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Iron redox transformation by the thermo-acidophilic archaea from the genus *Sulfolobus*

(Running title: Iron redox transformation by the genus *Sulfolobus*)

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Abstract

Iron redox transformations by five representative *Sulfolobus* strains (*S. metallicus* Kra23; *S. tokodaii* 7; *S. acidocaldarius* 98-3; *S. solfataricus* P1; *S. shibatae* B12) were studied to clarify the general trend of Fe metabolism across different species of the genus *Sulfolobus*. Negligible to major Fe(II) oxidation was detected in cell-suspensions of the strains. Fe(III)-reducing ability was differently expressed in each strain with dependence on the oxygen level and growth status: Growth-uncoupled cell-suspensions of all strains reduced Fe(III) under either anaerobic or micro-aerobic conditions, or under both conditions. A linear correlation between cell growth and Fe(III) reduction was also found in *S. tokodaii* 7, *S. solfataricus* P1 and *S. shibatae* B12. In addition to Fe redox behaviors, the strains were also tested for reduction of highly-toxic Cr(VI) to less toxic and soluble Cr(III), as an application possibility: The trend in degree of Cr(V) reduction did not necessarily correspond to that of Fe(III) reduction, suggesting the involvement of different reduction mechanisms.

Introduction

The genus *Sulfolobus* is generally recognized as a group of aerobic, thermo-acidophilic sulfur-oxidizing archaea. They naturally occur in a number of sulfur-rich acidic geothermal environments (Brock et al. 1972; Huber and Stetter 1991; Jan et al. 1999; Zillig et al. 1993). Consequently, utilization of S^0 (either autotrophically or heterotrophically) is widely seen in known *Sulfolobus* spp. (*S. tokodaii*, Suzuki et al. 2002; *S. metallicus*, Huber and Stetter 1991; *S. yangmingensis*, Jan et al. 1999; *S. shibatae*, Grogan et al. 1990; *S. tengchongensis*, Xiang et al. 2003), although with an exception of non-sulfur oxidizing *Sulfolobus* sp. GA1, as was recently reported (Masaki et al. 2016).

Given that microbial Fe(II) oxidation plays a major role in the geochemical Fe cycle of highly acidic environments, and that a number of *Sulfolobus* species are widely distributed in such environments, there are yet only limited studies evaluating Fe(II) oxidation ability in this genus (*S. metallicus*, *S. tokodaii*; Bathe and Norris 2007). Owing to its both Fe(II)- and S^0 -oxidizing metabolisms, *S. metallicus* has been employed to apply for high-temperature bioleaching (oxidative dissolution) of sulfide minerals such as pyrite and chalcopyrite (Sandström et al. 2005; Gautier et al. 2008; Vilcáez et al. 2008; Zhu et al. 2011). A comprehensive search for Fe(II)-oxidizing ability among different *Sulfolobus* species would therefore lead to better understandings of the role of this genus in natural

Fe cycles, as well as in possible utilities of the genus for application purposes.

So far, Fe(III)-reducing ability in the genus *Sulfolobus* is even less clear than its Fe(II)-oxidizing ability: *S. acidocaldarius* was reported to reduce soluble Fe(III) to Fe(II) under aerobic conditions in the presence of elemental sulfur or glutamate as electron donor (Brock and Gustafson 1976), although it was controversially pointed out that this archaeon does not grow autotrophically (Johnson et al. 2012). Our previous study detected the Fe(III)-reducing ability during growth under micro-aerobic conditions in recently isolated *Sulfolobus* sp. GA1 (Masaki et al. 2016). This observation thus further motivated us to investigate general trend of Fe(III)-reducing ability across different representative species of the genus *Sulfolobus*.

The possible importance of sulfur- and iron-oxidizing acidophiles in the natural geochemical Fe cycle by means of not only Fe(II) oxidation but also Fe(III) reduction was first pointed out by Brock and Gustafson (1976), by showing Fe(III)-reducing ability in *Acidithiobacillus thiooxidans*, *At. ferrooxidans* and *S. acidocaldarius*. Later studies in fact revealed a variety of acidophiles involved in microbial Fe(III) reduction: Anaerobic growth via Fe(III) respiration using S⁰ (or other reduced inorganic sulfur compounds) as electron donor was reported for *At. ferrooxidans* (Ohmura et al. 2002), *At. ferrivorans* (Hallberg et al. 2010), and *Acidiferrobacter thiooxydans* (Hallberg et al. 2011b). The

