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Determinants of Sulfur Chemolithoautotrophy in the Extremely Thermoacidophilic Sulfolobales

Benjamin M. Zeldes[1], Andrew J. Loder[1,^], James A. Counts[1], Mashkurul Haque[1], Karl A. Widney[1], Lisa M. Keller[1], Sonja-Verena Albers[2], and Robert M. Kelly[1,*]

[1]*Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695-7905*

[2]*Institute of Biology II - Microbiology, University of Freiburg, Freiburg, Germany*

^*Present address:* Novozymes North America Inc., Franklinton, NC 27525

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***Address correspondence to:** Robert M. Kelly
Department of Chemical and Biomolecular Engineering
North Carolina State University
Raleigh, NC 27695-7905
Phone: (919) 515-6396
Fax: (919) 515-3465
Email: rmkelly@ncsu.edu

Summary

Species in the archaeal order *Sulfolobales* thrive in hot acid and exhibit remarkable metabolic diversity. Some species are chemolithoautotrophic, obtaining energy through oxidation of inorganic substrates, sulfur in particular, and acquiring carbon through the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) CO_2 fixation cycle. The current model for sulfur oxidation in the *Sulfolobales* is based on biochemical analysis of specific proteins from *Acidianus ambivalens*, including sulfur oxygenase reductase (SOR) that disproportionates S° into H_2S and sulfite (SO_3^{2-}). Initial studies indicated SOR catalyzes the essential first step in oxidation of elemental sulfur, but an ancillary role for SOR as a "recycle" enzyme has also been proposed. Here, heterologous expression of both SOR and membrane-bound thiosulfate-quinone oxidoreductase (TQO) from *Sulfolobus tokodaii* 'restored' sulfur oxidation capacity in *Sulfolobus acidocaldarius* DSM639, but not autotrophy, though earlier reports indicate this strain was once capable of chemolithoautotrophy. Comparative transcriptomic analyses of *Acidianus brierleyi*, a chemolithoautotrophic sulfur oxidizer, and *S. acidocaldarius* DSM639 showed that while both share a strong transcriptional response to elemental sulfur, *S. acidocaldarius* DSM639 failed to up-regulate key 3-HP/4-HB cycle genes used by *A. brierleyi* to drive chemolithoautotrophy. Thus, the inability for *S. acidocaldarius* DSM639 to grow chemolithoautotrophically may be rooted more in gene regulation than biochemical capacity.

Originality-Significance Statement

The mechanisms by which extreme thermoacidophiles grow chemolithotrophically on inorganic energy sources are not well understood. Here, we use comparative genomics, molecular genetic tools and growth physiology analysis to examine why *Sulfolobus acidocaldarius* DSM 639 does not use S° as an energy source and identify possible reasons for why this growth mode is not operable in this archaeon, as it once might have been.

Introduction

The order *Sulfolobales* includes extremely thermoacidophilic archaea that thrive in hot acid (T_{opt} > 70°C, pH_{opt} < 3.5) and have remarkable metabolic diversity. Some *Sulfolobales* can grow chemolithoautotrophically, including members of the genera *Sulfolobus*, *Metallosphaera* and *Acidianus*. To date, all genome-sequenced *Sulfolobales* contain enzymes for the 3-hydroxypropionate (3-HP)/4-hydroxybutryrate (4-HB) carbon fixation cycle, along with indications of a common regulatory system for autotrophy (Leyn et al., 2015). There are, however, significant variations at the genus and species level with respect to lithotrophic energy substrates. For example, *Metallosphaera sedula* can utilize ferrous iron and molecular H_2 (Auernik and Kelly, 2010; Hawkins et al., 2013), while *Sulfolobus acidocaldarius* DSM639, a genetically tractable strain (Wagner et al., 2012), grows only heterotrophically, despite genomic signatures suggesting that sulfur oxidation and CO_2 fixation were once metabolic capabilities.

Acidianus ambivalens (previously *Desulfurolobus ambivalens*) (Zillig et al., 1986; Fuchs et al., 1996), a facultative anaerobe capable of autotrophic growth powered by either the oxidation or reduction of elemental sulfur, has served as a model for sulfur oxidation in the *Sulfolobales* (Kletzin, 2006). A key step in understanding acidophilic sulfur oxidation was the identification of cytoplasmic sulfur oxygenase reductase (SOR) in *A. ambivalens* and other *Acidianus* species; this enzyme catalyzes the oxygen-dependent disproportionation of inert elemental sulfur into more reactive sulfite and hydrogen sulfide (Kletzin, 1989; Veith et al., 2011). The cell derives no energy from this enzymatic transformation. However, *A. ambivalens* apparently processes the resulting reactive sulfur species through two parallel, energy-conserving pathways: the first, a series of membrane-associated oxidoreductases, which feed into the electron transport chain, and, second, a cytoplasmic pathway for substrate level phosphorylation which proceeds through an adenylsulfate intermediate (Zimmermann et al., 1999). The genes and proteins responsible for the cytoplasmic pathway have not been identified, but several membrane-associated enzymes of

A. ambivalens have been characterized. Sulfide-quinone oxidoreductase (SQO) (Brito et al., 2009) and thiosulfate-quinone oxidoreductase (TQO) (Müller et al., 2004) feed reduced quinones to one of several terminal quinol oxidases in the *Sulfolobales*, including CbsAB-SoxLN (Bandeiras et al., 2009), DoxBCE (Purschke et al., 1997), and the SoxABC and SoxM complexes (Lübben et al., 1994; Lubben et al., 1994). A tetrathionate hydrolase (TTH) localized to the outer cell surface has also been characterized and proposed to facilitate growth on tetrathionate by converting it to sulfur for further processing by SOR (Protze et al., 2011). In *M. cuprina*, enzymes encoded by *tusA* and *dsrE3A* were shown to transfer tetrathionate groups (Liu et al., 2014). Across the *Sulfolobales*, TusA and DsrE homologs are found adjacent to a large, highly conserved cluster with homology to the methanogenic heterodisulfide reductase (*hdr*) system, which oxidizes sulfur compounds to sulfate in bacteria (Koch and Dahl, 2018), and may provide cellular reducing equivalents by transferring electrons via lipoate to NADH (Cao et al., 2018). A model metabolic pathway based on the enzymes found in *A. ambivalens* has served as the basis for understanding sulfur oxidation in the *Sulfolobales* (Kletzin, 2006); an updated version of this model, including the more recently characterized enzymes, is shown in **Figure 1a**. **Figure 1c** lists gene homologs to proteins that have putative roles in sulfur biotransformations, including sulfur reduction, as well as genes encoding the terminal oxidases. Note that *S. acidocaldarius* DSM639, *S. solfataricus*, and the *Metallosphaera* species lack SOR and also do not oxidize elemental sulfur to any significant extent. Those *Sulfolobales* listed in **Figure 1** whose genomes encode SOR are known to oxidize elemental sulfur. The *sreABCD* genes are unique to several *Acidianus* species, and allow them to grow anaerobically on sulfur. Species in **Figure 1b** are arranged by 16S homology, but as the recent proposals to reclassify *Sulfolobus tokodaii* to *Sulfurisphaera tokodaii* (Tsuboi et al., 2018) and *Sulfolobus solfataricus* as *Saccharolobus solfataricus* (Sakai and Kurosawa, 2018) show, taxonomy within the order *Sulfolobales* remains in flux.

SOR is also found in certain members of the acidophilic genus *Acidithiobacillus* and a few other mesophilic, chemolithoautotrophic bacteria (Veith et al., 2011), suggesting that archaeal

and bacterial chemolithotrophs share a similar sulfur oxidation pathway. However, bacteria also exhibit distinct sulfur-oxidizing mechanisms, such as SoxYZ, located in the bacterial periplasm and therefore absent from archaea, and the Dsr system, which is not found in the *Sulfolobales* (Venceslau et al., 2014). Further complicating things, a SOR knockout strain of *Acidith. caldus* grew similarly to the parent strain on elemental sulfur, but surprisingly grew substantially faster on tetrathionate (Chen et al., 2012). The parent strain also produced SOR at higher levels during growth on tetrathionate than on S^0. These findings led to the hypothesis that, at least in *Acidith. caldus*, SOR is not responsible for the initial oxidation of sulfur, but is instead involved in recycling the sulfur species that form as a result of other biocatalytic steps (Chen et al., 2012). The supplementary, non-essential role of SOR in the S^0 oxidation pathways of *Acidith. caldus* differs from current models of sulfur oxidation in the *Sulfolobales*, raising the question of whether SOR plays different metabolic roles in sulfur-oxidizing bacteria and archaea (Wang et al., 2019). This issue was addressed here by examining the role of SOR in *Sulfolobales* species that natively either oxidize sulfur (*Acidianus brierleyi*) or likely had this metabolic capability in their evolutionary history (*S. acidocaldarius* DSM639). Furthermore, genomic determinants for sulfur oxidation and chemolithoautotrophy in the *Sulfolobales* were examined through the lens of comparative genomic and transcriptomic analysis.

Results

Growth of S. acidocaldarius DSM639 parent and mutant strains on S° and CO_2

Strains of *S. acidocaldarius* have been isolated from several sites that are globally distributed, but genetic tools have been developed only for the type strain DSM639 (Wagner et al., 2012), making it an important strain for studying metabolism and physiology in the *Sulfolobales*. However, in contrast to early studies using *S. acidocaldarius* strain 98-3 (Brock et al., 1972; Shivvers and Brock, 1973), eventually deposited as DSM639, studies spanning the past

few decades have found that this strain is no longer capable of chemolithoautotrophic growth on CO_2/S°, a result confirmed here. Therefore, *S. acidocaldarius* DSM639 could serve as a platform to decipher the determinants of chemolithoautotrophy by restoring this metabolic mode through insertion of specific genes related to this physiology. In heterotrophic media, growth of the genetic parent *S. acidocaldarius* strain (MW001, derived from DSM639 by deletion of uracil synthesis gene *pyrF* (Wagner et al., 2012)) was not affected by the addition of CO_2/S°, although some sulfur oxidation was observed, as indicated by increases in sulfate concentration and a decrease in pH (**Figure 2d, 2f**).

The *S. acidocaldarius* DSM639 genome encodes homologs of all genes hypothesized to comprise the 3-HP/4-HB cycle for CO_2 fixation (Hawkins et al., 2013). Thus, its inability to grow autotrophically could stem from an inability to utilize chemolithotrophic energy sources. The sulfur oxygenase reductase (SOR) has been proposed as the first enzymatic step during the oxidation of elemental sulfur by *A. ambivalens* and other *Sulfolobales*, since without it S° is too inert to be used as an energy source (Kletzin, 2006). As such, the gene encoding SOR from *S. tokodaii* (ST1127) was inserted into MW001 (along with Msed_0406 and concomitant deletion of Saci_1149, see Experimental Procedures) generating strain RK06. RK06 cells grew poorly when CO_2/S° was added to heterotrophic YE media (**Figure 2c, 2e**). In the presence of S°, cells exhibited higher sulfate production than MW001 on a per-cell basis (**Figure 2f**), but final sulfate concentration was lower than for the parent strain because of the dramatic growth defect. Reduced growth was not the result of external pH changes, since inhibition was noted even when pH was controlled at 3.0 in a bioreactor; note also that RK06 grew well in media lacking S° down to pH as low as 2.0.

