Acidithiobacillus caldus Sulfur Oxidation Model Based on Transcriptome Analysis between the Wild Type and Sulfur Oxygenase Reductase Defective Mutant

Linxu Chen¹, Yilin Ren², Jianqun Lin¹, Xiangmei Liu¹, Xin Pang¹, Jianqiang Lin¹

¹ State Key Lab of Microbial Technology, Shandong University, Jinan, China, ² School of Life Science, Shandong Normal University, Jinan, China

Abstract

Background: Acidithiobacillus caldus (A. caldus) is widely used in bio-leaching. It gains energy and electrons from oxidation of elemental sulfur and reduced inorganic sulfur compounds (RISCs) for carbon dioxide fixation and growth. Genomic analyses suggest that its sulfur oxidation system involves a truncated sulfur oxidation (Sox) system (omitting SoxCD), non-Sox sulfur oxidation system similar to the sulfur oxidation in A. ferrooxidans, and sulfur oxygenase reductase (SOR). The complexity of the sulfur oxidation system of A. caldus generates a big obstacle on the research of its sulfur oxidation mechanism. However, the development of genetic manipulation method for A. caldus in recent years provides powerful tools for constructing genetic mutants to study the sulfur oxidation system.

Results: An A. caldus mutant lacking the sulfur oxygenase reductase gene (sor) was created and its growth abilities were measured in media using elemental sulfur (S⁰) and tetrathionate (K₂S₄O₆) as the substrates, respectively. Then, comparative transcriptome analysis (microarrays and real-time quantitative PCR) of the wild type and the Δsor mutant in S⁰ and K₂S₄O₆ media were employed to detect the differentially expressed genes involved in sulfur oxidation. SOR was concluded to oxidize the cytoplasmic elemental sulfur, but could not couple the sulfur oxidation with the electron transfer chain or substrate-level phosphorylation. Other elemental sulfur oxidation pathways including sulfur dioxygenase (SDO) and heterodisulfide reductase (HDR), the truncated Sox pathway, and the S₄I pathway for hydrolysis of tetrathionate and oxidation of thiosulfate in A. caldus are proposed according to expression patterns of sulfur oxidation genes and growth abilities of the wild type and the mutant in different substrates media.

Conclusion: An integrated sulfur oxidation model with various sulfur oxidation pathways of A. caldus is proposed and the features of this model are summarized.

Introduction

Acidithiobacillus caldus (A. caldus), a gram-negative, acidophilic, obligately chemolithotrophic, moderately thermophilic bacterium [1,2] and an important member of a consortium of microorganisms used for industrial bioleaching [3], plays key roles together with iron-oxidizing bacteria in bio-leaching processes [4,5]. A. caldus has the capability of oxidizing elemental sulfur and a wide range of reduced inorganic sulfur compounds (RISCs), but could not oxidize ferrous iron. It uses energy and electrons derived from sulfur oxidation for carbon dioxide fixation and other anabolic processes [1,2,6,7].

Oxidation of elemental sulfur and RISCs happens normally in some chemolithotrophic bacteria and archaea [8–10]. The sulfur oxidizing (Sox) enzyme system in lithotrophic Paracoccus pantotrophus (P. pantotrophus), responsible for the oxidation of sulfide, elemental sulfur, thiosulfate, and sulfate to sulfate, accompanied by electron transfer to cytochrome c, has been well studied [8–12]. It is located in the periplasm and constituted generally by four proteins: SoxYZ, SoxAX, SoxB and Sox(CD)₂ [13]. Initially, SoxAX initiates the oxidation of thiosulfate (S₂O₃²⁻) producing SoxY-thiocysteine-S-sulfate (SoxYZ-S-SO₃²⁻) [14]; secondly, SoxB hydrolyzes sulfate (SO₄²⁻) from the thiocysteine-S-sulfate residue (SoxYZ-S-S-SO₄²⁻) producing S-thiocysteine (SoxYZ-S-S'); thirdly, Sox(CD)₂ may oxidize the outer sulfur atom of S-thiocysteine producing SoxYZ-cysteine-S-sulfate (SoxYZ-S-SO₃²⁻); finally, sulfate is hydrolyzed and removed by SoxB from SoxYZ-S-S- SO₄²⁻ and SoxYZ is regenerated. However, Sox(CD)₂ is absent in the so-called truncated Sox system of many prototypical α-Proteobacteria [8,10]. Another sulfur oxidation system based on the sulfur oxygenase reductase (SOR) has been elaborated in several acidophilic and thermophilic archaea [10,15]. SOR is able to catalyze the disproportionation of elemental sulfur, producing sulfite, thiosulfate, and sulfide [16,17]. The reaction has several characteristics: (1) it takes place in the cytoplasm; (2) it is dioxygen (O₂)-dependent with no external cofactors or electron donors.

Citation: Chen L, Ren Y, Lin J, Liu X, Pang X, et al. (2012) Acidithiobacillus caldus Sulfur Oxidation Model Based on Transcriptome Analysis between the Wild Type and Sulfur Oxygenase Reductase Defective Mutant. PLoS ONE 7(9): e39470. doi:10.1371/journal.pone.0039470

Editor: Nikolai Lebedev, US Naval Research Laboratory, United States of America

Received January 31, 2012; Accepted May 21, 2012; Published September 12, 2012

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Funding: This research was supported by the National Basic Research Program (http://www.973.gov.cn)(2010CB630902, 2004CB619202), the National Natural Science Foundation (http://www.nsfc.gov.cn)(31070034), and the Knowledge Innovation Program of CAS (http://www.cas.cn)(2AJKSCX2-YW-JS401) of China. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jianqunlin@sdu.edu.cn
required [16]; (3) sulfur oxidation is not coupled with electron transfer or substrate-level phosphorylation in Acidithiobacillus ambivalens (A. ambivalens) [19].

The sulfur oxidation system in acidophilic Acidithiobacillus genus is related to three representative species widely used in bioleaching, which are A. ferrooxidans, A. thiooxidans and A. caldus. Although, the sulfur metabolic mechanism in A. ferrooxidans has been studied for many years, it is still not completely understood. The elemental sulfur in nature consists of a stable octasulfane ring (S8), which forms orthorhombic crystals insoluble in water [19]. An elemental sulfur activation and oxidation model was proposed in Acidithiobacillus [20,21]. The S8 is firstly activated to become thiol-bound sulfane sulfur atoms (RS-SH) and then transported into the periplasm where it is oxidized by sulfur dioxygenase (SDO) [20,21]. SDO enzyme activity was detected from the crude cell extracts of A. ferrooxidans and A. thiooxidans, but SDO enzyme protein has not been purified and the gene(s) encoding for SDO activity not yet been identified [20,22]. Another elemental sulfur oxidation enzyme in A. ferrooxidans is the cytoplasmic heterodisulfide reductase complex (HdrABC), but it is a speculation from the genomic and transcriptomic analysis, not from biochemical experimental data [23]. The S8 pathway of thiolsulfate oxidation via the form of tetrathionate intermediates exists widely in Acidithiobacillus: the periplasmic thiosulfate is oxidized to tetrathionate by thiosulfate quinone oxidoreductase (TQO); then, tetrathionate is hydrolyzed by tetrathionate hydrolase (TetH) yielding thiosulfate and other products [10,24]. In addition, other RISC oxidation enzymes identified in Acidithiobacillus include: sulfide quinone reductase (SQR) being responsible for oxidation of hydrogen sulfide [25], and rhodanese or thiosulfate sulfurtransferase (TST) transferring a sulfur atom from thiosulfate to sulfur acceptors like cyanide and thiol compounds [26,27]. Recently, a model of sulfur oxidation in A. ferrooxidans was proposed, in which electrons from oxidation of RISCs are transferred via the quinol pool (QH2) to terminal oxidases to produce ATP or to NADH complex I to generate NADPH, coupling the oxidation of RISCs with the generation of energy or reducing power [23].

