represent SD of 3 replicates.

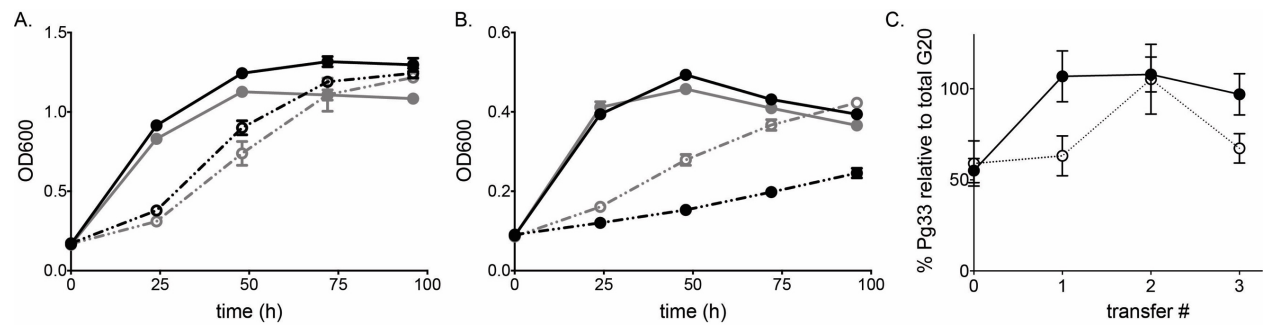


Figure 4.5: Growth of Pg11 (*sat*(709T>G), black) and Pg33 (Dde_0320::TN, grey) with (open symbols) and without (closed symbols) 100 mM perchlorate, in A) MOLS4 and B) APM medium. Bars represent SD of 3 replicates. C) Pg33 as a % of the total G20 population through three transfers in either monoculture (closed symbols) or co-culture with the fermentative fraction of the community (open symbols). Bars represent SD of 3 biological replicates. See Table 4.1 for strain information.

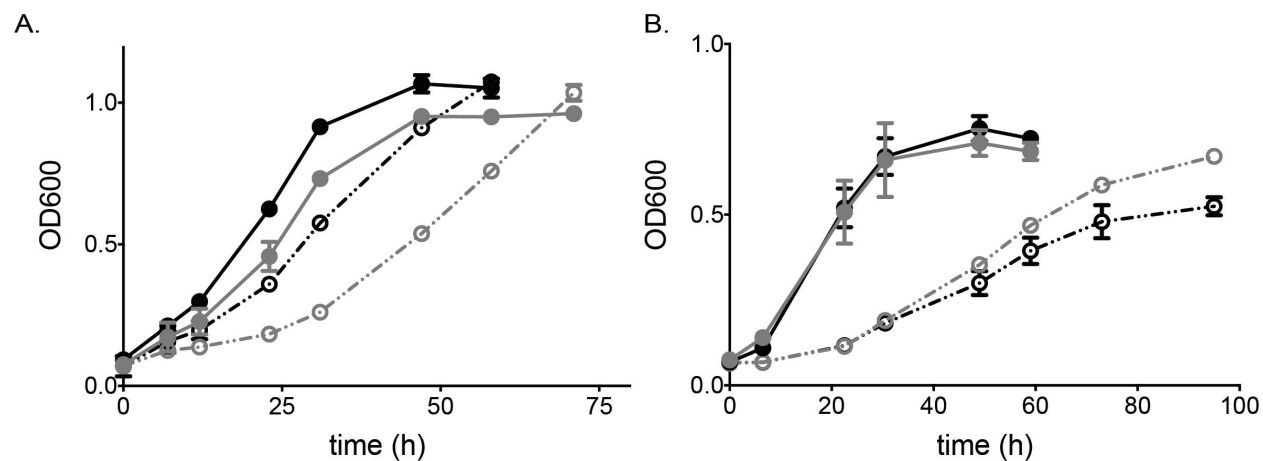


Figure 4.6: Growth of Pat3 (black) and Dde_0320::TN5 (grey) with (open symbols) and without (closed symbols) 100 mM perchlorate, in A) MOLS4 and B) APM media. Bars represent SD of 3 replicates. See Table 4.1 for strain information.

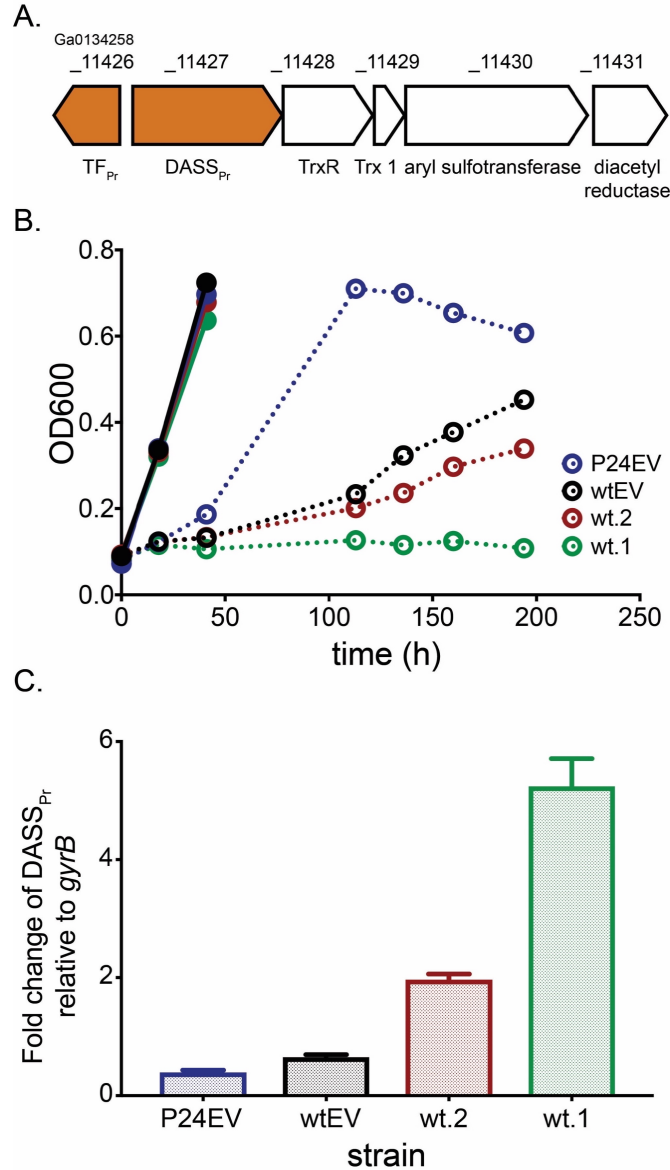


Figure 4.7: A. Genomic context of TF_{Pr}. Orange color indicates genes that are mutated in adapted strains. TF_{Pr} = transcriptional regulator, Ga0134258_11426; DASS_{Pr} = divalent-anion:sodium symporter, Ga0134258_11427. TrxR = thioredoxin reductase; Trx 1 = thioredoxin 1. B. Growth of overexpression strains with (open symbols) and without (closed symbols) 100 mM perchlorate. For strain description, see Table 4.1. Values are average of three biological replicates; SD bars smaller than symbols. C) Fold change in expression of DASS_{Pr} relative to *gyrB* across overexpression strains. Error bars represent SD of three biological replicates.

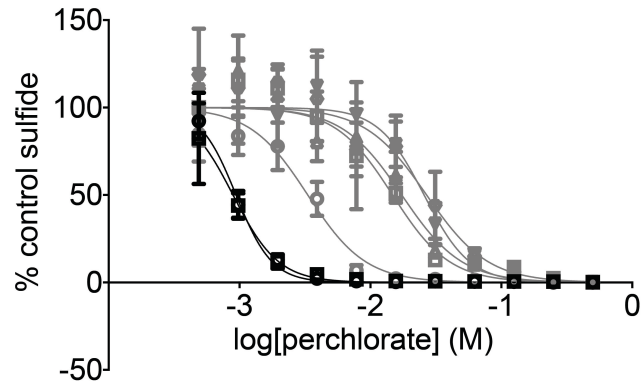


Figure 4.8: Dose-response curves against perchlorate for two pure-culture control populations (black) or five independently adapting populations (grey) of MKS. Error bars represent SD from 4 replicates. All dose-response curves generate significantly different IC₅₀ values, except control 1 vs. control 2 ($p < 0.0001$, extra sum-of-squares F test).

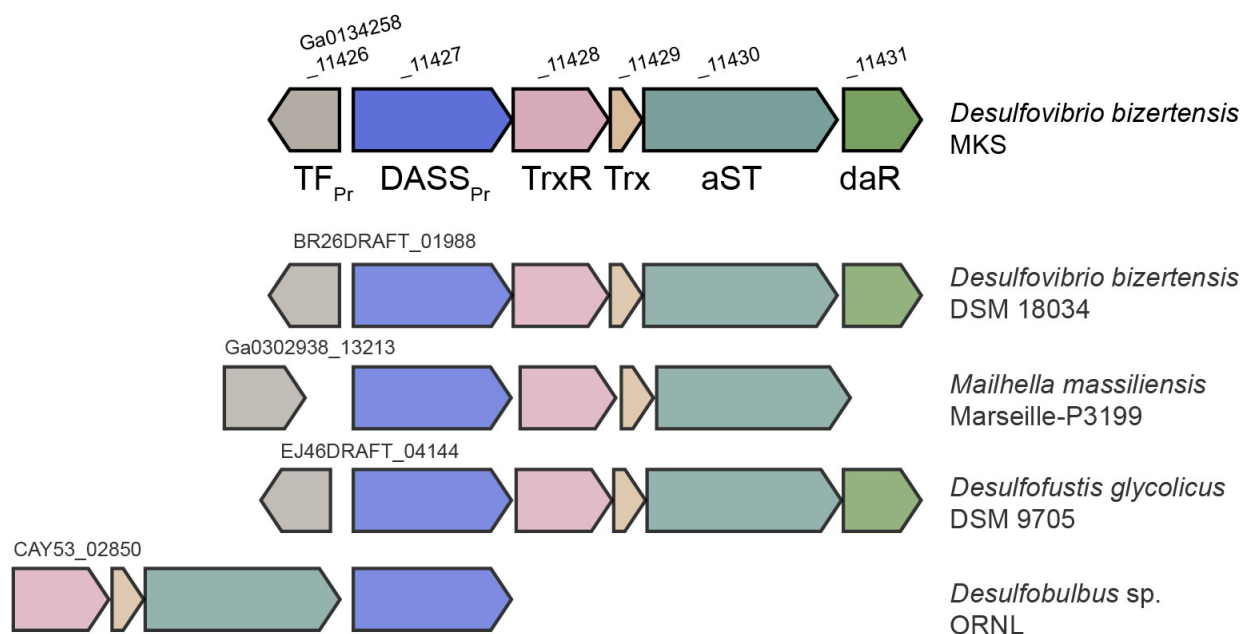


Figure 4.9: Genomic context of TF_{Pr} and $DASS_{Pr}$ in MKS, and orthologous operons in closely related SRM. Grey = cAMP receptor protein-like transcriptional regulator; blue = divalent-anion:sodium symporter (DASS)-family transporter; pink = TrxR, thioredoxin reductase; tan = Trx, thioredoxin 1; teal = aST, aryl sulfotransferase; green = daR, diacetyl reductase.

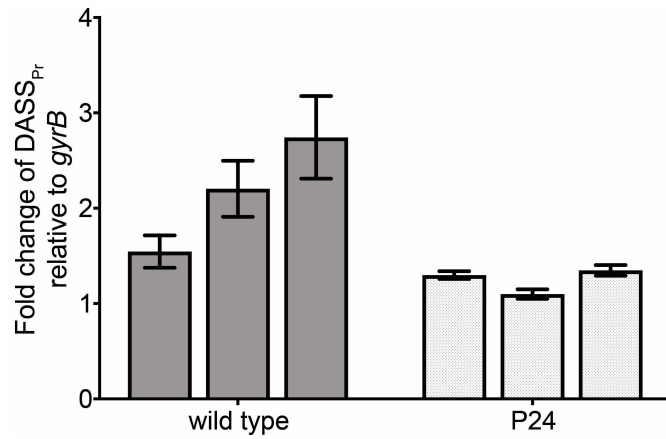


Figure 4.10: Fold change in expression of DASS_{Pr} relative to *gyrB* in triplicate wild type MKS (solid) and adapted strain P24 (patterned). Bars represent SD of three technical replicates.

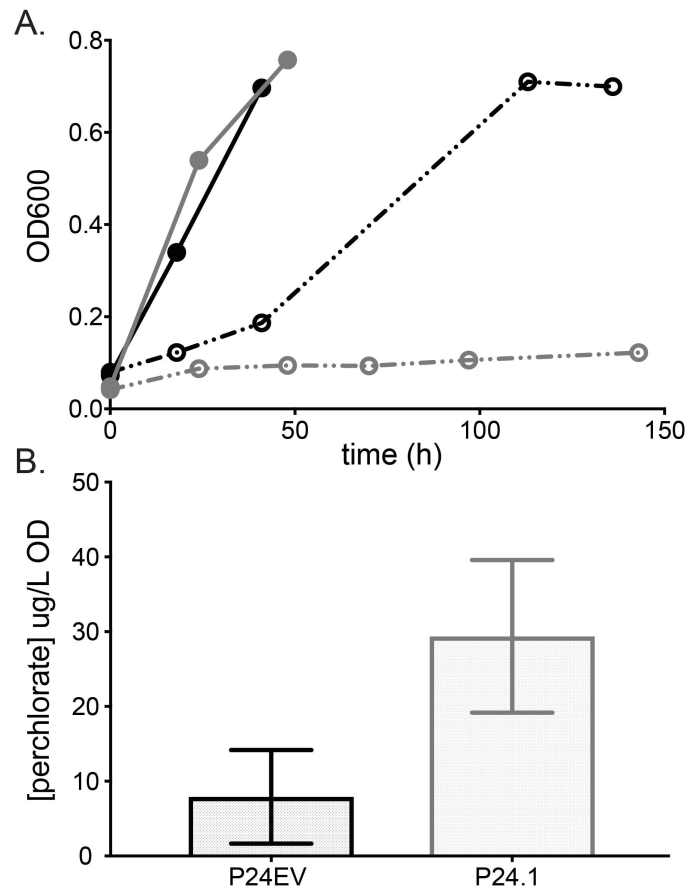


Figure 4.11: A. Growth of overexpression strain P24EV (black) and P24.1 (grey) with (open symbols) and without (closed symbols) 100 mM perchlorate. Values are average of three biological replicates; SD bars smaller than symbols. B. Average normalized intracellular perchlorate concentration of P24EV and P24.1. Error bars are SD of three biological replicates.