The poor growth of RK06 on YE/CO_2/S° suggested that the sulfur oxidation pathway remained incomplete and likely created cytoplasmic conditions (e.g., acidity, reactive sulfur compounds) with adverse consequences. Thiosulfate quinone oxidoreductase (TQO), which has no identifiable homolog in the *S. acidocaldarius* DSM639 genome, is known to connect sulfur

oxidation processes to cellular energy conservation (Müller et al., 2004). The possibility that the absence of TQO impacted RK06 viability was examined by inserting the gene encoding this protein from *S. tokodaii* into RK06, resulting in strain RK34 containing both heterologous SOR and TQO. Strain RK34 showed no growth defect in standard heterotrophic media, and also grew well on YE/CO_2/S°, producing substantially more sulfate than either MW001 or RK06 (**Figure 2f**). Airflow rates in most bioreactor runs were varied to maintain O_2 saturation (see Experimental Procedures), making direct comparisons of oxygen consumption difficult. However, when gas flow rates were held constant during exponential growth of RK34, O_2 consumption was higher in the presence of S°, although O_2 saturation declined for both sulfur and non-sulfur conditions (**Figure 2h**).

Next, the possibility that RK34, the strain capable of strong growth in the presence of CO_2/S°, could grow chemolithoautotrophically, was tested. The results here corroborated previous reports that *S. acidocaldarius* strain MW001 (the DSM 639 strain with a partial knockout of the *pyr*F gene Saci_1597 (Wagner et al., 2012)) failed to grow autotrophically on sulfur, even with a 10% CO_2 headspace, in media with uracil and 0.01% yeast-extract as a vitamin supplement. In addition, neither RK6 nor RK34 grew chemolithoautotrophically on CO_2/S°; growth was also not observed when S° was replaced with the more soluble tetrathionate.

Elemental sulfur oxidation by Acidianus brierleyi

To further examine chemolithoautotrophy in the *Sulfolobales*, heterotrophic and chemolithoautotrophic growth modes of *Acidianus brierleyi*, whose genome sequence was recently reported (Counts et al., 2018), were each examined through transcriptomic analysis. *A. brierleyi* (originally named *Sulfolobus brierleyi* (Zillig et al., 1980)) can grow aerobically by oxidizing S° or anaerobically by reducing S°, and this species was proposed to be the forerunner of sulfur-oxidizing archaea (Segerer et al., 1985). Certain details for CO_2 assimilation (Ishii et al.,

190 1996) (Kandler et al., 1981) and sulfur oxidation (Emmel et al., 1986) have been reported for this archaeon.

Species in the *Sulfolobales* often grow best when supplied with both chemolithotrophic (e.g., S°, Fe^{2+}, H_2) and organic (i.e., yeast extract (YE)) substrates as electron donors. For example, *M. sedula* grew faster on YE and H_2/CO_2 (t_d = 3 h) than on either YE alone (t_d = 5 h) or
195 H_2/CO_2 alone (t_d = 12 h) (Auernik and Kelly, 2010). A doubling time of 25 ± 5 h has been reported for autotrophic growth of *A. brierleyi* on tetrathionate, but growth on media containing both tetrathionate and YE was substantially faster (t_d = 9.5 h) (Wood et al., 1987). Here, *A. brierleyi* was grown chemolithoautotrophically (CO_2/S°) as well as heterotrophically on yeast extract (CO_2/YE). Surprisingly, chemolithoautotrophic growth for *A. brierleyi* was more rapid than
200 heterotrophic growth, with CO_2/S°-grown cells doubling in 19 ± 3 h, compared to 26 ± 2 h for YE grown cells, suggesting a preference for autotrophy. As with *S. acidocaldarius*, O_2 saturation declined faster in reactors containing CO_2/S° than YE alone, reflecting the bioenergetic burden related to autotrophy; note that in both cases cells were sensitive to excess O_2, so airflow had to be kept low in early growth phase (see Experimental Procedures). *A. brierleyi* grown
205 chemolithoautotrophically on CO_2/S° produced several times as much sulfate as RK34 (the sulfur-oxidizing *S. acidocaldarius* DSM639 strain) growing on YE + CO_2/S°, despite reaching average optical densities (OD_{600}) only one-fifth as high. This indicates the different extents to which energy is being conserved through sulfur oxidation processes in the two species.

The contrast between heterotrophic (CO_2/YE) and autotrophic (CO_2/S°) growth in *A.*
210 *brierleyi* was reflected in the transcriptome, where 100 ORFs were significantly differentially transcribed 2-fold or more (see **Table S1**). Nearly a third (29) of these ORFs are annotated as hypothetical proteins, reflecting the dearth of information on the physiology and metabolism of the *Sulfolobales*. Most of the 55 autotrophy-induced genes corresponded either to enzymes of the 3-HP/4-HB carbon fixation cycle or to pathways for biosynthesis of key cellular components (**Table**
215 **1**). The strong up-regulation of genes in the 3-HP/4-HB cycle in *A. brierleyi* growing on CO_2/S°

confirms that cycle's role as the primary form of carbon incorporation during autotrophy, and aligns with previous results in *M. sedula* (Hawkins et al., 2013). HhcR (DFR85_08180), a transcriptional regulator homologous to the global regulator for autotrophy previously identified in *M. sedula* (Leyn et al., 2015), was also up-regulated.

When S° replaced YE as an energy source, a homolog to *dsrE3A* (DFR85_11410), was up-regulated. DsrE3A has been implicated in thiosulfate transfer in *M. cuprina*, possibly even ferrying substrates to or from TQO (Liu et al., 2014). The adjacent Hdr operon (DFR85_114015-11450) was highly expressed in general, and appeared to be up-regulated on sulfur, despite not meeting statistical significance. Of the previously characterized sulfur-oxidizing enzymes from *A. ambivalens*, three homologs to SQO (DFR85_08380, DFR85_05280, DFR_06185) and two to TetH (DFR85_12520, DFR85_14790) were highly expressed during growth on S° (all five falling within the top 20% of all transcripts for that growth condition). Although these genes as well as those encoding SOR (DFR85_06255) and TQOa,b (DFR85_00360, DFR85_00355) were not specifically S°-responsive, they were all very highly transcribed under both conditions, suggesting that they may be expressed during aerobic growth regardless of whether sulfur is present. Homologs to the *A. ambivalens* sulfur-reducing complex SreABCD were transcribed at low levels under both growth conditions, as would be expected given that complex's specialized role in anaerobic growth. Two gene clusters containing membrane-associated quinone oxidases (DFR85_00210-20 and DFR85_00250-65), both with homology to SoxABC in *S. acidocaldarius* (Gleißner et al., 1997), were up-regulated on CO_2/S°. These clusters are closely related to each other, and to another group of cytochrome-associated genes (DFR85_00030-45), which was not transcriptionally responsive. Several sulfur-responsive genes, some of which may play a role in regulation (DFR85_04050, DFR85_01540) given their homology to known regulator families, were also noted.

There were few surprises among genes that were significantly down-regulated on S^0 (which can also be viewed as up-regulated on YE). Genes in catabolic pathways, such as the TCA-cycle's succinate-CoA ligase (DFR85_11300-05), several alcohol dehydrogenases (DFR85_12080, DFR85_15155), and a large operon of genes containing thiolase and DNA-binding domains (DFR85_13825-13850) were down-regulated. Many of the YE-induced genes have homologs in the genome of heterotrophic *S. acidocaldarius* DSM639, unlike the S^0-induced set (**Table 2**).

Sulfur response transcriptomes of parent (MW001) and mutant (RK34) S. acidocaldarius

The transcriptional response results from *A. brierleyi* were next used to examine *S. acidocaldarius* DSM639, with an eye towards understanding why the S°-oxidizing strain RK34 was incapable of chemolithoautotrophic growth. The transcriptomic differences between the strains can be broadly grouped into the following categories: 1) differences not related to presence of sulfur (**Figure 3a** along the diagonal); 2) differences triggered by presence of sulfur (**Figure 3b** along the axes); and, 3) shared responses to sulfur (**Figure 3b** along the diagonal). These categories are indicated by the highlighted color of ORFs in **Table S2**. The main focus was on the sulfur-induced strain differences to ascertain the impact of enabling sulfur oxidation.

The transcriptomes of MW001 and RK34 during growth on CO_2/S° + YE compared to YE identified 303 genes that were differentially transcribed 2-fold or more for at least one of four contrasts (**Figure 3**). The largest differences were for contrasts within strains when comparing growth with and without S°. Relatively few genes were differentially transcribed between strains when each was grown on sulfur.

During the construction of strains RK06 and RK34, the native Saci_1149 gene was knocked out, replaced with a homolog of this gene from *M. sedula* Msed_0406 (see Experimental Procedures). Therefore, the single largest transcriptional difference between the two strains is the down-regulation of Saci_1149 in RK34 relative to MW001 under both non-sulfur and sulfur

conditions. Other major differences between the strains included strong down-regulation of Saci_0619 in RK34, a homolog to a *S. tokodaii* enzyme (ST0452) involved in production of sugars for protein glycosylation (Zhang et al., 2010); this may be related to stress due to overexpression of the TQO and SOR genes, or due to the use of the strong promoter for the S-layer coat protein (potentially titrating S-layer associated transcription factors). Other stress-associated enzymes up-regulated in RK34 included transcription initiation factor IIB 2 (Saci_1341), and two operons encoding genes annotated as universal stress proteins (Saci_1357-58 and Saci_1638-40).

Another difference between the strains, which may be indicative of a sulfur-oxidation response, is the up-regulation of Saci_2101 and Saci_2106, which have weak homology to the SreB and SreD sulfur-reducing complex enzymes in *A. ambivalens* (Laska et al., 2003). Despite their sulfur-reducing role in *A. ambivalens*, SreB and SreD homologs in *M. sedula* (Msed_0815 and 0817) appear to have a sulfur oxidization role (Auernik and Kelly, 2008). Interestingly, Saci_2085-2093 (the SoxABCD-L oxidase cluster) was up-regulated in response to sulfur in the modified strain, but not the parent strain. This response mirrors the up-regulation of *A. brierleyi* SoxABCD homologs on sulfur (**Table 2**). Another sulfur response shared with *A. brierleyi* was up-regulation of the DsrE3A gene (Saci_0337, DFR85_11410). Expression of the next few Hdr-complex genes appears to mirror *A. brierleyi*, as the genes are highly expressed across all conditions (with minimal differential expression). However, a four-gene insertion (Saci_0330-0333) downstream of *hdrC1* (Saci_0334) appears to disrupt the operon because later genes, particularly *hdrA* (Saci_0328) are poorly expressed (**Figure S1**). Another major difference between the two species a putative transcriptional regulator, Saci_0006, is significantly down-regulated on sulfur, but the homologous *A. brierleyi* DFR85_01540 is up-regulated during sulfur autotrophy.