ability to use Fe(III) as sole electron acceptor during heterotrophic growth was reported in *Sulfobacillus* (*Sb.*) *acidophilus*, *Sb. thermosulfidooxidans*, *Acidimicrobium ferrooxidans* (Bridge and Johnson 1998) and *Acidicaldus organivorus* (Johnson et al. 2006). Reductive dissolution of Fe(III) minerals was observed in micro-aerobic cultures of heterotrophs, *Acidiphilium* spp. (Johnson and McGinness 1991; Johnson and Bridge 2002; Coupland and Johnson 2008), *Acidocella* spp. and *Acidobacterium* spp. (Coupland and Johnson 2008). Based on the above biodiversity found so far, it would be reasonable to expect that Fe(III)-reducing abilities could also exist across different *Sulfolobus* spp. coupled with growth on S⁰ and/or organic substrates.

In the viewpoint of biochemical Fe(II) oxidation mechanism, the responsible molecular complexes often seem quite versatile in different acidophiles: In the well-studied mesophilic Fe-oxidizing bacterium, *At. ferrooxidans*, electrons transfer from Fe(II) via cytochromes *c* and rusticyanin (Rus; a periplasmic blue copper protein), followed by the “downhill” and “uphill” pathways using a cytochrome *c* oxidase (Cox complex) and *bc*₁ complex (PetI), respectively. Unlike *At. ferrooxidans*, cytochrome *c* has not been detected in mesophilic Fe-oxidizing archaea *Ferroplasma* spp., but the terminal oxidase combines cytochrome *c* oxidase-like and *bc*₁ complex-like components. Also, a blue copper protein, sulfocyanin, may be serving as the branch point like

rusticyanin between downhill and uphill electron flows (Bonney and Holmes, 2012). In the case of iron-oxidizing thermophilic archaea, such as *Sulfolobus* spp. (She et al., 2001; Kawarabayashi et al., 2001; Hiller et al., 2003; Chen et al., 2005; Bathe and Norris, 2007), *Metallosphaera sedula* (Auernik et al., 2008) and *Acidans cophhuensis* (Urbiet al., 2017), again their genomic sequences have no evidence for cytochrome *c*, but the *fox* gene cluster (encoding FoxA/FoxB with identity to cytochrome *c* oxidase subunits, FoxC/FoxD with identity to cytochrome *b_{558/566}* subunits (CbsA-like) and others) was found, which may have an analogous role to the *rus* operon of *At. ferrooxidans* (Johnson et al., 2012). Likewise to *Ferroplasma* sp., sulfocyanin genes (*soxE*) were found in genomes of these thermophiles (cf. in the case of *A. cophhuensis*, an additional rusticyanin-like gene was also detected; Urbiet al., 2017).

Whilst Fe(III) reduction widely takes place among acidophilic prokaryotes, its biochemical mechanism is yet largely unknown, compared to the Fe(II) oxidation mechanism. So far most of the work focused on neutrophilic and mesophilic bacteria such as *Geobacter* sp. and *Shewanella* sp., and found that cytochromes *c* were responsible for their Fe(III) respiration (Lovely et al., 2011; Fredrickson et al., 2008). Compared to the neutrophilic counterparts, even fewer studies are available with acidophiles. In *At. ferrooxidans*, it was suggested that electrons travel from S^0 to Fe(III) via a respiratory

chain containing *bc*₁ complex and cytochrome *c* (Osorio et al., 2013). In thermo-
acidophilic archaea, the involvement of cytochrome *c* proteins seems to vary: Whilst a
number of other species have few to no cytochrome *c* genes, *Ferroglobus placidus* and
Geoglobus ahangari contained numerous cytochromes *c*. The importance of several
genes was suggested in Fe(III) respiration by *F. placidus* (i.e. three multiheme
cytochromes *c*, a menaquinol oxidoreductase Cbc4 complex, and a unique periplasmic
cytochrome *c* fused to a cytochrome *b*₅-type heme/steroid binding domain (CbcZ); Smith
et al., 2015).

From the viewpoint of biohydrometallurgical application, search for microbial
Fe(III)-reducing abilities would benefit the development of bio-processes such as
bioremediation of toxic metals (e.g., hexavalent chromium; Masaki et al. 2015;
Cummings et al. 2007) and reductive bioleaching of Fe(III)-minerals (e.g., nickel laterites
where Ni is associated with goethite; Hallberg et al. 2011a; Johnson 2012; Johnson et al.
2013; Nancucheo et al. 2014).