An unique sulfur oxidation system exists in A. caldus, which is quite different from that of A. ferrooxidans according to comparative genome analysis [28]. The sulfur oxidation system of A. caldus can be classified into three subsystems: the truncated Sox subsystem, non-Sox sulfur subsystem, and SOR subsystem. The truncated Sox subsystem in A. caldus contains two copies of essential sorABC genes but no sosCD genes. The non-Sox sulfur subsystem, similar to the sulfur oxidation system in A. ferrooxidans, contains the sulfur oxidation enzyme genes (tetH, sgr, rhd, and hddABC) and terminal oxidase genes. The SOR subsystem is characterized by the sulfur oxygenase reductase gene (sor) in A. caldus, which was only found in several acidophilic and thermophilic archaea but not in the two species A. ferrooxidans and A. thiooxidans [10,29,30]. Therefore, A. caldus has a complex and integrated sulfur oxidation system. The currently known sulfur oxidation pathways in A. caldus are mainly acquired from the genome sequence analysis, which are not clear and remain unanswered questions: (1) How is the elemental sulfur oxidized and how many elemental sulfur oxidizing pathways exist? (2) How does the truncated Sox subsystem work? (3) How do the various pathways interconnect to complete the sulfur oxidation? Although, a rough model for ISCs metabolism in A. caldus was proposed [31], the above questions are still awaiting clear answers.

Recently, a method for A. caldus mutant construction was developed in our laboratory, which is different from the previous reported gene knock-out method based on the homologous recombination in A. caldus [32]. In our experiments, construction of gene mutant via the transposition insertion mutagenesis of the insertion sequences (IS elements) in A. caldus was discovered for the first time. The first report about IS element transposition in Acidithiobacillus was the ISAfe1 insertional inactivation of mbB (a putative cytochrome c-type biosynthesis protein) producing the A. ferrooxidans mutant, which lost Fe (II) oxidation ability [33,34]. IS elements are also widely distributed in A. caldus [35]. The loss of the sor gene resulting from the ISAfe1 transposition in A. caldus SM-1 was proposed according to the comparison of A. caldus SM-1 genome sequence and the sor gene cloned from SM-1 [30,35].

In this research, A. caldus MTH-04 A sor mutant is constructed by electroporation of a suicide plasmid. The cell growth of the mutant is measured using S8 and K2S4O6 as the substrates. Then, comparative transcriptome analysis is carried out by whole-genome microarray and quantitative RT-PCR. Finally, an integrated sulfur oxidation model in A. caldus is proposed.

Results and Discussion

Construction of A sor mutant

The suicide plasmid pMD19 sor:Ω-Cm (Figure 1A) derived from pUC19 is unable to replicate in A. caldus. It carries the homologous sequence of the disrupted sor gene by insertion of chloramphenicol resistance gene (cat, 816 bp) generating two homologous sequences, the L-arm (1,321 bp) and the R-arm (1,332 bp) (Figure 1A). A. caldus MTH-04 was electroporated using the suicide plasmid and the mutants were screened using colony PCR, then the chromosomes of the screened mutants were extracted and analyzed by PCR (Figure 1B) and southern blot analyses (Figure 1D). As shown in Figure 1B, two sets of primers (SorA fwd and SorA rev, SorB fwd and SorB rev) specific to the sor gene and a set of primers (Big fwd and Big rev) specific to the homologous sequence were used to verify the mutant. Fragments of 684 bp (lane 2, Figure 1B) and 971 bp (lane 3, Figure 1B) specific to the sor gene were amplified from the chromosome of the wild type, but not from the mutant. An 8 kb fragment amplified from the mutant using the primers Big fwd and Big rev (lane 7, Figure 1B) was larger than the fragment of about 6 kb amplified from the wild type (lane 6, Figure 1B). The fragments of lane 6 and lane 7 in Figure 1B were sequenced for validation. The results are shown in Figure 1C: the sor gene region from 2,403 bp to 4,343 bp in A. caldus MTH-04 wild type was different from the corresponding region (from 2,403 bp to 6,537 bp) which was replaced by the IS element (ISAcal1) and the transposase gene in the A. caldus MTH-04 mutant. In order to further confirm the mutant, southern blot analysis was carried out using the sor gene as the probe. As shown in Figure 1D, an EcoRI fragment (6.67 kb) was isolated from the wild type chromosome, but not from the mutant. In addition, the analysis of the sor regions in different A. caldus strains is depicted in Figure 1C, ISAcal1 is positioned upstream of the sor gene in the wild type A. caldus MTH-04 genome, similar to A. caldus SM-1 but different from A. caldus ATCC 51765, while the sor gene region in the A sor mutant was replaced by the IS elements.

The hypothesis that IS element transposition results in the loss of sor gene in A. caldus SM-1 has been proposed according to sequence analysis [35]. Fortunately, it was confirmed for the first time in our experiments. Firstly, the same result was obtained in the repeated experiments and no mutant was obtained when plasmid pMD19 or pSDU1 was used, which showed that deletion of sor gene was not a natural occurrence. Secondly, no artificial or exogenous IS elements was introduced into the suicide plasmid. Finally, the IS element (ISAcal1) causing the loss of sor gene could be found on A. caldus MTH-04 wild type chromosome. For above
reasons, it is clear that the transposition of IS elements in _A. caldus_ MTH-04 chromosome leads to the loss of _sor_ gene. Moreover, it also implies the role of IS elements in gene evolution and metabolic diversity in _A. caldus_

**Growth curve analysis of the wild type and the _ Δsor_ mutant**

The growth curves of the wild type and the mutant in Starkey-SO or Starkey-K2S4O6 liquid media are shown in Figure 2. When SO was utilized as the sole substrate, the cell concentration of the mutant was a little higher than the wild type in the first six days, but was slightly lower for the next six days (Figure 2A). When K2S4O6 was used as the sole substrate, the mutant had an obvious growth advantage compared to the wild and its maximum cell concentration was 70% higher than the wild (Figure 2B). The mutant still had SO oxidation ability, indicating that SOR is not the sole and determinative elemental sulfur oxidation pathway and there are other additional elemental sulfur oxidation pathways in _A. caldus_. In addition, the maximum value of OD600 of _A. caldus_ in SO medium in Figure 2A could reach about 0.35 much higher than that in K2S4O6 medium (OD600=0.065), so it is suggested that _A. caldus_ has a high efficient elemental sulfur oxidizing ability to enable it grow well.

**Comparative transcriptome analysis**

Hybridization scheme, microarray experiments, and data analysis are described in materials and methods. Unsupervised hierarchical cluster analysis of sulfur oxidation genes are depicted in Figure 3. It shows the normalized transcription levels of sulfur oxidation genes for each sample compared to the common reference. Several remarkable differences are revealed in this figure: firstly, there was a bright green region related to the genes in the sox operons of the wild but a red color region of the mutant when K2S4O6 was applied; secondly, the bright green color appeared in the row of _sor_ gene of the mutant was resulted from the deletion of _sor_, moreover the _sor_ gene of the wild displayed red color in K2S4O6 medium and green color in SO medium, indicating the higher expression level of _sor_ in _K2S4O6_ medium; thirdly, the green region at the lower right corner of the figure indicated that genes involving _rhd, dsvE, and tusA_ had lower expression levels in the mutant when K2S4O6 was applied.

