Chapter

Two-Component Systems in the Regulation of Sulfur and Ferrous Iron Oxidation in Acidophilic Bacteria

Lifeng Li and Zhaobao Wang

Abstract

The two-component system (TCS) is a regulatory system composed of a sensor histidine kinase (HK) and a cytoplasmic response regulator (RR), which participates in the bacterial adaptation to external stimuli. Sulfur oxidation and ferrous iron oxidation are basic energy metabolism systems for chemoautotrophic acidophilic bacteria in acid mine environments. Understanding how these bacteria perceive and respond to complex environmental stimuli offers insights into oxidization mechanisms and the potential for improved applications. In this chapter, we summarized the TCSs involved in the regulation of sulfur and ferrous iron metabolic pathways in these acidophilic bacteria. In particular, we examined the role and molecular mechanism of these TCSs in the regulation of iron and sulfur oxidation in *Acidithiobacillus* spp.. Moreover, research perspectives on TCSs in acidophilic bacteria are discussed in this section.

Keywords: *Acidithiobacillus*, two-component system, ferrous iron oxidation, sulfur oxidation, transcriptional regulation

1. Introduction

Acidithiobacillus genus is composed of high acid-tolerance chemolithotrophic bacteria that can oxidize various reduced inorganic sulfur compounds (RISCs) and ferrous iron to obtain electrons for carbon dioxide fixation and energy production [1]. The composition and comparison of the members in this genus has been reviewed [2]. As reported, the bacteria can be classified into two groups based on their energy resources: the sulfur-oxidizing-only species and the sulfur- and ferrous- oxidizing species [3]. Sulfur-oxidizing-only bacteria include *Acidithiobacillus caldus*, *Acidithiobacillus thiooxidans* and *Acidithiobacillus albertensis*, whereas sulfur- and ferrous oxidizing bacteria include *Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferrivorans*, *Acidithiobacillus ferriphilus*, and *Acidithiobacillus ferridurans*. These bacteria are widespread in the bioleaching heap and acid mine drainage water environments and play critical roles in bioleaching and wastewater treatment [3–6]. Sulfur and iron oxidation capacities are critical physiological features of these bacteria, which are also the basis for their applications. The oxidation of reduced inorganic sulfur compounds can dissolve ore and produce sulfuric acid,

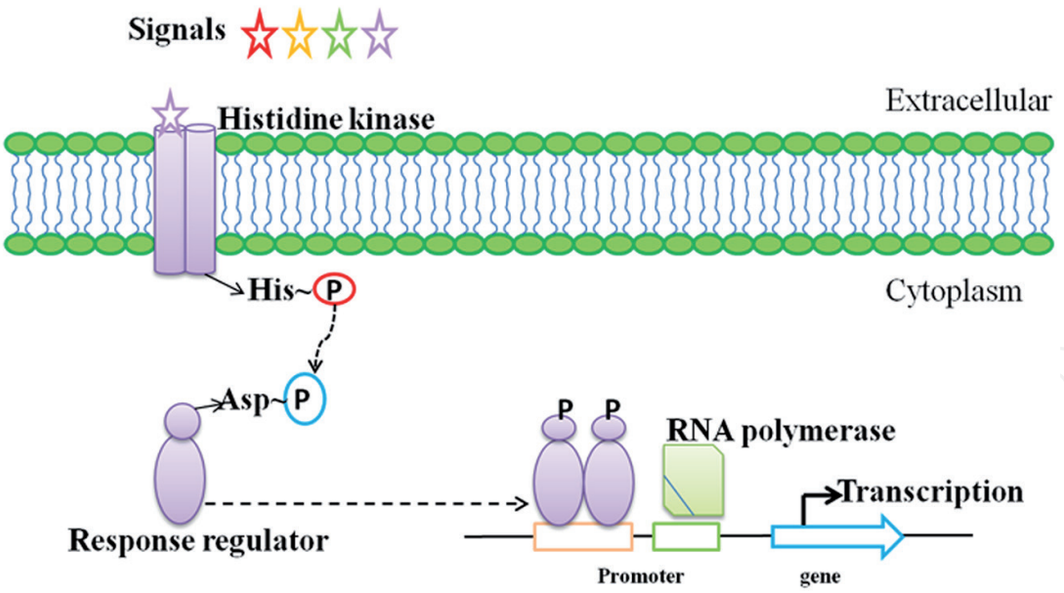

Figure 1.
Two-component system regulation mechanism.