With the aim to clarify the roles of *Sulfolobus* spp. in natural geochemical Fe
cycles, as well as to further explore their potentials in biohydrometallurgical applications,
this study has chosen representative strains; *S. metallicus* Kra23, *S. tokodaii* 7, *S.*
acidocaldarius 98-3, *S. solfataricus* P1 and *S. shibatae* B12, to test and compare their Fe

122 redox transformation capabilities as well as to search for a genetic clue to support their
123 Fe transformation behaviors by analyzing the available genome sequences. Additionally,
124 their abilities in Cr(VI) reduction were also investigated to compare with those in Fe(III)
125 reduction.

Materials and Methods

Microorganisms

S. metallicus Kra23^T (DSM 6482), *S. tokodaii* 7^T (DSM 16993), *S. acidocaldarius* 98-3^T (DSM 639), *S. solfataricus* P1^T (DSM 1616) and *S. shibatae* B12^T (DSM 5389) were purchased from the National Institute of Technology and Evaluation (Tokyo, Japan).

S. tokodaii 7 and *S. shibatae* B12 were maintained and pre-grown in 300 mL Erlenmeyer flasks containing 150 mL of heterotrophic basal salts (HBS) medium (pH 2.0 with H₂SO₄; Masaki et al. 2015) supplemented with 10 mM glucose and 0.01% (w/v) yeast extract. Elemental sulfur (S⁰; 0.1% (w/v)), instead of glucose, was added to HBS medium (pH 2.5 with H₂SO₄) for *S. metallicus* Kra23, *S. acidocaldarius* 98-3 and *S. solfataricus* P1. Cultures were incubated at 70°C, shaken at 120 rpm.

In all below experiments, water evaporation was compensated with sterile deionized water before each sampling. All of the experiments were conducted in duplicates.

Fe(II) oxidation test using growth-uncoupled cell-suspensions under aerobic conditions

Each of the five strains was pre-grown as described in the previous section except that 500 mL flasks containing 200 mL medium were used. The cultures were harvested at the late exponential phase by centrifugation (12,000 g, 10 min), washed twice, and finally re-suspended in 200 mL of fresh HBS medium (in 500 mL flasks; pH 2.0) containing 10 mM Fe(II) (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$): Densities of the resultant cell-suspensions were approx. 5×10^8 cells mL^{-1} for *S. tokodaii* 7 and *S. shibatae* B12, and 2×10^8 cells mL^{-1} for *S. metallicus* Kra23, *S. acidocaldarius* 98-3 and *S. solfataricus* P1. Cell-suspensions were incubated shaken (70°C, 120 rpm) and samples were regularly taken to monitor the concentrations of Fe(II) and total soluble Fe.

The Fe(II) oxidation rates (mg-Fe h^{-1} ; for comparison between abiotic and biotic Fe(II) oxidations) and specific Fe(II) oxidation rates ($\text{mg-Fe h}^{-1} \text{ cell}^{-1}$; for comparison between *Sulfolobus* strains) were calculated.

Fe(III) reduction test using growth-uncoupled cell-suspensions under anaerobic / micro-aerobic conditions

Anaerobic experiments: Each of the five strains was pre-grown, harvested, washed (as described in the previous section), and finally re-suspended (to a final cell density of 1×10^9 cells mL^{-1}) in 10 mL of HBS medium (in 25 mL serum bottles with

rubber stoppers; pH 2.0) containing 10 mM Fe(III) (as $\text{Fe}_2(\text{SO}_4)_3 \cdot n\text{H}_2\text{O}$). One millimolar glucose (N_2 -purged) and 0.01% S^0 were tested as electron donor (except for the case of *S. metallicus* Kra23, where only S^0 was used). All solutions used for anaerobic experiments were N_2 -purged until the DO (dissolved oxygen) level reached $< 0.1 \text{ mg L}^{-1}$ (DO-14P; TOA-DKK). After that, all media preparations were conducted in the anaerobic chamber (Coy laboratory products). Sealed serum bottles were taken out of the anaerobic chamber and incubated (unshaken, 70°C).

Micro-aerobic experiments: Each of the five strains was pre-grown, harvested, washed, and finally re-suspended (to a final cell density of $1 \times 10^9 \text{ cells mL}^{-1}$) in 15 mL of HBS medium (in 15 mL Falcon tubes to allow little headspace for aeration; pH 2.0) containing 10 mM Fe(III) (as $\text{Fe}_2(\text{SO}_4)_3 \cdot n\text{H}_2\text{O}$). Reagents were not N_2 -purged. One millimolar glucose was used as electron donor (except that 0.01% S^0 was used for autotrophic *S. metallicus* Kra23). The Falcon tubes were incubated unshaken at 70°C .

Samples were regularly taken (using syringe needles for anaerobic experiments) to monitor concentrations of Fe(II) and total soluble Fe. All of the experiments were conducted in duplicates.