The results of significance analysis of microarrays (SAM) for sulfur oxidation genes under four cases are shown in Table 1 (the numbers without brackets). The expression patterns observed with microarrays were validated by using real-time quantitative RT-PCR (qRT-PCR) for some sulfur oxidation genes (Table 1, the numbers in the brackets). The results from microarrays and qRT-PCR were highly consistent. Genes that met the criterions of fold change ≥1.3 with q-value ≤0.05 and fold change ≤0.67 with q-value ≤0.05 were up-regulated and down-regulated, respectively. The expression of sulfur oxidation genes showed distinct differences under the four experimental cases (Table 1). In the first case, the comparison was between the mutant and the wild using SO as sulfur source shown in the column of MT/WT (SO). No obvious difference was found in the expression levels of sulfur oxidation genes except that the expression of _sor_ was not detected in the mutant. The results implied that deletion of _sor_ had no obvious influence on the other sulfur oxidation pathways in SO.
medium at the early phase. In the second case, K2S4O6 was used as sulfur source to analyze the differential gene expressions between the mutant and the wild. The loss of sor led to significant gene expression changes in K2S4O6 medium, in particular: (1) the remarkable up-regulation of genes in the two sox operons; (2) the significant up-regulation of genes in tetrathionate hydrolase operon (tetH and doxD); (3) the obvious down-regulation of sulfur transferase genes, such as dsrE (ACAL_2473), tusC (ACAL_2474), and rhl (ACAL_2475, ACAL_2860). The results showed that deletion of sor stimulated the up-regulation of the sox pathway and the S4I pathway in K2S4O6 medium. In the third case, the effect of different sulfur sources on the gene expression of the wild type was showed in the column of S0/K2S4O6 (WT). Sulfur oxidation genes of the two sox operons, tetrathionate hydrolase operon, and heterodisulfide reductase complex operon had higher expression levels of varied degrees when S0 was added, in contrast, sor had a lower expression level. Therefore, the lower expression level of sor gene at the early fast growth phase in S0 medium and the better growth of the mutant at the early days in S0 medium implied that SOR does not play an important role at the early stage of the elemental sulfur oxidation process. The fourth case showed the differential gene expressions of the mutant in the two kinds of media. An obvious change compared to the third case was that the majority of sulfur oxidation genes of sox operons and tetrathionate hydrolase operon had higher expression levels in K2S4O6 medium than that in S0 medium. From above results together with the following facts that the lower expression levels of these genes of the wild type in the K2S4O6 medium (Table 1, column of S0/K2S4O6 (WT)) and the no differential expression of these genes between the mutant and the wild in S0 medium (Table 1, column of MT/WT (S0)), it can be concluded that there was a significant increase of the expression levels of above genes in the mutant compared with that in the wild type in K2S4O6 medium. Above conclusion showed that deletion of SOR pathway stimulated the sox and S4I pathways in K2S4O6 medium.

Model of the sulfur oxidation system in *A. caldus*

**The role of SOR in elemental sulfur oxidation.** Our experimental data can be explained when SOR is considered a cytoplasmic enzyme oxidizing elemental sulfur in the cytoplasm without coupling with the electron transfer chain or substrate-level phosphorylation in *A. caldus*. A hypothetical model is shown in Figure 4. Sulfur atoms (S) produced from other sulfur oxidation pathways in the periplasm can be accumulated in form of polymeric sulfur (Sn), then S or Sn are transported via an unknown mechanism into the cytoplasm where they are immediately oxidized by SOR. The higher expression level of sor gene in K2S4O6 medium can be explained by cytoplasmic located SOR. K2S4O6 is much easily and quickly entering into the periplasm, where it is hydrolyzed by TetH to produce sulfur atoms and transported into the cytoplasm inducing the expression of sor. While S0 needs to be activated before being transported into the periplasm, which is slower than that of K2S4O6, resulting in the lower expression level of sor in S0 medium. The role of SOR is supported by the facts that the sor (ACAL_1435) gene that encodes a sulfur oxygenase reductase with the ability to oxidize S0, has been cloned and expressed in *E. coli* and the enzymatic activity of S0 oxidation has been detected without addition of glutathione in our laboratory (unpublished data). The location of SOR in cytoplasm is in agreement with the reports that there are no intracellular sulfur globules in *A. caldus*, whereas sulfur globules are accumulated in *A. ferrooxidans* which acks sor gene [36], and SOR is reported existed in the cytoplasm in archaea [10].

SOR is not coupled with the electron transfer chain or substrate-level phosphorylation. The obvious growth advantage of the sor mutant in the first six days in S0 medium or during the whole growth period in K2S4O6 medium (Figure 2) should be caused by the sulfur oxidation pathway shifting from SOR to other pathways coupled with the electron transfer chain. Moreover, it is not an efficient way to produce electrons for sulfur atoms to be oxidized by SOR when sulfur atoms in cytoplasm are insufficient caused by the delay of sulfur activation and transportation at the early growth stage in S0 medium. In contrast, when sulfur atoms are sufficient at the late growth stage in S0 medium, SOR oxidizes the sulfur atoms to produce other sulfur compounds, which enter other sulfur oxidation pathways in the cytoplasm to produce electrons. These could be the reason for the lowered cell growth of the sor mutant in S0 medium than the wild type after six days of cultivation (Figure 2A).

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**Figure 2. Growth curves of *A. caldus* MTH-04 and the Δsor mutant (A) in Starkey-S0 medium and (B) in Starkey-K2S4O6 medium.** Each data point represents triplicate results. The error bars indicate standard deviations. doi:10.1371/journal.pone.0039470.g002
SOR plays a central role in the cytoplasmic sulfur oxidation pathways. The possible products from the SOR catalyzed reactions are thiosulfate, sulfite and sulfide [17,37]. Thiosulfate is considered to be catalyzed by rhodanese (TST) in the cytoplasm of A. caldus according to the reasons: (1) TST widely exists in the cytoplasm of both prokaryotes and eukaryotes, and it transfers a sulfur atom from thiosulfate to thiol compounds producing sulfane sulfate (RSSH) which is used as the substrate of the heterodisulfide reductase complex (HDR), thus a cycle is formed, in which RSSH obtains a sulfur atom from thiosulfate catalyzed by TST and then RSSH is oxidized by HDR to regenerated RSH. Sulfite is toxic to the cell and needs to be oxidized rapidly. An APS (adenosine-5'-phosphosulfate) pathway for sulfite oxidation maybe exists in A. caldus which is similar to the sulfite oxidation pathway in A. ferrooxidans [23]. A putative sulfate adenylyltransferase dissimilatory-type/denylsulfate kinase (sat) gene was discovered in A. caldus MTH-04 but the APS reductase gene that catalyzed sulfite to adenosine-5'-phosphosulfate (APS) was not determined (see Figure 4). Sulfide could be converted to hydrogen sulfide and then oxidized by sulfide:quinine reductase (SQR) located in the cytoplasmic membrane. Therefore, it is presumed that SOR plays a central role in the cytoplasmic elemental sulfur oxidation in A. caldus.

Other elemental sulfur oxidation pathways. An important elemental sulfur oxidation pathway in the periplasm near the outer membrane of A. caldus is hypothesized as shown in Figure 4: the extracellular elemental sulfur (S0) is activated by thiol groups of special outer-membrane proteins and transferred into the periplasmic space as persulfide sulfur (R-SH), and then oxidized by the sulfur dioxygenase (SDO), meanwhile hydrogen sulfide is produced during the activation of S0. Sulfur dioxygenase gene (sdo) in the A. caldus MTH-04 genome was discovered, cloned and expressed in E. coli in our laboratory, and the enzymatic activity of SDO was detected with the addition of glutathione (data to be published). Although several outer-membrane proteins related to sulfur oxidation in A. ferrooxidans have been reported, the thiol-bearing membrane proteins are not yet identified [39,40]. Another proposed elemental sulfur oxidation pathway locating in the periplasm near the inner membrane is shown in Figure 4: heterodisulfide reductase (HDR) catalyzes sulfuric sulfane (RSSH) to produce sulfite and regenerate RSH. Two subunits genes (hdrB and hdrC) of HDR were found in the draft genome sequence of A. caldus MTH-04 but all genes (hdrA, hdrB and hdrC) of HDR were found in A. caldus ATCC 51756 [31].