Other genes triggered by sulfur included Saci_0097-99, which is homologous to the DoxBCE terminal oxidase complex in *A. ambivalens* (Purschke et al., 1997). In *A. ambivalens*, DoxBCE subunits co-purified with the DoxDA subunits that make up TQO. The operon

Saci_1459-1464 was strongly up-regulated on sulfur and includes ribosomal proteins, chaperones, and other genes involved in production and translocation of membrane proteins. Homologous operons appear to be up-regulated under autotrophic growth in *M. sedula* (Msed_1629-1634) (GEO project: GSE39944) and *M. cuprina* (Mcup_0595-0600) (Jiang et al., 2014). Another cluster of ribosome-associated genes (Saci_0763-0769) was also up-regulated on sulfur.

Transcription of the recombinant genes in RK34 (not native to *S. acidocaldarius* and therefore not included in the microarray) was confirmed by qPCR (**Figure 4**). All the inserted genes (SOR, TQO, and Msed_0406) were transcribed much more strongly than the reference gene, as would be expected considering the use of strong constitutive promoters. The operon structure of the construct was also evident, with the Msed_0406 always transcribed more strongly than SOR, which follows it in the synthetic operon controlled by the *slaA* promoter. TQO, under the control of the *gdhA* promoter, is even more strongly transcribed, confirming that this promoter is appropriate when very high transcript levels are needed (Berkner et al., 2010).

Discussion

The absence of evidence for autotrophic growth in strain RK34, which contains a seemingly intact carbon fixation cycle and a (rudimentary) pathway for sulfur oxidation, begs the question – *What could be missing?* The initial premise was that *S. acidocaldarius* DSM639 was at some point an autotrophic sulfur oxidizer, and therefore minimal changes (provision of two known sulfur-oxidation enzymes that were missing) would be required to recover that capability. While chemolithoautotrophy was not restored, increased sulfur oxidation by both RK06 and RK34 strains supports the SOR-centric model of sulfur oxidation in the *Sulfolobales*, instead of the "recycle" model proposed for *Acidithiobacillus caldus* (Chen et al., 2012; Wang et al., 2019). It is also interesting that, at least in the *Sulfolobales* genomes sequenced so far, SOR is never found

without TQO (**Figure 1c**), thus the fitness issues related to SOR alone, as observed in RK06, may be universal. The reverse is not true, as many species contain versions of TQO without SOR.

While both SOR and TQO have previously been characterized *in vitro*, this report confirms that the two enzymes can function cooperatively *in vivo*, as evidenced by improved sulfur oxidation and improved fitness over SOR alone. While SOR was expressed recombinantly in *S. solfataricus* DSM639, this was done to produce the enzyme for biochemical characterization and growth in the presence of sulfur was not reported (Albers et al., 2006). Another interesting result from the RK06 and RK34 strains is the fact that both were capable of producing sulfuric acid, and thus possess the machinery to oxidize intermediate sulfur compounds to sulfate, and then export it, though the genes responsible are not known.

The transcriptomic analysis of *M. sedula* was key in efforts to determine the genes responsible for the enzymatic steps in the 3-HP/4-HB cycle of *Sulfolobales*, based on their response to autotrophic growth conditions (Hawkins et al., 2013). However, this approach was less successful here in identifying the comprehensive set of genes involved in S° oxidation, perhaps because this is a constitutive capability for *A. brierleyi*. None of the genes encoding homologs to already characterized sulfur-oxidizing enzymes were up-regulated in *A. brierleyi*, although nearly all were highly transcribed (**Table 2**). Only one S°-responsive gene (DsrE) seems likely to be directly involved in sulfur oxidation, although more candidates may be within the 22 hypotheticals up-regulated on S° (**Table S1**). Note that carbon fixation and amino acid synthesis genes were responsive to autotrophic growth conditions as expected, and several large membrane complexes that have been implicated in energy conservation also responded.

Four large terminal quinone oxidase membrane complexes have been identified the *Sulfolobales*. Two, SoxABCD (Lubben et al., 1994) and a second complex containing SoxM, a fusion of the normally distinct cytochrome I and III subunits (Lübben et al., 1994), have been found to be oppositely regulated and associated with autotrophic (SoxABC) and heterotrophic (SoxM) growth in several species. Both complexes transfer electrons from reduced caldariella quinone to

oxygen, and generate a transmembrane proton gradient. So, their role in any particular lithotrophic or heterotrophic metabolism must be indirect, i.e. simply converting reduced quinones from other enzymes into energy for the cell. These two operons are often found among the most significantly differentially transcribed genes in *Sulfolobales* grown in the presence of lithotrophic substrates (either with or without YE) (Kappler et al., 2005; Auernik and Kelly, 2010). Here, SoxABCD was up-regulated on sulfur in both *S. acidocaldarius* MW001 and *A. brierleyi*, while SoxM was up-regulated in RK34 without sulfur (*A. brierleyi* lacks a homolog to the SoxM fusion). The strong differential regulation of these complexes in response to lithotrophy makes sense in light of our observation that O_2 partial pressures are lower in cultures with lithotrophic substrates. The SoxM cluster was previously observed to be up-regulated in *S. solfataricus* grown under oxygen-rich conditions, while SoxABCD was up-regulated under low-oxygen conditions (Simon et al., 2009). Oxygen tension is known to affect the ratio of expression between high and low oxygen-affinity cytochrome oxidases in *E. coli* (Govantes et al., 2000), and it appears that SoxABCD and SoxM may function in a similar way in the *Sulfolobales*. Perhaps, the lack of a low-affinity SoxM in *A. brierleyi* accounts for its sensitivity to O_2. The other two terminal oxidases, CbsAB-SoxNL (Saci_1858-61) and DoxBCE (Saci_0097-99), show less consistent behavior both here and in previous studies (see **Table S3**), but the possibility that they are activated by a specific metabolism or environmental condition cannot be ruled out.

All of the facultatively anaerobic *Acidianus* species contain the SreABCD complex, which facilitates anaerobic growth on S°, while obligately aerobic *Metallosphaera* and *Sulfolobus* species contain weak homologs only to SreB and SreD (**Figure 1c**). *A. brierleyi* minimally transcribed the Sre complex genes under either aerobic growth condition tested, as would be expected, but *S. acidocaldarius* significantly up-regulated SreB and SreD in the presence of S^0, a result that has also been observed in *M. sedula*. If the facultative anaerobic lifestyle of *Acidianus* was shared by the common ancestor of all *Sulfolobales*, this suggests that the fully aerobic species have adapted by losing portions of the anaerobic machinery, and re-purposing other parts

to their new S°-oxidizing lifestyle. Whether the SreB and SreD homologs in aerobes are oxidizing sulfur directly for metabolic energy, or merely involved in some indirect aspect of sulfur oxidation (along the lines of SoxABCD) remains to be seen.

While obligately heterotrophic *S. acidocaldarius* and *S. solfataricus* appear to have lost several enzymes known to be important for sulfur oxidation (SOR, TetH, TQO), and the non-sulfur oxidizing *Metallosphaera* species lack SOR, it is interesting that all species contain the Hdr complex and associated DsrE and TusA, which actually exhibit less sequence divergence than the other genes in **Figure 1c**. The disruption of the *hdr* operonic structure (although not the individual genes) in *S. acidocaldarius* may partially explain its non-sulfur oxidizing phenotype, but cannot explain why *S. solfataricus* and *Metallosphaera* species also lack this ability. It is possible that the Hdr system is specialized for oxidation of sulfur compounds other than elemental sulfur, or has some other role in the non-sulfur oxidizing species, such as the known function of TusA in sulfur group transfer for tRNA and molybdenum cofactor production (Liu et al., 2014).

The transcriptomic response of the *S. acidocaldarius* strains to S° implies that sulfur oxidation was part of this archaeon's legacy, which may have been lost during frequent passages on heterotrophic media for several years prior to submission of the strain to DSMZ (Brock, 1978). Sulfur-responsive genes (yellow in **Table S2**) include transcriptional regulators (Saci_2219, 0006), and ribosome-associated operons (Saci_0763-0769 and 1459-63), suggesting that the cells are gearing up for a major metabolic re-restructuring. That genes associated with autotrophy in other species, such SoxABCD-L (Saci_2085-2092), SreBD (Saci_2101-2109), and Saci_1459-63, are also up-regulated on S°, especially in RK34, further supports this notion.

In contrast to the sulfur-induced response in *S. acidocaldarius*, the transcriptomes indicated no response from the in 3-HP/4-HB carbon fixation cycle, although many of these genes were differentially regulated under autotrophic conditions in *A. brierleyi* and *M. sedula* (Auernik and Kelly, 2010; Hawkins et al., 2014). A meta-analysis of transcriptional data for *M. sedula* (eleven growth conditions, 6 heterotrophic, 5 autotrophic), *S. acidocaldarius* (twelve conditions,

all heterotrophic) and *A. brierleyi* (the two conditions studied here) was used to determine the relative levels of transcripts within each transcriptome. This revealed that transcription levels of 3-HP/4-HB genes in *S. acidocaldarius* are similar to those of *A. brierleyi* and *M. sedula* grown heterotrophically, albeit with some differences (**Figure 5**). Two of the carboxylase (E1) subunits, as well as E4, E7, and E9 all average in the 30th percentile or below, dramatically lower than the other two species. In contrast, E3, E5, E10, and the regulator *hhcR* are consistently expressed at very high levels (90th percentile and above) in *S. acidocaldarius*. The other obligate heterotroph in the *Sulfolobales*, *S. solfataricus*, appears to use portions of the 3-HP/4-HB cycle during heterotrophic growth as a way to replenish TCA cycle intermediates (Wolf et al. 2016), and *S. acidocaldarius* may have a similar heterotrophic use for these three enzymes.

It is interesting to see that the autotrophy-associated transcriptional regulator *hhcR*, which is present throughout the *Sulfolobales* (Leyn et al., 2015), is so highly expressed in *S. acidocaldarius* (**Figure 5**). HhcR binds regulatory sequences upstream of most of the genes in the 3-HP/4-HB cycle. However, it lacks an effector binding site, so how this regulator detects autotrophic conditions and effects a transcriptional response is not clear. That *hhcR* is so strongly transcribed suggests that its role in heterotrophic *S. acidocaldarius* cells may actually be to repress autotrophy. Unlike *M. sedula* and *A. brierleyi*, in *S. acidocaldarius*, the *hhcR* gene is not preceded by an HhcR binding site, so the *S. acidocaldarius* version does not regulate its own expression, as has been proposed in most other *Sulfolobales* (Leyn et al., 2015). Therefore, one way to recover proper regulation of the carbon fixation cycle may be to replace the *S. acidocaldarius hhcR* gene, and all the corresponding regulatory sequences, with homologous versions from an autotrophic species.