The specific Fe(III) reduction rates ($\text{mg-Fe h}^{-1} (1 \times 10^{10} \text{ cells})^{-1}$ for anaerobic experiments; $\text{mg-Fe h}^{-1} (1.5 \times 10^{10} \text{ cells})^{-1}$ for micro-aerobic experiments) were calculated.

Growth-coupled dissimilatory Fe(III) reduction test under anaerobic conditions

In order to investigate whether or not Fe(III) can serve as sole electron acceptor for *Sulfolobus* growth, correlation between cell density and Fe(III) reduction was monitored after inoculation under strictly anaerobic condition. Each of the five strains was pre-grown and inoculated (to a final cell density of 1×10^7 cells mL⁻¹) into 20 mL of HBS medium (in 25 mL serum bottles with rubber stoppers; pH 2.0) containing either 0.01% S⁰ and trace elements (Masaki et al. 2016) (for *S. metallicus* Kra23 only), or 10 mM glucose and 0.01% yeast extract (for other 5 strains). The headspace of serum bottles were flushed with N₂(80%)-CO₂(20%) gas for obligately autotrophic *S. metallicus* Kra23 cultures. Different initial concentrations of Fe(III) (0, 5, 10, or 15 mM) were added to the growth media. All solutions were N₂-purged until the DO level reached < 0.1 mg L⁻¹ (DO-14P; TOA-DKK). After that, all media preparations were conducted in an anaerobic chamber (Coy laboratory products), until serum bottles were sealed and incubated (120 rpm, 70°C).

Samples were taken using N₂-purged syringe needles. The positive pressure inside the serum bottles automatically pushed out the liquid sample as the needle was stuck into the bottle, preventing contamination of oxygen. Cell densities (Thoma counting

chamber, phase contrast microscope) and concentrations of Fe(II) and total soluble Fe were regularly monitored from duplicate flasks.

Cr(VI) reduction test using growth-uncoupled cell-suspensions under micro-aerobic conditions

Cell-suspensions (1×10^9 cells mL⁻¹; in 50 mL HBS medium (pH 2.0) in 50 mL Falcon tubes) were prepared for each strain as described in previous sections. One millimolar glucose (or 0.01% S⁰ in the case of *S. metallicus* Kra23) and 0.2 mM Cr(VI) (as Na₂CrO₄·4H₂O) were added to the cell-suspensions, which were then incubated at 70°C without shaking. Samples were regularly taken to monitor concentrations of Cr(VI) and total soluble Cr. All of the experiments were carried out in duplicates.

Solution analysis

Liquid samples were filtered using 0.20-μm cartridge filters to determine concentrations of Fe(II) and total soluble Fe (*o*-phenanthroline method; Caldwell and Adams 1946, using ascorbic acid as a reducing agent), Cr(VI) (diphenylcarbazide method; Noroozifar and Khorasani-Motlagh 2003), and total soluble Cr (ICP-OES; SEIKO Vista-MPX).

Results and Discussion

Comparison of Fe(II) oxidation by five *Sulfolobus* strains in growth-uncoupled cell-suspensions under aerobic condition

Fe(II) oxidation in cell-suspensions of five *Sulfolobus* strains are compared in Figure 1. No further cell growth was observed in cell-suspensions during the experiments by cell counting (data not shown). A decrease in total Fe concentrations in cell-suspensions during Fe(II) oxidation experiments was attributed to Fe(III) precipitation (data not shown), rather than Fe sorption on cell surfaces. Thus, any decline in Fe(II) was attributed to oxidation of Fe(II) to Fe(III).

The Fe(II) oxidation rates (mg-Fe h⁻¹ for comparison between abiotic and biotic Fe(II) oxidations) and specific Fe(II) oxidation rates (mg-Fe h⁻¹ cell⁻¹ for comparison between *Sulfolobus* strains) were calculated (using the data points from designated hours in Figure 1) and listed in Table 1. *S. solfataricus* P1 and *S. shibatae* B12 showed only negligible Fe(II) oxidation, compared with the abiotic counterpart. The most effective Fe(II) oxidation was observed in cell-suspensions of *S. metallicus* Kra23, followed by *S. tokodaii* 7 (Figure 1). It should be noted that the final cell density regularly achieved in pre-grown cultures (for preparation of cell-suspensions) of strictly autotrophic *S. metallicus* Kra23 (approx. 2×10⁸ cells mL⁻¹) was much lower than that of heterotrophic