In summary, there are three elemental sulfur oxidation pathways in A. caldus: (1) the pathway based on SDO in the periplasm; (2) the pathway based on the HDR in cytoplasm near the inner membrane; and (3) the pathway based on the SOR in the cytoplasm.

The Sox pathway. The truncated Sox pathway in A. caldus MTH-04 contains two sox gene clusters, sox operon I (soxX-soxV-soxZ-soxA-hyp-soxB) and sox operon II (soxB-sox-ycdC-hyp-hyp-soxY-soxZ-soxA-hyp-resB-soxX-soxA-resC). There is also a strong connection between the expression of the sox genes and the terminal oxidase genes. As shown in the columns MT/WT (K2S3O8) and S0/K2S3O8 (WT) in Table 1, the expression of terminal oxidase genes, especially the cytochrome bo2 ubiquinol oxidase genes, upregulated significantly when sox genes had high expression levels. The well-studied Sox pathway in P. pantotrophus demonstrated that it couples the sulfur oxidation with the electron transfer [8]. Besides, the sox operon II together with a bo2 ubiquinol oxidase operon make up a big gene cluster on the chromosome, which implies the feasible regulation and control on their transcriptional level. The hypothetical pathway is shown in Figure 4: electrons produced from the Sox system via QH2 were transferred to the terminal oxidases (bd and bo3) and the NADH showed that the expressions of the two rhd genes down-regulated when sor was deleted. Consequently, a hypothetical thiosulfate oxidation pathway was put forward and shown in Figure 4: the thiol proteins (RSH) in the cytoplasm of A. caldus can be used as sulfur atom acceptors for the catalysis of thiosulfate by TST, producing sulfane sulfate (RSSH) which is used as the substrate of the heterodisulfide reductase complex (HDR), thus a cycle is formed, in which RSSH obtains a sulfur atom to form RSH catalyzed by TST and then RSH is oxidized by HDR to regenerate RSH. Sulfite is toxic to the cell and needs to be oxidized rapidly. An APS (adenosine-5'-phosphosulfate) pathway for sulfite oxidation may exist in A. caldus which is similar to the sulfite oxidation pathway in A. ferrooxidans [23]. A putative sulfate adenylyltransferase dissimilatory-type/denylsulfate kinase (sat) gene was discovered in A. caldus MTH-04 but the APS reductase gene that catalyzed sulfite to adenosine-5'-phosphosulfate (APS) was not determined (see Figure 4). Sulfide could be converted to hydrogen sulfide and then oxidized by sulfide:quinine reductase (SQR) located in the cytoplasmic membrane. Therefore, it is presumed that SOR plays a central role in the cytoplasmic elemental sulfur oxidation in A. caldus.

Figure 3. Hierarchical cluster analysis of genes involved in sulfur oxidation. The signals are shown in a red-green color scale, where red represents higher expression and green represents lower expression. Each column stands for a sample and each row stands for a gene.