whereas the oxidation of ferrous iron (Fe II) produces ferric iron (Fe III), in which sulfuric acid and ferric iron products can attack minerals, releasing metal ions [7]. Sulfur metabolism and iron oxidation are complicated and various metabolic genes are involved. Thus, the regulation and mechanism of the sulfur and iron oxidation in *Acidithiobacillus* spp. have drawn increasing attention.

Sensing and responding to environmental stimuli is necessary for bacteria to adjust the expression of related genes and adapt to changing habitats. The two-component systems (TCSs) are the most widespread regulation system in bacteria [8]. The TCS is mainly composed of two proteins, histidine kinase (HK) and their cognate response regulator (RR) (**Figure 1**). Histidine kinase is a membrane protein that can sense extracellular signals and autophosphorylate its histidine. The phosphorylated HK can transfer the phosphoryl group to its cognate RR protein leading to the phosphorylation of the RR protein at the aspartate residue (Asp) and the activation of RR protein. The activated RR protein is able to change its conformation by dimerization or multimerization and regulates the expression of its target genes. In general, the RR protein can regulate gene transcription by binding to specific sequences in the promoter region of related genes located upstream of the RNA polymerase binding region.

Although not completely understood, the study of molecular regulation mechanisms in acidophilic bacteria has recently been progressing. In this chapter, we discuss the occurrence of the TCS in these bacteria, the regulation mechanism of sulfur and iron oxidation, and the future prospects in the TCS regulation research.

2. Discovery of two-component system in acidophilic bacteria

The occurrence of the TCSs in the acidophilic bacteria was compared among different species on basis of the reported TspS-TspR, RsrS-RsrR, and RegB-RegA two-component systems [7, 9, 10] (**Figure 2**). The sulfur oxidization (Sox) system is a critical sulfur oxidization pathway of chemotrophic sulfur-oxidizing bacteria, and the regulation of the Sox system in *A. caldus* by the TspS-TspR two-component system has been reported [10]. Meanwhile, genome sequences were used to