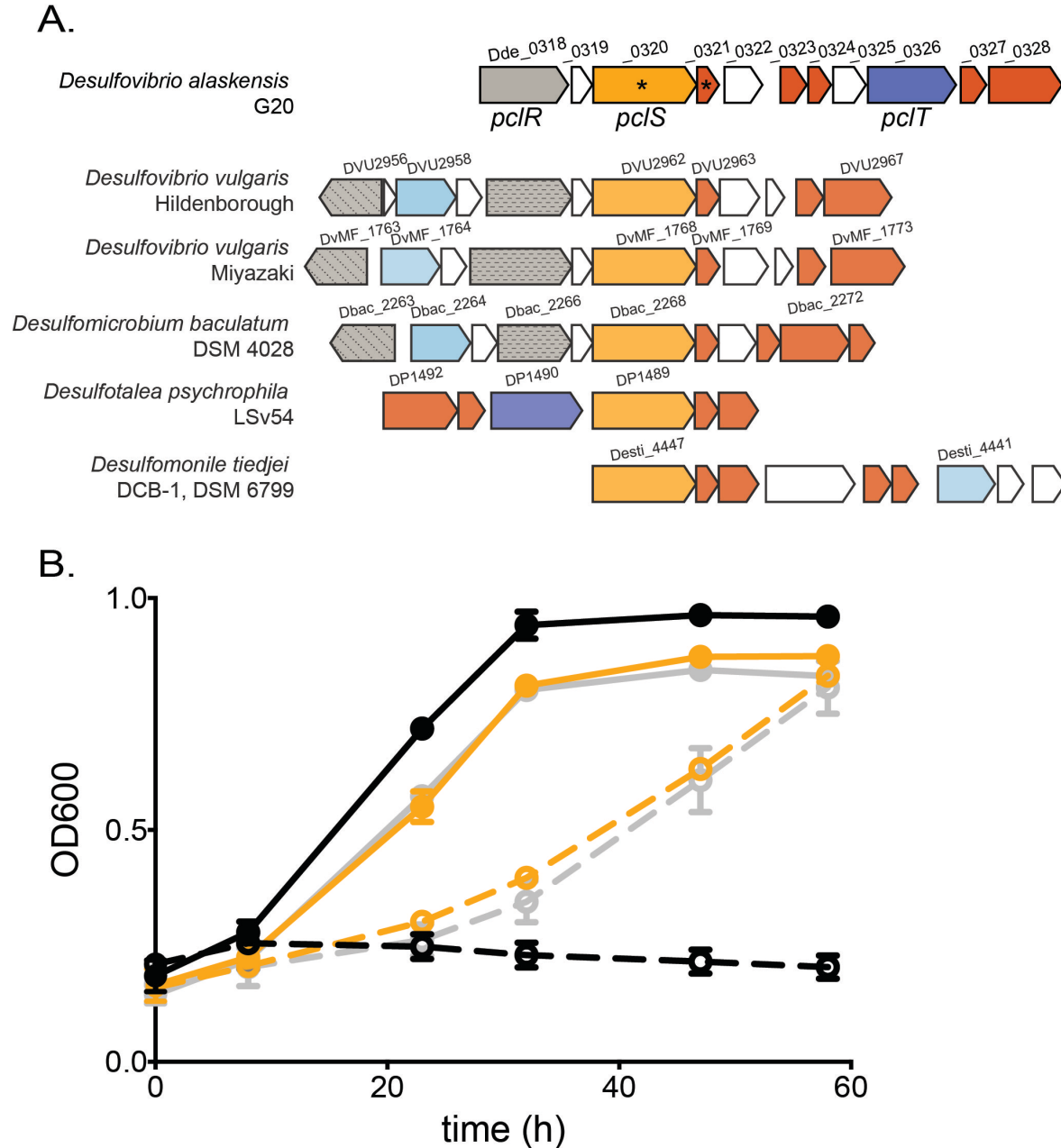


Figure 4.12: A. The “perchlorate cluster” and homologous operons in closely related SRM. *pclR* = σ^{54} -dependent Fis family transcriptional regulator (Dde_0318); *pclS* = histidine kinase (Dde_0320); *pclT* = divalent-anion:sodium symporter (DASS) family transporter (Dde_0326); grey = σ^{54} -dependent Fis family transcriptional regulator, similarly patterned are orthologous; yellow = histidine kinase; orange = protein with response regulator receiver domain; dark blue = DASS family transporters light blue = TSUP family transporter. *=sustained mutations during adaptation to perchlorate. B. Growth in MOLS4 medium of wild type G20 (black), the Dde_0320::TN5 strain (yellow) and the Pg33 adapted strain (grey) with (open symbols) and without (closed symbols) 100 mM perchlorate. Bars represent SD of 3 replicates.

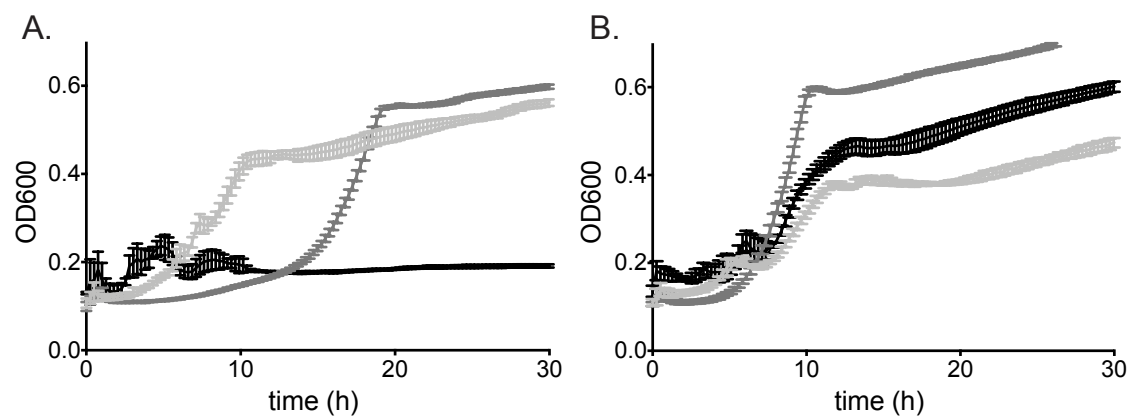


Figure 4.13: Growth of strains jwEV (black), jw.1 (light grey), and jw.2 (dark grey) in M63 minimal medium with sulfur sources: A. 250uM sulfate or B. 250uM cysteine. For description of strains, see Table 4.1.

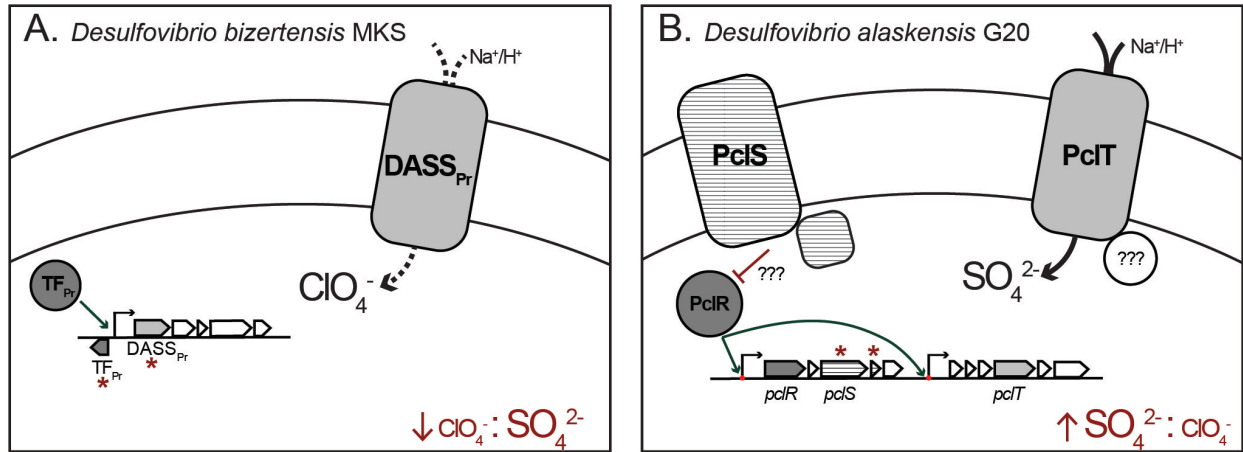


Figure 4.14: Adaptation to perchlorate targets anion transport in both *Desulfovibrio bizertensis* MKS and *Desulfovibrio alaskensis* G20, increasing intracellular sulfate:perchlorate ratios and alleviating effects of competitive inhibition. A. In MKS, the TF_{Pr} regulates expression of the downstream DASS_{Pr} , which is leaky towards perchlorate. Inactivation of the transporter or TF leads to decreased perchlorate entry into cells. B. In G20, PclS represses the activity of PclR (directly or via a regulatory cascade); PclR activates transcription of the perchlorate cluster, including sulfate transporter, PclT . Inactivation of pclS thus causes increased sulfate entry into cells. Other genes in the cluster, or elsewhere in the genome may modulate the transport activity of PclT . * = genes in which mutations were sustained over the course of adaptation to perchlorate; red dot = σ^{54} -dependent promoters.

Table 4.1: Plasmids/strains used in this study

Plasmid/Strain Name	Description	Source
Plasmids		
pBMK7	Parental plasmid; used as overexpression vector for MKS; Kan ^R	from Arash Komeili; [202, 217]
pMS1	pBMK7 with wild type MKS CRP-like transcriptional regulator (TF _{Pr} , Ga0134258_11426) on its native promoter	This study
pMS2	pBMK7 with the P24 mutant TF _{Pr} (L209*) on its native promoter	This study
pMS3	pBMK7 with the MKS DASS _{Pr} (Ga0134258_11427) on a separate constitutive promoter (promoter for Ga0134258_10740).	This study
pOW107	Parental plasmid used for complementation of <i>E. coli</i> CysA KO strain; Amp ^R ; weak constitutive promoter BBa_J23103 (Chris Anderson, http://parts.igem.org/Promoters/Catalog/Anderson)	from Ouwei Wang and John Dueber [218]
pOW107.1	pOW107 with wild type G20 gene <i>pclT</i> (Dde_0326)	This study
pOW107.2	pOW107 with wild type G20 genes of the perchlorate cluster, Dde_0323 to Dde_0328	
<i>Desulfovibrio bizertensis</i> MKS strains		
P24	Adapted strain of MKS with a mutation in the HTH motif of the TF _{Pr} (L209*)	This study
wtEV	Wild type MKS with pBMK7	This study
wt.1	Wild type MKS with pMS1	This study
wt.2	Wild type MKS with pMS2	This study
P24EV	Adapted MKS strain P24 with pBMK7	This study
P24.1	Adapted MKS strain P24 with pMS3	This study
<i>Desulfovibrio alaskensis</i> G20 strains		
G20	Wild type <i>D. alaskensis</i> G20	from Adam Arkin
Dde_0320::TN5	G20 TN5 mutant; transposon insertion in <i>pclS</i> (Dde_0320)	[92]
Dde_0321::TN5	G20 TN5 mutant; transposon insertion in Dde_0321	[92]
Dde_0326::TN5	G20 TN5 mutant; transposon insertion in <i>pclT</i> (Dde_0326)	[92]
Pat3	G20 strain with <i>sat</i> (709T>G) mutation; from previous pure-culture adaptation	[190]
Pg11	G20 community-adapted strain with <i>sat</i> (709T>G) mutation	This study
Pg33	G20 community-adapted strain with transposon insertion in <i>pclS</i> (Dde_0320)	This study
<i>E. coli</i> JW2415 strains (CysA KO)		
jwEV	JW2415 with pOW107	This study
jw.1	JW2415 with pOW107.1	This study
jw.2	JW2415 with pOW107.2	This study

Table 4.2: Primers used in this study

Primer name	Sequence ¹	Purpose
MKS_gyrB_F MKS_gyrB_R	ACGAGAACCTAAGGAGGTT TTCGGATTCTCCGGCTTTT	qPCR primer set for amplifying part of <i>gyrB</i> (Ga0134258_12211) from MKS
MKS_sulsymp_F MKS_sulsymp_R	GGGTGCTGCTTTCCATCTCT CCTTTGGCATTCAGCGTAG	qPCR primer set for amplifying part of DASS family transporter (DASS _{Pr} , Ga0134258_11427) from MKS
Tn0320_F Tn0320_R	AGGACGGTTCATGACGAAGTC GCTATAAAAGCCACGGAAGCC	qPCR primer set spanning TN5 insertion site in Dde_0320; used to amplify the Dde_0320::TN5 g20 mutant Pg33.
G20_gyrB_F G20_gyrB_R	TCACCGTTCTTGAGGGGTTG TCAAGATGCAGCTTCACGGT	qPCR primer set for amplifying part of <i>gyrB</i> (Dde_0003) from g20.
MKS_insert_F MKS_insert_R	tgcatgcctgcaggtcgactgcgaATGCAACAAGAAGAAAATCATAAC actatagggaattcgagctcTTAGTAAACGAGTTCTTCTCTTCGTG	Primer set for amplifying CRP-like transcriptional regulator (TR _{Pr} , Ga0134258_11426) and its native promoter from MKS.
pBMK7_lin_F pBMK7_lin_R	GAGCTCGAATTCCTATAGTGAGTC AGTCGACCTGCAGGCATG	Primer set for linearizing vector pBMK7
MKS_insert2_F MKS_insert2_R	ATGCAAGATAGACTTCGAAATGTCTG actatagggaattcgagctcCTAGCTGAATAATATTTGTGAGAGAGGAAG	Primer set used to amplify DASS _{Pr} (Ga0134258_11427) from MKS
MKS_promoter_F MKS_promoter_R	tgcatgcctgcaggtcgactGGAGCGTGGATTGAACTGTTC tttcgaagtctatcttgcattGGCGTGCTCAATGTTGGATG	Primer set used to amplify the promoter (500bp upstream of start codon) of a CRP/FNR family transcriptional regulator (Ga0134258_10740) from MKS
pOW_lin_F pOW_lin_R	ATAGCAACAAAAGCTGGATGTGA AGATCTTTTCTCCTCTTTGCTAGCG	Primer set for linearizing vector pOW107
pOW_326_F pOW_326_R	gcaaagaggagaaaagatctATGTCTACCAAAGCTCAATCGACA catccagcttttggctatTTACATGCCTACTCTGGGCAC	Primer set used to amplify <i>sulT</i> (Dde_0326) from G20, for cloning into pOW107
pOW_323_F pOW_328_R	gcaaagaggagaaaagatctATGAAAAACGCTGTGGTGCT catccagcttttggctatTCAGGCGCTTTTCAGGTTCTG	Primer used to amplify Dde_0323-Dde_0328 from G20 (for pOW107)
pOW_13_F pOW_13_R	CGCTAGCAAAGAGGAGAAAAAGATCT TCACATCCAGCTTTTGTGCTAT	Primer set for screening colonies with pOW107 constructs

¹lowercase sequence represents overhang region (complementary to vector) used for Gibson assembly

Table 4.3: Genome-wide mutations in adapted MKS isolates¹

Locus Tag	Gene annotation	Frequency ²	Mutation ³	Isolate ⁴ (s)	Notes ⁵
Ga0134258_11426 (TF _{Pr})	CRP-like transcriptional regulator	6/8 community 4/5 pure culture; 1/1 G20-augmented;	L209*	P24,P25,PPg11,P3	34% id. to DVU0379 from <i>D. vulgaris</i> Hildenborough (DvH); 35% id. to Dde_0463 from G20; regulon of 7-8 genes; important for uranium reduction [212]; closer homologs discussed in main text
			Frameshift @ 196	PP41	
			F190L	PP22	
			Deletion @ 154	PP1, PP41	
			L119R	P34, P35	
			Large insertion @ 129	P14	
			Frameshift @ 103	P22	
Ga0134258_11427 (DASS _{Pr})	DASS family transporter	2/8 community; 1/5 pure culture;	G25*	P33, P32	No homolog in G20 or DvH; other homologs discussed in main text
			Frameshift @ 41	PP57	
Ga0134258_103337	hypothetical protein;	2/8 community; 1/5 pure culture;	silent	P14, P22, PP41	radical SAM superfamily
Ga0134258_102194	regulatory protien, Fis family	2/8 community;	L44F	P33, P32	70% id. to DvMF_1324 from <i>D. vulgaris</i> Miyazaki (DvM); detrimental to perchlorate stress
Ga0134258_10824	hypothetical protein	1/8 community;	silent	P14	
Ga0134258_102316	heterodisulfide reductase subunit B	1/8 community;	Frameshift @ 27	P22	52% id. to DvMF_0323 from DvM; important for perchlorate stress
Ga0134258_103300	transcriptional regulator, Lrp family	1/8 community;	W110C	P34	
Ga0134258_10167/8	hypothetical proteins	1/8 community;	Large deletion	P14	Covers majority of both genes
Ga0134258_112	<i>intergenic</i>	2/8 community;	deletion 1350-1351	P33, P32	promoter region for Ga0134258_1122 (hypothetical protein)
Ga0134258_141	<i>intergenic</i>	1/5 pure culture;	deletion 3047-3658	PP22	between two putative transposases
Ga0134258_103	<i>intergenic</i>	1/5 pure culture;	G402511T	PP57	promoter region for Ga0134258_103374 (Fusaric acid resistance family protein)