doi:10.1371/journal.pone.0039470.g003

Acidithiobacillus caldus Sulfur Oxidation Model
| ID      | Gene                      | Function                                      | MT/WT (S⁰) | MT/WT (K₂S₄O₆) | S⁰/K₂S₄O₆ (WT) | S⁰/K₂S₄O₆ (MT) |
|---------|---------------------------|-----------------------------------------------|------------|-----------------|----------------|----------------|
| Sox operon I |                           |                                               |            |                 |                |                |
| ACAL_2486 | soxX                      | cytochrome c class I                          | NC         | 3.86±0.01       | 2.19±0.03      | NC             |
|          | ACAL_2487 | soxY                  | sulfur covalently binding protein              | NC         | 12.92±0.08      | 10.89±0.01     | NC             |
|          | ACAL_2488 | soxZ                  | Sulfur compound chelating protein              | NC         | 1.96±0.06       | 0.43±0.07      | NC             |
|          | ACAL_2489 | soxA                  | cytochrome c (diheme)                         | NC         | 13.68±0.14      | 4.35±0.02      | NC             |
|          | ACAL_2491 | soxB                  | sulfite thiol esterase                         | NC         | 10.64±0.13      | 0.09±0.09      | NC             |
| Sox operon II and cytochrome bo |               |                                               |            |                 |                |                |
| ACAL_2515 | soxB                      | cytochrome c oxidase, subunit II              | NC         | 5.40±0.01       | 18.36±0.02     | 3.65±0.02      |
|          | ACAL_2516 | cox                   | cytochrome c oxidase                          | NC         | 22.26±0.01      | 53.67±0.05     | 2.36±0.03      |
|          | ACAL_2517 | cyoC                  | cytochrome quinol oxidase subunit 3           | NC         | 9.61±0.01       | 25.37±0.02     | 2.32±0.01      |
|          | ACAL_2518 | hyp                   | hypothetical protein                           | NC         | 11.24±0.01      | 33.64±0.01     | 2.34±0.01      |
|          | ACAL_2519 | hyp                   | hypothetical protein                           | NC         | 13.93±0.05      | 10.40±0.01     | NC             |
|          | ACAL_2520 | soxY                  | sulfur covalently binding protein              | NC         | 29.24±0.04      | 29.98±0.06     | NC             |
|          | ACAL_2521 | soxZ                  | sulfite thiol esterase                         | NC         | 17.12±0.03      | 21.94±0.01     | NC             |
|          | ACAL_2522 | soxB                  | sulfite thiol esterase                         | NC         | 16.66±0.03      | 21.80±0.01     | NC             |
|          | ACAL_2523 | hyp                   | hypothetical protein                           | NC         | 31.41±0.07      | 24.64±0.02     | NC             |
|          | ACAL_2524 | resB                  | cytochrome c-type maturation protein           | NC         | 7.04±0.02       | 5.36±0.02      | NC             |
|          | ACAL_2525 | soxY                  | cytochrome c class I                           | NC         | 10.06±0.21      | 17.66±0.01     | NC             |
|          | ACAL_2526 | soxA                  | cytochrome c (diheme)                         | NC         | 22.44±0.02      | 32.03±0.01     | NC             |
|          | ACAL_2527 | resC                  | cytochrome c-type maturation protein           | NC         | 17.33±0.04      | 19.03±0.03     | NC             |
| Tetrasulfonate hydrolase operon |               |                                               |            |                 |                |                |
| ACAL_1013 | tetH                      | tetrionate hydrolase                          | NC         | 57.02±0.02      | 7.73±0.05      | NC             |
|          | ACAL_1014 | dsoD                  | Thiosulfate oxidoreductase subunit             | NC         | 18.41±2.74      | 8.91±0.02      | NC             |
| Sulfur oxygenase reductase |               |                                               |            |                 |                |                |
| ACAL_1435 | sor                       | sulfur oxygenase reductase                     | NC         | 2.64±0.04       | 2.24±0.01      | NC             |
|          | ACAL_1436 | sqr                   | sulfide quinone reductase                      | NC         | 2.66±0.06       | 2.24±0.01      | NC             |
|          | ACAL_2678 | sqr                   | sulfide quinone reductase                      | NC         | 0.83±0.01       | NC             | NC             |
| Heterodisulfide reductase complex operon |               |                                               |            |                 |                |                |
| ACAL_1042 | hdcC                      | heterodisulfide reductase subunit C            | NC         | 0.65±0.06       | 3.77±0.10      | NC             |
|          | ACAL_1043 | hdb                   | heterodisulfide reductase subunit B            | NC         | 0.65±0.06       | 3.77±0.10      | NC             |
|          | ACAL_2473 | dxsE                  | hypothetical protein (sulfur transference)    | NC         | 0.20±0.01       | 0.17±0.06      | NC             |
|          | ACAL_2474 | tsaA                  | hypothetical protein (sulfur transference)    | NC         | 0.07±0.01       | 0.06±0.01      | NC             |
|          | ACAL_2475 | rhd                   | rhodanese (sulfur transference)                | NC         | 0.21±0.04       | 0.56±0.04      | NC             |
| Rhodanese (sulfur transference) |               |                                               |            |                 |                |                |
| ACAL_0894 | rhd                       | rhodanese (sulfur transference)                | NC         | 1.27±0.01       | NC             |
|          | ACAL_1407 | rhd                   | rhodanese (sulfur transference)                | NC         | 1.27±0.01       | NC             |
|          | ACAL_2860 | rhd                   | rhodanese (sulfur transference)                | NC         | 0.16±0.05       | 0.50±0.05      | NC             |
| Cytochrome bd ubiquinol oxidase |               |                                               |            |                 |                |                |
| ACAL_0179 | cydA                      | cytochrome d ubiquinol oxidase, subunit I      | NC         | 0.59±0.05       | NC             |
|          | ACAL_0180 | cydB                  | cytochrome d ubiquinol oxidase, subunit II     | NC         | 2.09±0.15       | NC             |
|          | ACAL_1110 | cydB                  | cytochrome d ubiquinol oxidase, subunit II     | NC         | 0.61±0.01       | NC             |
|          | ACAL_1111 | cydA                  | cytochrome d ubiquinol oxidase, subunit I      | NC         | 6.25±0.01       | NC             |
|          | ACAL_1252 | cydB                  | cytochrome d ubiquinol oxidase, subunit II     | NC         | 2.42±0.14       | NC             |
| ID      | Gene | Function                                      | MT/WT (S<sub>0</sub>) | MT/WT (K<sub>2</sub>S<sub>4</sub>O<sub>6</sub>) | S<sub>0</sub>/K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> (WT) | S<sub>0</sub>/K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> (MT) |
|---------|------|-----------------------------------------------|-----------------------|-----------------------------------------------|------------------------------------------------|------------------------------------------------|
| ACAL_1253 | cydA | cytochrome d ubiquinol oxidase, subunit I     | NC                    | 1.86±0.15                                      | NC                                             | NC                                             |
| ACAL_2185 | cydA | cytochrome d ubiquinol oxidase, subunit I     | UD                    | UD                                             | UD                                             | UD                                             |
| ACAL_2186 | cydB | cytochrome d ubiquinol oxidase, subunit II    | UD                    | UD                                             | UD                                             | UD                                             |
| ACAL_2017 | cydB | cytochrome d ubiquinol oxidase, subunit II    | NC                    | 2.09±0.04                                      | 2.02±0.02                                      | NC                                             |
|        |      | Cytochrome bo₃ ubiquinol oxidase              |                       |                                                |                                                |                                                |
| ACAL_1757 | cyaB | cytochrome o ubiquinol oxidase, subunit II    | NC                    | 3.31±0.01                                      | 9.61±0.01                                      | 2.79±0.01                                      |
| ACAL_1758 | hyp  | hypothetical protein                         | NC                    | NC                                             | 3.33±0.11                                      | 3.69±0.02                                      |
| ACAL_1759 | cyaA | cytochrome o ubiquinol oxidase, subunit I    | NC                    | 15.28±0.01                                     | 30.69±0.00                                     | 1.76±0.01                                      |
| ACAL_1760 | cyaC | cytochrome o ubiquinol oxidase, subunit III  | 0.73±0.08                         | 2.16±0.01                                      | 9.27±0.00                                      | 3.11±0.01                                      |
| ACAL_1761 | hyp  | hypothetical protein                         | 0.74±0.05                         | 5.96±0.00                                      | 21.87±0.00                                     | 2.71±0.02                                      |
| ACAL_1762 | cyoD | cytochrome o ubiquinol oxidase, subunit IV    | NC                    | 10.49±0.00                                     | 25.81±0.00                                     | 2.40±0.01                                      |
|        |      | Cytochrome c protein                         |                       |                                                |                                                |                                                |
| ACAL_0446 | resC | cytochrome c-type maturation protein          | NC                    | 20.67±0.21                                     | 5.34±0.00                                      | 0.24±0.21                                      |
| ACAL_1072 | resC | cytochrome c-type maturation protein          | NC                    | 17.35±0.21                                     | 2.54±0.01                                      | 0.13±0.09                                      |
|        |      | NADH complex I operon                        |                       |                                                |                                                |                                                |
| ACAL_0727 | nuaA | NADH ubiquinone oxidoreductase A subunit      | NC                    | NC                                             | NC                                             | NC                                             |
| ACAL_0728 | nubB | NADH ubiquinone oxidoreductase B subunit      | NC                    | 0.61±0.08                                      | 0.67±0.01                                      | NC                                             |
| ACAL_0729 | nubC | NADH ubiquinone oxidoreductase C subunit      | NC                    | 0.43±0.06                                      | 0.35±0.03                                      | NC                                             |
| ACAL_0730 | nudD | NADH ubiquinone oxidoreductase D subunit      | NC                    | 0.60±0.04                                      | NC                                             | 1.79±0.03                                      |
| ACAL_0731 | nueE | NADH ubiquinone oxidoreductase E subunit      | NC                    | 0.71±0.00                                      | NC                                             | 1.57±0.01                                      |
| ACAL_0732 | nufF | NADH ubiquinone oxidoreductase F subunit      | NC                    | 2.15±0.02                                      | 2.88±0.01                                      | NC                                             |
| ACAL_0733 | nugG | NADH ubiquinone oxidoreductase G subunit      | NC                    | 3.66±0.03                                      | 3.68±0.11                                      | NC                                             |
| ACAL_0734 | nuhH | NADH ubiquinone oxidoreductase H subunit      | NC                    | 3.35±0.11                                      | 3.15±0.10                                      | NC                                             |
| ACAL_0735 | nuol | NADH ubiquinone oxidoreductase L subunit      | NC                    | NC                                             | 0.38±0.54                                      | ND                                             |
| ACAL_0736 | nuom | NADH ubiquinone oxidoreductase K subunit      | NC                    | NC                                             | NC                                             | NC                                             |
| ACAL_0737 | nuoJ | NADH ubiquinone oxidoreductase J subunit      | NC                    | NC                                             | 1.88±0.04                                      | 1.35±0.02                                      |
| ACAL_0738 | nuol | NADH ubiquinone oxidoreductase L subunit      | NC                    | NC                                             | NC                                             | ND                                             |
| ACAL_0739 | nuom | NADH ubiquinone oxidoreductase M subunit      | ND                    | ND                                             | ND                                             | ND                                             |
| ACAL_0740 | nuon | NADH ubiquinone oxidoreductase N subunit      | NC                    | 6.29±0.15                                      | 5.53±0.02                                      | NC                                             |
|        |      | ATP synthetase complex operon                |                       |                                                |                                                |                                                |
| ACAL_2147 | atpB | ATP synthase F0, A subunit                   | NC                    | 0.34±0.60                                      | 0.26±0.55                                      | NC                                             |
| ACAL_2148 | atpE | ATP synthase F0, C subunit                   | NC                    | NC                                             | NC                                             | NC                                             |
| ACAL_2149 | atpF | ATP synthase F0, B subunit                   | NC                    | NC                                             | NC                                             | 1.53±0.01                                      |
| ACAL_2150 | atpH | ATP synthase F1, delta subunit               | NC                    | 0.52±0.48                                      | NC                                             | 1.67±0.01                                      |
complex to produce ATP and NADPH, respectively. However, an important question is raised as to how the truncated Sox system works in *A. caldus* in the absence of SoxCD. Two possible ways to regenerate SoxYZ are put forward. One way is that the sulfur atom of the sulfane intermediate (SoxYZ–S–S\(^2\)) is dropped from SoxYZ, which has been reported in other bacteria [10,41,42]. Another way is that the sulfur atom of the sulfane intermediate is oxidized by sulfur dioxygenase (SDO), because SoxY is actually a kind of thiol-bearing protein that may be used as an oxidation substrate of SDO. In addition, SoxY is presumed to play a role in the activation of S\(_8\) for the reasons that elemental sulfur (S\(_8\)) assembled on the thiol of SoxY via nonenzymatic conjugation has been reported [10,42], and the expression of soxY gene of the wild type is much higher in S\(_0\) medium than that in K\(_2\)S\(_4\)O\(_6\) medium (Table 1). Therefore, the Sox system in *A. caldus* has capabilities to oxidize a wide range of sulfur compounds including sulfite, thiosulfate, sulfide and elemental sulfur and couple the process

