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The substrate-dependent regulatory effects of the AfeI/R system in *Acidithiobacillus ferrooxidans* reveals the novel regulation strategy of quorum sensing in acidophiles

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Summary

A LuxI/R-like quorum sensing (QS) system (AfeI/R) has been reported in the acidophilic and chemoautotrophic *Acidithiobacillus* spp. However, the function of AfeI/R remains unclear because of the difficulties in the genetic manipulation of these bacteria. Here, we constructed different *afeI* mutants of the sulfur- and iron-oxidizer *A. ferrooxidans*, identified the N-acyl homoserine lactones (acyl-HSLs) synthesized by AfeI, and determined the regulatory effects of AfeI/R on genes expression, extracellular polymeric substance synthesis, energy metabolism, cell growth and population density of *A. ferrooxidans* in different energy substrates. Acyl-HSLs-mediated distinct regulation strategies were employed to influence bacterial metabolism and cell growth of *A. ferrooxidans* cultivated in either sulfur or ferrous iron. Based on these findings, an energy-substrate-dependent regulation mode of AfeI/R in *A. ferrooxidans* was illuminated that AfeI/R could produce different types of acyl-HSLs and employ specific acyl-HSLs to regulate specific genes in response to different energy substrates. The discovery of the AfeI/R-mediated substrate-dependent regulatory mode expands our knowledge on the function of QS system in the chemoautotrophic sulfur- and ferrous iron-oxidizing bacteria, and provides new insights in understanding energy metabolism modulation, population control, bacteria-driven bioleaching process, and the coevolution between the acidophiles and their acidic habitats.

Introduction

Acidophiles, a class of important extremophiles and geomicrobes, are widely distributed in the hot springs and acid mines. In these natural habitats, the chemoautotrophic acidophiles participate in the global element cycles of sulfur and iron via the oxidation of reduced inorganic sulfur compounds (RISCs) to sulfate and the conversions between ferrous and ferric ions (Menzel et al., 2015; Quatrini and Johnson, 2016). The acidophile-driven bioleaching process has given rise to a worldwide problem of water and solid contaminations in natural and man-made mine environments (Chen et al., 2014). On the other hand, this process has been advantageously utilized in the biomining industry for the recovery of valuable metals from sulfide ores, such as copper or gold (Rawlings, 1998; Chen et al., 2014). Thus, the researches on the bioleaching microbes and their metabolism and regulation mechanisms are of significance for the treatments of acid mine contaminations and the development of high-efficient biological metallurgy technology. *Acidithiobacillus* spp., a group of acidophilic chemolithoautotrophic Gram-negative bacteria, are prevalent in the sulfur- and ferrous iron-contained acidic ecosystems, and are the predominant player in acidophile communities structures in acid mine drainages (AMD) and terrestrial hot springs (Rawlings, 1998; Liljeqvist et al., 2015; Quatrini and Johnson, 2018). They
are the most active and wide-used bioleaching bacteria in the biomining industry (Olson et al., 2003; Rohwerder et al., 2003). All Acidithiobacillus strains are capable of oxidizing various RISCs for autotrophic growth, and some of them can use ferrous iron as an energy substrate (Bosecker, 1997; Rohwerder et al., 2003). Seven species have been identified in the Acidithiobacillus genus, including four sulfur- and ferrous iron-oxidizing species (A. ferrooxidans, A. ferridurans, A. ferriphilus and A. ferrivorans), and three sulfur-oxidizing-only species (A. thiooxidans, A. caldus and A. albertensis).

Acidithiobacillus. ferrooxidans has become an important model bacterium for the researches of the acidophilic bacteria on physiological biochemistry, molecular biology, microbial mineralogy and so on (Sugio et al., 1987; Rawlings, 2002). It can gain energy by the oxidation of ferrous iron and reduced sulfur compounds at the aerobic condition, and can also obtain energy via anaerobic metabolisms including the oxidation of sulfur and hydrogen by using ferric iron as an electron acceptor and the oxidation of hydrogen by using sulfur as electron acceptor (Ohmura et al., 2002). For the variety of RISCs, the sulfur metabolism is achieved by different kinds of enzymes located in different cellular compartments in A. ferrooxidans, such as thiosulfate dehydrogenase, thiosulfate oxidoreductase, tetrathionate hydrolase (TetH) in periplasmic space; persulfide dioxygenase (formerly named as sulfur dioxygenase, SDO), HDR, Hdr-like complex in the cytoplasm; and sulfide:quinone oxidoreductase (SQR) located in the inner membrane (Ng et al., 2000; Sugio et al., 2009; Wang et al., 2019). Ferrous iron oxidation in A. ferrooxidans involves the petI and rus operons, two transcripational units that mediate downhill and uphill electron pathways to generate ATP and NADH respectively (Quatini et al., 2009). Therefore, A. ferrooxidans exhibits distinct physiological characteristics and gene expression profiles depending on the availability of these two energy substrates.