¹only mutations in adapted strains that are NOT in control strains are included²frequencies listed as “community” when referring to the isolates isolated from adapted native communities, “pure culture” when referring to isolates from pure culture MKS adaptations; and “G20-augmented” when referring to the isolate from G20-augmented replicate community 2³refers to position of mutation in amino acid sequence; for intergenic mutations, listed as scaffold: mutation and position; all information publicly available on IMG (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>); only mutations that are *absent* from control strains are listed⁴isolates are annotated as P when originating from perchlorate-adapting communities (P1, P2 or P3); PP when originating from perchlorate-adapting pure cultures (PP1, PP2, PP3, PP4 or PP5); and PPg when originating from G20-augmented perchlorate-adapting communities (PPg1 is the only community where MKS was sustained)⁵fitness data from Microbes Online (<http://www.microbesonline.org/>) and Fitness Browser (<http://fit.genomics.lbl.gov/>); regulon data from RegPrecise (<http://regprecise.lbl.gov/>)

Table 4.4: Genome-wide mutations in adapted G20 isolates¹

Locus Tag	Gene annotation	Frequency	Mutation ²	Isolates ³	Notes ⁴
Dde_0320 (<i>pclS</i>)	periplasmic sensor signal transduction histidine kinase	7/13	Large insertion @79 (Dde_0486; transposase, IS5 family) Large insertion @539 Large insertion @104	Pg31, Pg32, Pg33, Pg34, Pg14, Pg22, Pg24	24% id. To DvMF1768 from <i>D. vulgaris</i> Miyazaki; detrimental under antibiotic and perchlorate stress; important under starvation conditions and nitrate; detrimental for G20 under perchlorate stress [28]; σ^{54} promoter
Dde_0321	response regulator receiver domain	3/13	Large insertion @64	Pg21, Pg25, Pg26	same as Dde_0320
Dde_2265	Sulfate adenylyltransferase	3/13	S237A	Pg11, Pg12, Pg13	previously shown in 10/10 independently adapted to 100mM perchlorate pure cultures of G20 [190]
Dde_1741	Prophage antirepressor-like	4/13	Large insertion @72	Pg31, Pg32, Pg33, Pg34	
Dde_1459	cytidine deaminase/ amino acid ABC transporter, PAAT family	3/13	Large insertion @111	Pg11, Pg12, Pg13	
Dde_2962	hypothetical protein	3/13	Large insertion @113	Pg31, Pg33, Pg34	
Dde_3261	methyl-accepting chemotaxis sensory transducer with Cache sensor	3/13	Large insertion @508	Pg11, Pg12, Pg13	
Dde_0292	Hypothetical protein	1/13	Large insertion @214	Pg22	
Dde_0734	Hypothetical protein	1/13	S46L	Pg34	polysaccharide deacetylase domain
Dde_1985	ABC transporter permease protein	1/13	Large insertion @224	Pg14	
Dde_2442	DNA repair protein RadC	1/13	Synonymous mutation	Pg24	
Dde_2870	DEAD/DEAH box helicase-like	1/13	Large insertion @717	Pg24	
Dde_3039	Pyruvate formate-lyase	1/13	Large insertion @341	Pg32	
Dde_3362	transposase (class V)	1/13	Approx. 2x increased coverage	Pg31, Pg33, Pg34	transposase and inactivated derivatives
	<i>Intergenic</i>	3/13	C2709684T	Pg25, Pg26, Pg21	directly after Dde_2707 (tRNA pseudouridine synthase A)

¹ only mutations that are *absent* from control strains are listed

² refers to position of mutation in amino acid sequence; for intergenic mutations, mutation at genomic position; all information publicly available on IMG (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>);

³ isolates are annotated based on the perchlorate-adapting community they originate from (Pg1, Pg2 or Pg3);

⁴ fitness data from Microbes Online (<http://www.microbesonline.org/>) and Fitness Browser (<http://fit.genomics.lbl.gov/>); domain predictions from Interpro (<https://www.ebi.ac.uk/interpro/>)

Table 4.5: TF_{Pr} (Ga0134258_11426) orthologs across SRM genomes

Organism	NCBI accession/ IMG genome ID	Regulator	Regulated operon	Putative binding site for CRP regulator
<i>Desulfovibrio bizertensis</i> MKS	2687453693	Ga0134258_11426	Ga0134258_11427- Ga0134258_11431	AcAAccTAGtctgagCTActTTtT
<i>Desulfovibrio bizertensis</i> DSM 18034	FUYA01000006	SAMN02745702_ 01988	SAMN02745702_01987 - SAMN02745702_01983	
<i>Desulfotrustis glycolicus</i> DSM 9705	FQXS01000041	SAMN02745124_ 04144	SAMN02745124_04145 - SAMN02745124_04149	cAAAcATAGtCcaGgCTATtTTTc
<i>Mailhella massiliensis</i> strain Marseille-P3199	NZ_LT706952	CZ345_RS12845	CZ345_RS12850 - CZ345_RS12865	cAAcACTAGTtTAgACTAGTtTTc
<i>Desulfobulbus</i> sp. ORNL	CP021255	--	CAY53_02850 CAY53_02865	-

Table 4.6: Set of sites for construction of position weight matrix of the PclR (Dde_0318) binding motif

Protein ID ¹	Site sequence	Function	Organism
WP_051445054	cgtttcaaaatgtttcataa	Fis family transcriptional regulator	Desulfocurvus vexinensis DSM 17965
BBD08009	tgttcattctgttcaaga	Fis family transcriptional regulator	Desulfovibrio ferrophilus
BBD08011	tggttcgattgtttcaaaa	response regulator receiver protein	Desulfovibrio ferrophilus
WP_028586772	tgttcattgtgtttcataa	response regulator receiver protein	Desulfocurvus vexinensis DSM 17965
WP_012612352	cgatgcaaaatgttgcaaaa	Fis family transcriptional regulator	Desulfovibrio vulgaris str. 'Miyazaki F'
WP_051384352	tggtgcataatgttgcaaaa	Fis family transcriptional regulator	Desulfovibrio termitidis H11
WP_027368940	tgttcgttctgttccatgg	Fis family transcriptional regulator	Desulfovibrio africanus subsp. africanus DSM 2603
WP_014260523	tgttcgttctgttccatgg	Fis family transcriptional regulator	Desulfovibrio africanus str. Walvis Bay
WP_005988910	tgttcgttctgttccatgg	Fis family transcriptional regulator	Desulfovibrio africanus PCS
WP_007523476	cgatgcaaaacgttgcaaaa	Fis family transcriptional regulator	Desulfovibrio sp. A2
GBO95138	cgatgcaaaacgttgcaaaa	Fis family transcriptional regulator	Desulfovibrio sp. HK-II
WP_084448523	tgttcattgtgacgcaaaa	Fis family transcriptional regulator	Desulfovibrio inopinatus DSM 10711
WP_011366465	tgattttttgtttcatca	response regulator receiver protein	Desulfovibrio alaskensis G20
WP_073040584	tgtatcatactgtctcatgc	response regulator receiver protein	Desulfacinum infernum DSM 9756
WP_011366460	tgtatcaaaactttgcaata	Fis family transcriptional regulator	Desulfovibrio alaskensis G20
WP_073040591	cgttcagtcggtttcatcc	Fis family transcriptional regulator	Desulfacinum infernum DSM 9756
WP_084287933	cttttcataatgtgtataa	DASS family transporter	Desulfovermiculus halophilus DSM 18834

¹**bold** = First gene of the operon encoding DASS family transporter

Table 4.7: Predicted binding sites for PclR (Dde_0318)

Gene	Product	Position	Score	Site sequence	Predicted promoter	σ^{54} -dependent
Dde_0323		-265	5.04	TGaTTtATttTGTTTCATcA	-188	=>
		-119	4.87	ggfTGcgACATaATGgAACg	gTTGGCACGtTcCTTGcT	
Dde_0191	kinase	-270	4.77	TTATatAACAgATGAtACA	-226	=>
					gTTGGCACGgcagTTGCTa	
Dde_0218	methyl-accepting chemotaxis sensory transducer	-323	4.75	TGTTTCaATGTTTCAggt		
Dde_0683	cytochrome c family protein	-360	4.74	cagTGcAACAgATGcAACA		
Dde_0685	formylmethanofuran dehydrogenase subunit	-104	4.74	TGTTgCATtcTGTTgCActg	-53	=>
					TgcGGCgCGCagTTGtTg	
Dde_0318		-102	4.65	TGTaTCAaAActTTgCAatA	17	=>
					aaaGGCACGgcTtcTGCTg	

Table 4.8: Summary of RNAseq data pertaining to predicted regulon of Dde_0318

Synonym	Product	No stress experiment			Stress experiment		
		Expression WT	Expression TN	qValue TN vs WT	Expression WT	Expression TN	qValue TN vs WT
predicted RNA	*upstream of Dde_0318	22	4690	0.0E+00	8	2437	0.0E+00
Dde_0318	Fis family transcriptional regulator	31	75	1.7E-02	27	106	5.5E-47
Dde_0319	hypothetical protein	55	91	5.9E-01	32	109	1.1E-30
Dde_0320	integral membrane sensor signal transduction histidine kinase	57	77	4.6E-01	50	58	1.0E+00
Dde_0321	response regulator receiver protein	62	37	4.1E-03	115	8	0.0E+00
Dde_0322	histidine kinase A domain-containing protein	38	41	3.5E-01	112	33	2.5E-113
Dde_0323	response regulator receiver protein	40	63	6.8E-01	58	235	2.1E-57
Dde_0324	response regulator receiver protein	36	61	5.9E-01	103	223	1.9E-08
Dde_0325	hypothetical protein	44	71	7.3E-01	111	191	2.8E-02
Dde_0326	sodium/sulfate symporter	45	53	1.5E-01	96	207	2.2E-05
Dde_0327	response regulator receiver protein	80	40	1.1E-04	272	219	4.2E-06
Dde_0328	response regulator receiver protein	239	106	2.6E-13	634	263	6.3E-34
Dde_0218	methyl-accepting chemotaxis sensory transducer	17	166	1.0E-107	28	244	0.0E+00
Dde_0191	hypothetical protein	127	195	7.4E-01	276	443	1.0E-02
Dde_0685	formylmethanofuran dehydrogenase subunit E	56	79	6.0E-01	129	111	6.8E-09
Dde_0678	rhodanese-like protein	12	13	5.6E-01	2	4	1.0E+00
Dde_0679	rhodanese-like protein	31	41	8.3E-01	29	14	1.0E+00
Dde_0680	cytochrome b/b6 domain-containing protein	15	22	9.1E-01	9	7	1.0E+00
Dde_0681	4Fe-4S ferredoxin	9	8	3.4E-01	5	4	1.0E+00
Dde_0682	nitrate reductase	7	11	7.3E-01	4	9	1.0E+00
Dde_0683	cytochrome c family protein	15	33	1.6E-01	18	56	1.0E+00

*expression values calculated from raw counts by Rockhopper software; WT= wild type G20; TN = Dde_0320::TN5 strain

Table 4.9: Consistently significantly up/down genes in both RNAseq datasets¹

		No stress				Stress			
Up-regulated in Dde_0320::TN5 strain relative to WT ²									
Synonym	Product	WT	TN	qValue	TN/WT	WT	TN	qValue	TN/WT
Dde_1250	electron bifurcation transhydratase 2, alpha subunit	102	302	4.4E-02	3.0	27	64	3.0E-11	2.4
Dde_1251	electron bifurcation transhydratase 2, beta subunit	67	192	3.1E-03	2.9				
Dde_1253	Malate:NADPH oxidoreductase, decarboxylating					160	424	7.8E-08	2.7
Dde_1254	fumarate hydratase, beta subunit	101	273	4.5E-03	2.7	106	321	8.5E-20	3.0
Dde_1255	fumarate hydratase, alpha region	126	329	4.6E-02	2.6	103	242	2.6E-08	2.3
Dde_1256	fumarate reductase, alpha subunit	73	200	7.5E-03	2.7				
Dde_1258	Fumarate reductase, cytochrome b subunit	224	730	1.5E-02	3.3				
Dde_0109	Fis family transcriptional regulator	24	60	1.6E-02	2.5	34	68	2.4E-06	2.0
Dde_0110	nitrogen specific signal transduction histidine kinase NtrB	13	39	2.8E-03	3.0	32	81	7.3E-12	2.5
Dde_0111	zinc resistance-associated protein	301	1312	1.3E-04	4.4	904	2455	9.5E-11	2.7
Dde_2819	hypothetical protein	94	452	1.2E-11	4.8	451	308	2.3E-05	0.7
Dde_2641	hybrid cluster protein	172	581	6.5E-03	3.4	525	2660	6.2E-22	5.1
Dde_2642	4Fe-4S ferredoxin	37	109	6.1E-04	2.9	55	1076	0.0E+00	19.6
Dde_0503	HPP family protein	4	13	1.2E-02	3.3				
Dde_2303	cupin	22	62	1.3E-02	2.8	450	1402	2.0E-14	3.1
Dde_2389	4Fe-4S ferredoxin	11	40	1.8E-05	3.6	20	201	0.0E+00	10.1
Dde_2390	nitrite and sulfite reductase 4Fe-4S region	40	134	9.2E-05	3.4	72	263	2.0E-41	3.7
Down-regulated in Dde_0320::TN5 strain relative to WT									
Synonym	Product	WT	TN	qValue	TN/WT	WT	TN	qValue	TN/WT
Dde_1791	ferroxidase	277	93	1.1E-24	3.0	299	18	0.0E+00	16.6
Dde_1792	2-oxoacid:acceptor oxidoreductase subunit alpha	290	123	2.5E-14	2.4	197	38	0.0E+00	5.2
Dde_1793	pyruvate ferredoxin/flavodoxin oxidoreductase subunit beta	255	116	9.0E-16	2.2	138	58	7.3E-89	2.4
Dde_0098	UspA domain-containing protein	319	154	4.0E-12	2.1	365	35	0.0E+00	10.4
Dde_0102	glutamine synthetase	83	29	7.4E-26	2.9	101	11	0.0E+00	9.2
Dde_0210	thioredoxin-disulfide reductase	89	44	2.6E-12	2.0	206	32	0.0E+00	6.4
Dde_0298	flagellar hook-basal body protein	114	57	5.8E-13	2.0	62	31	4.5E-54	2.0
Dde_0327	response regulator receiver protein	80	40	1.1E-04	2.0	272	219	4.2E-06	1.2
Dde_0328	response regulator receiver protein	239	106	2.6E-13	2.3	634	263	6.3E-34	2.4
Dde_1018	response regulator receiver protein	821	392	1.2E-11	2.1	173	79	3.3E-37	2.2

Dde_1442	signal peptide peptidase SppA, 36K type	316	143	1.0E-12	2.2	187	24	0.0E+00	7.8
Dde_1533	GTP-binding protein TypA	121	45	5.8E-26	2.7	71	3	0.0E+00	23.7
Dde_1553	alanine dehydrogenase	43	11	9.7E-22	3.9	98	80	8.6E-06	1.2
Dde_1704	hypothetical protein	495	239	1.0E-09	2.1	297	215	2.3E-12	1.4
Dde_2007	peptidase S16 lon domain-containing protein	88	42	6.6E-13	2.1	109	15	0.0E+00	7.3
Dde_2313	redoxin domain- containing protein	367	158	2.8E-18	2.3	555	51	0.0E+00	10.9
Dde_3119	thiamine biosynthesis Thi4 protein	92	44	2.2E-11	2.1	80	39	3.1E-66	2.1
Dde_3555	zinc transporter ZupT	89	42	2.8E-09	2.1	80	5	0.0E+00	16.0

¹table excludes significant hits presented in Table 4.8

²values represent expression (normalized counts, Rockhopper software); WT = wild type G20; TN = Dde_0320::TN5 strain;

Table 4.10: Intracellular sulfate and perchlorate measurements in wild type and Dde_0320::TN5 G20 strains

	OD	ClO ₄ ⁻ (ug/l)	ClO ₄ ⁻ ng/L OD	SO ₄ ²⁻ (mg/l)	SO ₄ ²⁻ ug/L OD
Wild type rep. 1	68	3.4	50	<20	<294
Wild type rep. 2	91	2.1	23.1	<20	<220
Wild type rep. 3	98	1.7	17.3	<20	<204
Dde_0320::TN5 rep. 1	76	15.9	209.2	41	539.5
Dde_0320::TN5 rep. 2	62	2.7	43.5	42	677.4
Dde_0320::TN5 rep. 3	68	3.4	50.0	38	558.8

Chapter 5:

Cellular targets of sulfate-analogs in sulfate-reducing microorganisms

Abstract

Previous chapters have focused on the sulfate analog perchlorate, its mechanism of inhibition of SRM and how SRM adapt to the presence of high extracellular concentrations of perchlorate across pure-culture and community-embedded conditions. This chapter aims to expand our understanding of specific inhibition and its cellular targets by examining three additional inhibitors: nitrate, which like perchlorate, functions as a dead-end inhibitor of the sulfate adenylyltransferase (Sat); monofluorophosphate (MFP), which is a substrate for Sat, forming a stable product of catalysis; and tungstate, which like MFP is a substrate for Sat, but forms an unstable end-product. Cellular targets of these stressors were identified by adapting *Desulfovibrio alaskensis* G20 to each inhibitor. Whole-genome sequencing of adapted strains revealed a set of genes targeted by each compound. Across all stressors, mutations in anion transport systems were a primary mechanism of resistance. Inactivation of nitrate transport (via the nitrate cluster) was sufficient for robust growth in 100 mM of the inhibitor. Adaptation to MFP required both changes in transport as well as overexpression of the MFP cluster, putatively involved in detoxification of fluorinated compounds resulting from MFP turnover in cells. Adaptation to tungstate additionally targeted several key residues in the Sat, implying that tungstate stress must be overcome by diminishing Sat-catalyzed tungstate turnover. Thus, we identify a set of mutations allowing G20 to respire sulfate in the presence of equivalent (or higher) concentrations of sulfate analogs, and hence provide insight into key determinants of the specificity of this metabolism.