S. tokodaii 7 (approx. 5×10^8 cells mL⁻¹). Therefore, the specific Fe(II) oxidation rate (mg-
 Fe h⁻¹ cell⁻¹) was over 4 times greater with *S. metallicus* Kra23 than with *S. tokodaii* 7
 (Table 1). *S. acidocaldarius* 98-3 was less effective Fe(II) oxidizer than *S. metallicus*
 Kra23 and *S. tokodaii* 7, but marginally more effective than the rest (Figure 1; Table 1).
 Theoretical conversion from per cell to per g-protein (Table 1) found that the specific
 Fe(II) oxidation rate (mg-Fe h⁻¹ g-protein⁻¹) of *S. metallicus* Kra23 is still much smaller
 than those of Fe(II) oxidizing bacteria (Johnson et al., 2012), although a simultaneous
 comparison test would be needed to confirm this observation. Bathe and Norris (2007)
 reported that *S. tokodaii* readily oxidized Fe(II) during heterotrophic growth on yeast
 extract, but its autotrophic Fe(II) oxidation was much weaker than that by *S. metallicus*.
 Our results demonstrated that *S. metallicus* Kra23 and *S. tokodaii* 7 oxidize Fe(II) much
 more readily than the others tested. Since *S. metallicus* is the only known strictly
 autotrophic type strain in the genus (Huber and Stetter 1991), it is reasonable that this
 species oxidizes Fe(II) much more effectively than all the others to support its growth.

Putative genes possibly involved in Fe(II) oxidation / Fe(III) reduction in
Sulfolobus spp. were summarized in Supplemental Table 1. Based on the available
 genomic sequences, a series of *sox*, *cbs* and *ods* genes (likely involved in the respiratory
 chains) were evident in *Sulfolobus* spp. including both Fe(II)-oxidizing (*S. tokodaii*) and

non- or minor-Fe(II) oxidizing (*S. solfataricus* or *S. acidocaldarius*, respectively) strains.

A clear difference was the presence of the *fox* gene cluster solely in Fe(II)-oxidizing strains (*S. metallicus* and *S. tokodaii*), as was previously suggested by Bathe and Norris (2007). Whether or not Sox, Cbs, Ods and/or proteins with some homology to Fe(II)-upregulated genes of *At. ferrooxidans* (Quairini et al., 2009; Supplemental Table 1) are possibly responsible for any minor Fe(II) oxidation (e.g., by *S. acidocaldarius*) is yet unclear.

Comparison of Fe(III) reduction by five *Sulfolobus* strains in growth-uncoupled cell-suspensions under anaerobic / micro-aerobic conditions

Among the five *Sulfolobus* strains tested, only *S. metallicus* Kra23 is known to grow autotrophically on S⁰ as energy source (Huber and Stetter 1991). Other five strains grow heterotrophically on glucose as sole electron donor (Grogan 1989; Grogan et al. 1990; Suzuki et al. 2002; Masaki et al. 2016). The following Fe(III) reduction experiments therefore used S⁰ and glucose as electron donor for *S. metallicus* Kra23 and the rest, respectively.

Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains under anaerobic and micro-aerobic conditions are compared in Figure 2 and 3, respectively. No further

cell growth was seen in cell-suspensions during the experiments by cell counting (data not shown). The rates of Fe(III) reduction (mg-Fe h^{-1}) and specific Fe(III) reduction ($\text{mg-Fe h}^{-1} \text{ cell}^{-1}$) were calculated (using the data points from designated hours in Figure 2 and 3) and listed in Table 2.

S. metallicus Kra23, the strongest Fe(II) oxidizer among the five strains, was shown to be also the most effective Fe(III) reducer under anaerobic conditions (Figure 2a; Table 2). When tested micro-aerobically, however, its Fe(III)-reducing ability was almost totally suppressed (Figure 3a; Table 2).

S. tokodaii 7 readily reduced Fe(III) under anaerobic conditions with both S^0 and glucose as electron donor (Figure 2b; Table 2). Under micro-aerobic conditions, however, Fe(III) reduction started off to build up Fe(II) for the first 20 hours, but later switching to an apparent Fe(II) oxidation phase (20-65 hours), which was finally followed solely by steady Fe(III) reduction (Figure 3b). The Fe(II) hump between 0-65 hours in glucose-free controls mimicked (but about half) that with glucose addition (Figure 3b). This is likely due to the presence of residual intracellular electron carriers, such as NADH, accumulated during pre-growth (Magnuson et al. 2000; Okibe et al., 2017). Therefore, this observation may be explained by a gradual and steady depletion of oxygen in the system as follows: During 0-20 hours, electrons (deriving from externally added glucose plus residual

intracellular NADH) were supposedly accepted by both oxygen and some Fe(III). Build-up of a certain amount of the resultant Fe(II) may have triggered Fe(II) oxidation with the residual oxygen (20-65 hours). Eventually, the oxygen was consumed to support steady Fe(III) reduction (after 65 hours).