![Figure 4. Model of sulfur oxidation in *A. caldus*.](image-url)

Table 1. Cont.

| ID     | Gene | Function                  | MT/WT (S\(_0\)) | MT/WT (K\(_2\)S\(_4\)O\(_6\)) | S\(_0\)/K\(_2\)S\(_4\)O\(_6\) (WT) | S\(_0\)/K\(_2\)S\(_4\)O\(_6\) (MT) |
|--------|------|---------------------------|-----------------|-----------------|-------------------------------|---------------------------------|
| ACAL_2151 | atpA | ATP synthase F1, alpha subunit | 0.74±0.01 | NC               | 2.49±0.23                    | 1.37±0.01                      |
| ACAL_2152 | atpG | ATP synthase F1, gamma subunit | 0.79±0.01 | NC               | 1.54±0.19                    | NC                             |
| ACAL_2153 | atpD | ATP synthase F1, beta subunit | NC             | NC               | 2.59±0.15                    | 1.94±0.05                      |
| ACAL_2154 | atpC | ATP synthase F1, epsilon subunit | NC             | 4.94±0.03        | 4.56±0.12                    | NC                             |

Fold Change ≥1.5, q-value≤0.05: up-regulation, Fold Change ≤0.667, q-value≤0.05: down-regulation; NC: not credible, q-value>0.05; UD: undetected.

Numbers without brackets from microarrays; numbers in brackets from qRT-PCR.

doi:10.1371/journal.pone.0039470.t001

Figure 4. Model of sulfur oxidation in *A. caldus*. The sulfur oxidation system involves varied sulfur oxidation pathways and the electron transfer system in different cellular compartments. Starting from the extracellular elemental sulfur (S\(_8\)), it is activated and transported into the periplasmic space as persulfide sulfur (R-SH), and then oxidized by the sulfur dioxygenase (SDO) to produce SO\(_3\)^2\(^{-}\); SO\(_3\)^2\(^{-}\) can enter into Sox pathway or combine with sulfur atoms to form S\(_2\)O\(_3\)^2\(^{-}\) via a nonenzymatic reaction; S\(_2\)O\(_3\)^2\(^{-}\) has two destinies, one is to be oxidized by the Sox pathway, the other is to form S\(_2\)O\(_3\)^2\(^{-}\) by thiosulfate quinone oxidoreductase (TQO); S\(_2\)O\(_3\)^2\(^{-}\) is hydrolyzed by tetrasulfonate hydrolase (Teth) producing S\(_2\)O\(_3\)\(^{-}\), SO\(_3\)\(^{-}\), and S\(_8\); produced from hydrolysis of S\(_2\)O\(_3\)\(^{-}\), oxidation of H\(_2\)S by sulfide quinone reductase (SQR) or from truncated oxidation of S\(_2\)O\(_3\)\(^{-}\) by the Sox pathway can be accumulated in the form of polymeric sulfur (S\(_n\)) in the periplasm and transferred into the cytoplasm; the cytoplasmic elemental sulfur (S\(_8\)) is oxidized by sulfur oxygenase reductase (SOR) producing S\(_2\)O\(_3\)\(^{-}\), SO\(_3\)\(^{-}\), and H\(_2\)S, which stimulate the cytoplasmic sulfur pathways including the metabolism of S\(_2\)O\(_3\)\(^{-}\) by rhodanese (TST) and heterodisulfidereductase (HDR) and the oxidation of SO\(_3\)^2\(^{-}\) via the APS pathway. Two methods of SoxYZ regeneration are proposed, with one being the sulfur atom is provided from the sulfane intermediate (SoxYZ–S–S\(^2\)) and the other being oxidation of SoxYZ–S–S\(^2\) by SDO to complete the Sox sulfur oxidation pathway. Electrons from SQR, TQO, HDR and SoxAX are mediated by the quinol pool in the inner membrane, then are utilized by terminal oxidases bd or bo to produce a proton gradient to generate ATP or by the NADH complex I to generate reducing power.

doi:10.1371/journal.pone.0039470.g004
with electron transfer, which make the Sox pathway play a central role in the periplasmic sulfur oxidation system.

The S, I pathway. The reaction cycle linking the hydrolysis of tetrathionate and the oxidation of thiosulphate is an important sulfur oxidation pathway in the periplasm of *A. caldus*. Tetrathionate hydrolyase (TetH), a soluble periplasmic enzyme, is responsible for the hydrolysis of tetrathionate in *A. caldus* [43]. One of the hydrolysatases of tetrathionate catalyzed by TetH is thiosulfate, but the other products are uncertain [43,44]. The higher expression level of the *sox* gene in K_{2}S_{4}O_{6} medium than that in S^{0} medium (Table 1) implied that sulfur atoms (S) may be one of the products [44]. Thiosulfate quiname oxidoreductase (TQO), which is constituted of subunits DoxX and DoxD, catalyzes thiosulfate producing tetrathionate. The expression levels of *tetH* and *doxD* of the wild type in S^{0} medium were much higher than that in K_{2}S_{4}O_{6} medium (Table 1), suggesting that a mass of S_{4}O_{6}^{2−} and S_{2}O_{3}^{2−} were produced during elemental sulfur oxidation. Therefore, the pathways are proposed: S_{2}O_{3}^{2−} is synthesized by nonenzymatic reaction from sulfite and a sulfur atom, then oxidized by TQO, producing S_{2}O_{3}^{2−} in the cytomembrane (Figure 4). However, only *doxD* gene was found in all published *A. caldus* genomes, while *doxX* was not found. The thiosulfate oxidation process by TQO was illustrated in *A. ambivalens*: DoxD catalyzes S_{2}O_{3}^{2−} to S_{2}O_{2}^{−} and gains two electrons, meanwhile, DoxX transfers electrons to the quinine [45]. Both subunits are thought to be constituents of the terminal oxidase and the function of DoxX is to transfer electrons, so it is hypothesized that other terminal oxidase in place of DoxX combines with DoxD to constitute TQO in *A. caldus*. In addition, due to the instability of S_{2}O_{2}^{−} and the stability of S_{2}O_{3}^{2−} in the acidic environment, the produced S_{2}O_{3}^{2−} in the periplasm is rapidly oxidized by the Sox system or entered into the S_{I} pathway. For this reason, formation of S_{2}O_{3}^{2−} via the S_{I} pathway may be important for sulfur storage.