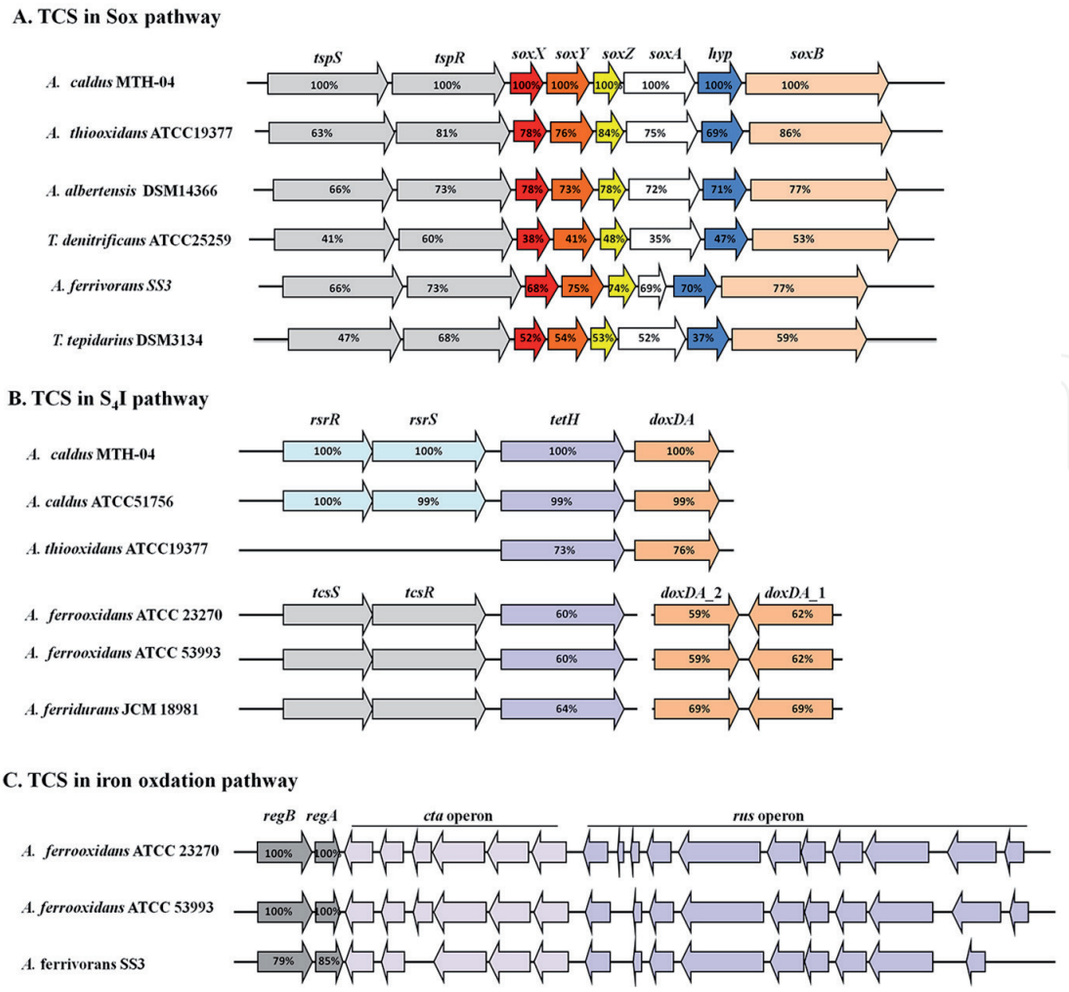

Figure 2.
Distribution of two-component system in acidophilic bacteria. The identities of corresponding protein were indicated by the percentage values with the first line of each part set as 100%. Accession numbers (GenBank) for proteins in Sox pathway are as follows, A. caldus *MTH-04, sox (A5904_11270–11305); A. thiooxidans ATCC19377, sox (ATHIO_RS0101665-RS0101630);* A. albertensis *DSM 14366, sox (BLW97_RS11430-RS11465);* A. ferrivorans *SS3, sox (Acife_2487–2494). Accession numbers (GenBank) for proteins in S_4I pathway are as follows,* A. caldus *MTH-04, RsrR (ANJ65973.1), RsrS (ANJ65974.1), TetH (OAN03451.1), DoxDA (OAN03452.1) (GenBank: MK165448);* A. caldus *ATCC 51756, RsrR (ABP38227.1), RsrS (ABP38226.1), TetH (ABP38225.1), DoxDA (ABP38224.1);* A. thiooxidans *ATCC 19377, TetH (WP_029316048.1), DoxDA (WP_010638552.1);* Acidithiobacillus ferrooxidans *ATCC 23270, TcsS (ACK79489.1), TcsR (ACK79259.1), TetH (ACK80599.1), DoxDA_2 (ACK79881.1), DoxDA_1 (ACK78481.1);* A. ferrooxidans *ATCC 53993, TcsS (ACH82290.1), TscR (ACH82291.1), TetH (ACH82292.1), DoxDA_2(ACH82311.1), DoxDA_1(ACH82307.1);* Acidithiobacillus ferridurans *JCM 18981, TcsS (BBF65177.1), TcsR (BBF65176.1), TetH (BBF65175.1), DoxDA_2 (BBF65156.1), DoxDA_1 (BBF65160.1). Accession numbers (GenBank) for proteins in iron oxidation pathway are as follows,* Acidithiobacillus ferrooxidans *ATCC 23270, AFE_RS14375-AFE_RS16285;* A. ferrooxidans *ATCC 53993, LFERR_RS13505-LFERR_RS15550;* A. ferrivorans *SS3, ACIFE_RS08920-ACIFE_RS09010.*

compare the occurrence of TCS similar to that of TspS-TspR in the *Acidithiobacillus* spp.. *tspS-tspR-sox* gene clusters were found in all the sulfur-oxidizing bacteria (*Acidithiobacillus caldus*, *Acidithiobacillus thiooxidans* and *Acidithiobacillus albertensis*), and in one type of the sulfur- and ferrous- oxidizing bacteria, *Acidithiobacillus ferrivorans*. The amino acid similarity of the TspS/TspR ranged from 63% to 81%.