S. solfataricus P1 also displayed fairly strong Fe(III)-reducing ability (more readily with glucose than S^0 as electron donor) under anaerobic conditions (Figure 2d; Table 2), which was slightly negatively affected by the presence of residual oxygen when glucose was used as electron donor (Figure 3d; Table 2).

S. acidocaldarius 98-3 and *S. shibatae* B12 showed the least Fe(III) reduction among the five strains under anaerobic conditions (glucose was the better electron donor than S^0 for Fe(III) reduction in both strains) (Figure 2c, e; Table 2). However, Fe(III) reduction by the two strains were greatly facilitated under micro-aerobic conditions, showing the most effective Fe(III) reduction among the five strains (Figure 3c, e; Table 2). Again, this was not caused by a cell number increase in the presence of residual oxygen, since there was no further cell growth in cell-suspensions during the experiment (by cell counting; data not shown). A similar observation was reported with Fe(III) reduction by *Acidiphilium* spp.: Whilst most strains do not grow under strictly anoxic conditions in the presence of Fe(III), growth-coupled Fe(III) reduction was most readily observed under

micro-aerobic conditions (Johnson and McGinness, 1991; Bridge and Johnson,2000). Fe(III) reduction was constitutive in *A. cryptum* SJH but was inducible in *A. acidophilum* and low oxygen concentrations, rather than Fe(III) was suggested to induce a putative “iron reductase” in the latter (Bridge and Johnson,2000; Johnson et al., 2012). This could possibly be the case also for some *Sulfolobus* spp.. Relatively high standard redox potential of Fe(III)/Fe(II) (+0.76V; pH 2.0), comparable to that of 1/2O₂/H₂O (+0.82V), makes iron favorable alternative electron acceptor for acidophiles (Madigan and Martinko 2006). The results suggested that this is also applicable to *Sulfolobus* spp. under both/either microaerobic and/or anaerobic condition.

Theoretical conversion of the specific Fe(III) reduction rate from per cell to per g-protein (Table 2) indicated that Fe(III) reduction by *S. metallicus* Kra23 (anaerobic condition) is comparable to that reported in other bacterial counterparts (Johnson et al., 2012). However, again, a simultaneous comparison is necessary to verify this (Johnson et al., 2012).

Cell growth-coupled dissimilatory Fe(III) reduction in *Sulfolobus* spp. under anaerobic conditions

Linear correlations between cell densities and the amounts of Fe(III) reduced

were observed in *S. tokodaii* 7 (Figure 4b), *S. solfataricus* P1 (Figure 4d) and *S. shibatae* B12 (Figure 4e) (Figure 4f), indicating that cell growth of these three strains is supported by Fe(III) reduction using glucose as electron donor.

In the case of other two strains, *S. metallicus* Kra23 and *S. acidocaldarius* 98-3, Fe(III) reduction did occur by the existing cells, but no correlation between cell growth and Fe(III) reduction was found (Figure 4a, c). Interestingly, the microbial capability of growth-coupled Fe(III) respiration (Figure 4) did not necessarily correspond to efficient Fe(III) reduction in its growth-uncoupled cell suspensions (Figures 2, 3). The results from a series of Fe(III) reduction experiments using cell-suspensions (Figure 2, 3) revealed that all tested *Sulfolobus* spp. showed major Fe(III) reduction compared to sterile controls, either/both under anaerobic (Figure 2) or/and micro-aerobic (Figure 3) conditions, as summarized as follows:

(i) *S. metallicus* Kra23 (Figures 2-4a): Strong Fe(III) reducer under anaerobic conditions, though incapable of anaerobic growth using S^0 and Fe(III) as sole electron donor and acceptor, respectively; residual oxygen significantly suppresses its Fe(III) reduction. (ii) *S. tokodaii* 7 (Figures 2-4b): Readily reduces Fe(III) under anaerobic conditions; its Fe(II) oxidation can overtake Fe(III) reduction in the presence of residual oxygen, exhibiting apparent alternate switch on/off of Fe(III) reduction and Fe(II)