Electron transfer from sulfur oxidation to respiratory system. Little is known about the electron transfer chain in *A. caldus*, but it has been well studied in *A. ferrooxidans*. One of the electron transfer chains in *A. ferrooxidans* is that electrons from Fe (II) oxidation flow through Ccyc2 to rusticycin, and then reduce oxygen via a cytochrome aa3 complex (downhill pathway) or reduce NADH via a bc1/quinone pool/NADH complex (uphill pathway) [23,46,47]. The other one is that electrons from RISCs oxidation are transferred via the quinol pool (QH2) either to terminal oxidases bd, bo3 and aa3 to produce a proton gradient, or to NADH complex I to generate reducing power [23,46]. *A. caldus* only has the sulfur oxidation system and its genome information shows that four copies of *bd* ubiquinol oxidase genes (*cydAB*), two gene clusters encoding *bo3* ubiquinol oxidase (*coxB* and *coyc*) and a cytochrome c oxidase gene (*cox, ACAE_2516*) exist in the *A. caldus* MTH-04 draft genome. SoxAX, a c-type cytochrome complex that consists of SoxX as a diheme subunit and SoxO as a monoheme [48], can carry two electrons during the catalyzing process in the Sox pathway. The comparative transcriptome analysis showed that once the expression of genes in Sox pathway upregulated, the expression of *bd* and *bo3* oxidases genes were also upregulated (Table 1), which implied that electrons could be transported from the Sox pathway to *bd* and *bo3* oxidases in *A. caldus*. Therefore, a hypothesized electron transfer chain based on the quinol pool in *A. caldus* is depicted in Figure 4: electrons from SQO, TQO, HDR and SoxAX are transferred via the quinol pool either directly to terminal oxidases *bd* or *bo3* to produce the proton gradient to generate ATP, or directly to NADH complex I to generate reducing power. Furthermore, there are several features of the electron transfer in *A. caldus* including: (1) SoxAX connects the Sox pathway with the electron transfer chain, so that makes the Sox pathway become an important sulfur oxidation pathway for producing mass electrons; (2) Quinol terminal oxidases (*bd* and *bo3*) are the dominant terminal oxidases as more electrons are produced from S^{0} oxidation than that from Fe (II) oxidation; (3) The quinol pool (QH_{2}) located in the cytoplasmic membrane may play a regulatory role in the electron transfer chain. Overall, a powerful electron transfer and respiratory system including the quinol pool (QH_{2}), terminal oxidases and NADH complex exist in *A. caldus*.

Conclusions

The growth measurements of the wild type and the mutant, and the comparative transcriptome analysis extend our understanding of the complicated sulfur oxidation system in *A. caldus*. Several distinctive features of the sulfur oxidation system of *A. caldus* are summarized as follows: (1) The sulfur oxidation system of *A. caldus* involves the Sox subsystem, a non-Sox sulfur subsystem similar to that in *A. ferrooxidans*, and SOR which work together to complete the oxidation of elemental sulfur and various sulfur compounds, so that *A. caldus* has a growth advantage over any other bacteria in the bio-mining reactor [33]. (2) Regional division of the sulfur oxidation system in *A. caldus* is an obvious characteristic. The first region is the outer membrane of the periplasm where the extracellular elemental sulfur (S_{0}) is activated and transported into the periplasmic space as persulphide sulfur (R-SH). The second region is the periplasm where the Sox pathway, SDO and TetH perform their functions, and this is an important place for sulfur oxidation. Moreover, nonenzymatic reactions producing thiosulfate from sulfite and sulfur atoms and forming polysulfur (S_{n}) are complementary to the periplasmic sulfur oxidation system. The third region is the cytoplasmic membrane involving SQO, TQO and HDR with a common feature of coupling sulfur oxidation with electron transport. The fourth region is the cytoplasm with an SOR-based sulfur oxidation system. (3) There are complex controls on the sulfur oxidation process. On one hand, the sulfur oxidation related genes belong to different pathways regulated at the transcriptional level to adapt to the production and consumption of various sulfur compounds during the process of elemental sulfur oxidation. On the other hand, metabolic control on the substrate level may be an important regulatory method, as the poly-sulfur (S_{n}) and poly-thionates (S_{2}O_{3}^{2−}) accumulated in the periplasm could be the main forms for energy storage.

In summary, an integrated sulfur oxidation model of *A. caldus* is proposed based on comparative transcriptomic analysis, which provides new insights and guides for the future study of the sulfur oxidation metabolism. In view of the diversity of the RISCs and the complexity of the sulfur oxidation system of *A. caldus*, many fundamental questions such as identification of sulfur oxidation genes and determination of enzyme activities remain to be resolved. Fortunately, the establishment of genetic manipulation of *A. caldus* provides effective and powerful tools for elucidation of the sulfur oxidation mechanism of *A. caldus* [32,49,50].

Methods

Bacteria and culture conditions

The bacterial strains and plasmids used are listed in Table 2. The media of Luria broth (LB) or agar plate for *E. coli* were described in reference [51]. Liquid Starkey-S_{0} and Starkey-K_{2}S_{4}O_{6} inorganic medium and solid Starkey-Na_{2}S_{2}O_{3} medium for cultivation of *A. caldus* MTH-04 were prepared as described in reference [52]. Elemental sulfur (S^{0}) (boiling sterilized, 20 g/L) and K_{2}S_{4}O_{6} (membrane filtration, 5 g/L) was added before
A. caldus MTH-04 was isolated from Tengchong area, Yunnan province, China [58].

A. caldus MTH-04 Δsor mutant

This study

Plasmids

pSIMP1E19 EcoR V/BAP

Ap'; lacZ'; ColE1 replicon; blunt-tailed PCR product cloning vector

TaKaRa Biotechnology Co. China

pSIMP1E1hsdMFe±Cm

Ap' Cm'; suicide plasmid containing the hsdM gene inserted by the chloromycetin resistance gene

Our laboratory

pSDU1

Cm'; IncQ; mob'

Our laboratory

pJR215-tac-sor

Sm', Km'; IncQ, Mob'; tac promoter,sor

Our laboratory

pMD19sor:±Cm

Ap' Cm'; suicide plasmid containing the sor gene inserted by the chloromycetin resistance gene

This study

doi:10.1371/journal.pone.0039470.t002

Table 2. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype or description | Source or reference |
|-------------------|------------------------|---------------------|
| A. caldus MTH-04  | Isolated from Tengchong area, Yunnan province, China | [58] |
| A. caldus MTH-04  | Δsor mutant           | This study |
| E. coli DH5a      | F-::p80d lacZ::M15T flacZYA-argF U169 end A1 recA1 hsdR17(rK- mK+ sM46) supE44 thi-1 gyr96 relA1 phoA | TransGen Biotech Corp. China |

Generation of mutant

The suicide plasmid was constructed as follows. The essential part of the suicide plasmid was amplified from pSIMP1E19hsdMFe±Cm using the primers of PMID fwd and PMID rev, then digested with Sal I and Not I. A 2.6 kb homologous sequence arm holding the sor gene was amplified from A. caldus MTH-04 chromosome using the primers of Whol fwd and Whol rev, digested with Sal I and Not I, and ligated to the essential part of the suicide plasmid. The resulting plasmid was named pMD19sorΔ, then linearized in the middle of the sor gene, using the primers of Mid fwd and Mid rev. The linear plasmid pMD19sorΔ was digested with Kpn I and Bgl II, and ligated to the chloramphenicol resistance gene (cat) amplified from plasmid pSDU1 using primers of Cat fwd and Cat rev digested with Kpn I and Bgl II. The generated plasmid carrying the mutant allele of sor disrupted by the cat was the suicide plasmid pMD19sorΔ:±Cm. The suicide plasmid was sequenced by Invitrogen Corp. for sequence confirmation. The sequences of the primers are listed in Table 3. The restriction enzymes and Prime STAR HS Taq were purchased from TaKaRa Corp. The suicide plasmid was electroporated into A. caldus MTH-04 using the methods described in the reference [49].

Colonial PCR was used to screen the mutants using the primers of Clnfwd and Cln rev. The mutants were incubated in liquid Starkey-S0 medium with bacterial loading at 10% (vol/vol). Cells were harvested at the stationary growth phase. The solid sulfur in the cultivation broth was removed by low-speed centrifugation (100 x g) before cell harvesting. Then, the cells were harvested by high-speed centrifugation (10,000 x g), washed twice using deionized distilled H2O (ddH2O), and diluted to the final inoculation. Chloromycetin (Cm) was added to the final concentrations of 34 μg/ml in LB medium, 60 μg/ml in liquid Starkey-S0 medium and solid Starkey-Na2S2O3 medium, respectively. The cultivation temperatures were 37°C for E. coli and 40°C for A. caldus MTH-04. The shaking speed for liquid cultivation of A. caldus MTH-04 was 125 r/min if not specifically stated.