The S_4I pathway is also an important thiosulfate oxidization pathway composed of tetrathionate hydrolase (TetH) and thiosulfate: quinone oxidoreductase (DoxDA), and its regulation by the RsrS-RsrR system was reported [9, 11]. However, a similar distribution of this gene cluster was only found in *A. caldus*. No regulatory system was found in *A. thiooxidans*. In contrast, a different kind of TCS

was found before the *tetH* gene in *Acidithiobacillus ferrooxidans* and *Acidithiobacillus ferridurans* with two *doxDA* genes separated in a different gene cluster.

The RegB-RegA is a well-studied global redox responding regulatory system in *A. ferrooxidans*, and plays roles in the iron and sulfur oxidization regulation [12, 13]. RegB-RegA located in the *cta* and *rus* operon, which was composed of genes in the biogenesis of aa3 type oxidase and iron oxidation pathway. Similarly, the *regB-regA-cta-rus* cluster was only found in the sulfur- and ferrous- oxidizing bacteria *A. ferrooxidans* and *A. ferrivorans* with high identity.

Hence, the TCSs are widespread in the sulfur and iron oxidization bacteria, while different distributions are revealed by bioinformatics analysis and different regulation mechanism maybe adapted, which deserves further studies.

3. Roles of two-component system in sulfur oxidation

Gene transcription is a fundamental process in bacteria, which is carried out by multi-subunit RNA polymerase (RNAP). σ factors determine transcription specificity by recognizing specific promoter sequences. Bacterial σ factors can be divided into two distinct classes: σ^{70} and σ^{54} [14]. σ^{70} recognizes the consensus −10 and − 35 regions and recruits RNAP to a specific promoter region to initiate gene transcription [15]. σ^{70} controls transcription of most housekeeping genes, whereas σ^{54} regulates the genes involved in nitrogen assimilation [16], phage shock response [17], infection [18], and other cellular stresses [19, 20]. σ^{54} recognizes distinct sequences in the −12 (GC) and − 24 (GG) regions of the promoter. The requirement of the bacterial enhancer binding proteins (bEBPs) is a remarkable feature of σ^{54}-dependent transcription initiation [20]. Accordingly, two kinds of transcription regulation were reported in acidophilic bacteria (**Figure 3**).

The *rsrS-rsrR-tetH-doxDA* gene cluster in *A. caldus* was reported in 2007 [11]. The genes in this cluster were proven to be cotranscribed using RT-PCR (Reverse transcription PCR), and the results of quantitative PCR and Western blot indicated that the gene cluster was tetrathionate induced. The promoter before *tetH* gene was mapped by primer extension. The verification of the regulation role and mechanism of the S_4I pathway by the RsrS-RsrR two component system was reported in 2016 [9]. Δ*rsrR* and Δ*rsrS* strains were constructed using a marker-less gene knockout method in *A. caldus*. The transcription levels of *rsrS*, *rsrR*, *tetH*, and *doxDA* were analyzed by RT-qPCR under the stimulation of $K_2S_4O_6$ in the wild type and two gene knockout strains, and the results indicated that the RsrS-RsrR regulated the transcription of *tetH* and *doxDA* in a $K_2S_4O_6$–dependent manner. The regulatory protein RsrR was expressed and purified to verify protein and promoter DNA binding using electrophoretic mobility shift assays (EMSAs). A 19 bp (AACACCTGTTACACCTGTT) inverted repeat sequence (IRS) was identified to be the binding motif of RsrR through EMSA and promoter probe plasmid analysis *in vitro* and *in vivo*, respectively. Hence, as summarized in **Figure 3**, the RsrS can sense the extracellular tetrathionate signal and autophosphorylated, then RsrR is activated by receiving the phosphate from RsrS, the phosphorylated RsrR dimerizes and binds on the IRS region of *tetH* operon and initiates the transcription of the genes together with the RNA polymerase.