This work serves to highlight the many common features of *Sulfolobales*, such as the presence (if not function) of 3-HP/4-HB carbon fixation machinery, as well as substantial metabolic and transcriptional responses to the presence of sulfur. However, the unique genus-level phenotypes proved to be more dramatic than expected. Despite its strong sulfur oxidizing

heritage, even recombinant SOR and TQO expression in RK34 failed to allow obligately heterotrophic *S. acidocalarius* DSM639 to make use of sulfur as anything more than perhaps a minor energy input, although the strong phenotypes do validate the importance of SOR for sulfur oxidation. On the other hand, the facultatively anaerobic *A. brierleyi* lacks a terminal oxidase appropriate for high-oxygen levels, and turns off the anaerobic sulfur reductase SreABCD complex when oxygen is present; obligately aerobic *Sulfolobus* and *Metallosphaera* species appear to have found new uses for elements of this complex, up-regulating SreBD under aerobic conditions when sulfur compounds are present.

While what unifies the *Sulfolobales* is their carbon metabolism, their unique lithotrophic pathways differentiate otherwise highly-related species in dramatic and unexpected ways. Genetic tools continue to be developed and improved (Straub et al., 2017; Straub et al., 2018), and additional functional genomics experiments are needed to expand on hard-won biochemical insights from previous efforts, and shed additional light on life in hot acid.

Experimental Procedures

Cultivation of Sulfolobales

Media for all *Sulfolobales* species consisted of Brock's salts (DSM medium #88 minus yeast extract) containing per liter: 1.3 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 4.5 mg $Na_2B_4O_7 \cdot 10H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CuCl_2 \cdot 2H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg $VOSO_4 \cdot 2H_2O$, and 0.02 mg $CoSO_4 \cdot 7H_2O$. A low sulfate Brocks salts formulation was used for experiments monitoring sulfate production, which substituted $MgCl_2 \cdot 6H_2O$ for $MgSO_4$. Heterotrophic media contained 1 g NZ amine (0.1 g if sulfur oxidation was being monitored by pH change) and 2 g sucrose for *S. acidocaldarius,* 1 g YE for other species. A low carbon formulation of *S. acidocaldarius* medium used one-tenth as much NZ amine and sucrose (0.1 and 0.2 g). Autotrophic media contained 10 g steam-sterilized elemental sulfur or 2.5 g sodium tetrathionate supplemented with 0.01 or 0.1 g

YE (negligible growth was observed at either level of YE in the absence of sulfur). Uracil auxotrophic strains of *S. acidocaldarius* were supplemented with 0.01 g uracil in all media. Media was adjusted with concentrated sulfuric acid to pH 3.3 for *S. acidocaldarius*, and pH 2 for *A. brierleyi, S. metallicus*, and *M. sedula* (the last two used as positive controls for autotrophic growth). Plate media consisted of a 2x concentrated media solution, pre-heated and mixed with an equal volume of 1.2% phytagel. Cells were grown at 70-75°C. Small scale cultures were in 150 mL serum bottles with either foam stoppers or needles through rubber stoppers for air exchange. Autotrophic growth was in sealed bottles with air and 5% (vol) CO_2 headspace replaced daily. Larger cultures used 3-L glass bioreactors (Applikon Biotechnology, Delft, Netherlands) with a 2-L working volume, sparged with variable flow-rates (10 to 100 mL/min) of air supplemented with ~5% CO_2. Autoclavable probes (Applikon) were used to monitor pH and dissolved oxygen. For some *S. acidocaldarius* bioreactors, 1M stocks of sodium hydroxide or hydrochloric acid were used for automatic pH control, maintaining cultures at pH 3.2 ± 0.2. Freshly inoculated cells showed sensitivity to high sparging rates, especially *A. brierleyi*, so flow rates started low (10 mL/min for *A. brierleyi*, 25 mL/min for *S.acidocaldarius*) until O_2 saturation started to decline, indicating growth. Bioreactors were inoculated from bottles with identical media, except that S° was omitted from inocula going into S° reactors for MW001, RK06, and RK34. Routine cell growth was monitored by optical density (OD_{600}). For media containing sulfur, samples in 1.7 mL tubes were spun for 10 seconds in a table-top centrifuge to pellet out sulfur, and the supernatant immediately transferred to a cuvette to measure OD.

Generation of recombinant S. acidocaldarius DSM639 strains

The published genome sequence of *S. acidocaldarius* DSM639 (Chen et al., 2005) indicates a frame-shift mutation in Saci_1149, a candidate for E10 from the 3-HP/4-HB cycle. Therefore, the recombinant *S. acidocaldarius* constructs were cloned into this site, deleting the gene and replacing it with a known functional homologue Msed_0406. Subsequent resequencing

of the Saci_1149 gene found it to be intact, so this modification was unnecessary, but it served as a convenient control for microarray studies. To generate the cloning construct for RK06, genes encoding E10 from *M. sedula* (Msed_0406) and SOR from *S. tokodaii* (ST1127) were cloned into strain MW001 under the control of the strong constitutive promoter of the S-layer subunit SlaA (the 201 bp upstream of Saci_2355), with the SlaB ribosome binding site (12bp upstream of Saci_2354) in between. Flanking regions of approximately 500 bp upstream and downstream were used to insert the synthetic operon into the Saci_1149 locus, with concomitant deletion of Saci_1149. The uracil prototrophy marker *pyrBEF* (Sso0614-16) was used for selection. All cloning fragments were amplified from the appropriate genomic DNA using the primers listed in **Table S4** and assembled onto a plasmid backbone amplified from pUC19 using Gibson assembly master mix (New England Biolabs). The Gibson assembly reaction was used to transform One Shot Top10 chemically competent *E. coli* (ThermoFisher Scientific), and the plasmid was sequence-verified. Plasmid maps of the two cloning constructs are shown in **Figure S2**.

To generate the cloning construct for strain RK34, genes for the two subunits *doxD* and *doxA* from *S. tokodaii* (ST1855-1856) were assembled into a pUC19 backbone containing the *pyrBEF* selection cassette under the control of the strong constitutive promoter from the glutamate dehydrogenase (Saci_0155) gene (Berkner et al., 2010). Flanking regions were used to locate the construct immediately after the inserted SOR gene in strain RK06 (so strain RK34 had strain RK06 as a parent, rather than MW001). Since the *doxDA* genes were amplified from *Stok* gDNA together, the original operon structure was preserved.

Genetic methods in *S. acidocaldarius* relied on the uracil auxotrophic mutant MW001, as described in (Wagner et al., 2012). Briefly, electrocompetent *S. acidocaldarius* parent strains were transformed with methylated cloning plasmids (generated in an *E. coli* strain expressing the methylase EsaBC4I, which modifies the last cytosine in the recognition sequence GGCC Morgan, 2009 #48}) and plated on uracil-free medium to select for chromosomal integration. Colonies were sub-cultured in uracil-free liquid media and screened by PCR, then plated on medium containing

uracil and 0.1g/L 5-fluoroorotic acid to select for removal of the *pyrBEF* marker. Colonies were sub-cultured in medium containing uracil, screened by PCR, and the sequence of the Saci_1149 region was verified (Genewiz).

Monitoring sulfur oxidation

Sulfate concentrations in culture supernatant were measured using a turbidimetric assay modified from (Lundquist et al., 1980). First, samples were centrifuged at 15,000g for 5 minutes to remove sulfur and cells, then diluted to fall within range of the standard curve (0-10 mM Na_2SO_4). The assay reagent stock solution contained (per liter): 35 g $BaCl_2 \cdot 2H_2O$, 75 g polyethylene glycol (MW 8000), and 20 mL concentrated HCl. Assay reagent was prepared by adding 50 µL of 10 mM Na_2SO_4 to 10 mL of reagent stock solution. In a 96-well plate, 100 µL sample was mixed with 75 µL of freshly-prepared assay reagent. The absorbance at 600 nm was read on a Synergy MX plate reader (Biotek Instruments, Winooski, VT). A cubic polynomial fit of the standard curve was used to determine sample concentrations.

RNA extraction

To collect RNA for transcriptomic analysis (RNAseq, Microarray, qPCR), 300-600 mL of culture was harvested from early-exponential phase bioreactors and rapidly cooled using a dry ice-ethanol bath. Sulfur particles were removed by slow centrifugation (500 × g for 2 min) or allowing to settle on ice for several minutes after chilling, then sulfur-free cell culture was decanted into new bottles. Cells were pelleted by 4°C centrifugation at 10,000 × g for 20 min. Cell pellets were stored at -80°C prior to RNA extraction. RNA was extracted using Trizol reagent (Ambion) and the RNeasy RNA isolation kit (Qiagen), according to vendor instructions, and stored at -80°C prior to analysis. RNA quality was confirmed by the presence of intact 16S and 23S bands on an agarose gel.

Oligonucleotide microarray

RNA was reverse-transcribed (Superscript III, Invitrogen) using a dNTP mix with aminoallyl-dUTP in place of some of the dTTP, to provide a reactive site for later dye-labeling. The resulting cDNA was hydrolyzed and cleaned up in QIAquick PCR purification columns (Qiagen). At this point, cDNA from each condition was separated into two tubes, pooled with a biological replicate, labeled with Cy3 or Cy5 dye (GE Healthcare, Chicago, IL), then mixed with an oppositely labeled tube from a different condition, and hybridized to microarray slides overnight. Spotted whole-genome 60-mer oligonucleotide microarray slides were used, based on 2278 protein-coding open reading frames (ORFs) from the *S. acidocaldarius* DSM 639 genome. Spots were printed in five replicates onto SuperChip aminopropylsilane coated slides (ThermoFisher) using a Qarray Mini microarray printer (Genetix) and UV crosslinked (600mJ).

Slides were washed and scanned with a GenePix 4000B microarray scanner (Molecular Devices). Spots were quantified using GenePix Pro v7. JMP Genomics 9 was used for ANOVA and Loess normalization, and a mixed-effects ANOVA model was used to determine differential gene-expression. The Bonferroni correction was used to account for multiple comparisons, with alpha set to 0.05 expression differences with $-\log_{10}$(p-value) greater than 5.5 are considered significant.

qPCR analysis

Quantitative reverse-transcription PCR was done using SsoFast Evagreen Supermix (Bio-Rad, Hercules, CA), with the *S. acidocaldarius* gene encoding the DNA polymerase sliding clamp (Saci_0817) used as reference. Total RNA previously isolated for microarray analysis was reverse-transcribed using iScript Reverse Transcription supermix (Bio-Rad). cDNA corresponding to ~5 ng total RNA was used in each 20-ul qPCR reaction. qPCR reactions were run in triplicate with one no-RT control and one no-template control. Primers are listed in **Table S4**.

