Iron Redox Transformation by the Thermo-Acidophilic Archaea from the Genus Sulfolobus

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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

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

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

60 The possible importance of sulfur- and iron-oxidizing acidophiles in the natural
61 geochemical Fe cycle by means of not only Fe(II) oxidation but also Fe(III) reduction
62 was first pointed out by Brock and Gustafson (1976), by showing Fe(III)-reducing ability
63 in *Acidithiobacillus thiooxians*, *At. ferrooxidans* and *S. acidocaldarius*. Later studies in
64 fact revealed a variety of acidophiles involved in microbial Fe(III) reduction: Anaerobic
65 growth via Fe(III) respiration using S^0 (or other reduced inorganic sulfur compounds) as
66 electron donor was reported for *At. ferrooxidans* (Ohmura et al. 2002), *At. ferrivorans*
67 (Hallberg et al. 2010), and *Acidiferrobacter thiooxydans* (Hallberg et al. 2011b). The

68 ability to use Fe(III) as sole electron acceptor during heterotrophic growth was reported
69 in *Sulfobacillus* (*Sb.*) *acidophilus*, *Sb. thermosulfidooxidans*, *Acidimicrobium*
70 *ferrooxidans* (Bridge and Johnson 1998) and *Acidicaldus organivorus* (Johnson et al.
71 2006). Reductive dissolution of Fe(III) minerals was observed in micro-aerobic cultures
72 of heterotrophs, *Acidiphilium* spp. (Johnson and Mcginness 1991; Johnson and Bridge
73 2002; Coupland and Johnson 2008), *Acidocella* spp. and *Acidobacterium* spp. (Coupland
74 and Johnson 2008). Based on the above biodiversity found so far, it would be reasonable
75 to expect that Fe(III)-reducing abilities could also exist across different *Sulfolobus* spp.
76 coupled with growth on S^0 and/or organic substrates.

77 In the viewpoint of biochemical Fe(II) oxidation mechanism, the responsible
78 molecular complexes often seem quite versatile in different acidophiles: In the well-
79 studied mesophilic Fe-oxidizing bacterium, *At. ferrooxidans*, electrons transfer from
80 Fe(II) via cytochromes *c* and rusticyanin (Rus; a periplasmic blue copper protein),
81 followed by the "downhill" and "uphill" pathways using a cytochrome *c* oxidase (Cox
82 complex) and *bc*1 complex (PetI), respectively. Unlike *At. ferrooxidans,* cytochrome *c*
83 has not been detected in mesophilic Fe-oxidizing archaea *Ferroplasma* spp., but the
84 terminal oxidase combines cytochrome *c* oxidase-like and bc_1 complex-like components.
85 Also, a blue copper protein, sulfocyanin, may be serving as the branch point like

86 rusticyanin between downhill and uphill electron flows (Bonnefoy and Holmes, 2012). In
87 the case of iron-oxidizing thermophilic archaea, such as *Sulfolobus* spp. (She et al., 2001;
88 Kawarabayashi et al., 2001; Hiller et al., 2003; Chen et al., 2005; Bathe and Norris, 2007),
89 *Metallosphaera sedula* (Auernik et al., 2008) and *Acidanus cophhuensis* (Urbieta et al.,
90 2017), again their genomic sequences have no evidence for cytorhrome *c,* but the *fox* gene
91 cluster (encoding FoxA/FoxB with identity to cytochrome *c* oxidase subunits,
92 FoxC/FoxD with identity to cytochrome $b_{558/566}$ subunits (CbsA-like) and others) was
93 found, which may have an analogous role to the *rus* operon of *At. ferrooxidans* (Johnson
94 et al., 2012). Likewise to *Ferroplasma* sp., sulfocyanin genes (*sox*E) were found in
95 genomes of these thermophiles (cf. in the case of *A. cophhuensis*, an additional
96 rusticyanin-like gene was also detected; Urbieta et al., 2017).

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

104 chain containing bc_1 complex and cytochrome *c* (Osorio et al., 2013). In thermo-
105 acidophilic archaea, the involvement of cytochrome *c* proteins seems to vary: Whilst a
106 number of other species have few to no cytochrome *c* genes, *Ferroglobus placidus* and
107 *Geoglobus ahangari* contained numerous cytochromes *c*. The importance of several
108 genes was suggested in Fe(III) respiration by *F. placidus* (i.e. three multiheme
109 cytochromes *c*, a menaquinol oxidoreductase Cbc4 complex, and a unique periplasmic
110 cytochrome *c* fused to a cytochrome b_5-type heme/steroid binding domain (CbcZ); Smith
111 et al., 2015).