The RR protein of TCS can function as the activator of σ^{54}-dependent transcription initiation, which converts the closed RNAP-σ^{54} holoenzyme complex to open state to initiate transcription. σ^{54} -dependent RR proteins have been reported in several bacteria [21–23]. It was reported that the two-component system TspS-TspR could regulate the sulfur oxidization (Sox) system in *Acidithiobacillus caldus* and some chemolithotrophic bacteria in a σ^{54}-dependent manner [10]. RT-PCR was used to analyze the

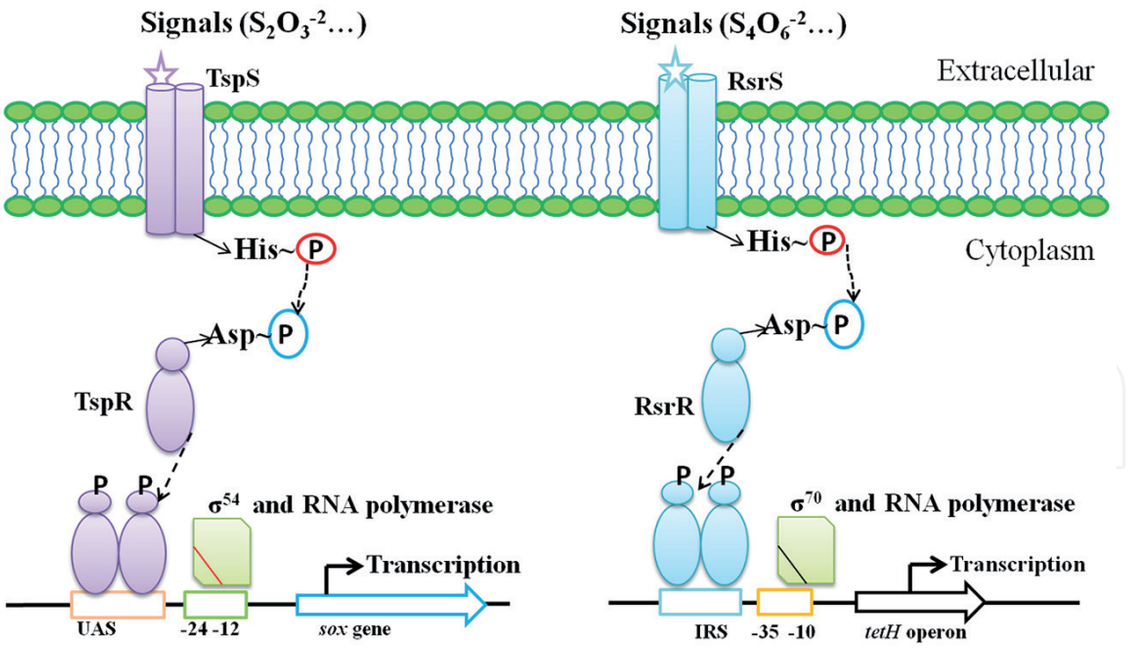

Figure 3.
Different TCS regulation mechanisms between the sox system and the S_4I pathway. Sox system and the S_4I pathway are important sulfur oxidation system in A. caldus*, and the regulation mechanism of which has been revealed. The regulation mechanism was summarized in this diagram, the left part represented the regulation mechanism of TspS-TspR on the Sox system in* A. caldus *summarized according to literature [10]. The right part was the model of RsrS-RsrR regulating the S_4I pathway in* A. caldus *[9]. The activation signals, the interaction between the regulators and key binding motifs of the promoters were showed.*