5.1 Introduction

Previous chapters examined the sulfate analog perchlorate: how perchlorate acts to inhibit sulfidogenesis in marine communities; how *D. alaskensis* G20 adapts to perchlorate stress in pure culture; and the mechanisms of adaptation to perchlorate in the context of sulfate-reducing communities. From work presented here and elsewhere, it is apparent that inhibition by perchlorate largely acts to slow the growth of SRM, likely by slowing sulfate turnover by the sulfate adenylyltransferase (Sat) [28]. SRM, in turn, adapt to the presence of perchlorate via specific point mutations in Sat (e.g. Sat(S237A)), which decrease perchlorate-mediated inhibition of this enzyme, or via inactivation of PclS, which increases sulfate import by PclT.

As described in detail above (see: *Chapter 1*), apart from various uncharacterized transport systems, Sat is the only enzyme of the sulfate-reduction pathway to directly interact with sulfate. Sulfate analogs can be classified on the basis of their ability to be substrates of Sat. Monovalent oxyanions (e.g. nitrate, perchlorate) are dead-end inhibitors: they bind Sat and inhibit sulfate turnover, but cannot be substrates themselves. Divalent oxyanions (e.g. monofluorophosphate, molybdate, tungstate, selenate), on the other hand, are substrates of Sat and form APS analogs with varying degrees of stability. Molybdate and tungstate degrade immediately, and hence can cycle through the Sat-catalyzed reaction infinitely, depleting cellular ATP stores. Selenate and MFP form semi-stable to stable APS analogs, with uncharacterized downstream effects [27, 76]. Thus, although the above chapters define the mechanisms of perchlorate stress, *alternative substrates* remain uncharacterized with respect to their cellular targets. These stressors are more potent *and* more specific inhibitors of SRM [10]. A mechanistic understanding of how SRM cope with these stressors will inform on the fundamental physiology and determinants of specificity of sulfate reduction.

In this study, we aim to address the mechanism of SRM inhibition across three sulfate analogs, from three different “classes”: nitrate (dead-end inhibitor), MFP (alternative substrate, stable APS analog) and tungstate (alternative substrate, APS analog degrades immediately). The mechanism of nitrate stress has been best characterized in G20 and DvH using barcoded transposon insertion mutant libraries [17]: inactivation of genes in the “nitrate cluster” are sufficient for growth in 100 mM nitrate. The nitrate cluster encodes genes for signal transduction as well as, importantly, a CysZ-type permease. This permease is likely leaky towards nitrate; its inactivation thus prevents nitrate entry into cells. Less is known about the targets of MFP and tungstate. Unlike perchlorate or nitrate stress, MFP and tungstate stress cannot be overcome by overexpression of *sat*; more Sat enzymes (Δrex strain) instead renders G20 more sensitive to both inhibitors, likely as a result of increased turnover of these alternative/futile substrates. For the same reason, and again in contrast to perchlorate and nitrate, both analogs are also potent inhibitors of fermenting G20 cells [10]. MFP stress has been linked to intracellular fluoride release: the transposon insertion mutant of the fluoride specific efflux pump, *crcB*, shows increased sensitivity to MFP [10]. Tungstate stress is thought to act largely via Sat-mediated turnover and ATP depletion; across the phylogenetic tree, SRM have tungstate-responsive regulators which putatively decrease the influx of tungstate and increase sulfate import in response to high extracellular tungstate and molybdate concentrations [68]. Tungstate stress may also have additional targets: studies of molybdate stress in *Desulfovibrio vulgaris* Hildenborough (DvH) show that molybdate is toxic even in a Δsat strain (Grant Zane, Judy Wall, personal communications). This implies the presence of other targets of molybdate stress in DvH, perhaps in the form of additional enzyme that can turnover molybdate at the expense of ATP.

In order to define a set of targets for each stressor, we adapted model SRM *Desulfovibrio alaskensis* G20 to 30 mM MFP, 10 mM tungstate, or 100 mM nitrate, all concentrations inhibitory to wild type cells. Adaptation of independent replicate G20 populations via serial transfers in increasing concentrations of inhibitor yielded strains capable of robust growth in the final stress condition. Whole-genome sequencing of single-colony adapted isolates and comparison to the ancestral strain as well as isolates from control populations, transferred in the absence of inhibitor, revealed a set of mutations unique to each adaptation. Several common trends emerge: all inhibitors, regardless of their class, target sulfate or anion transport. MFP stress, likely as a result of the stability of the APS analog formed, also targets a specific cluster of genes, termed the MFP cluster, likely involved in coping with fluoride toxicity. Tungstate stress primarily acts on Sat, changing specific conserved residues, putatively determinants of Sat specificity. Thus, we identify a set of mutations and targets that allow G20 to respire sulfate in the presence of equivalent (or higher) concentrations of sulfate analogs.

5.2 Methods

Media and growth conditions

All strains of *Desulfovibrio alaskensis* G20 (G20) [154] were grown in anaerobic MOLS4 medium, as described in previous chapters [175]. Inhibitors or antibiotics were added as necessary.

Adaptive evolution experiments

G20 was adapted as described previously (see: Chapter 3). Briefly, triplicate G20 populations were independently transferred into increasing concentrations of the inhibitor, starting at/or below the IC₅₀ concentration (defined in [10]) and ending at 100 mM nitrate, 30 mM MFP and 10 mM tungstate. Growth of G20 was monitored via OD₆₀₀ and upon reaching early log phase (OD₆₀₀ ~0.8), populations were transferred into sequentially higher increments of inhibitor, at a 10% inoculum. Once the target final inhibitor concentration was reached, populations were transferred in the absence of inhibitor three times, and then one final time in the presence of the final concentration of inhibitor. Triplicate populations were also transferred for the same number of transfers as the tungstate adapting strains, in the absence of a stressor.

Quantifying resistance

G20 strains were assayed for resistance to inhibitors via dose-response (either end-point assays or based on area-under-the-curve data (AUC) generated from growth curves), as described in previous chapters.

Whole-genome sequencing and variant analysis

Single colony adapted and control isolates of G20 were obtained by plating on MOLS4 medium with 1.5% agar [151, 197]. Fifteen MFP-adapted isolates were obtained (five from each of three independently MFP-adapted populations), ten tungstate-adapted isolates (five from two independent populations; the third population crashed), and fourteen nitrate-adapted isolates (four

from one population and five from the remaining two populations). Ten isolates across two independent control populations were also obtained.

All isolates were grown in MOLS4 + inhibitor and genomic DNA (gDNA) was extracted from 1 mL of each respective culture, using the Qiagen DNeasy Kit (Qiagen, Redwood City, CA) following the protocol for Gram-negative bacteria (with RNase treatment). Libraries were prepared by the Functional Genomics Lab (QB3, Berkeley, CA) and sequencing was done on an Illumina HiSeq 4000 100PE lane by the Vincent J. Coates Genomic Sequencing Laboratory (QB3, Berkeley, CA). Sequencing data was processed as previously outlined [190] (see: Chapter 3). Briefly, reads were mapped to the reference *Desulfovibrio alaskensis* G20 using Bowtie2 [198], and processed using Picard (<http://broadinstitute.github.io/picard>) [199]. The average genome coverage in the resulting alignment files was 380x for MFP-adapted strains, 314x for tungstate-adapted strains, 320x for nitrate adapted strains and 345x for control strains. Variant calling was performed using the Genome Analysis Toolkit (GATK) HaplotypeCaller. SNPs and INDELs were filtered by quality of depth (QD <10), strand bias (FS>60) and mapping quality (MQ<40). Genome-wide coverage was assessed as previously outlined [170].

RNA extractions and RT-qPCR

Cultures for RNA were grown in triplicate, to mid-log and 1 mL was pelleted and stored in 0.75 mL TRIzol reagent (Invitrogen) at -80°C for RNA extractions. Extractions were performed as per manufacturer's instructions (TRIzol Reagent user manual) and resulting RNA was treated with TURBO DNase (Invitrogen) twice and purified over an RNA column (Allprep DNA/RNA Mini Kit, Qiagen). cDNA (first strand) synthesis was performed using SuperScript (Invitrogen) and this reaction was used as a template for RT-qPCR. Triplicate technical replicates were set up for each biological replicate, as well as a no-RT control per sample and triplicate negatives per primer set. See Table 5.3 for a list of primers. RT-qPCR was performed using the SYBR qPCR Master Mix (Thermo Scientific) as per manufacturer's instructions, on a StepOnePlus qPCR machine (Applied Biosciences).

Construction of G20 overexpression strains

Overexpression strains of G20 (Table 5.2) were constructed on the pMO9075 plasmid, using methods previously described for *Desulfovibrio* [219] (see: Chapter 4). Briefly, pMO9075 was linearized via PCR. Inserts were amplified from G20 gDNA, gel purified, cloned into the pMO9075 backbone using the Gibson Assembly 2x MasterMix (NEB), and transformed into chemically competent XL1 Blues cells (UCB DNA Sequencing Facility). Positive colonies were identified by PCR screening with the M13F/R primer set and subsequently mini-prepped using the QIAprep Spin Miniprep Kit (Qiagen). The resulting plasmids were transformed into chemically competent *E. coli* strain WM3064. Transformation into G20 was accomplished via conjugation with WM3064, as previously described [202] (see: Chapter 4). Single colony trans-conjugants were obtained by plating on MOLS4 + Spec (100ug/mL) solid medium. For a full list of primers, see Table 5.3. Constructs and strains are listed in Table 5.2.

Construction of E. coli over-expression strains

The pOW107 plasmid, which contains a weak constitutive promoter [220] was used for all overexpression in *E. coli*. Constructs were made using methods outlined above for pMO9075 (see Table 5.3 for primers) and XL1-Blues colonies were screened using the pOW_13_F/R primer set. Positive colonies were grown in 5 mL LB + 50 ug/mL carbenicillin and mini-prepped using the QIAprep Spin Miniprep Kit (Qiagen). The constructs were then transformed into either chemically competent *E. coli* strain DH5- α , or the *E. coli* CysA knock-out (KO) strain JW2415. JW2415 was transformed via electroporation [220] (see: Chapter 4). Single colonies isolates were confirmed via Sanger sequencing (UCB DNA Sequencing Facility). DH5- α strains (Table 5.2) were phenotyped by defining dose-response curves under tungstate, molybdate, selenate and fluorophosphate stress. JW2415 strains (Table 5.2) were tested for their ability to complement the sulfate uptake deficiency phenotype of the control (jwevc) strain. Briefly, strains were inoculated in LB, or M63 or M9 minimal media, with either 250 uM sulfate, 2 mM sulfate or 250 uM cysteine as a sulfur source and growth was monitored as OD600 (Tecan Sunrise microplate reader). For a list of all constructs/strains and primers, see Table 5.2 and Table 5.3, respectively.

5.3 Results

Model SRM *Desulfovibrio alaskensis* G20 was adapted to a suite of sulfate analog inhibitors: 30 mM MFP, 10 mM tungstate, and 100 mM nitrate, whole-genome sequenced and compared to whole-genome sequenced control strains transferred in the absence of inhibitor. This allowed us to identify cellular targets of sulfate analog stress.

Monofluorophosphate adaptation targeted the previously characterized “perchlorate cluster” and a novel cluster of genes, the “MFP cluster”

All fifteen sequenced MFP-adapted G20 isolates contained a nonsense mutation inactivating *pclS* (Dde_0320) (Table 5.1). PclS is a histidine kinase with an extracellular sensory domain, characterized in Chapter 4. It is the sensory component of the perchlorate cluster (Dde_0318-Dde_0328), a cluster of genes involved in perchlorate resistance and encoding proteins for signal transduction and PclT, a DASS family sulfate transporter (Dde_0326). All five isolates from one of the three MFP-adapting populations (M1-1 – M1-9) also contained substitutions in PclT. In addition to the mutation inactivating PclS, all MFP-adapted isolates contained a mutation in or directly downstream of Dde_2195, encoding a 42-amino acid long peptide with no annotated domains. Finally, three other genes were targeted by adaptation to MFP in only a single isolate: gene Dde_0257, encoding another histidine kinase, Dde_0572, encoding a carboxynorspermidine dehydrogenase, and Dde_2777, encoding a protein with a permease-like domain.

Because of the consistency with which mutations were sustained in Dde_2195 and *pclS*, we hypothesized that these genes are causal to G20 adaptation to 30 mM MFP. Moreover, fitness data from a G20 barcoded TN5 insertion library suggests that *pclS* and the downstream response regulator, Dde_0321, are detrimental to MFP stress [53, 92]. There is no fitness data for Dde_2195 but data for the downstream Dde_2193 shows it is very important for MFP stress: inactivation of Dde_2193 causes a strong fitness defect in 1-2 mM MFP, a fitness cost which is unique to this condition. To confirm these phenotypes, TN5 mutants in Dde_2193, *pclS* and Dde_0321 (strains Dde_2195::TN5, Dde_0320::TN5 and Dde_0321::TN5, respectively, Table 5.2) were obtained from the Adam Arkin Laboratory (UC Berkeley) and for each strain, the concentration of MFP inhibiting 50% of growth over 48h (IC50) was measured (Figure 5.1A). As expected, inactivation

of *pclS*, and to a lesser extent of Dde_0321 rendered these strains significantly more resistant to MFP: the IC₅₀ increased by ~5x and ~3x, respectively, from 1.8 mM (95%CI 1.7 – 2.0 mM) for the wild type strain to 8.9 mM (95%CI 7.8 - 10.4 mM) for Dde_0320::TN5 and 4.9 mM (95%CI 4.2 – 5.7 mM) for Dde_0321::TN5. Conversely, the TN5-mediated inactivation of Dde_2193 rendered this strain significantly more sensitive to MFP, decreasing the IC₅₀ to 1.3 mM (95%CI 1.2 - 1.5mM) ($p < 0.001$ for all comparisons, Extra sum of squares F-test). This increase in sensitivity was further exemplified when growth in 2 mM MFP is compared between the wild type and Dde_2193::TN5 strains (Figure 5.1B). The Dde_2193::TN5 strain did not show altered sensitivity to other oxyanions tested: tungstate, nitrate or perchlorate (data not shown).