112 From the viewpoint of biohydrometallurgical application, search for microbial
113 Fe(III)-reducing abilities would benefit the development of bio-processes such as
114 bioremediation of toxic metals (e.g., hexavalent chromium; Masaki et al. 2015;
115 Cummings et al. 2007) and reductive bioleaching of Fe(III)-minerals (e.g., nickel laterites
116 where Ni is associated with goethite; Hallberg et al. 2011a; Johnson 2012; Johnson et al.
117 2013; Ñancucheo et al. 2014).

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

redox transformation capabilities as well as to search for a genetic clue to support their Fe transformation behaviors by analyzing the available genome sequences. Additionally, their abilities in Cr(VI) reduction were also investigated to compare with those in Fe(III) 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 H_2SO_4; Masaki et al. 2015) supplemented with 10 mM glucose and 0.01% (w/v) yeast extract. Elemental sulfur (S^0; 0.1% (w/v)), instead of glucose, was added to HBS medium (pH 2.5 with H_2SO_4) 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

144 Each of the five strains was pre-grown as described in the previous section except
145 that 500 mL flasks containing 200 mL medium were used. The cultures were harvested
146 at the late exponential phase by centrifugation (12,000 g, 10 min), washed twice, and
147 finally re-suspended in 200 mL of fresh HBS medium (in 500 mL flasks; pH 2.0)
148 containing 10 mM Fe(II) (as $FeSO_4 \cdot 7H_2O$): Densities of the resultant cell-suspensions
149 were approx. 5×10^8 cells mL^{-1} for *S. tokodaii* 7 and *S. shibatae* B12, and 2×10^8 cells mL^{-1}
150 for *S. metallicus* Kra23, *S. acidocaldarius* 98-3 and *S. solfataricus* P1. Cell-suspensions
151 were incubated shaken (70°C, 120 rpm) and samples were regularly taken to monitor the
152 concentrations of Fe(II) and total soluble Fe.
153 The Fe(II) oxidation rates (mg-Fe h^{-1}; for comparison between abiotic and biotic
154 Fe(II) oxidations) and specific Fe(II) oxidation rates (mg-Fe h^{-1} $cell^{-1}$; for comparison
155 between *Sulfolobus* strains) were calculated.
156
157 **Fe(III) reduction test using growth-uncoupled cell-suspensions under anaerobic /**
158 **micro-aerobic conditions**
159 Anaerobic experiments: Each of the five strains was pre-grown, harvested,
160 washed (as described in the previous section), and finally re-suspended (to a final cell
161 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 $Fe_2(SO_4)_3 \cdot nH_2O$). 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 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 1×10^9 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 $Fe_2(SO_4)_3 \cdot nH_2O$). 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 (mg-Fe h^{-1} $(1\times10^{10}$ cells$)^{-1}$ for anaerobic experiments; mg-Fe h^{-1} $(1.5\times10^{10}$ 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% S^0 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 N_2(80%)-CO_2(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 N_2-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 N_2-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

198 chamber, phase contrast microscope) and concentrations of Fe(II) and total soluble Fe
199 were regularly monitored from duplicate flasks.
200
201
202 **Cr(VI) reduction test using growth-uncoupled cell-suspensions under micro-aerobic**
203 **conditions**
204 Cell-suspensions (1×10^9 cells mL^{-1}; in 50 mL HBS medium (pH 2.0) in 50 mL Falcon
205 tubes) were prepared for each strain as described in previous sections. One millimolar
206 glucose (or 0.01% S^0 in the case of *S. metallicus* Kra23) and 0.2 mM Cr(VI) (as
207 $Na_2CrO_4 \cdot 4H_2O$) were added to the cell-suspensions, which were then incubated at 70°C
208 without shaking. Samples were regularly taken to monitor concentrations of Cr(VI) and
209 total soluble Cr. All of the experiments were carried out in duplicates.
210
211 **Solution analysis**
212 Liquid samples were filtered using 0.20-μm cartridge filters to determine concentrations
213 of Fe(II) and total soluble Fe (*o*-phenanthroline method; Caldwell and Adams 1946, using
214 ascorbic acid as a reducing agent), Cr(VI) (diphenylcarbazide method; Noroozifar and
215 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. 2×10^8 cells mL^{-1}) was much lower than that of heterotrophic