composition of the *sox* operon. Results indicated that the genes in the *sox* operon were cotranscribed whereas the transcription of *tspR* was independent. The activation of σ^{54} on the transcription of the *sox* genes was verified by the higher transcripts of the operon genes in the constructed *rpoN*-overexpression strain. Following, the transcription initiation site (TSS) of the first gene (*sox*-X) in the operon was verified using 5'RACE (Rapid amplification of cDNA ends). Upstream of the TSS (G, +1), the potential −12 (GC) and − 24 (GG) sites were also identified, which was a typical feature of the σ^{54}-dependent promoter. Promoter-probe plasmids were constructed to analyze the promoter activity in *A. caldus* by comparing the wild type (P1) and the mutated promoter (GG/GC mutated to AA, P_{12M}, P_{24M}, and $P_{12/24M}$) containing strains. Hence, the σ^{54}-dependent promoter was verified by 5'RACE and promoter-probe plasmid activity analysis. As reported, the σ^{54}-dependent transcription requires binding of the enhancer binding protein (EBP) to upstream activator sequences (UASs) to activate transcription initiation. TspR protein was then expressed and purified to analyze the binding of P1 promoter by EMSA. The binding of TspR protein with different length promoters with two different predicted UASs was analyzed, and only the promoter containing UAS_1 (TGTCCCAAATGGGACA) showed a shift lane on the native PAGE. To verify the critical sites in UAS_1, UAS_{M1} and UAS_{M2} mutants were constructed, which converted the bases TGT/ACA to GAG/GTG and changed the variable bases CCC to TTG, respectively. TGT/ACA was identified as the critical sites of UAS_1 by analyzing the activity of the wild type, UAS_{M1} and UAS_{M2} mutants. Thus, the experiments confirmed that the Sox system was regulated by the σ^{54}-dependent two-component system TspS/TspR. A signal transduction and transcriptional regulation model for the Sox system in *A. caldus* is depicted in **Figure 3**. TspS can sense the signal stimuli such as thiosulfate and other sulfur substrates, and phosphorylate at a proposed conserved His residue. TspS is activated TspR by the transferring phosphoryl group from its His residue to the conserved Asp residue of TspR. Subsequently, the activated TspR is dimerized and binds to the UAS sequence of promoter P_1, meanwhile changing the

conformation of the holoenzyme (σ^{54}-RNA polymerase), which binds to the −12/−24 region to activate the transcription of *sox* genes. Interestingly, potential −12/−24 region and UAS sequences were predicted in other bacteria with similar *tspS-tspR-sox* gene composition, which may indicate the importance of TCS in the regulation the Sox oxidation system.

4. Roles of two-component system in ferrous iron oxidation

A. ferrooxidans is an important iron and sulfur oxidizing bacterium in the *Acidithiobacillus* genus, which can oxidize Fe (II) and reduced sulfur compounds to obtain energy for growth. Compared with Fe (II), sulfur seems to be a better energy source because it can provide more ATP at the same molar level [24]. Understanding the function and regulation mechanism of the two energy production systems is critical in coordinating sulfur and iron oxidation process to avoid the S^0 deposition and improve the efficiency of bioleaching.