Mutations in *pclS* and Dde_2195 co-occurred in all adapted genomes, defying attempts to distinguish between their relative importance. However, mutations in *pclT* occurred only in MFP-adapted isolates from one population, allowing us to assess the importance these substitutions relative to mutations in the two other dominant targets. We defined dose-response curves for representative isolates that contained either *only* the Dde_2195 and *pclS* mutations (M2-1, M2-7, M3-5), or Dde_2195/*pclS* and additionally *pclT* mutations (M1-1, M1-3, M1-5) (Figure 5.2A, see Table 5.1 and Table 5.2 for mutations and strains). These dose-response curves were compared to ones generated for the wild type and Dde_0320::TN5 strains. Inactivation of *pclS*, as quantified above, increased resistance to MFP. Adding the Dde_2195 mutation in this background had an even more dramatic effect on resistance (IC₅₀s of ~60mM for all six representative strains). No significant difference in resistance was apparent among strains with or without the additional mutations in *pclT*. This was further evidenced upon growing these strains in 30 mM MFP (Figure 5.2B): inactivation of *pclS* led to only very slow growth after a lag of >50h. However, strains with both a *pclS* null and the Dde_2195 mutation were capable of robust growth in 30 mM MFP. Again, no difference in growth kinetics was observed in strains containing the additional *pclT* mutations. Thus, it appears that the specific substitutions acquired in *pclT* are of secondary importance for growth in MFP stress.

As discussed above, inactivation of *pclS* was not sufficient for growth in 30 mM MFP; mutations in Dde_2195 played a critical role in the resistance phenotype. Dde_2195 is a short peptide with no annotated domains; further, MFP-resistant strain M2-3 sustained an insertion *after* the stop codon of this gene. Together with the increased sensitivity to MFP of the Dde_2193::TN5 strain, we hypothesized that the important gene is not Dde_2195, but rather Dde_2193, perhaps in combination with other genes of the Dde_2192-Dde_2195 operon (termed “MFP cluster”). Mutations in and around Dde_2195 may have changed expression of the MFP cluster. In accordance with this, three MFP-adapted strains (M2-1, M3-5 and M2-7) showed significantly increased expression of Dde_2193 compared to wild type G20 (three to five-fold, $p = 0.0031$, $p = 0.00002$, and $p = 0.015$, t-test of M2-1, M3-5 or M2-7, vs. wild type) (Figure 5.3). The Dde_0320::TN5 strain also showed a slight but significant upregulation of Dde_2193 (1.5x, $p = 0.03$). The upstream gene, Dde_2196 encoding DNA polymerase III, showed no significant change in expression compared to wild type, across all strains tested ($p = 0.181$, $p = 0.652$, $p = 0.978$ and $p = 0.178$, t-test of M2-1, M3-5, M2-7 or Dde_0320::TN5 vs. wild type). Thus, it seems that the Dde_2195 mutations act to increase transcription of the MFP cluster.

The MFP cluster consists of four genes: Dde_2195, Dde_2194, encoding a hypothetical protein with cytoplasmic, transmembrane and extracellular domains, Dde_2193, encoding a protein with an HD domain, and Dde_2192, encoding an aminotransferase (Figure 5.4A). To test whether increased expression of the MFP cluster is sufficient for robust growth in 30 mM MFP, we created G20 strains overexpressing either Dde_2195 (strain 2195), Dde_2193 (strain 2193),

Dde_2193-Dde_2194 (strain 2193-4), or Dde_2192-Dde_2194 (strain 2192-4) or the entire cluster (strain 2192-5) (Table 5.2). All test strains, with the exception of strain 2195 and the control strain (G20evc), eventually grew in 30 mM MFP (Figure 5.4B). Strain 2192-5 came up first, then strain 2192-4, strain 2193-4, and last, strain 2193. These strains, however, did not show the robust growth of the originally adapted strains; overexpression of the MFP cluster allowed for eventual growth in 30 mM MFP but was not sufficient for robust growth at this concentration of inhibitor.

We also overexpressed the same set of genes in the Dde_0320::TN5 mutant strain (Table 5.2, strains TN2193, TN2193-4, TN2192-4, TNevc). In this background, overexpression of Dde_2192-Dde_2194 had a completely different effect (Figure 5.4C): strains TN2193-4 and TN2192-4 grew with identical kinetics in the presence and absence of 30 mM MFP. Strain TN2193 grew slightly slower with 30 mM MFP as compared to without, but still grew to the same final OD within 65 h. Thus, inactivation of *pclS* and overexpression of Dde_2193 are sufficient for robust growth in 30 mM MFP.

Tungstate adaptation targeted sulfate symporter, SulP1 and the sulfate adenylyltransferase (Sat)

Adaptation of G20 to tungstate caused the largest number of genomic changes across adapted isolates, suggesting that 10 mM tungstate was a stronger selective pressure as compared to 30 mM MFP or 100mM nitrate (Table 5.1). All ten tungstate-adapted strains contained mutations in *Sat*: all strains from the first population had substitutions V172S and V310A, while all strains from the second population had substitutions A207T, F317S and I322S (Figure 5.5). The V172L substitution was less dramatic, considering both residues are hydrophobic and certain *Desulfovibrio* contain leucine at that position. Substitutions at position 207, 310, 317 and 322 changed a nonpolar, hydrophobic conserved residue (alanine, valine, phenylalanine or isoleucine, respectively) to a smaller, or polar residue (threonine, alanine or serine). These changes were in the catalytic domain of the enzyme, in close proximity to putative active site residues. The effect of these changes on enzyme specificity remains to be addressed, but presumably they collectively diminished the turnover of tungstate relative to sulfate.

All tungstate-adapted strains also showed a specific mutation in Dde_1102 (henceforth “*sulP1*”), one of three annotated SulP-family transporters in the G20 genome. The G259R substitution switched glycine (a small, non-polar residue) for a positively charged, much larger arginine, in the 7th transmembrane helix. Again, the effect this change has on transport is unknown, but likely rendered SulP1 more specific towards sulfate or less leaky towards tungstate. We expressed both the wild type and mutated SulP1 in *E. coli* DH5- α (strains dhSulP1, dhSulP1*, Table 5.2). In LB rich medium, the mutated SulP1 was less affected by tungstate (Figure 5.6). In minimal M9 medium, overexpressing either version of SulP1 rendered *E. coli* much less sensitive to tungstate, compared to the control (empty vector) strain (Figure 5.7); strain dhSulP1*, however, displayed slower growth under all concentrations of tungstate when compared to strain dhSulP1.

Another dominant mutation occurring in nine out of ten tungstate-adapted strains was an insertion in the Dde_1728 gene, encoding a hypothetical protein with a zinc ribbon domain. Dde_1728 is part of a putative two-gene operon; Dde_1729 encodes a GTP cyclohydrolase 1 type 2 which is very important for G20 under nitrate stress (MicrobesOnline). From here on, the two populations diverged in terms of the mutational profile of sequenced strains. All adapted isolates from the first population contained an insertion in Dde_0352, encoding the flagellar motor switch protein, FliG. Three out of five strains from this population also contained one of two substitutions (R223W or R227W) in Dde_0697, a putative response regulator. Dose-response curves of the

Dde_0697::TN5 mutant compared to wild type G20 showed that inactivation of Dde_0697 did not change sensitivity of G20 to tungstate (Figure 5.8).

All strains from the second tungstate-adapted population contained a substitution (W221C) in Dde_0535, encoding a histidine kinase, and insertions into Dde_0604, encoding a pyruvate-water dikinase, and Dde_2882, encoding a hypothetical protein with a methyltransferase type II domain. Four out of five isolates also contained an insertion in *qrcB* (Dde_2933), a cytochrome c3:menaquinone oxidoreductase. Interestingly, Dde_0604 is part of the “nitrate cluster” previously characterized as detrimental to growth of G20 in 100 mM nitrate; the Dde_0604::TN5 strain (inactivation of Dde_0604) is sufficient for growth in this condition. The mechanism of resistance conferred by the nitrate cluster is thought to be decreased nitrate entry into cells via Dde_0598, a CysZ family transporter. We phenotyped the Dde_0598::TN5 strain via dose-response to tungstate, and there was no change in sensitivity compared to wild type G20 (Figure 5.8). Finally, the V479A substitution in Dde_0978 and the insertion in Dde_1291 each occurred only in one strain. Collectively, these data suggest that SulP1 and Sat were primary targets of 10 mM tungstate stress in G20, and that the severity of this stress may have required additional mechanisms for robust resistance.

SulP permeases: sulfate as a substrate and the specificity of transport

Tungstate-adaptation targeted SulP1. There are two additional genes annotated as SulP in the G20 genome: Dde_2506 (henceforth *sulP2*) and Dde_3641 (henceforth *sulP3*). None of the three are essential for sulfate respiration (all support TN5 insertions) and the main sulfate transporters are not known [53, 92]. In order to address this knowledge gap, we heterologously expressed all three SulP genes in CysA *E. coli* knock out strain JW2415 (strains jwSulP1, jwSulP2 and jwSulP3). JW2415 is deficient in sulfate uptake and hence cannot grow in minimal medium with sulfate and the sole sulfur source. Strain jwSulP3 complemented the sulfate uptake deficiency phenotype of the jwevc (JW2415 empty vector control) at both high (2 mM) and low (250 μ M) external sulfate concentrations (Figure 5.9). JwSulP1 could also uptake sulfate, but only when it was provided at 2 mM. Finally, strain jwSulP2 did not complement sulfate-uptake at either sulfate concentration. Thus, SulP3 (Dde_3641) and SulP1 (Dde_1102) are sulfate transporters, though SulP1 may only function at higher sulfate concentrations (i.e. a low affinity transporter).

Regardless of the intended substrate of these SulP-family permeases, they may be leaky towards oxyanion inhibitors. In order to begin to assess the specificity of these transporters, we expressed all three *sulP* genes in *E. coli* DH5- α and monitored growth at various concentrations of selenate, tungstate, molybdate and MFP, in minimal medium M63 with either cysteine or sulfate as a sulfur source (at 100 μ M). Strikingly, under sulfate assimilation conditions, strain dhSulP1 was resistant to all concentrations of tungstate and molybdate tested, and showed increased resistance to selenate, relative to the control dhevc strain (Figure 5.10). Conversely, strain dhSulP3 showed increased sensitivity to MFP, but increased resistance to selenate, when compared to dhevc. DhSulP3 also grew to a lower OD than the control strain under molybdate stress; the shape of the dose-response curve, however, was similar. Finally, strain dhSulP2 behaved exactly like the control dhevc in all four oxyanion stress conditions. Growth curves at all concentrations of all inhibitors were also generated in minimal medium with 2 mM sulfate. Inhibition under these conditions followed the same patterns as in 100 μ M sulfate, but the potency of all inhibitors was decreased (data not shown). Growth was also monitored at all concentrations of inhibitors, for all strains, in minimal medium with cysteine as the sulfur source; in these conditions, sulfate analogs

should be minimally inhibitory. Indeed, dose-response curves could not be generated for most strains under most conditions (growth was similar irrespective of the concentration of inhibitor). The exception was dhSulP3, which showed sensitivity towards tungstate and MFP and slightly increased sensitivity to molybdate. From these data and data generated in the JW2415 strain, we hypothesize (1) that SulP1 functions as a sulfate transporter that can rescue inhibition by several sulfate analogs, (2) SulP2 is not a sulfate transporter and is not permeable to other oxyanions tested, (3) SulP3 is a sulfate transporter functioning at a range of sulfate concentrations, but its expression increased sensitivity to MFP suggesting it is perhaps leaky towards this substrate.

Nitrate adaptation targeted nitrate transport, via the previously characterized “nitrate cluster”

Nitrate adaptation over the course of our study primarily targeted genes of the “nitrate cluster”: in 13/14 strains, there was an insertion in the Dde_0601 gene, encoding a protein with periplasmic binding domain, and in the last strain there was an insertion in Dde_0597. Both mutations likely act to stop nitrate entry into cells, via Cys-Z type transporter, Dde_0598. Indeed, strains Dde_0598::TN5 and Dde_0601::TN5 showed increased resistance (3 to 4x) to nitrate compared to wild type G20 (Figure 5.11). An additional mutation in a non-coding region (2657199G>T) occurred in 8/14 nitrate-adapted strain. It is unclear whether this mutation conferred any benefit. The final two mutations occurred only in one strain each: an insertion in Dde_1177, encoding an Ala/Glu epimerase/enolase (in N2-6) and a frameshift mutation in Dde_1868, encoding a hypothetical protein (in N2-9). It is unlikely that mutations other than the ones in the nitrate cluster played a critical role in resistance; inactivation of the nitrate cluster has previously been shown as sufficient for robust growth of closely related SRM, *Desulfovibrio vulgaris* Hildenborough [17].

5.4 Discussion

In this study, we identified targets of sulfate analog stress in model SRM *Desulfovibrio alaskensis* G20. Sulfate analogs targeted component of the SR pathway that directly interact with sulfate: sulfate/anion transport systems, and the sulfate adenylyltransferase (*sat*). Depending on the stability and toxicity of sulfate analogs once cytoplasmic, additional mechanisms were required to cope with each stressor.

Adaptation to MFP targeted the previously characterized perchlorate cluster, specifically PclS and the PclT. PclS inactivation likely acted to increase sulfate transport into cells via PclT; this mechanism is sufficient for growth in 100 mM of the sulfate analog perchlorate, as described in Chapter 4. Previous adaptations, although inactivating PclS or the downstream response regulator, did not cause mutation of PclT. Here, MFP adaptation also targeted PclT directly, supporting the current hypothesis that the mechanism of resistance is indeed related to sulfate symport via PclT.

Inactivation of PclS enhanced MFP resistance in G20 but was not sufficient for robust growth in the presence of 30 mM of the inhibitor. MFP adaptation additionally required increased expression of the MFP cluster (Dde_2195-Dde_2192). Increased expression was achieved by mutation of Dde_2195 or via an insertion directly after this gene. How either of these mutations increased transcription of the cluster remains unclear; overexpression of Dde_2195 alone did not recapitulate the resistance phenotype, implying that Dde_2195 is cis-acting. The mechanism of resistance of the MFP cluster is also unclear. MFP toxicity has been attributed largely to

intracellular release of fluoride, with evidence coming from the increased sensitivity of fluoride-efflux deficient mutants to MFP [10]. Homologs of MFP cluster genes Dde_2194 and Dde_2193 exist in *Desulfovibrio vulgaris* Miyazaki, DvMF_0131-DvMF_0132, and are detrimental to starvation conditions and growth on a variety of carbon sources, including casamino acids and L-lactate, as well as growth with nutrients L-serine, L-methionine, or L-threonine [54]. In G20, however, the operon may have a different function: there is no significant fitness data under these conditions and instead the operon is very important uniquely under MFP stress [53].

The MFP cluster may have a direct role in the turnover of MFP or of the fluorinated APS analog (ADP β F) generated when MFP is activated by Sat. Dde_2193 has an HD domain, with predicted phosphohydrolase activity. HD domain proteins have been implicated in the metabolism of nucleotides as well as in signal transduction [221]. Although the majority of HD domain proteins are uncharacterized, recently membrane bound enzymes with cytoplasmic HD domains have been linked to the hydrolysis of second messenger c-di-AMP to 5'-phosphadenylyl-adenosine in *Bacillus subtilis*, *Listeria monocytogens* and *Staphylococcus aureus* (PgpH / GdpP) [222–225]. Interestingly, the adjacent gene, Dde_2194, encodes a hypothetical protein with cytoplasmic, transmembrane, and extracellular domains. Dde_2194 may sense an unknown signal, and via protein-protein interactions, modulate the phosphohydrolase activity of Dde_2193. The final gene of the operon, Dde_2192, encodes a pyridoxal phosphate-dependent transferase. Downstream of the MFP cluster in G20 and downstream of the homologous operons in *D. vulgaris* Hildenborough and *D. vulgaris* Miyazaki, is a gene encoding is an S-adenosyl-L-methionine-dependent methyltransferase (Dde_2191/DVU1357/DvMF_0133). This class of methyltransferases has been implicated in fluorination reactions, generating 5'-fluoro-5'-deoxyadenosine intermediates which are then transformed into fluoroacetate or fluorothreonine [226]. Thus, the MFP cluster may protect cells from ADP β F and/or intracellular fluoride or via reactions that sequester this ion.