Quorum-sensing (QS) is a cell-to-cell communication mechanism that enables bacteria to control gene expression in response to changes in cell density (Parsie and Greenberg, 2000; Wackett, 2008). QS regulation depends on the production, release, accumulation and detection of signaling autoinducers, and this process is generally mediated by the autoinducer synthase and cognate autoinducer receptor (An et al., 2006; Schaefer et al., 2008). QS has been widely identified in Gram-negative and Gram-positive bacteria and is fundamental for cell-to-cell communication (Juhás et al., 2005; Kai and Bassler, 2016). Hundreds of traits can be regulated via QS in both pathogenic and environmental bacteria, such as EPS synthesis, biofilm formation, cell colonization, bioluminescence and the secretion of virulence factors (Goo et al., 2015; Ben-Yaakov and Salomon, 2019). QS can also regulate metabolic processes in some bacteria, such as sugar and phosphate metabolism, as well as secondary metabolites (Goo et al., 2015; Certner and Vollmer, 2018; Ha et al., 2018). Moreover, QS has been also observed in many extremophiles; however, such studies are limited by the difficulty of genetic manipulation in these bacteria (Inaba et al., 2018).

A LuxI/R-like QS system (AfeI/R), encoded by the afeI-orf3-afeR operon, was discovered in A. ferrooxidans (Farah et al., 2005; Rivas et al., 2005). Similar to the prototype LuxI/R-like system in many Gram-negative bacteria, the QS system in A. ferrooxidans consists of a LuxI-type autoinducer synthase (AfeI) and a LuxR-type receptor (AfeR) that mediates the production of N-acyl homoserine lactones (acyl-HSLs) and controls genes expression by binding signaling molecules respectively (An et al., 2006; Schaefer et al., 2008). Additionally, another potential acyl-HSL synthetase (Act) in A. ferrooxidans was discovered in an operon. This operon encompasses four co-transcribed genes (glyQ, glysS, gph and act), which encode for the α and β subunits of glycine tRNA synthetase, a phosphatase and an acyltransferase respectively (Rivas et al., 2007). Given that Act has only been previously confirmed to produce C_{14}-HSL in Escherichia coli in the absence of a corresponding signal molecule receptor gene in the operon, the role of the Act-like QS system in A. ferrooxidans remains largely unclear (Rivas et al., 2007; Gonzalez et al., 2013).

Nine acyl-HSLs have been identified from A. ferrooxidans cultures, including C_{12}-HSL, C_{14}-HSL, 3-OH-C_{10}-HSL, 3-OH-C_{12}-HSL, 3-OH-C_{14}-HSL, 3-OH-C_{16}-HSL, 3-O-C_{12}-HSL and 3-O-C_{14}-HSL (Farah et al., 2005). The addition of exogenous acyl-HSLs led to notable phenotypic changes. For instance, the addition of C_{12}/C_{14}-HSL mixtures or acyl-HSL analogs promoted biofilm formation on the surfaces of elemental sulfur and pyrite (Gonzalez et al., 2013; Bellenberg et al., 2014). Moreover, the addition of a C_{14}-HSLs mixture also improved A. ferrooxidans electroactivity on an inert carbon electrode (Chabert et al., 2017). Furthermore, more than 100 genes were differentially expressed in A. ferrooxidans exposed to a tetracellic acid-HSL analog (tetracellic 9c), of which 60 were involved in biofilm formation (Marnani et al., 2016). Exposure to a synthetic QS blocker also revealed that AfeI/R mediates Cu^{2+} resistance in A. ferrooxidans (Wenbin et al., 2011). In addition, the over-expression of afeI/R operon suggested the important roles of AfeI/R in the growth of A. ferrooxidans in S^{2-}-enriched media and in improving the bioleaching efficiency of A. ferrooxidans to ores (Gao et al., 2020). However, although some functions of AfeI/R have been identified via the addition assays of exogenous signal molecules, the understanding of the roles of AfeI/R in A. ferrooxidans has not been fully achieved. For example, the acyl-HSLs
synthesized by AfeI were not determined due to the interference of the Act system. Meanwhile, the roles of AfeI-produced acyl-HSLs were also unclear.

The question that whether AfeI/R has a regulatory function in Fe²⁺-cultivating *A. ferrooxidans* remains to be answered. Unlike elemental sulfur, ferrous iron exists as an ion in bacterial cultures (Quatrini et al., 2009). The utilization of elemental sulfur by *A. ferrooxidans* requires EPS-mediated attachment, while ferrous iron metabolism employs very different pathways (Gehrke et al., 1998; Hameit et al., 2006; Quatrini et al., 2009). Notably, a lower transcriptional level of the afeI/R operon has been observed in Fe²⁺-enriched media compared with S⁰-enriched media (Farah et al., 2005). However, the role of AfeI/R in *A. ferrooxidans* when Fe²⁺ is used as the energy source is not clear.