oxidation; anaerobic cell growth coupled with Fe(III) reduction using glucose as sole electron donor. (iii) *S. solfataricus* P1 (Figures 2-4d): Readily reduces Fe(III) under both anaerobic and micro-aerobic conditions; anaerobic cell growth likely coupled with Fe(III) reduction using glucose and Fe(III) as sole electron donor and acceptor, respectively. (iv) *S. acidocaldarius* 98-3 (Figures 2-4c): No clear evidence for anaerobic Fe(III) reduction, whilst micro-aerobic conditions significantly facilitate its Fe(III)-reducing ability; no anaerobic growth observed using glucose and Fe(III) as sole electron donor and acceptor, respectively. (v) (Figures 2-4e) *S. shibatae* B12: Anaerobic Fe(III) reduction is negligible, whilst micro-aerobic conditions significantly accelerate its Fe(III)-reducing ability; capable of anaerobic growth using Fe(III) as sole electron acceptor (glucose as electron donor).

Since genomes of *Sulfolobus* spp. do not possess cytochromes *c*, their Fe(III) reduction mechanisms seem to take place differently from that of *F. placidus* (Supplemental Table 1; Smith et al., 2015). However, genomic sequence analysis detected Ferp_1268 (4Fe-4S ferredoxin iron-sulfur binding domain protein) and Ferp_1269 (NrfD; polysulphide reductase)-like proteins in *S. tokodaii*, *S. acidocaldarius* and *S. solfataricus* (Supplemental Table 1). By utilizing different cytochrome systems (e.g., cytochrome *b_{558/566}*, as was suggested in Fe(II) oxidation mechanism) it may still be possible that 4Fe-

4S ferredoxin and/or polysulphide reductase-type protein are involved in the Fe(III) reduction mechanism of *Sulfolobus* spp.. Nonetheless, such hypothesis requires detailed biochemical studies to be clarified.

Comparison of Cr(VI) reduction by five *Sulfolobus* strains in growth-uncoupled cell-suspensions under micro-aerobic condition

Following the previous observation that Fe(III)-reducing *Sulfolobus* sp. GA1 is also capable of Cr(VI) reduction to Cr(III) (Masaki et al. 2016), this study tested five *Sulfolobus* strains to investigate whether or not Cr(VI)-reducing ability is also widely found across different representative *Sulfolobus* species.

No further cell growth was observed in cell-suspensions during the experiments (by cell counting; data not shown). Since no changes in total soluble Cr concentration were observed throughout the experiment (data not shown), Cr(VI) reduced by *Sulfolobus* strains remained soluble mostly in the form of Cr(III). Liquid media were free of any trace of iron and no noticeable abiotic reduction of Cr(VI) was observed in sterile controls (Figure 5).

All five strains were found to reduce Cr(VI), though to a different extent (Figure 5) and with different specific Cr(VI) reduction rates (Table 3). Interestingly, the trend in

degree of Fe(III) reduction by five strains (under the same micro-aerobic conditions) did not necessarily correspond to that of Cr(VI) reduction: e.g., *S. acidocaldarius* 98-3 and *S. shibatae* B12 showed the highest Fe(III) reduction under micro-aerobic conditions (Figure 3c, e, respectively). Nonetheless, the former was the least effective Cr(VI) reducer while the latter was the most effective among the five strains.

Oxido-reduction of metal species is often mediated by cytochromes in electron transport chains: In the case of acidophilic bacteria, cytochromes *c* in cell membranes were found to be involved in anaerobic Fe(III) reduction by *At. ferrooxidans* (Ohmura et al. 2002). Cr(VI) reduction by Fe(III)-respiring acidophile *A. cryptum* was also reported to involve cytochromes *c* (Magnuson et al. 2010). In the case of Fe(III)-reducing neutrophiles, cytochromes *c* were found to function in Fe(III) reduction by *Geobacter sulfurreducens* (Magnuson et al. 2000) and both Fe(III) and Cr(VI) reduction by *Shewanella putrefaciens* (Beliaev et al. 2001). In the latter bacterium, Cr(VI) reductase was reported to be distinct from Fe(III) reductase, and was not irreversibly inhibited by oxygen (Myers et al. 2000). The fact that the trend in Fe(III) reduction by five *Sulfolobus* strains does not always match that in Cr(VI) reduction implies that different mechanisms may be involved in microbial reduction of the two metals in *Sulfolobus* spp., as was the case with *Shewanella putrefaciens* (Beliaev et al. 2001).