MFP stress necessitated *both* overexpression of the MFP cluster *and* inactivation of PclS, suggesting that cells required a two-fold approach: G20 coped with intracellular fluoride release via the MFP cluster *and* competition from MFP for Sat active sites, via increased sulfate transport. Interestingly, fitness data in G20 shows a strong inverse correlation between fitness profiles of *pcrS* and of Dde_2193, suggesting these two proteins may share additional interactions, direct or indirect.

Sulfate analog inhibitors can be classified as *alternative substrates*, if they are substrates of Sat (e.g. MFP, tungstate, molybdate, selenate), or *dead-end* inhibitors, if they are not substrates of Sat and rather competitively bind and inhibit sulfate-turnover (e.g. nitrate, perchlorate) (*see*: Chapter 1). This distinction between alternative substrates and dead-end inhibitors played a role in the mechanism of resistance. For dead-end inhibitors (nitrate, perchlorate), increased sulfate symport or decreased inhibitor import was sufficient to overcome inhibition. For alternative substrates, additional mechanisms of resistance were required. The stability of the Sat-catalyzed end-product (APS analog) was also critical: MFP putatively forms a stable APS analog, ADP β F, with uncharacterized downstream effects that likely involve the evolution of intracellular fluoride, as discussed above [76]. Thus, MFP adaptation required the MFP cluster. Tungstate is also an alternative substrate for Sat, but forms the unstable product, APW [27]. Because APS formation requires the input of ATP, this instability results in rapid depletion of intracellular ATP. Tungstate adaptation thus required a decrease in tungstate turnover, i.e. altered specificity of Sat. In accordance with this, in both tungstate-adapted G20 populations, mutations in Sat were fixed. Although the actual effect of these mutations remains to be addressed, they hint at residues putatively involved in determining the specificity of this critical enzyme.

All sulfate analogs enter cells via anion transport systems. The importance of these various transport systems is obvious across the sulfate analog stressors we examined: MFP stress targeted the sulfate DASS family transporter, PclT (Dde_326), nitrate adaptation, the CysZ-family transporter, Dde_0598, and tungstate stress, the SulP-family transporter, SulP1 (Dde_1102). Transport systems represent the first line of defense and are likely easy targets for adaptation, as a result of their redundancy and, in certain cases, the plethora of genes necessary for their activation/regulation (e.g. perchlorate and nitrate clusters). Here, we have begun to characterize transport across the SulP family: we confirmed that SulP3 can transport sulfate at both 250uM and 2mM external sulfate; SulP1 can transport sulfate at high external concentrations (2 mM) and SulP2 does not function as a sulfate transporter, in line with predictions from a recent comparative genomics analysis [51]. SRM *Desulfobacterium autotrophicum* HRM2 similarly has 3 annotated *sulP* genes; according to a recently study, the expression of one is limited to high sulfate conditions, the second is expressed both at high and low external sulfate, and expression of the last was not detected at all [50]. We also noted changes in the sensitivity of *E. coli* strain DH5- α over-expressing SulP1, SulP2 or SulP3, hinting at the importance of further defining the specificity transport systems across SRM. Future studies will need to address the native substrates and regulation in the native hosts. This insight will be critical in defining the metabolic flexibility of SRM and their innate sensitivity to sulfate analogs. Similarly, the determinants of the specificity of Sat require further investigation and can lend key insights into inhibition of sulfate turnover across the three domains of life.

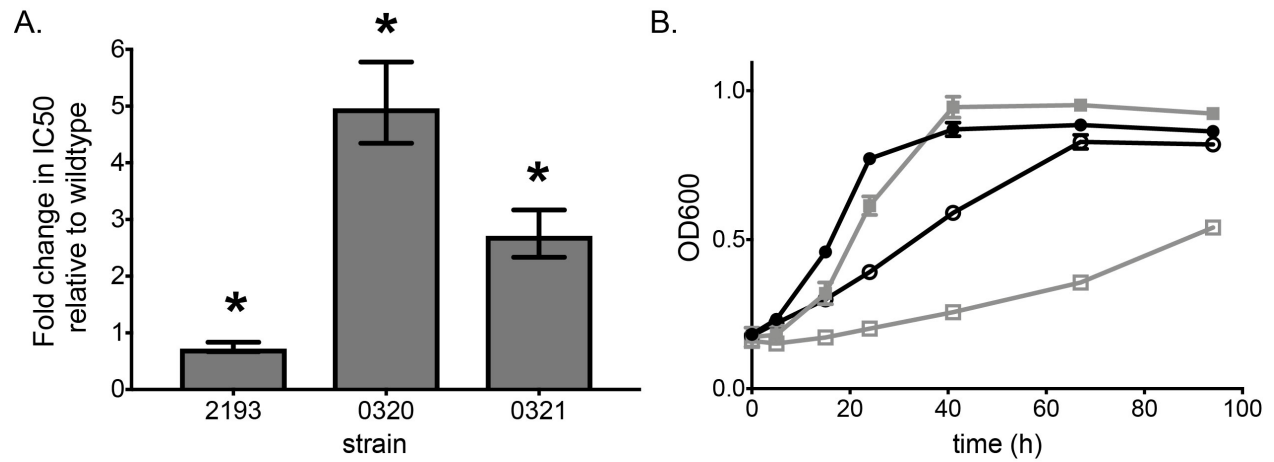


Figure 5.1: A. IC₅₀ against MFP of strains Dde_2193::TN5 (2193), Dde_0320::TN5 (0320) and Dde_0321::TN5 (0321). Bars represent 95% CI. All IC₅₀s are significantly different wild type ($p < 0.001$, Extra sum of squares F-test) B. Growth of wild type G20 (black circles) and the Dde_2193::TN5 strain (grey squares) in the absence (solid symbols) and presence (open symbols) of 2 mM MFP. Bars represent SD of 3 replicates.

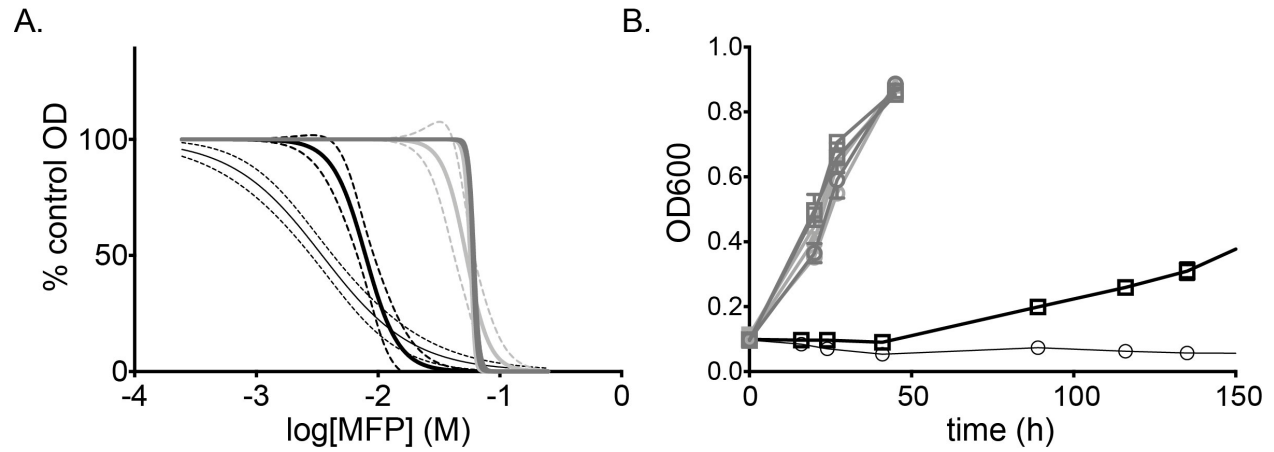


Figure 5.2: A. Dose-response curves against MFP for wild type G20 (thin black), Dde_0320::TN5 strain (thick black), M1-1, M1-3 and M1-5 strains (dark grey) and M2-1, M2-7 and M3-5 (light grey). Dashed lines represent 95% CI for dose-response curves. B. Growth in MOLS4 + 30 mM MFP of wild type (thin black circles), Dde:0320::TN5 (thick black squares), M1-1, M1-3 and M1-5 strains (dark grey), and M2-1, M2-7 and M3-5 (light grey). Error bars represent SD of triplicate cultures. For strain description, see Table 5.2.

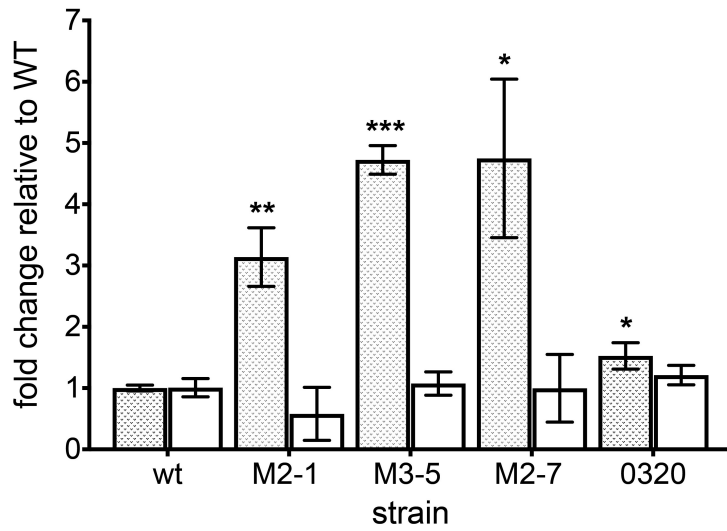


Figure 5.3: Fold change in the expression Dde_2193 (patterned) or Dde_2196 (open) relative to wild type G20 (wt) in strains M2-1, M3-5, M2-7 and Dde_0320::TN5 (0320). Comparisons labeled as significant * are between wt expression and expression in the respective strains. Bars represent SD from 3 biological replicates. *= $p < 0.05$, **= $p < 0.005$, ***= $p < 0.0001$. For strain description, see Table 5.2.

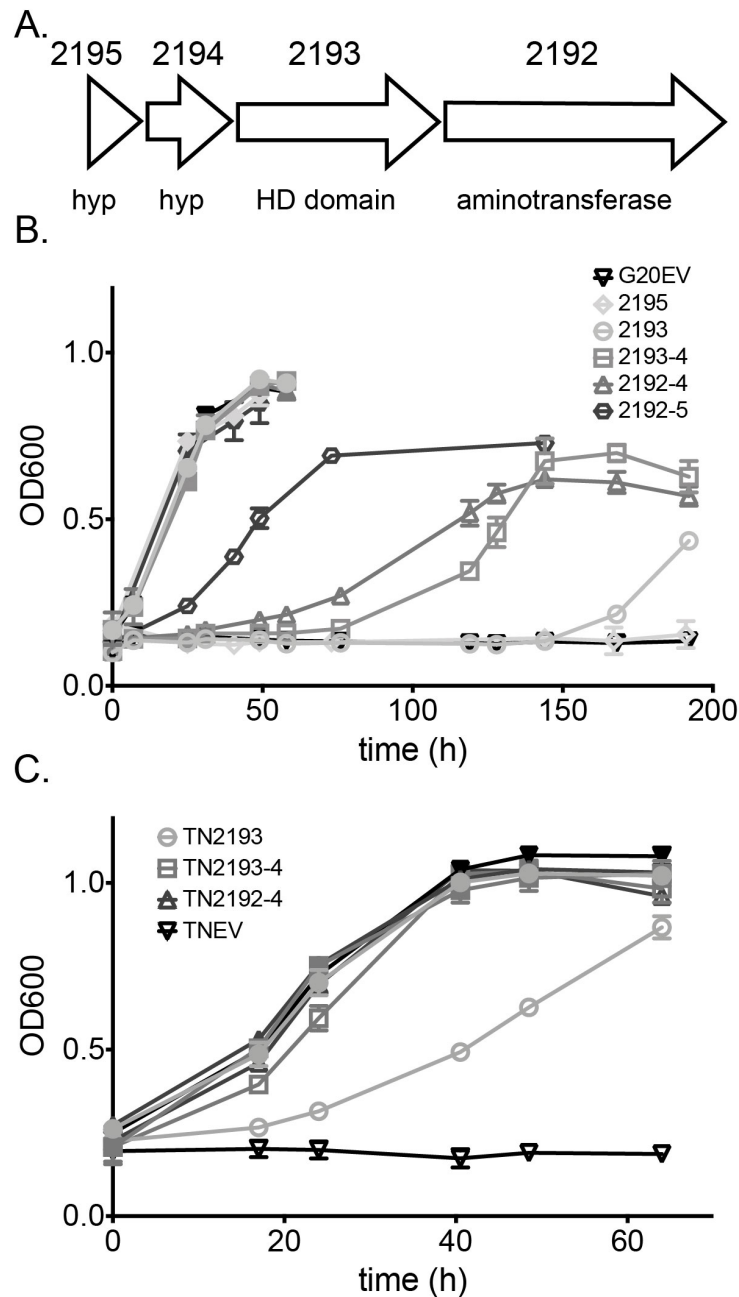


Figure 5.4: A. Genomic context for Dde_2195; B. Growth in the absence (filled symbols) and presence (open symbols) of 30 mM MFP of wild type G20 strains overexpressing genes from the MFP cluster. C. Same constructs as panel B., overexpressed in the Dde_0320::TN5 mutant G20 background. For strain description, see Table 5.2. Error bars represent SD from triplicates.

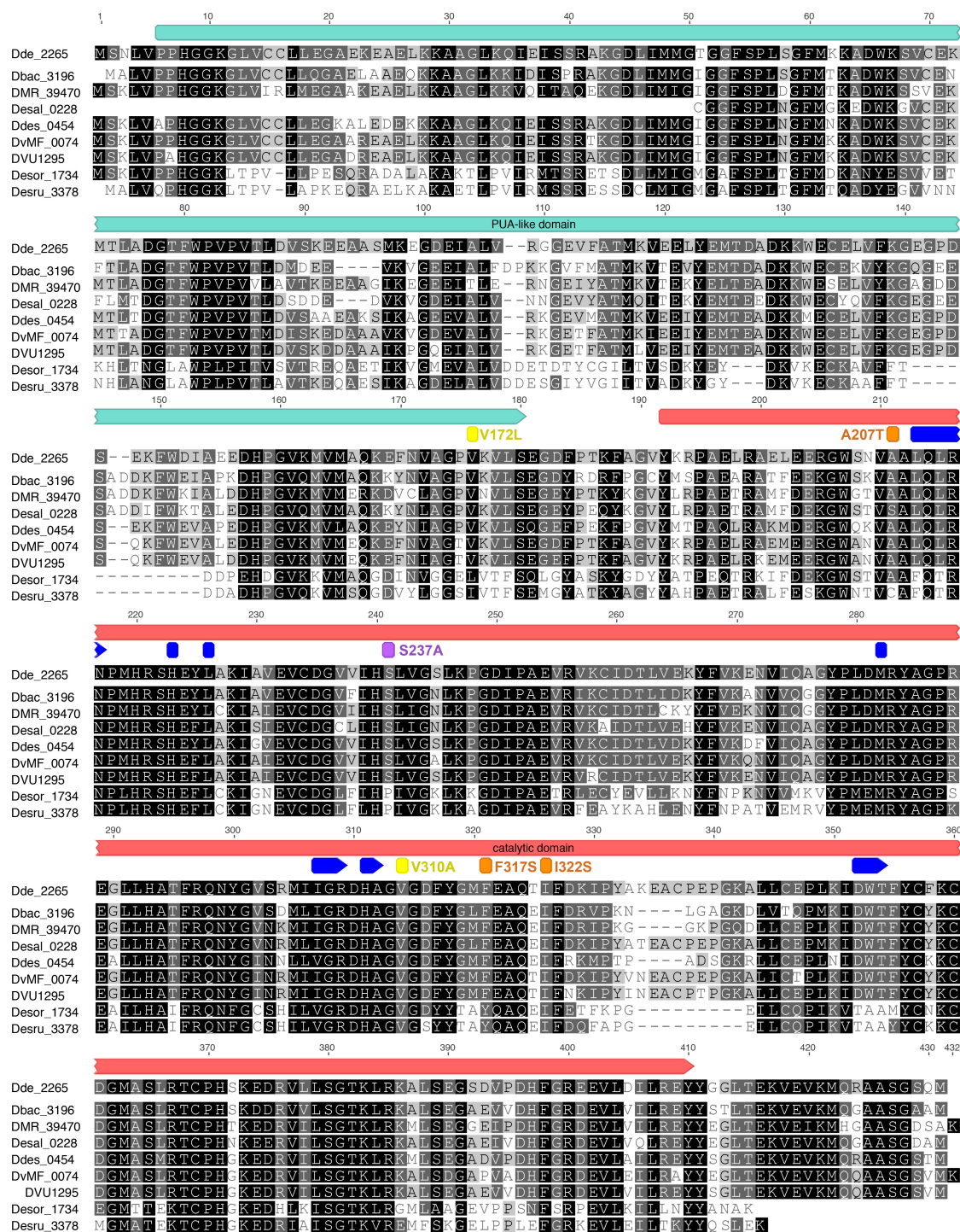


Figure 5.5: Alignment of *sat* from G20 and closely related SRM. Blue = active site (CDD); Residues mutated in all tungstate-adapted strains from population 1 (yellow color) and population 2 (orange color) are indicated (annotation based on position in G20). Residue annotated in purple is a previous substitution arising consistently in perchlorate-adapted G20 (see: Chapter 3). Domain annotation from InterPro (IPR025980 and IPR024951).