Mutagenesis of the QS genes has become a powerful and effective approach to study the biological functions and regulation mechanisms of QS in many bacteria. In this study, we explored the distribution of AfeI/R-like QS system in *Acidithiobacillus* and other acidophiles. Several mutants of the acyl-HSLs synthetase genes were successfully constructed and used to study the effect of gene knockout and overexpression on acyl-HSLs synthesize, energy metabolism, cell growth, EPS secretion and gene transcript profile in *A. ferrooxidans* cultivated with different energy substrates. Moreover, the acyl-HSLs produced by AfeI were identified, and two key acyl-HSLs were found to influence *A. ferrooxidans* growth. Therefore, our results revealed that AfeI/R-mediated regulation effects in *A. ferrooxidans* were versatile and substrate-dependent. The results in this study provide new insights in understanding the QS-mediated regulation in acidophilic sulfur-oxidizing and/or ferrous iron-oxidizing bacteria.

## Results

### Distribution of AfeI/R-like QS system in Acidithiobacillus and other acidophiles

The protein sequences of AfeI, AfeR and Orf3 from *A. ferrooxidans* ATCC 23270 were used to explore the homologous proteins in *Acidithiobacillus* and other acidophiles based on the reported acidophilic species and their published genome sequences on the NCBI database (Quatrini and Johnson, 2016). As shown in Fig. 1, AfeI/R-like QS system could be identified from *A. ferrooxidans*, *A. ferridurans*, *A. ferrivorans* and *A. thiooxidans* in the genus of *Acidithiobacillus*. AfeI/R system was found in all the sulfur- and ferrous iron-oxidizing species except *A. ferriphilus* that did not have the published genomic information, while only *A. thiooxidans* possesses the QS system in the three reported sulfur-oxidizing-only species of *Acidithiobacillus*. The result indicated the distribution of AfeI/R in the sulfur- and ferrous iron-oxidizing species is more pervasive than that in sulfur-oxidizing-only species of *Acidithiobacillus*. AfeI/R-like QS system also found in the genus of *Thiomonas* that is a group of chemoautotrophic sulfur-oxidizing-only bacteria. AfeI/R system showed some...
variations at the gene arrangement and protein sequence in *Acidithiobacillus*. The *afeR-orf3-afeI* operon could be identified from the species of *A. ferrooxidans*, *A. ferridurans* and *A. thiooxidans*, while *A. ferrivorans* only possesses two separated genes *afeI* and *afeR* encoding the proteins with low identities to that from *A. ferrooxidans* ATCC 23270. Although almost all of the strains in *A. thiooxidans* have the conserved *afeR* operon, *afeR* gene and the *afe-orf3* are separated on the genome of *A. thiooxidans* ATCC 19377. A truncated AfeR and the low-identity AfeI and orf3 are found in *A. thiooxidans* ATCC 19377, in contrast with those in other *A. thiooxidans* strains. Therefore, the gene arrangement and protein sequence of AfeI/R system in *Acidithiobacillus* would be variant in different species or strains.

### Generation of *A. ferrooxidans* *afeI* mutants

To characterize the biological function of the AfeI/R QS system, the acyl-HSL synthase gene (*afeI*, AFE_1999) was targeted to generate *afeI* deletion and overexpression mutant strains. The *afeI* knockout strains were screened and identified by PCR using different primer sets (Fig. 2A and B). The Δ*afeI* strain was a markerless in-frame Afel...
mutation with a 552-bp deletion from the start site (ATG) to the stop codon (TAA). The *afeI* expression plasmid was constructed using a *tac* promoter to initiate gene transcription, as well as the autonomously replicating plasmid pJRD215 as the backbone of the expression plasmid (Fig. 2C). The constructed *afeI* expression plasmid and the backbone plasmid were respectively conjugated into wild type *A. ferrooxidans* strain WT, generating the *afeI* overexpression strain OEafeI and the wild-type control strain WT(pJRD215) (Fig. 2D).

To identify the molecules synthesized by AfeI, a 639-bp sequence of the acyltransferase gene (*act*) was deleted from strains of WT and ΔafeI, generating Δact and ΔafeI&act respectively (Fig. 2E and G). Furthermore, the *afeI* expression plasmid (pJRD215-P-tac-afeI) and the empty plasmid (pJRD215) were conjugated into Δact and ΔafeI&act, resulting in the *afeI*-expression-only strain ΔafeI and ΔafeI&act deletion strain ΔafeI&act(215) respectively.