RNA sequencing

A. brierleyi cells were collected from heterotrophic and autotrophic cultures each grown in triplicate at bioreactor scale, and RNA extracted as described above. Ribosomal RNA was removed using the bacterial Ribo-Zero rRNA removal kit (Illumina), followed by library construction using NEBNext Ultra Directional RNA Library Prep Kit (NEB) and sequencing on an Illumina Hiseq2500 instrument (125 bp read length) by the North Carolina State University Genomic Sciences Laboratory (Raleigh, NC). Reads were aligned to the *A. brierleyi* reference genome (CP029289), and the resulting normalized read counts (RPKM) calculated in Geneious version 8.1.9 (http://www.geneious.com, (Kearse et al., 2012)). Statistical analysis was performed in JMP genomics 9 using the standard Next-Generation sequencing analysis pipeline. Briefly, data were filtered to remove genes where two or more samples had RPKM values below 2, then normalized by Trimmed Mean of *M*-component (removes large transcript bias inherent in RPKM). Differences in gene expression between autotrophic and heterotrophic conditions were determined by ANOVA. Statistical significance defined by a false discovery rate (FDR) with α=0.05 to account for multiple comparisons, meaning $-\log_{10}$(p-values) greater than 2.35 were considered significant.

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FIGURE CAPTIONS

Figure 1. Overview of sulfur oxidation in the Sulfolobales a) Updated model of sulfur oxidation pathways in the *Sulfolobales*. b) 16S tree of relatedness among a subset of the *Sulfolobales*. c) Sulfur oxidizing enzymes and their % identity at the amino acid level (tblastn, all have at least 80% coverage) within the *Sulfolobales. A. ambivalens* and *M. cuprina* genes are used as query for sulfur oxidizing enzymes, *S. acidocaldarius* genes for the terminal oxidases. Enzyme abbreviations: TetH = tetrathionate hydrolase, SQO = sulfide:quinone oxidoreductase, SOR =sulfur oxygenase-reductase, TQO = thiosulfate:quinone oxidoreductase, SAOR = sulfite:acceptor oxidoreductase, APSR = APS reductase, APAT = adenylylsulfate:phosphate adenyltransferase, AK = adenylate kinase, HDR = heterodisulfide reductase. Enzymes outlined in red have been confirmed to function in a member of the *Sulfolobales*.

Figure 2. Growth and sulfur oxidation in recombinant *S. acidocaldarius* strains (heterotrophic media). Representative time course of culture OD_{600} (a) and pH (b). Final values for OD_{600} (c) and net change in pH (d) in bioreactors without pH control (using 10% of YE for standard media). Final OD_{600} (e) and specific sulfate production (net sulfate produced/final OD_{600} value) (f) in pH-controlled bioreactors. Time course of RK34 growth (g) and oxygen consumption (h) in bioreactors with matched airflow rates (cell MW = parent strain; RK06 = SOR Knock-In, RK34 = SOR + TQO Knock In). All experiments done in 3 L bioreactors, with 2 L working volume, with heterotrophic media; (+S) conditions have 10 g/L sulfur added. Line graphs are of single runs, while bars are averages of two runs (four for MW-controls) with error bars representing standard deviation.

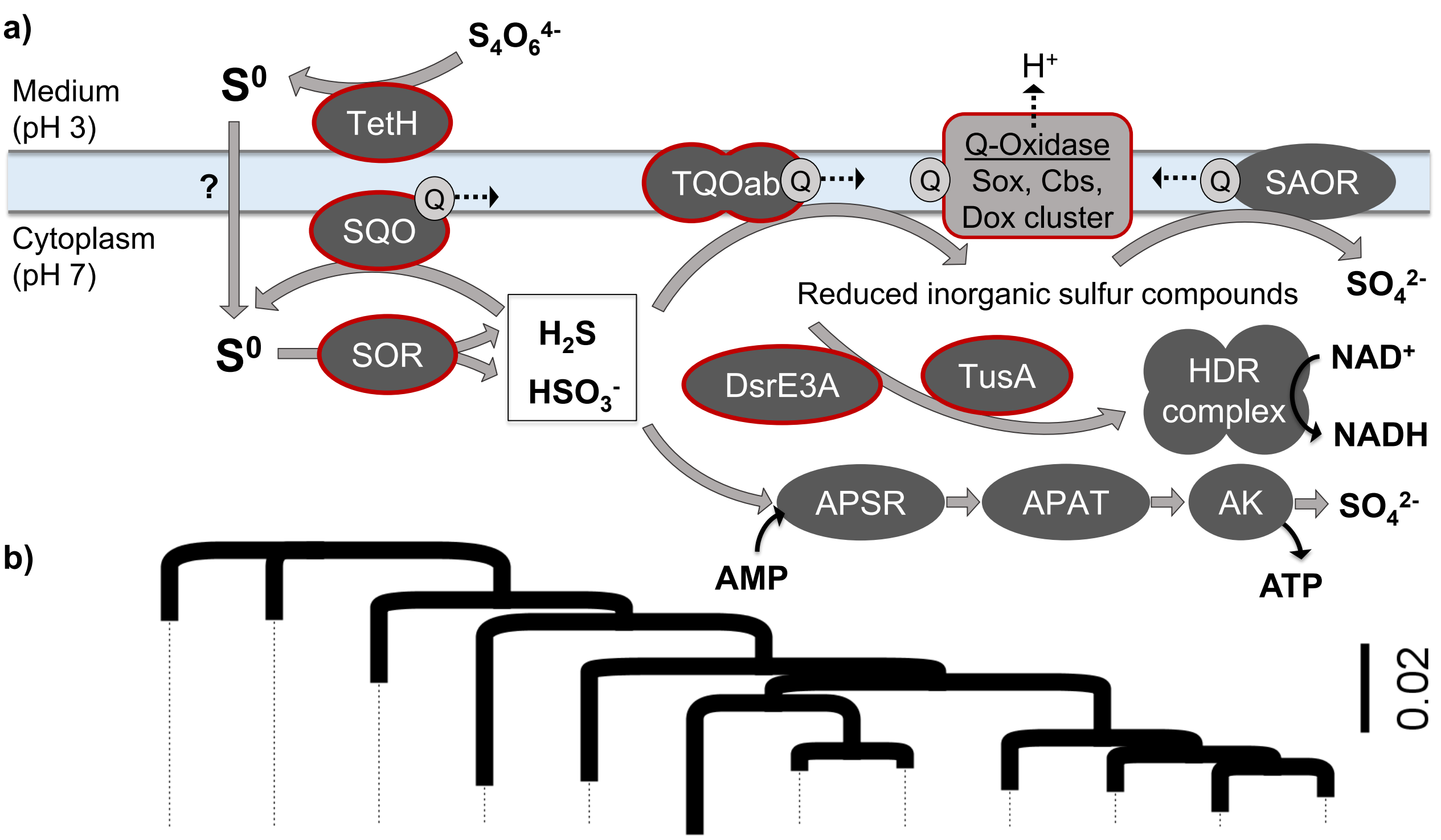
Figure 3. Significantly differentially transcribed genes from *S. acidocaldarius* Microarray. (a) Genes differing between strains MW001 and RK34 (SOR, TQO knock-in. (b) Significant

differences within strains in response to sulfur. The colored ovals indicate groups of co-regulated genes (usually operons) – Additional details in **Table S2.** Dashed lines box-in regions with difference of treatment less than 2 and 4 (4- and 16-fold changes), and indicate the diagonal line corresponding to y = x. (note diff of trt has been converted to fold-change in **Table S2**). Symbols indicate significant comparison is: (a) o (34)-(MW), □ (34 S)-(MW S), + both, and (b) ∆ (MW)-(MW S), ◊ (34)-(34 S), × both.

Figure 4. qPCR confirmation for transcription of recombinant genes in *S. acidocaldarius* 639 strain RK34. DNA polymerase sliding clamp (Saci_0817) gene is used as reference gene. The native 4-hydroxybutyrate:CoA ligase Saci_1149 was replaced by equivalent gene Msed_0406 in RK34. ST1127 = *S. tokodaii* SOR gene, ST1856 = TQO-beta gene. MW = parent *S. acidocaldarius* acceptor strain, 34 = RK34 strain with SOR and TQO Knock Ins. All data from replicate bioreactors is shown, so MW001 cells grown without sulfur are MWn1 & MWn2

Figure 5. Transcription profiles (reported as percentile of all ORFs in transcriptome) for 3-HP/4-HB genes in *Sulfolobus acidocaldarius* DSM 639 (Saci), *Metallosphaera sedu*la DSM 5348 (Msed), and *Acidianus brierleyi* DSM 1651 (Abr). For Msed and Abr both autotrophic "A" and heterotrophic "H" transcription levels are reported. LEGEND: E1αβγ, acetyl-CoA/propionyl-CoA carboxylase; E2, malonyl-CoA/succinyl-CoA reductase; E3, malonate semialdehyde reductase; E4, 3-hydroxypropionate:CoA ligase; E5, 3-hydroxypropionyl-CoA dehydratase; E6, acryloyl-CoA reductase; E7, methylmalonyl-CoA epimerase; E8αb, methylmalonyl-CoA mutase; E9, succinate semialdehyde reductase; E10, 4-hydroxybutyrate:CoA ligase; E11, 4-hydroxybutyrl-CoA dehydratase; E12, crotonyl-CoA hydratase/(*S*)-3-hydroxybutyrl-CoA dehydrogenase; E13, acetoacetyl-CoA β-ketothiolase. *Abr* and *Saci* genes are also listed in **Table 1**.

a)