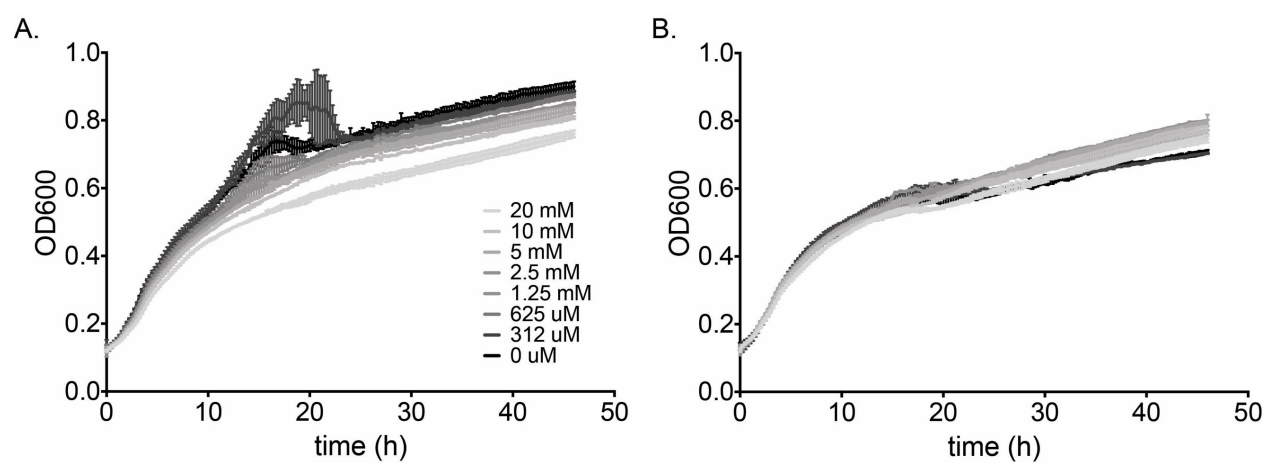


Figure 5.6: Growth of *E. coli* strains dhSulP1 (A.), dhSulP1* (B.) LB and various concentrations of tungstate. Bars represent SD of three replicates; for strain information, see Table 5.2.

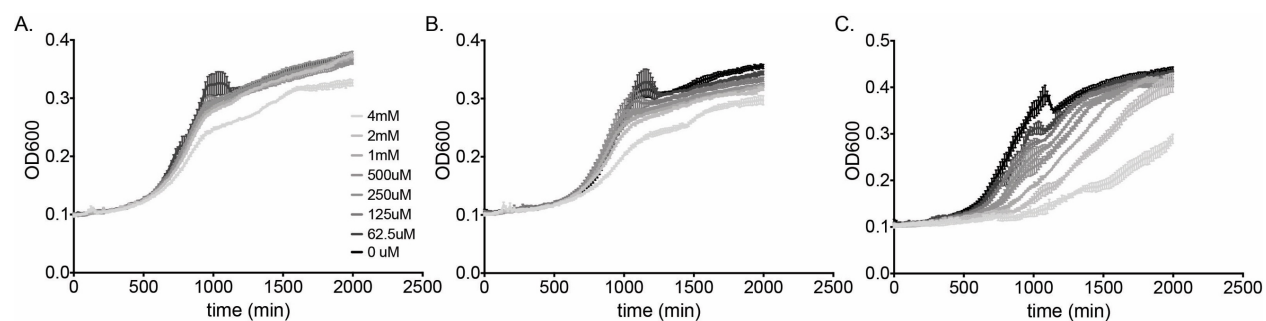


Figure 5.7: Growth of *E. coli* strains dhSulP1 (A.), dhSulP1* (B.) or dhevc (C.) in M9 medium with sulfate as a sole sulfur source and various concentrations of tungstate. Bars represent SD of three replicates; for strain information, see Table 5.2.

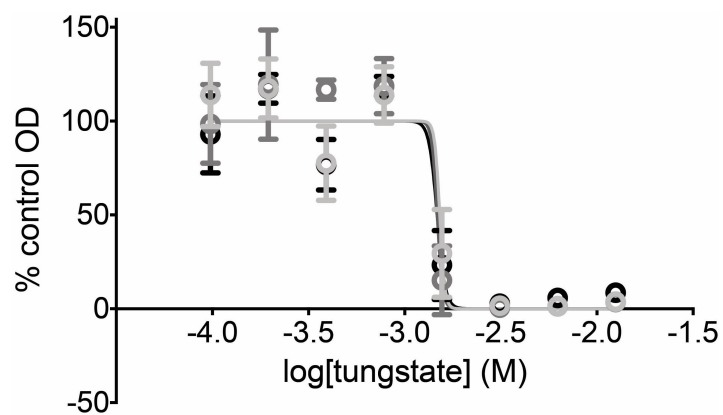


Figure 5.8: Dose-response curves against tungstate for wild type G20 (black), strain Dde_0697::TN5 (dark grey), and strain Dde_0598::TN5 (light grey). Error bars represent SD of four replicates. See Table 5.2 for strain information.

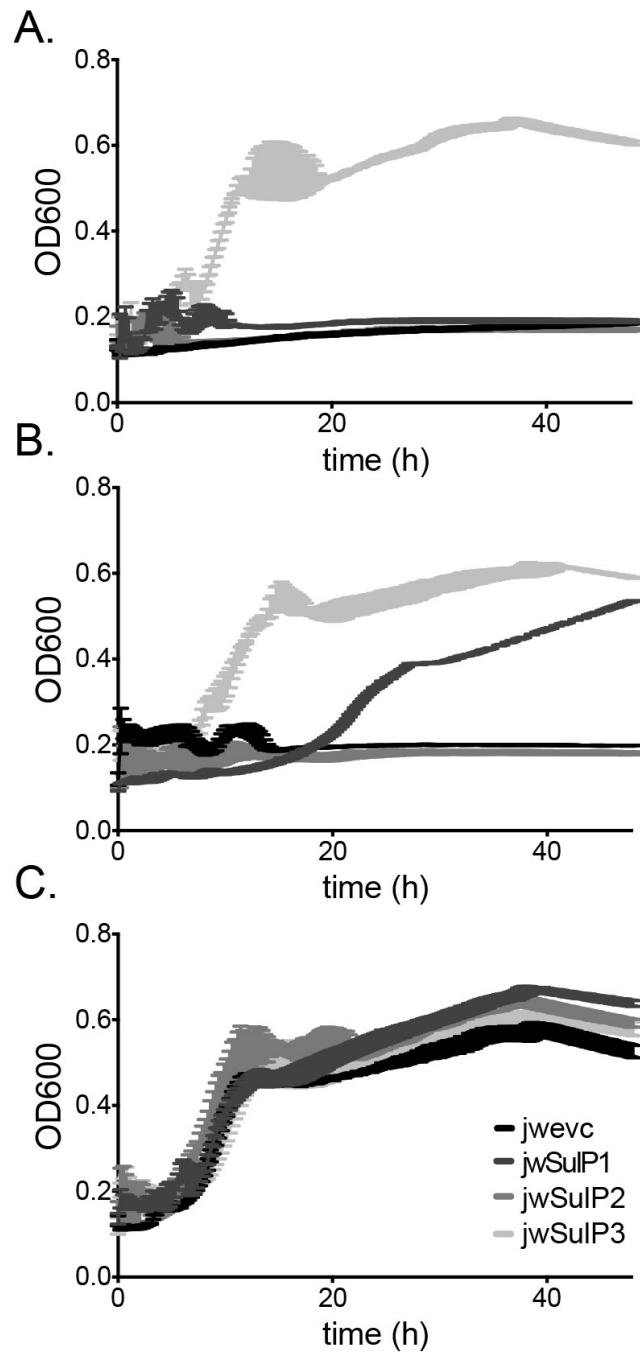


Figure 5.9: Growth of JW2415 overexpressing an empty vector (black) or SulP1 (jwSulP1, darkest grey), SulP2 (jwSulP2, medium grey) or SulP3 (jwSulP3, lightest grey) in M63 medium with the sole sulfur source as either A. 2mM sulfate, B. 250uM sulfate or C. 250uM cysteine. Bars represent SD of three replicates. For strain description, see Table 5.2.

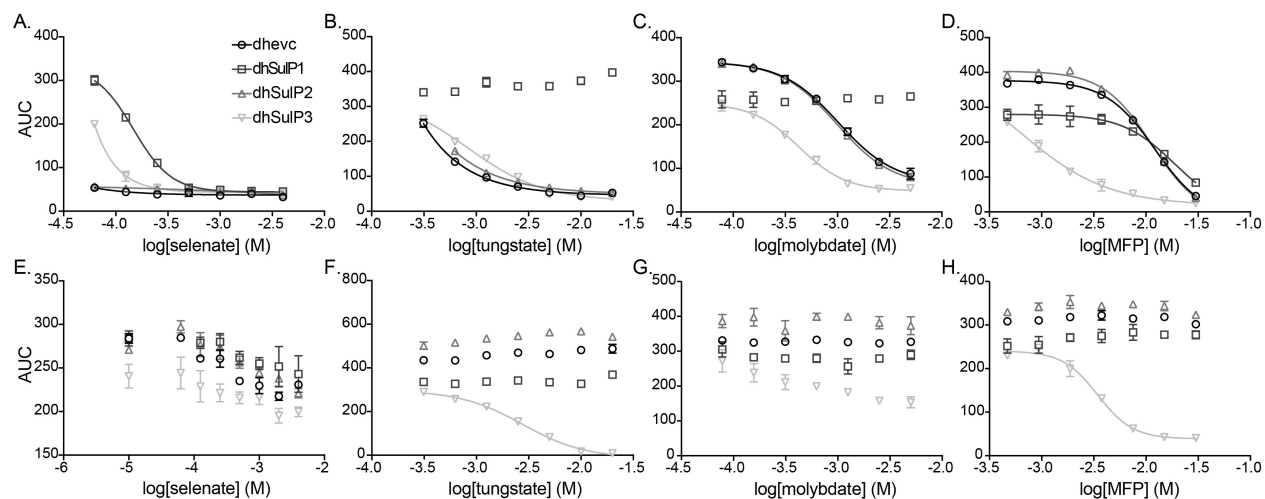


Figure 5.10: Area under the curve (AUC) for growth curves of *E. coli* DH5- α strains over-expressing SulP1, SulP2 or SulP3 in M63 minimal medium with 100 uM sulfate as the sole sulfur source (A-D) or 100 uM cysteine as the sole sulfur source (E-F). AUC values are plotted against inhibitor concentrations. Bars represent SD of three replicates. When possible, dose-response curves are fit to the data.

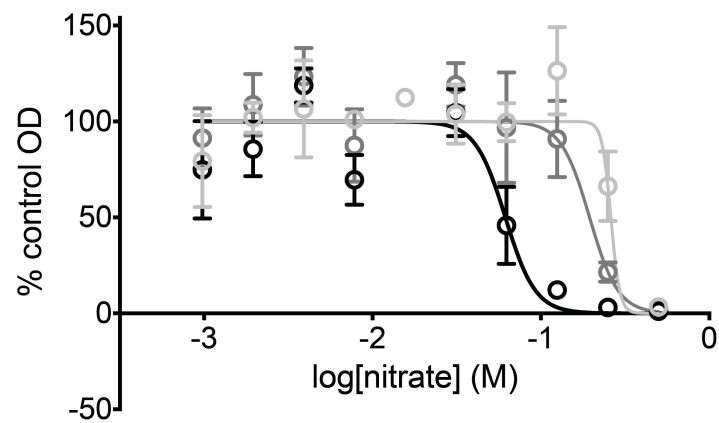


Figure 5.11: Dose-response curves against nitrate for wild type G20 (black), Dde_0601::TN5 strain (dark grey), and the Dde_0598::TN5 strain (light grey). Error bars represent SD of four replicates. See Table 5.2 for strain information.

Table 5.1: Genome-wide mutations in inhibitor-adapted G20 isolates

Position ¹	Mutation ²	Nucleotide change	A.A. change	Gene	Gene annotation	Frequency ³	Strains	Notes ⁴
<i>MFP adaptation</i>								
310619	nonsense	(+) 1267G>T	E423*	Dde_0320	periplasmic sensor signal transduction histidine kinase	15/15	M1-1, M1-3, M1-5, M1-7, M1-9, M2-1, M2-3, M2-5, M2-7, M2-9, M3-1, M3-3, M3-5, M3-7, M3-9	Detrimental to perchlorate and MFP stress; previously seen in perchlorate adaptation [220]
2234324	substitution	(-) 82C>T	H28Y	Dde_2195	hypothetical protein	14/15	M1-1, M1-3, M1-5, M1-7, M1-9, M2-1, M2-5, M2-7, M2-9, M3-1, M3-3, M3-5, M3-7, M3-9	Downstream operon is important to fitness in MFP
2234435	Insertion	after *		Dde_2195	hypothetical protein	1/15	M2-3	Same as above
314574 315095	substitution	(+) 632C>T (+) 1153G>A	A211V V385M	Dde_0326	sodium sulfate symporter	5/15	M1-5, M1-7 M1-1, M1-3, M1-9	
240403	substitution	(-) 1876G>A	E626K	Dde_0257	histidine kinase	1/15	M3-9	
597198	substitution	(+) 67G>A	A23T	Dde_0572	carboxynorspermidine dehydrogenase	1/15	M2-9	
2779691	insertion			Dde_2777	hypothetical, permease-like	1/15	M1-7	
<i>Tungstate adaptation</i>								
2287699	SNP	(+) 514G>C	V172L	Dde_2265	sulfate adenylyltransferase	10/10	T1-1, T1-3, T1-5, T1-7, T1-9	
2288114	SNP	(+) 929T>C	V310A					
2287804	SNP	(+) 619G>A	A207T					
2288135	SNP	(+) 950T>C	F317S				T3-1, T3-3, T3-5, T3-7, T3-9	
2288150	SNP	(+) 965T>G	I322S					
1130389	SNP	(-) 775G>A	G259R	Dde_1102	sulfate transporter (SulP family)	10/10	T1-1, T1-3, T1-5, T1-7, T1-9, T3-1, T3-3, T3-5, T3-7, T3-9	
1786259	insertion			Dde_1728	hypothetical protein	9/10	T1-1, T1-3, T1-5, T1-7, T1-9, T3-1, T3-5, T3-7, T3-9	Zn-ribbon domain
342736	insertion			Dde_0352	Flagellar motor switch protein FliG	5/10	T1-1, T1-3, T1-5, T1-7, T1-9	Affected by NaCl tolerance in G20 [184]
544977	SNP	(-) 663G>C	W221C	Dde_0535	signal transduction histidine kinase	5/10	T3-1, T3-3, T3-5, T3-7, T3-9	
627711	insertion			Dde_0604	pyruvate/water dikinase	5/10	T3-1, T3-3, T3-5, T3-7, T3-9	part of "nitrate cluster" [17];
2871202	insertion			Dde_2882	hypothetical protein	5/10	T3-1, T3-3, T3-5, T3-7, T3-9	methyltransferase family
2913764	insertion			Dde_2933	putative molybdopterin oxidoreductase	4/10	T3-1, T3-5, T3-7, T3-9	
712123	SNP	(+) 667C>T	R223W	Dde_0697	response regulator receiver domain protein	3/10	T1-1, T1-5	
712135		(+) 679C>T	R227W				T1-3	
1005902	SNP	(-) 1436T>C	V479A	Dde_0978	metal dependent phosphohydrolase	1/10	T3-3	
1314831	insertion			Dde_1291	hypothetical protein	1/10	T1-7	
<i>Nitrate adaptation</i>								