When *A. ferrooxidans* was cultivated in the presence of both Fe (II) and S^0 as electron donors, the Fe (II) concentration, bacterial concentration, and pH were measured along with the growth process [7]. The results indicated that ferrous iron was oxidized before S^0. The redox potential increased in the Fe (II) oxidization process, while kept stable during sulfur oxidation. Additionally, RT-qPCR analysis showed that the Fe (II) oxidization genes (*rus, cup, petC1* and *cta*) transcribed before the sulfur oxidation genes (*cyoB, hdrA, hdrC, hdrB, sqr* and *tetH*). The sensor/regulator two-component signal transducing system RegBA consisting of a redox-sensing RegB and a DNA binding RegA, located near the *rus* operon, was also studied. The recombinant RegA was produced and purified and was used in the EMSA experiments to analyze its binding with related gene promoters. The retarded bands could be detected with the regulatory regions of the Fe (II) oxidization genes (*rus, petI, cta*) and sulfur oxidation genes (*cyo, hdr, hdrB, tetH* and *sqr*), which indicated the regulation roles of RegA on these genes. As a result, an initial model of the RegBA regulation on *A. ferrooxidans* sulfur and iron oxidation was proposed. Both the full-length RegA and the DNA binding domain of RegA could bind the *rus* and *hdr* operon regulatory regions in the phosphorylated and unphosphorylated state [13]. However, the recombinant RegA tagged with six histidines had signs of aggregation and precipitation. Moinier et al. attempted to purify the RegA protein using the SUMO tag and compared its binding affinity to the target genes in four different states [12]. Similarly, different forms of RegA (DNA binding domain, wild-type, unphosphorylated and phosphorylated-like forms) are able to specifically bind to the regulatory region of the *rus, cta, petI, reg, tet, cyo, hdr,* and *sqr* genes/operons, and the binding of the target genes leads to the formation of multimeric complexes as shown by EMSA results. Further, dynamic light scattering (DLS) analysis also confirmed that 6His-SUMO-RegA protein was polydispersed according to the increased size of hydrodynamic diameters. The protein in the solution (94.6%) had a mean diameter of 7.58 nm, indicating that it was a stable dimer without target binding, whereas it multimerized in the presence of its target DNA, which was consistent with the EMSA analysis. Acetyl phosphate and amino acid mutation was used to change the phosphorylation state of RegA and all the treatments showed that the phosphorylation state of RegA had no effect on the binding affinity for the targets. TSS was determined for the iron oxidation genes (*rus, cta, petI,* and *reg* operons) and RISC oxidation genes (*tetH, cyo, hdr, hdrB, cyd, sqr* and *doxII* operons) using 5′ RACE experiments. The main promoters were σ^{70}-dependent whereas the *tetH* gene and the *cyd* operons had predicted σ^{54}-dependent promoters and the *cyd* operon also had a σ^{70}-dependent promoter. The sequences

and the downstream sequences of the RNA polymerase binding site of each gene were amplified and used in the analysis. Results indicated that RegA mainly binded upstream of the −10 (−12) /−35 (−24) region, except for the PI promoter of the *rus* operon and the *tetH* operon promoter. However, no RegA binding motifs could be found in the binding gene promoter region using several bioinformatics analysis methods. Hence, the RegBA is a global regulatory system regulating the expression of genes involved in the energy production.

Moreover, other regulatory proteins may be involved in the regulation of these genes. The transcription factor Fur was proven to control the transcription of *petI* operon by binding to the promoter region in EMSA experiments [25]. Fur may inhibit the binding of RNA polymerase and repress the transcription of *petI* in the presence of high intracellular levels of Fe (II). RegA may impede Fur binding on the regulatory region of the *petI* operon when Fe (II) is present and activate its transcription. However, the interaction between RegA and Fur requires further studies. Interestingly, the identification of σ^{54}-dependent promoters in *tetH* operon was parallel to the occurrence of a σ^{54}-dependent transcriptional response regulator and cognate histidine kinase at the upstream of *tetH* operon whereas the role of the TCS has not been verified. A σ^{54}-dependent transcriptional regulator was also predicted at the upstream of the *cyd* operon consistent with the existence of this type promoter whereas no histidine kinase was found near the gene [26].

Based on the reported results, the regulation model for RegBA two-component system is portrayed in **Figure 4**. When Fe (II) is used as the electro donor, RegB is able to sense the low potential state and activate through autophosphorylation. It then activates the RegA protein by transferring the phosphoryl group to the conserved Asp residue of RegA. Phosphorylated RegA protein multimerizes and binds to the promoter region of the target genes, which may activate iron oxidation genes by repressing the binding of other repressor proteins such as Fur for *petI* as well as