396

397 **Conclusions**

398 This study clarified the presence and the degree of Fe oxido-reduction abilities in five
399 representative *Sulfolobus* strains (*S. metallicus* Kra23; *S. tokodaii* 7; *S. acidocaldarius*
400 98-3; *S. solfataricus* P1; *S. shibatae* B12). The degree of Fe(II)-oxidizing abilities were
401 found to differ largely between the strains. Three strains (*S. tokodaii* 7, *S. solfataricus* P1
402 and *S. shibatae* B12) were capable of growth via Fe(III) respiration. All five strains
403 displayed Fe(III)-reducing abilities in growth-uncoupled cell-suspensions: Nonetheless,
404 Fe(III)-reducing ability in each strain responded significantly differently to the oxygen
405 level. Highly-toxic Cr(VI) was also reduced in growth-uncoupled cell-suspensions of all
406 strains, though to a varying degree. The trend of Fe(III) reduction in five strains did not
407 correspond to that of Cr(VI), suggesting different mechanisms being involved in Fe(III)
408 and Cr(VI) reduction in *Sulfolobus* spp. For being a major member in the acidophile
409 community, Fe redox transformation in the genus *Sulfolobus* remained so far largely
410 ambiguous. The results of this study provide comprehensive understandings over the
411 genus *Sulfolobus* on its Fe redox transformation capabilities and the potential utility of
412 the *Sulfolobus* spp. for biohydrometallurgical applications.

413

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625

Figure Legends

Figure 1.

Growth-uncoupled Fe(II) oxidation in cell-suspensions of five *Sulfolobus* strains (pH 2.0, 70°C, aerobic conditions): *S. metallicus* Kra23 (▲, △); *S. tokodaii* 7 (■, □); *S. acidocaldarius* 98-3 (◆, ◇); *S. solfataricus* P1 (▼, ▽); *S. shibatae* B12 (●, ○); sterile controls (+, ×) (duplicate data are individually plotted). The initial cell densities of cell-suspensions (naturally achieved after pre-growing each strain) were 2×10^8 cells mL⁻¹ (*S. metallicus* Kra23; *S. acidocaldarius* 98-3; *S. solfataricus* P1) and 5×10^8 cells mL⁻¹ (*S. tokodaii* 7; *S. shibatae* B12).

Figure 2.

Growth-uncoupled Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains (pH 2.0, 70°C, anaerobic conditions): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S. acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12, (f) sterile controls. Glucose (●, ○) or elemental sulfur (▲, △) was used as electron donor. Solid or broken lines indicate concentrations of total soluble Fe or Fe(II), respectively. Duplicate data are individually plotted.

Figure 3.

Growth-uncoupled Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains (pH 2.0, 70°C, micro-aerobic conditions): (a) *S. metallicus* Kra23 (0-25 h), (b) *S. tokodaii* 7 (0-25 h), (c) *S. acidocaldarius* 98-3 (0-100 h), (d) *S. solfataricus* P1 (0-25 h), (e) *S. shibatae* B12 (0-100 h), (f) sterile controls (0-100 h). Glucose (●, ○) or elemental sulfur (▲, △) was used as electron donor. Electron-donor-free controls (■, □) were also prepared. Solid or broken lines indicate concentrations of total soluble Fe or Fe(II), respectively. Duplicate data are individually plotted.

Figure 4.

Correlation between anaerobic cell growth and the amount of Fe(III) reduced by five *Sulfolobus* strains (pH 2.0, 70°C): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S. acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12. The initial Fe(III) concentration used were 0 (▼), 5 (●), 10 (■), or 15 (▲) mM. The number indicated for each plot indicates the time for sampling. Data points are the average of two measurements from duplicate flasks (error bars show the average difference in the two values).

Figure 5.

Cr(VI) reduction in cell-suspensions of five *Sulfolobus* strains (pH 2.0, 70°C, micro-aerobic conditions): *S. metallicus* Kra23 (▲,△); *S. tokodaii* 7 (■,□); *S. acidocaldarius* 98-3 (◆,◇); *S. solfataricus* P1 (▼,▽); *S. shibatae* B12 (●,○); sterile controls with glucose (+, ×) or elemental sulfur (/, \) as electron donor. Duplicate data are individually plotted.

Table 1.

Fe(II) oxidation rates and specific Fe(II) oxidation rates determined for five *Sulfolobus* strains in growth-uncoupled cell suspensions.

Table 2.

Fe(III) reduction rates and specific Fe(III) reduction rates determined for five *Sulfolobus* strains in growth-uncoupled cells suspensions under anaerobic or micro-aerobic conditions.

Table 3.

Cr(VI) reduction rates and specific Cr(VI) reduction rates determined for five *Sulfolobus*

680 strains in growth-uncoupled cell suspensions under micro-aerobic condition.

681

682 **Supplemental Table 1.**

683 Putative genes possibly involved in Fe(II) oxidation / Fe(III) reduction in *Sulfolobus* spp.

684 searched using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database.