234 *S. tokodaii* 7 (approx. 5×10^8 cells mL^{-1}). Therefore, the specific Fe(II) oxidation rate (mg-
235 Fe h^{-1} $cell^{-1}$) was over 4 times greater with *S. metallicus* Kra23 than with *S. tokodaii* 7
236 (Table 1). *S. acidocaldarius* 98-3 was less effective Fe(II) oxidizer than *S. metallicus*
237 Kra23 and *S. tokodaii* 7, but marginally more effective than the rest (Figure 1; Table 1).
238 Theoretical conversion from per cell to per g-protein (Table 1) found that the specific
239 Fe(II) oxidation rate (mg-Fe h^{-1} g-$protein^{-1}$) of *S. metallicus* Kra23 is still much smaller
240 than those of Fe(II) oxidizing bacteria (Johnson et al., 2012), although a simultaneous
241 comparison test would be needed to confirm this observation. Bathe and Norris (2007)
242 reported that *S. tokodaii* readily oxidized Fe(II) during heterotrophic growth on yeast
243 extract, but its autotrophic Fe(II) oxidation was much weaker than that by *S. metallicus*.
244 Our results demonstrated that *S. metallicus* Kra23 and *S. tokodaii* 7 oxidize Fe(II) much
245 more readily than the others tested. Since *S. metallicus* is the only known strictly
246 autotrophic type strain in the genus (Huber and Stetter 1991), it is reasonable that this
247 species oxidizes Fe(II) much more effectively than all the others to support its growth.
248 Putative genes possibly involved in Fe(II) oxidation / Fe(III) reduction in
249 *Sulfolobus* spp. were summarized in Supplemental Table 1. Based on the available
250 genomic sequences, a series of *sox*, *cbs* and *ods* genes (likely involved in the respiratory
251 chains) were evident in *Sulfolobus* spp. including both Fe(II)-oxidizing (*S. tokodaii*) and

252 non- or minor-Fe(II) oxidizing (*S. solfataricus* or *S. acidocaldarius*, respectively) strains.
253 A clear difference was the presence of the *fox* gene cluster solely in Fe(II)-oxidizing
254 strains (*S. metallicus* and *S. tokodaii*), as was previously suggested by Bathe and Norris
255 (2007). Whether or not Sox, Cbs, Ods and/or proteins with some homology to Fe(II)-
256 upregulated genes of *At. ferrooxidans* (Quairini et al., 2009; Supplemental Table 1) are
257 possibly responsible for any minor Fe(II) oxidation (e.g., by *S. acidocardarius*) is yet
258 unclear.
259
260 **Comparison of Fe(III) reduction by five *Sulfolobus* strains in growth-uncoupled cell-**
261 **suspensions under anaerobic / micro-aerobic conditions**
262 Among the five *Sulfolobus* strains tested, only *S. metallicus* Kra23 is known to
263 grow autotrophically on S^0 as energy source (Huber and Stetter 1991). Other five strains
264 grow heterotrophically on glucose as sole electron donor (Grogan 1989; Grogan et al.
265 1990; Suzuki et al. 2002; Masaki et al. 2016). The following Fe(III) reduction
266 experiments therefore used S^0 and glucose as electron donor for *S. metallicus* Kra23 and
267 the rest, respectively.
268 Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains under anaerobic
269 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 (mg-Fe h^{-1} $cell^{-1}$) 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

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

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

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

306 micro-aerobic conditions (Johnson and McGinness, 1991; Bridge and Johnson,2000).
307 Fe(III) reduction was constitutive in *A. cryptum* SJH but was inducible in *A. acidophilum*
308 and low oxygen concentrations, rather than Fe(III) was suggested to induce a putative
309 "iron reductase" in the latter (Bridge and Johnson,2000; Johnson et al., 2012). This could
310 possibly be the case also for some *Sulfolobus* spp.. Relatively high standard redox
311 potential of Fe(III)/Fe(II) (+0.76V; pH 2.0), comparable to that of $1/2O_2/H_2O$ (+0.82V),
312 makes iron favorable alternative electron acceptor for acidophiles (Madigan and Martinko
313 2006). The results suggested that this is also applicable to *Sulfolobus* spp. under
314 both/either microaerobic and/or anaerobic condition.

315 Theoretical conversion of the specific Fe(III) reduction rate from per cell to per
316 g-protein (Table 2) indicated that Fe(III) reduction by *S. metallicus* Kra23 (anaerobic
317 condition) is comparable to that reported in other bacterial counterparts (Johnson et al.,
318 2012). However, again, a simultaneous comparison is necessary to verify this (Johnson et
319 al., 2012).