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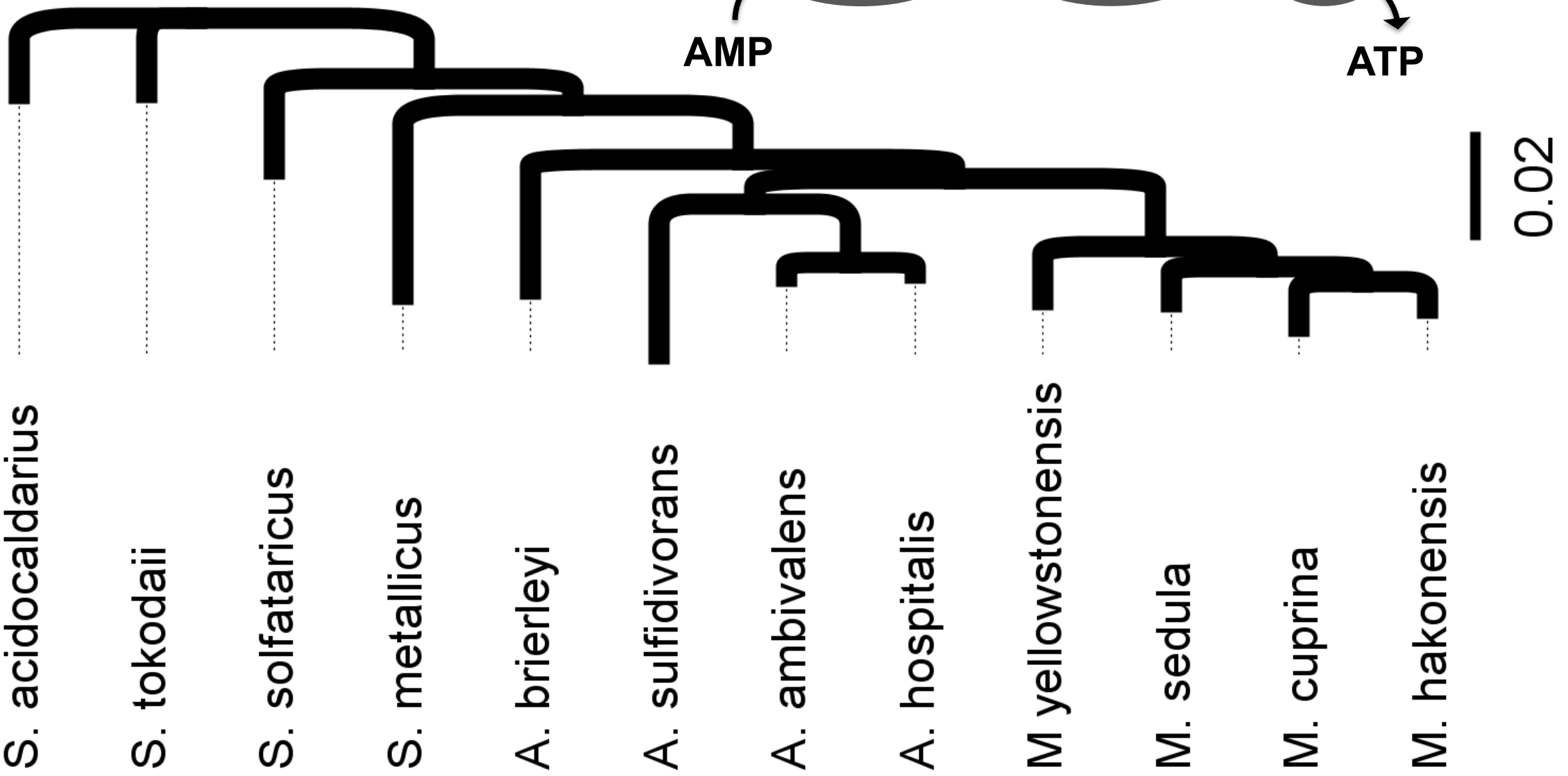


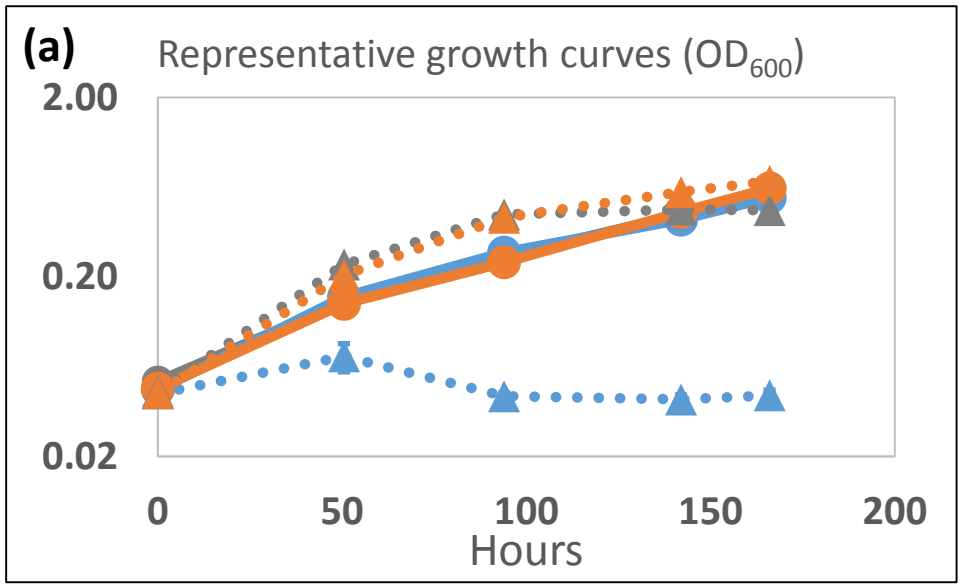
c)

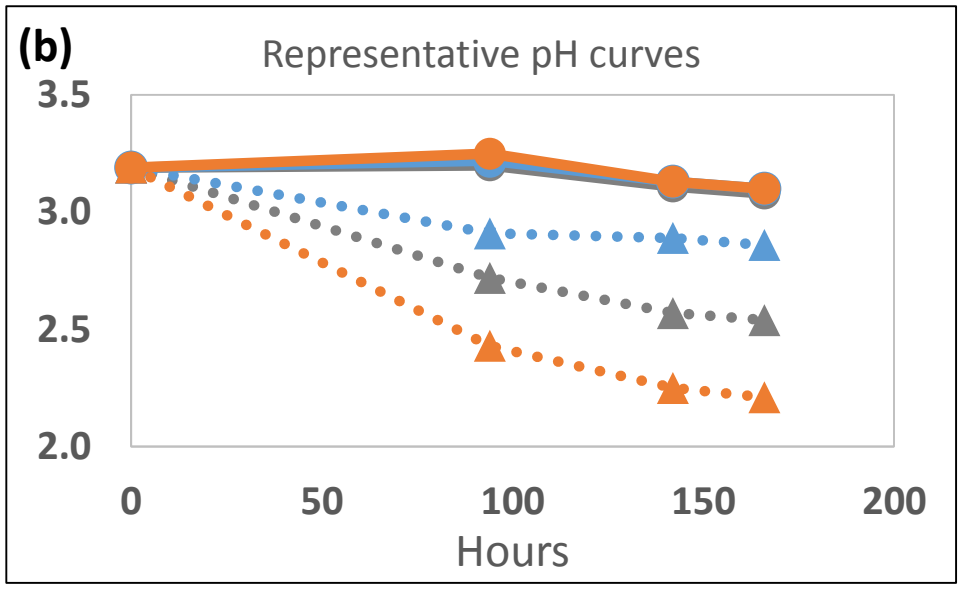
SOR	0	69	0	68	88	80	100	100	0	0	0	0
TQOa	0	91	72	61	84	92	100	100	78	85	81	82
TQOb	0	66	71	48	68	85	100	98	65	70	65	65
TetH	0	61	0	67	74	72	100	98	60	58	57	59
SQO*	0	80	76	82	88	90	100	99	80	80	79	79
SreA	0	0	56	0	56	55	100	0	56	0	0	0
SreB	30	30	83	28	88	85	100	34	83	29	29	29
SreC	0	0	66	0	73	63	100	0	66	0	0	0
SreD	24	22	47	22	61	49	100	0	48	0	22	21
SoxA	100	64	76	58	58	60	#	33	57	56	59	55
SoxB	100	59	82	56	58	58	#	87	59	61	59	28
SoxC	100	63	79	59	60	61	#	60	64	64	64	64
SoxM	100	59	73	0	0	0	#	0	56	58	58	58
CbsA	100	74	75	62	70	63	62	62	64	67	64	66
CbsB	100	44	57	32	36	34	36	39	34	36	38	36
SoxL	100	76	80	62	70	70	62	69	73	72	70	70
SoxN	100	69	73	64	69	69	63	64	67	67	65	67
DoxB	100	67	64	58	62	61	65	65	64	62	62	63
DoxC	100	60	50	48	46	46	46	45	44	45	44	44
DoxE	100	67	66	68	62	61	61	59	61	61	66	58
DsrE3A	85	86	90	87	93	93	#	91	98	99	100	99
DsrE2B	81	84	83	79	80	81	#	80	93	94	100	92
TusA	83	90	86	84	88	88	#	75	93	94	100	93
HdrC1	83	82	84	81	88	86	#	85	94	97	100	98
HdrB1	84	87	88	84	90	90	#	88	93	97	100	96
HdrA1	82	86	84	85	90	91	#	87	95	98	100	97
HdrC2	77	86	84	88	91	90	#	84	95	97	100	97
HdrB2	79	83	80	80	85	87	#	79	91	94	100	96

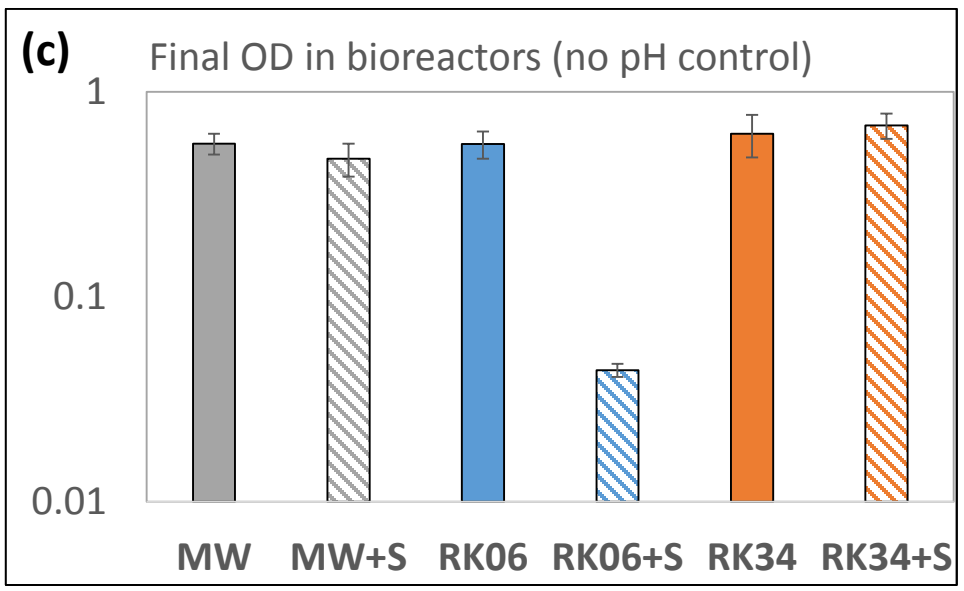
*Amb species have second SQO hits ID>70, cov=100, #= no genome available