624299 /624196	Insertions		Dde_0601	phosphonate-binding periplasmic protein	13/1 4	N1-1, N1-5, N1-7, N2-1, N2-3, N2-5, N2-6, N2-9, N3-1, N3-3, N3-5, N3-7, N1,9	Detrimental to fitness on nitrate; "nitrate cluster" [17]
620617	insertion		Dde_0597	hypothetical protein	1/14	N3-9	Detrimental to fitness on nitrate; "nitrate cluster" [17]
2657199	SNP	G>T	IGR	noncoding	8/14	N2-1, N2-3, N2-5, N2-6, N2-9, N3-1, N3-5, N3-7	b/w Dde_2639 Ser-tRNA hydrolase and Dde_R0057 tRNA-Val- GAC
1204452	insertion		Dde_1177	Ala/Glu epimerase/enolase	1/14	N2-6	
1921073- 4	frameshift		Dde_1868	hypothetical protein	1/14	N2-9	

¹ coordinates based on reference (IMG genome ID 637000095)

² only mutations that are *absent* from control strains are listed

³ proportion of sequenced isolates containing the mutation

⁴ fitness data from Microbes Online (<http://www.microbesonline.org/>) and Fitness Browser (<http://fit.genomics.lbl.gov/>); domain predictions from Interpro (<https://www.ebi.ac.uk/interpro/>)

Table 5.2: Strains and constructs used in this study

Name	Description	Source
Plasmids		
pOW107	Parental plasmid used for complementation of <i>E. coli</i> CysA KO strain; Amp ^R ; weak constitutive promoter Bba_J23103 (Chris Anderson, http://parts.igem.org/Promoters/Catalog/Anderson)	From [220]; originally John Dueber [218]
pOW107.4	pOW107 with wild type G20 gene <i>sulP1</i> (Dde_1102)	This study
pOW107.5	pOW107 with wild type G20 gene <i>sulP2</i> (Dde_2506)	This study
pOW107.6	pOW107 with wild type G20 gene <i>sulP3</i> (Dde_3641)	This study
pOW107.7	pOW107 with <i>SulP1</i> from tungstate-adapted G20, contains mutation G259R	This study
pMO9075	Parental plasmid; used for overexpression in G20; P _{npt} ; Sp ^R	Gift from Wall lab [219]
pMS3	pMO9075 with wild type G20 gene Dde_2193	This study
pMS4	pMO9075 with wild type G20 gene Dde_2193-Dde_2194	This study
pMS5	pMO9075 with wild type G20 gene Dde_2192-Dde_2194	This study
pMS6	pMO9075 with wild type G20 gene Dde_2192-Dde_2195	This study
pMS7	pMO9075 with wild type G20 gene Dde_2195	This study
<i>Desulfovibrio alaskensis</i> G20 strains		
G20	Wild type <i>D. alaskensis</i> G20	from Adam Arkin
Dde_0320::TN5	G20 TN5 mutant; transposon insertion in Dde_0320	[92]
Dde_0321::TN5	G20 TN5 mutant; transposon insertion in Dde_0321	[92]
Dde_0326::TN5	G20 TN5 mutant; transposon insertion in Dde_0326	[92]
Dde_2193::TN5	G20 TN5 mutant; transposon insertion in Dde_2193	[92]
Dde_0598::TN5	G20 TN5 mutant; transposon insertion in Dde_0598	[92]
Dde_0697::TN5	G20 TN5 mutant; transposon insertion in Dde_0697	[92]
Dde_0601::TN5	G20 TN5 mutant; transposon insertion in Dde_0601	[92]
M1-1	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 (E423*), a substitution in Dde_0326 (V385L) and a substitution in Dde_2193 (28H>Y)	This study
M1-3	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 (E423*), a substitution in Dde_0326 (V385L) and a substitution in Dde_2193 (28H>Y)	This study
M1-5	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 (E423*), a substitution in Dde_0326 (A211V) and a substitution in Dde_2193 (28H>Y)	This study
M2-1	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 and a substitution in Dde_2193 (28H>Y)	This study
M2-7	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 and a substitution in Dde_2193 (28H>Y)	This study
M3-5	Adapted to 30 mM MFP G20 strain containing non-sense mutation in Dde_0320 and a substitution in Dde_2193 (28H>Y)	This study
G20EV	Wild type G20 with pMO9075	This study
2193	Wild type G20 with pMS3	This study
2193-4	Wild type G20 with pMS4	This study
2192-4	Wild type G20 with pMS5	This study
2192-5	Wild type G20 with pMS6	This study
2195	Wild type G20 with pMS7	This study
TNEV	The Dde_0320::TN5 G20 strain with pMO9075	This study
TN2193	The Dde_0320::TN5 G20 strain with pMS3	This study
TN2193-4	The Dde_0320::TN5 G20 strain with pMS4	This study
TN2192-4	The Dde_0320::TN5 G20 strain with pMS5	This study
<i>E. coli</i> JW2415 strains (CysA KO)		
dhevc	DH5- α <i>E. coli</i> with pOW107	This study
dhSulP1	DH5- α <i>E. coli</i> with pOW107.4	This study

dhSulP1*	DH5- α <i>E. coli</i> with pOW107.7	This study
dhSulP2	DH5- α <i>E. coli</i> with pOW107.5	This study
dhSulP3	DH5- α <i>E. coli</i> with pOW107.6	This study
jwevc	<i>E. coli</i> JW2415 with pOW107	This study
jwSulP1	<i>E. coli</i> JW2415 with pOW107.4	This study
jwSulP2	<i>E. coli</i> JW2415 with pOW107.5	This study
jwSulP3	<i>E. coli</i> JW2415 with pOW107.6	This study

Table 5.3: Primers used in this study

Primer name	Sequence ¹	Purpose
pOW_lin_F pOW_lin_R	ATAGCAACAAAAGCTGGATGTGA AGATCTTTTCTCCTCTTGCTAGCG	Primer set for linearizing vector pOW107
pOW_1102_F pOW_1102_R	gcaaagaggagaaaagatctATGAAAACGGGCATCGCAG catccagctttgttgctatTTAATGGTGGTGGTGATGATGCTCCGCGGTCAATTCCGG	Primer set used to amplify Dde_1102 from G20, for cloning into pOW107
pOW_2506_F pOW_2506_R	gcaaagaggagaaaagatctATGCATTCTCAAATGTGGCCTC catccagctttgttgctatTCAATGGTGGTGGTGATGATGGGAAAGGGAAGAAGCGGTG	Primer set used to amplify Dde_2506 from G20, for cloning into pOW107
pOW_3641_F pOW_3641_R	gcaaagaggagaaaagatctATGCTGACGAGAGTCTTCCC catccagctttgttgctatCTAATGGTGGTGGTGATGATGCGTCAATGCGCAGTAGGTG	Primer set used to amplify Dde_3641 from G20, for cloning into pOW107)
pOW_13_F pOW_13_R	CGTAGCAAAGAGGAGAGAAAAGATCT TCACATCCAGCTTTTGTGCTAT	Primer set for screening colonies with pOW107 constructs
pMO9075_lin_F pMO9075_lin_R	TAAACTGGATGGCTTTCTtGCC CTTGCAGGGCTTCCCAAC	Primer set for linearizing vector pMO9075
pMO_2193_F pMO_2193_R	gggtgggaagccctgcaaagTATCGCGGAAGGGACTGAAC caagaaagccatccagttaGTTTTCCATTATACGGCAACAGC	Primer set used to amplify Dde_2193 from G20 for cloning into pMO9075
pMO_2194_F	gggtgggaagccctgcaaagCTGCAACACTATGGCGCAAG	Forward primer for amplifying Dde_2194-Dde_2193 from G20, for cloning into pMO9075
pMO_2192_R	caagaaagccatccagttaTCTGCGCCGCCCATAG	Reverse primer for amplifying Dde_2194-Dde_2192 from G20, for cloning into pMO9075
pMO_2195_F pMO_2195_R	gggtgggaagccctgcaaagGCACTCAATTGCCTAAATGTAAATGG caagaaagccatccagttaCTTGCGCCATAGTGTTGCAG	Forward set for amplifying Dde_2195 from G20, for cloning into pMO9075; forward primer for Dde_2195-Dde_2192
pMO_13_F pMO_13_R	GTTGGGAAGCCCTGCAAAG GGCAAGAAAGCCATCCAGTTTA	Primer set for screening colonies with pMO9075 constructs
G20_gyrB_F G20_gyrB_R	TCACCGTTCTTGAGGGGTTG TCAAGATGCAGCTTCACGGT	qPCR primer set for amplifying part of <i>gyrB</i> (Dde_0003) from g20 for RT-qPCR
Dde_2193_qPCR_F Dde_2193_qPCR_R	CCTTGC GTTCTCTCGGAGTT GTCGGCATCTCTGACCACAT	qPCR primer set for amplifying part of Dde_2193 from g20 for RT-qPCR
Dde_2196_qPCR_F Dde_2196_qPCR_R	TTACAAGTCGCCGACGAAA CCTTCCGGCAGTTCGTAGTT	qPCR primer set for amplifying part of Dde_2196 from g20 for RT-qPCR

¹lowercase sequence represents overhang region (complementary to vector) used for Gibson assembly; **bold** = His-tag

Chapter 6: Conclusions and Future Perspectives

The aim of this dissertation was to elucidate the inhibitory mechanisms and targets of specific inhibitors in pure culture and community-embedded SRM. Sulfate analog inhibitors enter cells through sulfate (or other anion) transport systems and are either dead-end inhibitors or alternative substrates of the sulfate adenylyltransferase (Sat). Dead-end inhibitors such as perchlorate compete with and slow sulfate turnover, which led to a reduced growth rate and increased washout of SRM in planktonic systems. This inhibitory effect depended on several additional factors, such as the area available for surface attachment, the presence of dissimilatory perchlorate-reducing microorganisms, and the donor used for sulfate-reduction. Isolation, combined with the creation and characterization of synthetic co-cultures, defined a set of controls on perchlorate inhibition in pure culture and in mixed communities.

For a complete understanding of inhibition, this dissertation also examined adaptation to high external perchlorate concentrations across both pure culture and community-embedded SRM. In pure cultures, *D. alaskensis* G20 consistently adapted to perchlorate via a mutation in a conserved residue of the Sat, which decreased the perchlorate-mediated inhibition of this enzyme. In contrast, in the context of a sulfate-reducing community, both *D. alaskensis* G20 and environmental isolate *D. bizertensis* MKS adapted to perchlorate by altering anion transport systems directly, or the regulatory systems behind them. Changes in transport led to an increased intracellular sulfate to perchlorate ratio, in this way overcoming the competitive effects of perchlorate inhibition.

Finally, sulfate analogs can also be alternative substrates of Sat, and have downstream effects based on the stability of the Sat-catalyzed sulfate-analog product. To identify additional targets of sulfate analog stress in SRM, *D. alaskensis* G20 was adapted to nitrate, monofluorophosphate and tungstate. This work confirmed the importance of transport systems across all sulfate analogs studied. Moreover, monofluorophosphate stress was found to necessitate a cluster of genes putatively involved in the detoxification of fluorinated compounds, whereas adaptation to tungstate identified critical residues in Sat, likely responsible for conferring specificity against tungstate. Thus, this dissertation has provided a detailed account of cellular targets of specific inhibitors in SRM, illustrating how this ubiquitous group of microorganisms copes with sulfate analog stress.

The potency and specificity of inhibitors across diverse SRM

There are several remaining questions in the field that require specific investigation. This dissertation has focused largely on *Desulfovibrio* spp. However, previous research hinted at the varying susceptibilities of SRM across the phylogenetic tree (e.g. archaeal or thermophilic species). Although Sat is a primary target of sulfate-analog stress and is conserved among SRM, varying susceptibilities may result from diverse sulfate/sulfate-analog transport systems as well as “off-target” or “alternative” effects. This has recently been suggested for molybdate stress. Quantitatively and mechanistically examining the sensitivity of diverse SRM to inhibitors will not only shed light onto this key metabolism but will also help in defining the *specificity* of inhibition: at what concentration are these compounds inhibitory to sulfate-reduction vs. to general microbial growth. Such quantification is critical in order to make use of SRM-specific inhibitors. Future work will need to define concentration ranges for specificity across various environments. Finally,

this dissertation has noted different evolutionary outcomes in sulfate-reducing communities as compared to sulfate-reducing pure-cultures. This represents another interesting area for future research: comparing susceptibilities and mechanisms of inhibition in pure-culture, vs. co-culture will help define a set of parameters that govern inhibition in environmentally-relevant contexts.

The Sulfate Adenylyltransferase

Although the Sat from various sulfate assimilating organisms has been purified and characterized *in vitro*, there is no such published characterization for dissimilatory Sat enzymes from SRM. Adaptation of *D. alaskensis* G20 to both perchlorate and tungstate necessitated certain specific changes to Sat. Continuing to probe determinants of Sat specificity using adaptive laboratory evolution is critical to understanding and optimizing the function of this enzyme and to developing specific inhibitors across both assimilatory and dissimilatory sulfate-reducing organisms.

The perchlorate, nitrate and MFP clusters

Adaptation of *D. alaskensis* G20 to specific inhibitors has also revealed several clusters of genes, whose function remains unresolved. The perchlorate cluster represents a two-operon set of genes regulated by PclR, and involved in signal transduction (PclS is the sensory component) and sulfate transport (via PclT). The signal for the activation of this system remains to be discovered, as does the signaling cascade leading to changes in sulfate transport. The results presented herein may suggest cross-talk between PclS/PclR and other regulatory systems. Further, the nitrate cluster is similarly involved in the regulated transport of an as-of-yet undetermined substrate. Closely related SRM *D. vulgaris* Hildenborough has 72 response regulators, only 29 of which have DNA-binding domains. Many contain CheY-like domains and are predicted to act via protein-protein interactions. Understanding these regulatory networks and the phenotypes they generate is of obvious importance to understanding how SRM cope with stress.

The native function of the MFP cluster and how it pertains to MFP stress also remains to be elucidated. It is possible that this cluster is involved more broadly with halogen stressors. The potential interaction between this gene cluster and the perchlorate cluster also warrants further investigation.

Transport as the final frontier

Throughout this dissertation, sulfate transport has emerged as a largely uncharacterized function in SRM, and one that is critical to overcoming specific inhibition. The complexity of sulfate transport and its regulation, thus, represents the final, as-of-yet undefined aspect of dissimilatory sulfate-reduction. Putative anion transporters across SRM genomes have been computationally identified. Future research will need to undertake thorough biochemical characterization of the substrates and specificity of each transport system, as well as an understanding of which systems are active under which conditions, and how the redundancy in sulfate transport allows SRM to maintain this function across sulfate concentrations that differ by multiple orders of magnitude, as well as in the presence of equivalent or greater concentrations of sulfate analog stressors.

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