320

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

323 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)

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

353 Since genomes of *Sulfolobus* spp. do not possess cytochromes *c*, their Fe(III)
354 reduction mechanisms seem to take place differently from that of *F. placidus*
355 (Supplemental Table 1; Smith et al., 2015). However, genomic sequence analysis detected
356 Ferp_1268 (4Fe-4S ferredoxin iron-sulfur binding domain protein) and Ferp_1269 (NrfD;
357 polysulphide reductase)-like proteins in *S. tokodaii*, *S. acidocaldarius* and *S. solfataricus*
358 (Supplemental Table 1). By utilizing different cytochrome systems (e.g., cytochrome
359 $b_{558/566}$, as was suggested in Fe(II) oxidation mechanism) it may still be possible that 4Fe-

360 4S ferredoxin and/or polysulphide reductase-type protein are involved in the Fe(III)
361 reduction mechanism of *Sulfolobus* spp.. Nonetheless, such hypothesis requires detailed
362 biochemical studies to be clarified.
363
364 **Comparison of Cr(VI) reduction by five *Sulfolobus* strains in growth-uncoupled cell-**
365 **suspensions under micro-aerobic condition**
366 Following the previous observation that Fe(III)-reducing *Sulfolobus* sp. GA1 is
367 also capable of Cr(VI) reduction to Cr(III) (Masaki et al. 2016), this study tested five
368 *Sulfolobus* strains to investigate whether or not Cr(VI)-reducing ability is also widely
369 found across different representative *Sulfolobus* species.
370 No further cell growth was observed in cell-suspensions during the experiments
371 (by cell counting; data not shown). Since no changes in total soluble Cr concentration
372 were observed throughout the experiment (data not shown), Cr(VI) reduced by *Sulfolobus*
373 strains remained soluble mostly in the form of Cr(III). Liquid media were free of any
374 trace of iron and no noticeable abiotic reduction of Cr(VI) was observed in sterile controls
375 (Figure 5).
376 All five strains were found to reduce Cr(VI), though to a different extent (Figure
377 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).

Conclusions

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

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417

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625

626 **Figure Legends**

627 **Figure 1.**

628 Growth-uncoupled Fe(II) oxidation in cell-suspensions of five *Sulfolobus* strains (pH 2.0,
629 70°C, aerobic conditions): *S. metallicus* Kra23 (▲, △); *S. tokodaii* 7 (■, □); *S.*
630 *acidocaldarius* 98-3 (◆, ◇); *S. solfataricus* P1 (▼, ▽); *S. shibatae* B12 (●, ○); sterile
631 controls (+, ×) (duplicate data are individually plotted). The initial cell densities of cell-
632 suspensions (naturally achieved after pre-growing each strain) were 2×10^8 cells mL^{-1} (*S.*
633 *metallicus* Kra23; *S. acidocaldarius* 98-3; *S. solfataricus* P1) and 5×10^8 cells mL^{-1} (*S.*
634 *tokodaii* 7; *S. shibatae* B12).

635

636 **Figure 2.**

637 Growth-uncoupled Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains (pH 2.0,
638 70°C, anaerobic conditions): (a) *S. metallicus* Kra23, (b) *S. tokodaii* 7, (c) *S.*
639 *acidocaldarius* 98-3, (d) *S. solfataricus* P1, (e) *S. shibatae* B12, (f) sterile controls.
640 Glucose (●, ○) or elemental sulfur (▲, △) was used as electron donor. Solid or broken
641 lines indicate concentrations of total soluble Fe or Fe(II), respectively. Duplicate data are
642 individually plotted.

643

644 **Figure 3.**

645 Growth-uncoupled Fe(III) reduction in cell-suspensions of five *Sulfolobus* strains (pH 2.0,
646 70°C, micro-aerobic conditions): (a) *S. metallicus* Kra23 (0-25 h), (b) *S. tokodaii* 7 (0-25
647 h), (c) *S. acidocaldarius* 98-3 (0-100 h), (d) *S. solfataricus* P1 (0-25 h), (e) *S. shibatae*
648 B12 (0-100 h), (f) sterile controls (0-100 h). Glucose (●, ○) or elemental sulfur (▲,
649 △) was used as electron donor. Electron-donor-free controls (■, □) were also prepared.
650 Solid or broken lines indicate concentrations of total soluble Fe or Fe(II), respectively.
651 Duplicate data are individually plotted.

652

653 **Figure 4.**

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

661

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.