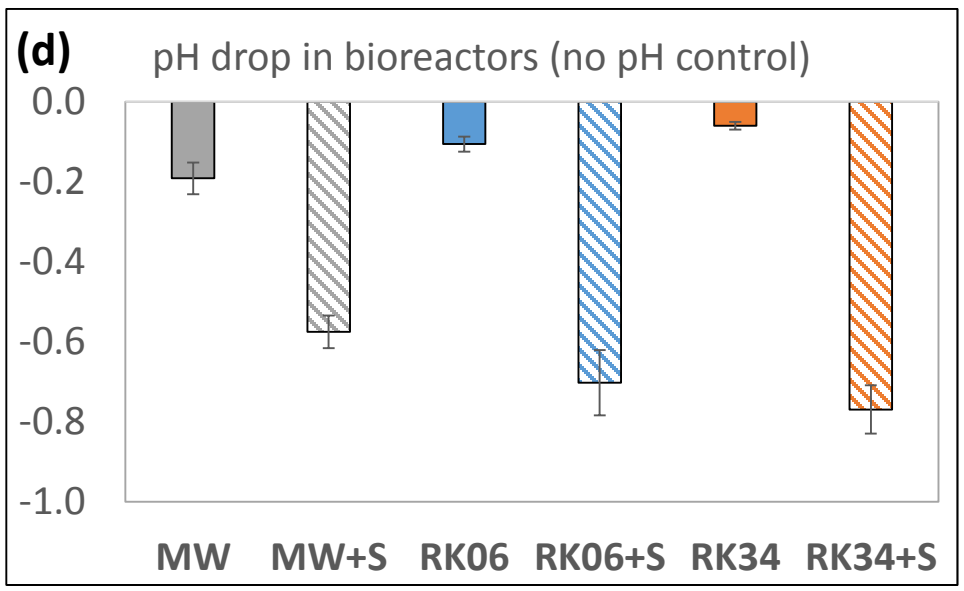
Cell growth

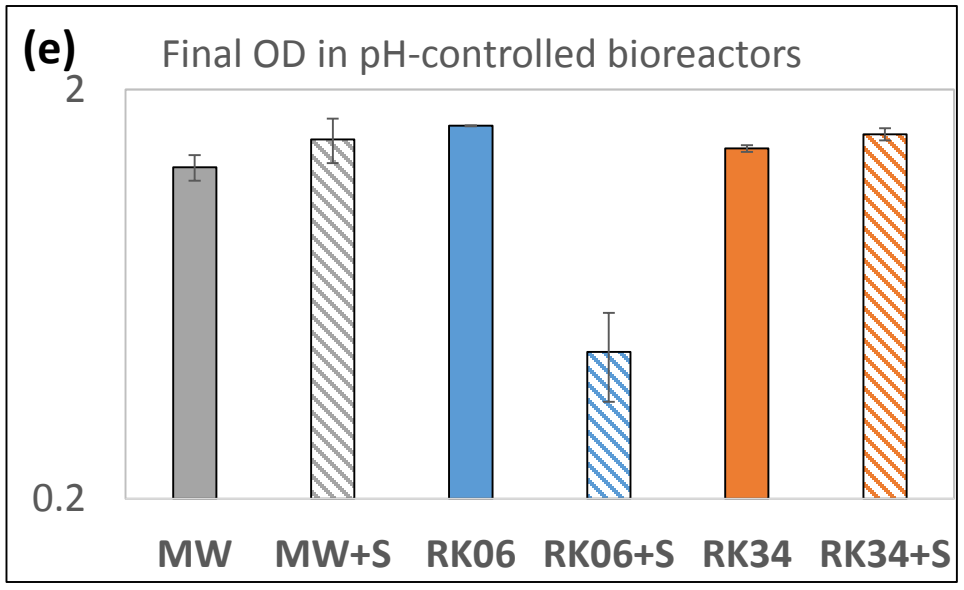
Sulfur oxidation

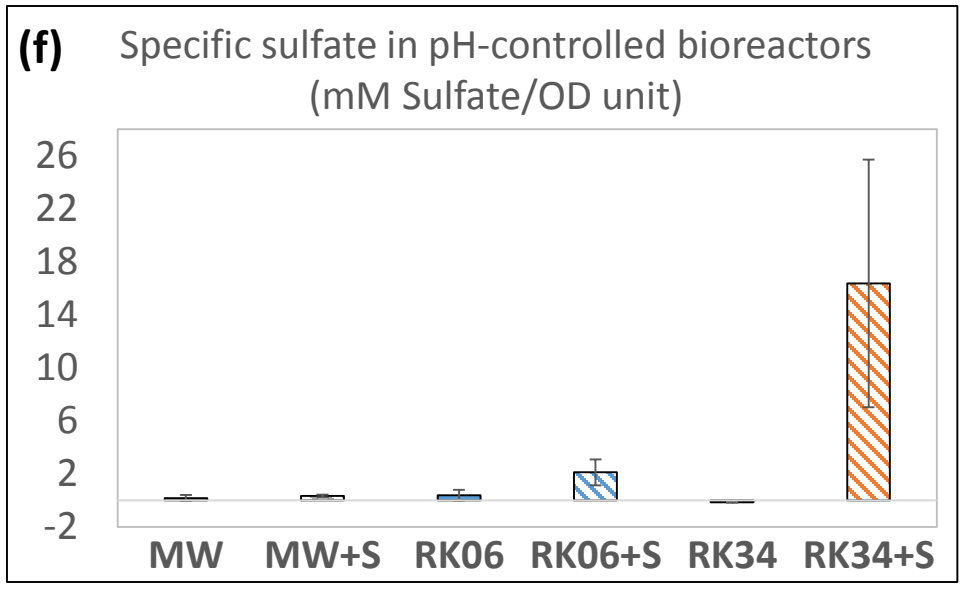


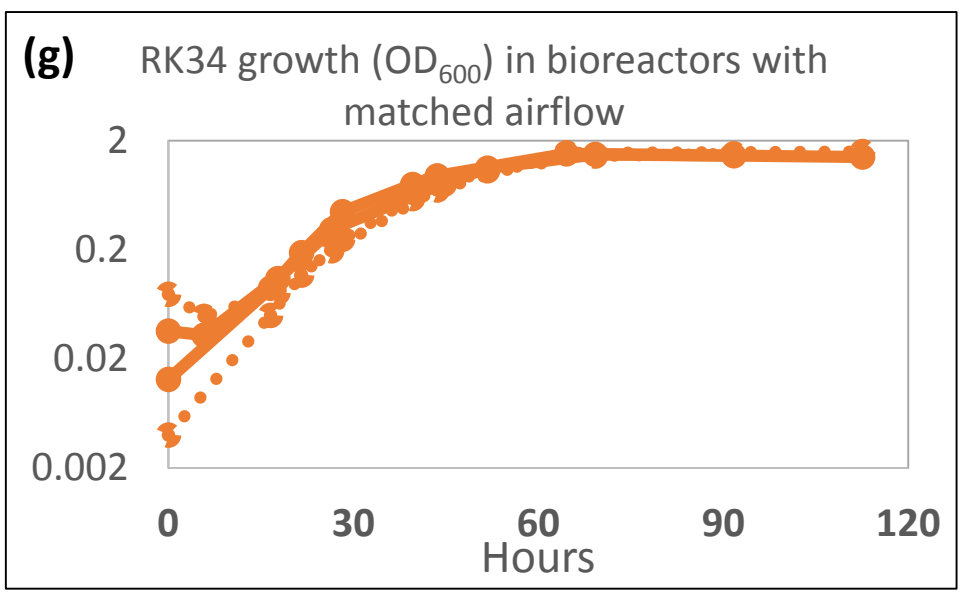


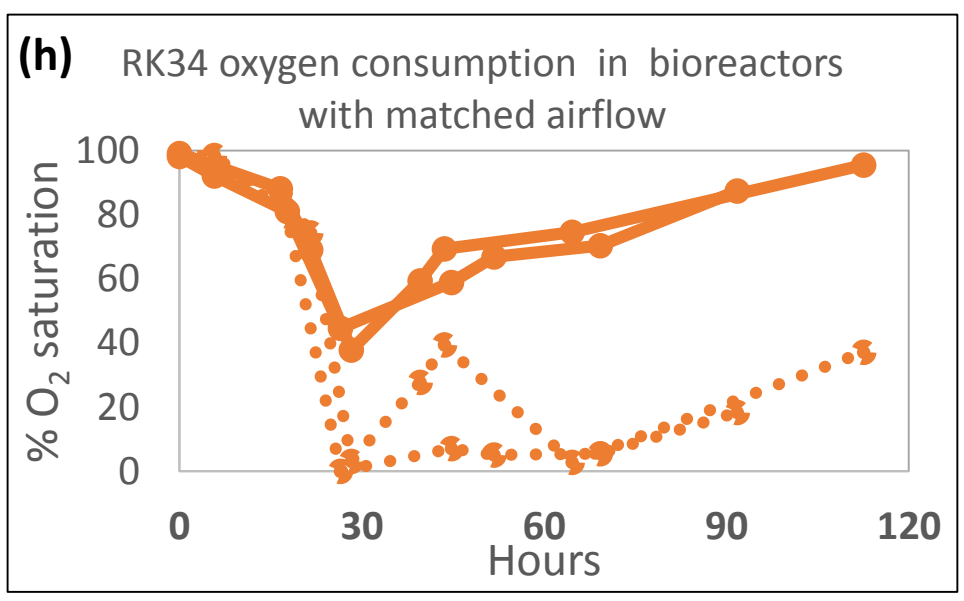




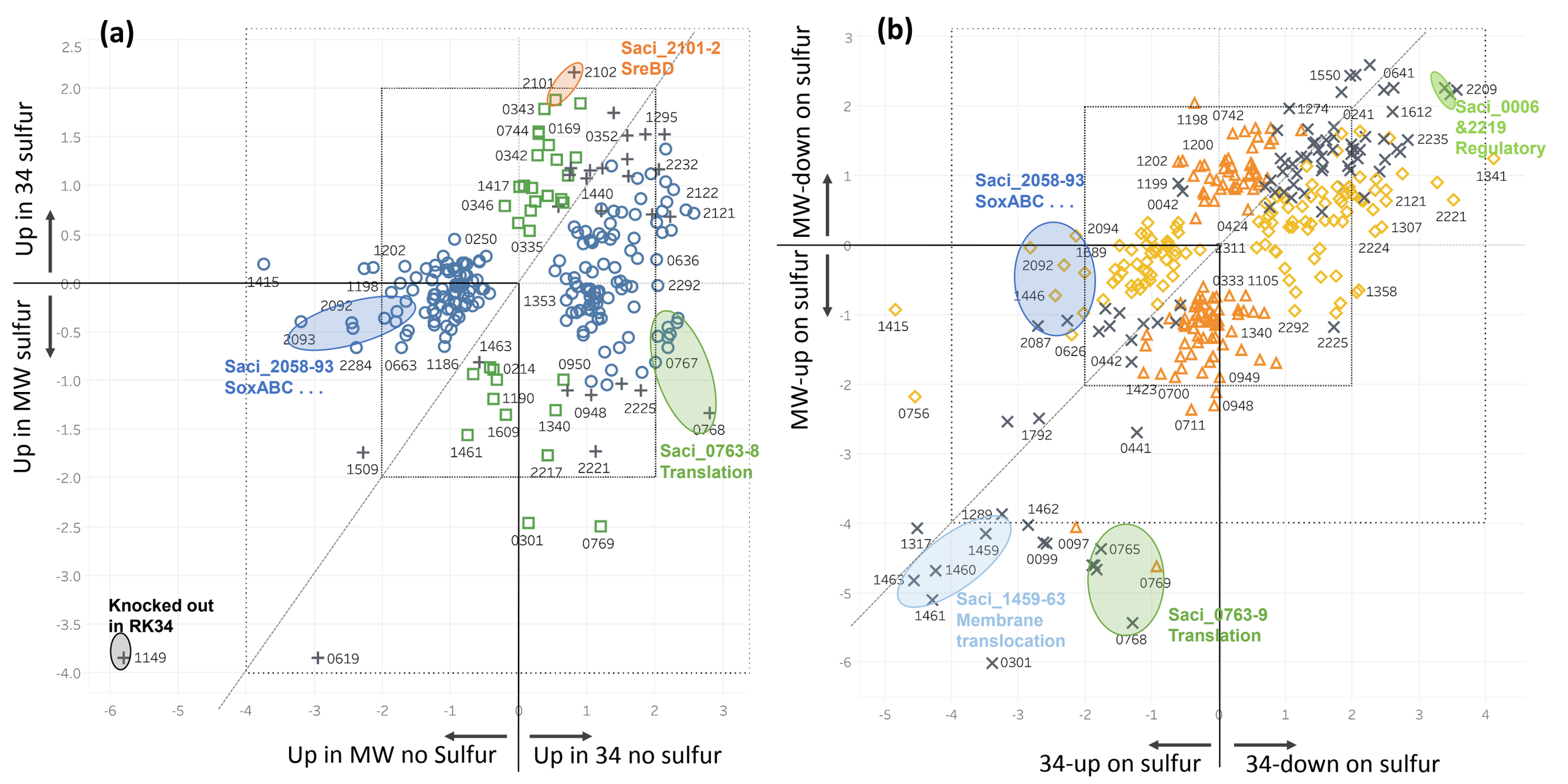


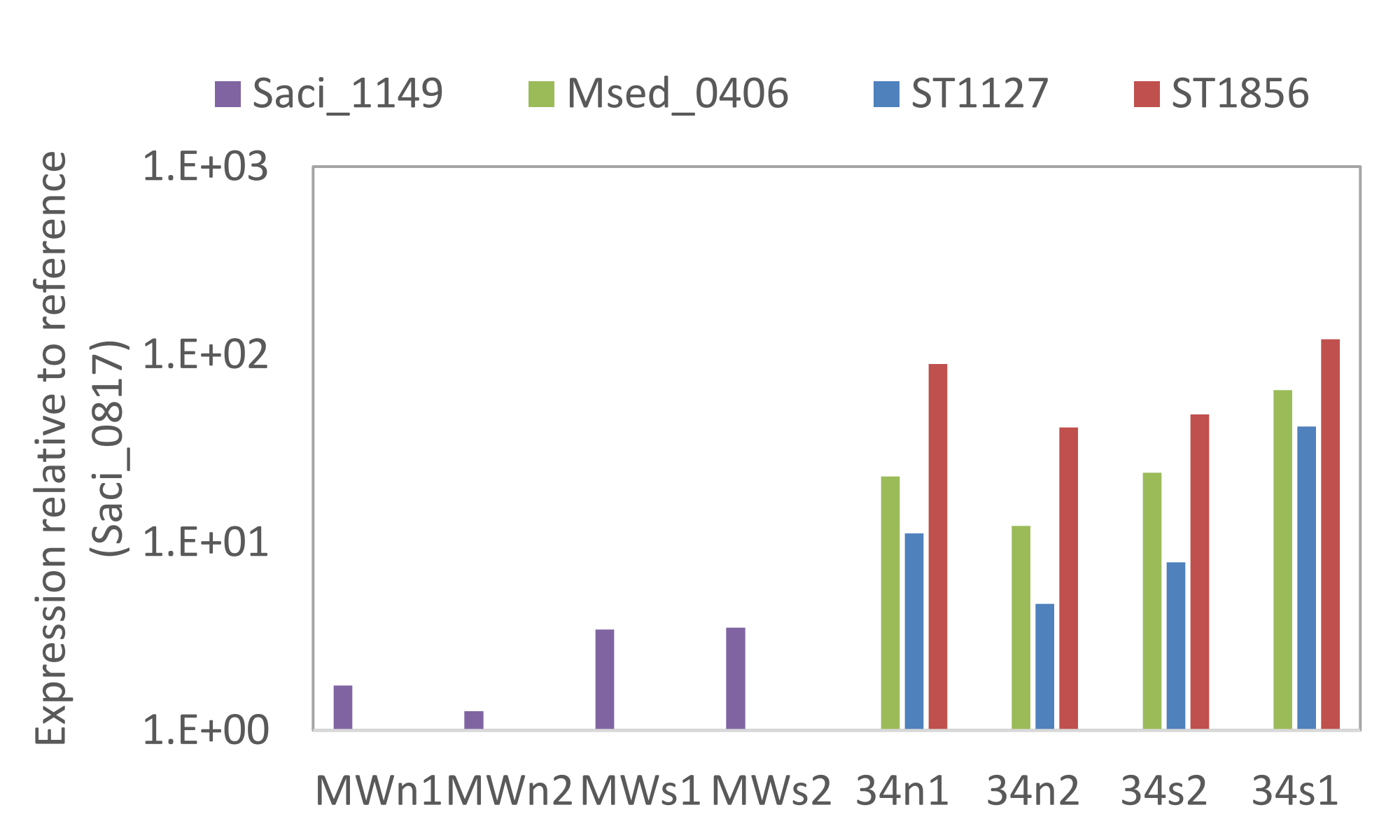


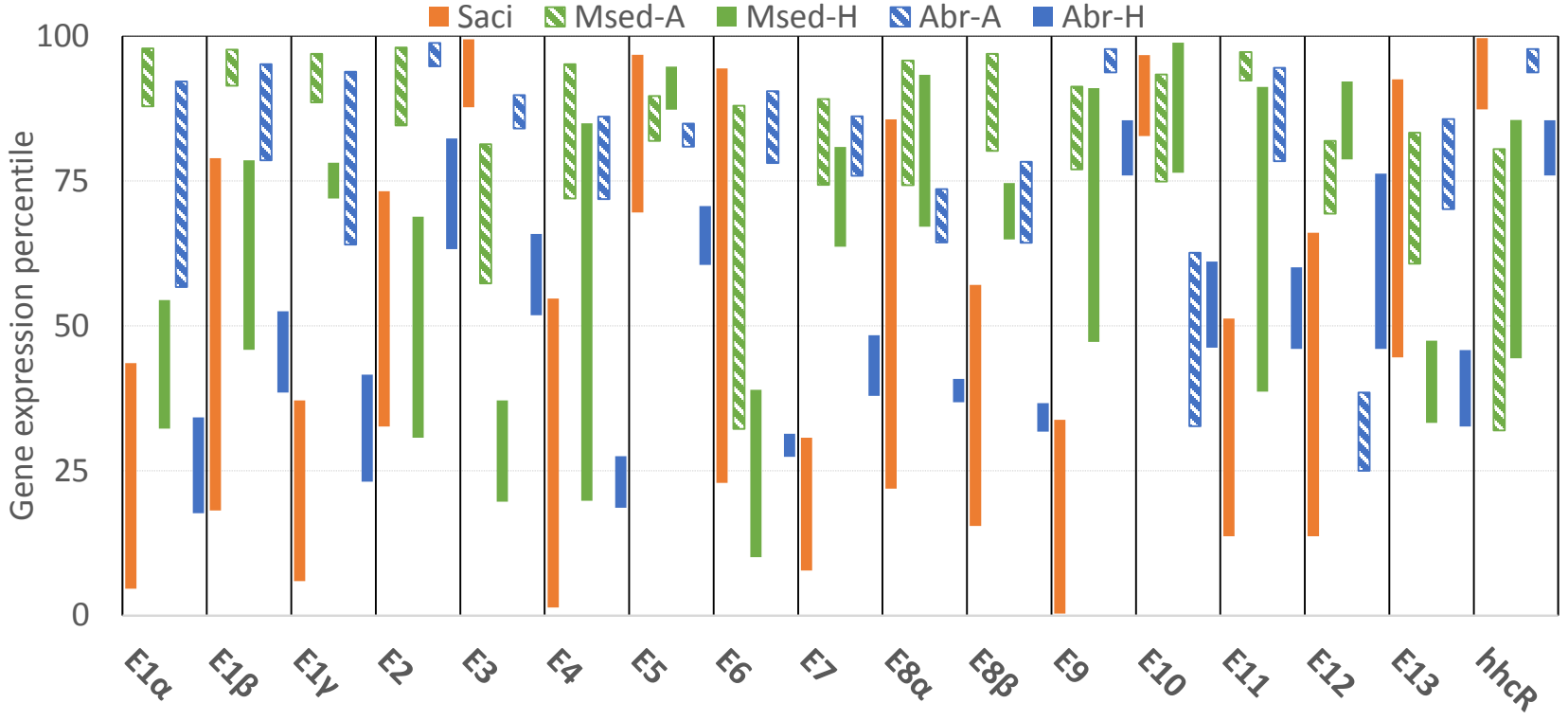




MW MW+S RK06 RK06+S RK34 RK34+S


(a)
Up in 34 sulfur
Up in MW sulfur
Up in MW no Sulfur
Up in 34 no sulfur
Saci_2101-2
SreBD
Saci_2058-93
SoxABC . . .
Saci_0763-8
Translation
Knocked out
in RK34
(b)
MW-up on sulfur
MW-down on sulfur
34-up on sulfur
34-down on sulfur
Saci_2058-93
SoxABC . . .
Saci_0006
&2219
Regulatory
Saci_1459-63
Membrane
translocation
Saci_0763-9
Translation


Saci_1149
Msed_0406
ST1127
ST1856
Expression relative to reference (Saci_0817)
1.E+03
1.E+02
1.E+01
1.E+00
MWn1
MWn2
MWs1
MWs2
34n1
34n2
34s2
34s1


Saci
Msed-A
Msed-H
Abr-A
Abr-H
Gene expression percentile
100
75
50
25
0
E1α
E1β
E1γ
E2
E3
E4
E5
E6
E7
E8α
E8β
E9
E10
E11
E12
E13
hncR