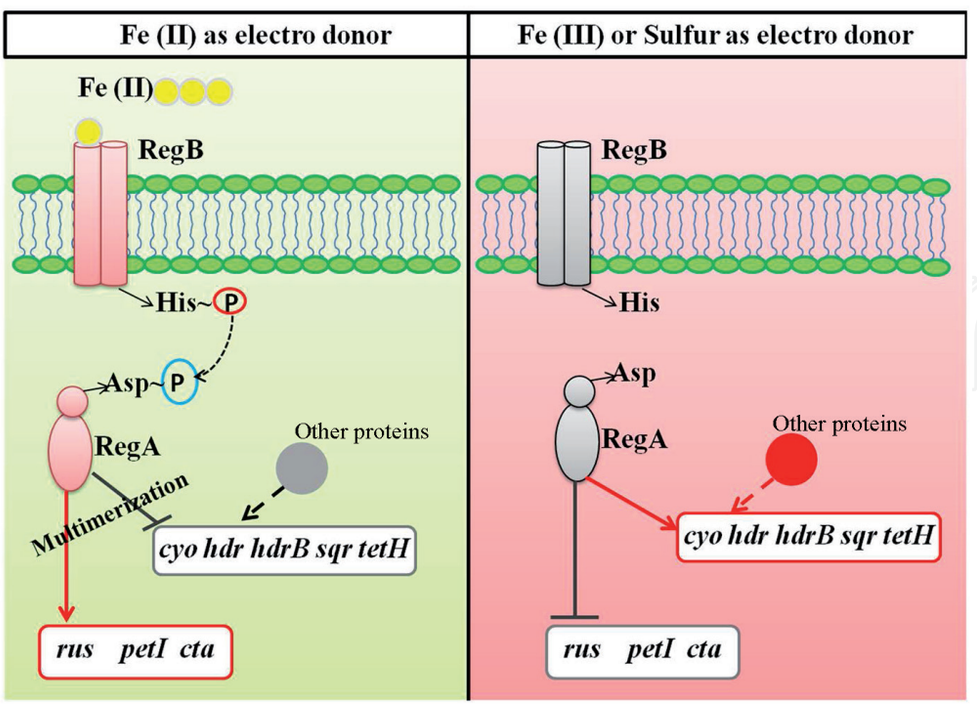

Figure 4.
Regulation of sulfur and ferrous iron oxidation by the TCS system. The regulation in A. ferrooxidans *is complicated because it can oxidize Fe (II) and reduced sulfur compounds to obtain energy for growth. Hence, the regulation in this bacterium can be divided according to the energy resources used. The left part represents the regulation mechanism when Fe is the electro donor, and the right part represents the case when sulfur is the electro donor. Several genes are involved in the regulation summarized according to the studies reported [7, 12, 13, 25, 27–29].*

repress sulfur oxidation by interaction with other activator proteins such as σ^{54}-dependent transcriptional regulator for *tetH*. In the absence of Fe (II), RegB is not activated and other proteins may act together with RegA, leading to the activation of sulfur oxidation genes and repression of the iron oxidation genes. The interaction between RegBA and other regulatory proteins should be studied further to fully understand the regulation mechanism of the iron and sulfur oxidation pathways.

5. Conclusions

Two component systems possess critical roles in the regulation of sulfur and iron oxidation in acidophilic bacteria. In the sulfur oxidizing species *A. caldus*, two typical regulatory modes were identified, the σ^{54}-dependent TCS regulation in the Sox system and the σ^{70}-dependent TCS regulation in the S_4I pathway. Meanwhile, research on the global regulatory RegBA system indicates that it could control the transcription of several important genes relevant to iron and sulfur oxidation pathways in *A. ferrooxidans*. Although it has been verified that three different two-component systems can participate the regulation of energy production processes in *Acidithiobacillus* spp., further studies are required in the following aspects: (i) the distribution of similar regulatory systems such as TspS-R, RsrS-R, and RegB-A were identified, but the verification of their regulatory roles in relative genes awaits further research; (ii) the detailed regulation mechanism of the different two-component systems in the iron oxidation and sulfur oxidation bacteria merits investigation, for example, the σ^{54}-dependent TCS in the *tetH* operon in *A. ferrooxidans*; (iii) studies should examine the interactions between the TCS systems and other regulatory proteins to understand the concrete mechanism of energy regulation in *Acidithiobacillus* spp.; (iv) studies should identify the signaling molecules and reveal the interaction between the signals and the response proteins; (v) the structural studies of the TCSs in *Acidithiobacillus* spp. await further research. Therefore, studies on important TCSs in acidophilic bacteria will benefit the understanding of the mechanisms of their environmental adaption and growth as well as facilitate applications that take advantage their special properties.

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Conflict of interests

The authors declare no conflict of interests.

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