Growth measurements of the wild type and the Δsor mutant

A. caldus MTH-04 and the Δsor mutant were inoculated into 150 ml of fresh Starkey-S0 medium with bacterial loading at 10% (vol/vol). Cells were harvested at the stationary growth phase. The solid sulfur in the cultivation broth was removed by low-speed centrifugation (100 x g) before cell harvesting. Then, the cells were harvested by high-speed centrifugation (10,000 x g), washed twice using deionized distilled H2O (ddH2O), and diluted to the final

| Name        | Sequence                                   |
|-------------|--------------------------------------------|
| PMID fwd    | ATTAAGATTCGGCCGCGGTATATCCGTATCCAC          |
| PMID rev    | TCCGGGAATCCCGTGCGACTAATGGTTGTACAGCGTGAAGT |
| Wholfwd     | TGCGGAATCCCGTGCGACTAATGGTTGTACAGCGTGAAGT |
| Wholrev     | ATTAAGATTCGGCCGCGGTATATCCGTATCCAC          |
| Mid fwd     | TGCGGATACCCGTCCGTATCCGTATCCGTATCCAC        |
| Mid rev     | TGGAAAGATCTTCCATCCGCTGACCAATCCGTGACCAATCC |
| Cat fwd     | CTGAGATCTTCATGGTTGACGCT                  |
| Cat rev     | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| Clnfwd      | CTGAGATCTTCATGGTTGACGCT                  |
| Cln rev     | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| Sorfwd      | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| Sorrev      | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| SorB fwd    | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| SorB rev    | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |
| Big fwd     | CGAGTCTCCGCGCGTCTGTTGACGCTGACGCTGACGCTGAC |
| Big rev     | ATCCCGTCTACCGCTCGTTGACGCTGACGCTGACGCTGAC |

doi:10.1371/journal.pone.0039470.t003

Table 3. Primers used in constructing the suicide plasmid and the mutant.

Generated by capillary blotting to positively charged Hybond-N membranes (Roche Corp.). The sor probe was obtained by incorporation of alkali-labile DIG-IUTP (Roche Corp.) during PCR elongation with primers SorAfwd and SorA rev. Hybridization was carried out under stringent conditions as recommended by the manufacturer.
concentration with OD_{600} at 1.0. An aliquot (400 μl) of the diluted cells was inoculated into 150 ml Starkey-S0 or Starkey-K2S4O6 medium and incubated at 40 °C, 125 r/min. Each condition was replicated three times and the medium without inoculation was used as the control. An aliquot (250 μl) of each sample was taken, stand for 5 minutes, and then 200 μl was taken from each of the 250 μl sample and used for OD measurement using a Microplate Spectrophotometer (Molecular Devices) at the wavelength of 600 nm. Samples were taken and measured every 24 hours until the 12th day.

**Construction of the whole-genome microarray**

An *A. caldus* genome 60 bp oligonucleotide microarray was obtained from CapitalBio Corporation (Beijing, China) [53]. Briefly, the *A. caldus* genome oligonucleotide set consisting of 5′ amino acid-modified 60 bp probes and representing 3,603 ORFs was synthesized by Biosune Corporation. The 3,603 ORFs consist of 2,972 ORFs from *A. caldus* ATCC 51756 (GenBank: ACVD00000000). Then oligonucleotides were dissolved in EasyArray™ spotting solution (CapitalBio Corp.) at 40 μM concentration, and printed in triplicate on PolymerSlide, in which the surface is covered by a thin layer of aldehyde group modified three-dimensional polymer chain (CapitalBio Corp.). On the slide, there are 48 blocks and each block has 18 columns and 15 rows.

**Design of the hybridization scheme on the gene chips**

A complex hybridization scheme was designed using two-color platforms and common reference samples to determine the levels of the differential expressions between the group pairs [53]. As shown in Table 4, there were four experimental groups: WT (S0); the wild type in S0 medium, WT (K2S4O6); the wild type in K2S4O6 medium, MT (S0); the mutant in S0 medium, and MT (K2S4O6); the mutant in K2S4O6 medium. Each group contained triplicate biological repeats, so there were totally 12 samples. An aliquot of each sample was taken, mixed and used as common reference. Samples labeled with Cy5-dCTP and common reference labeled with Cy3-dCTP were hybridized on a chip. The gene expression level in the samples was determined based on the common reference. Therefore, there were four cases based on group pairs, which were the gene differential expression in S0 medium between the mutant and the wild type (MT/WT (S0)), the gene differential expression in S0 medium between the mutant and the wild type (MT/WT (S0)), the gene differential expression in S0 medium between the mutant and the wild type (MT/WT (S0)), the gene differential expression in S0 medium between the mutant and the wild type (MT/WT (S0))
mutant and the wild type (MT/WT (K2S4O6), the gene differential expression of the wild type between S0 medium and K2S4O6 medium (S0/K2S4O6 (WT)), and the gene differential expression of the mutant between S0 medium and K2S4O6 medium (S0/K2S4O6 (MT)).

RNA isolation and microarray experiments

The wild type and theΔssr mutant were cultured as described in the section of growth measurements of the wild type and theΔssr mutant. Cells were collected at exponential growth phase (4th day), washed twice using sterile RNase-free ddH2O and treated with RNa protect Bacteria Reagent (Qiagen Corp.). Total RNA was extracted using RNasy Mini Kit (Qiagen Corp.) in accordance with the manufacturer's instructions. Total RNA was purified using Nucleospin® RNA Clean-up (MN Corp.) and with polyA using Poly (A) Polymerase (Ambion Corp.). The purified RNA was transcribed using M-MLV Reverse Transcriptase (Invitrogen Corp.) and tailed with polyA using Poly (A) Polymerase (Ambion Corp.). Cy5/cy3-labeled cDNA were hybridized with the Affymetrix Genechip at 42 °C for 16 h. Following hybridization, the arrays were washed using two consecutive solutions (0.2% SDS, 2× SSC for 5 min at room temperature). Arrays were scanned using a confocal LuxScan™ scanner and the images obtained were then analyzed using LuxScan™ software. For individual channel data extraction, faint spots with intensities below 400 units after background subtraction in both channels (Cy3 and Cy5) were removed. A space- and intensity-dependent normalization based on a LOWESS program was employed [54]. The raw data were Log 2 transformed and median centered by arrays and genes using the Adjust Data function of CLUSTER 3.0 software and then further analyzed using hierarchical clustering with average linkage [55]. To determine the significant differentially expressed genes whose ratio changes ≥1.5 fold with p≤0.05, Significance Analysis of Microarrays (SAM, version 3.02) was performed using two-class unpaired comparison in the SAM procedure [56].

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted as described above and was treated with RNase-Free DNase Set (Qiagen Corp.) to eliminate the traces of genomic DNA. The total RNA of 2 μg was reversely transcribed using M-MLV Reverse Transcriptase (Invitrogen Corp.) under the following conditions: 25°C for 10 min, 37°C for 60 min, and 70°C for 10 min. RT reaction products of 1 μl were used for PCR amplification using Power SYBR Green PCR Master Mix (Applied Biosystems). The conditions for the PCR reaction were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using a 7900 HT Fast RealTime PCR system (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase gene (gapdh) was used as the reference gene for normalization. The relative expression was calculated using the comparative ΔΔCT method, and the values were expressed as 2−ΔΔCT [57]. Primers used in this study are listed in Table 5.

Acknowledgments

The authors would like to thank Dr. Pamela Holt of Shandong University for proofreading the manuscript.

Author Contributions

Conceived and designed the experiments: LC Jianqun Lin Jianqiang Lin. Performed the experiments: LC YR. Analyzed the data: LC. Contributed reagents/materials/analysis tools: XL XP. Wrote the paper: LC. Revised the manuscript: Jianqun Lin Jianqiang Lin.

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