**Substrate-dependent regulatory effects of the AfeI/R on *A. ferrooxidans* growth and energy metabolism**

When cells were grown in Fe²⁺-enriched media, the cell growth rate, maximum cell density and ferrous iron oxidation rate of the *afeI* overexpression strain were dramatically decreased, and the maximum cell density of the *afeI* overexpression strain reached only approximately 70% of that of the control strain (Fig. 3A and C). In contrast, almost no difference was observed between the *afeI* knockout and the WT strain in terms of cell growth and ferrous iron oxidation (Fig. 3B and D). These results indicate that overexpression of *afeI* could inhibit *A. ferrooxidans* ferrous iron oxidation and cell population in Fe²⁺-enriched media.

When elemental sulfur was used as an energy substrate, overexpression of *afeI* significantly increased the cell density in the lag and exponential growth phases of the *afeI* overexpression strain compared with the wild-type control strain WT (pJRD215) (Fig. 3E), and simultaneously increased sulfate production on days 3, 6, 8 and 10 (Fig. 3G). However, the enhanced growth caused by *afeI* overexpression decreased and ultimately disappeared when the cells entered the late exponential and stationary growth phases (Fig. 3E and G). Deletion of *afeI* did not distinctly affect cell density, although a slight decrease in sulfate production was observed on days 8 and 10 for the ΔafeI mutant (Fig. 3F and H). These results indicated that a high level of AfeI expression could enhance sulfur metabolism and cell growth in *A. ferrooxidans*, and this AfeI/R-mediated regulation was dependent on bacterial growth stage in the S⁰-enriched media.

**Substrate-dependent influences of AfeI/R on EPS synthesis in *A. ferrooxidans***

In Fe²⁺-enriched media, the main EPS components (proteins and carbohydrates) of *afeI* knockout and overexpression strains had no significant difference compared with that of the control strains (Fig. 4A). The results indicated that the regulatory effect of AfeI/R on *A. ferrooxidans* EPS synthesis did not occur in the Fe²⁺-enriched media.

In S⁰-enriched media, the levels of the EPS components of the *afeI* overexpression strain were more than

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*Fig 3. Analyses of the growth and metabolism of engineered *A. ferrooxidans* strains. Growth (A, B) and ferrous oxidation (C, D) of the *afeI* overexpression (OEafeI) and knockout (ΔafeI) strains in Fe²⁺-enriched media. Growth (E, F) and sulfate production (G, H) curves for the *afeI* overexpression (OEafeI) and knockout (ΔafeI) strains S⁰-enriched media. CK indicates control. NS indicates no significant difference. [Color figure can be viewed at wileyonlinelibrary.com]*

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threefold higher than those of the control strain in the exponential growth phase (Fig. 4B), whereas the difference disappeared in the stationary phase (Fig. 4C). The deletion of *afeI* did not result in a significant change in EPS protein and carbohydrate content in S⁰-enriched media (Fig. 4B and C). Scanning electron microscopy (SEM) results showed that the surfaces of sulfur coupons cultivated with the *afeI* overexpression strain were uneven, bumpy and gully-like, and the cells tended to aggregate and form biofilms (Fig. 4D). In contrast, the results for the Δ*afeI* and WT strains were considerably different, with smooth-surface sulfur coupons and scattered cells (Fig. 4D).

These results suggested that the regulation of AfeI/R on the EPS synthesize was dependent on the energy substrates.

**Substrate-dependent regulatory role of acyl-HSLs synthesized by AfeI on the growth of A. ferrooxidans**

The significant influence of *afeI* overexpression on *A. ferrooxidans* growth in Fe²⁺- or S⁰-enriched media implied that the acyl-HSLs synthesized by AfeI could influence cell growth. To further validate this speculation, add-back assays with *A. ferrooxidans* WT strain were carried out using acyl-HSLs extracted from the culture broth of the *afeI* overexpression or deletion strains. When Fe²⁺ was used as the sole energy substrate, the addition of acyl-HSLs extracted from S⁰- or Fe²⁺-enriched *afeI* overexpression strain cultures suppressed cell growth and final bacterial population (Fig. 5A and B). Cell growth was enhanced in *A. ferrooxidans* cultivated in S⁰-enriched media upon the exogenous addition of acyl-HSLs from S⁰-enriched *afeI* overexpression strain cultures.

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at the initial or mid-growth phase (Fig. 5C and D). However, no growth advantage occurred when the acyl-HSLs were added in the stationary growth phase (Fig. 5E). No apparent change in cell growth was observed upon the addition of extracts from ΔafeI cultures (Fig. 5). Thus, the regulatory effects of the extracted acyl-HSLs were consistent with the effects obtained by over-expressing afeI, indicating the influence of the AfeI-synthesized acyl-HSLs on cell growth of A. ferrooxidans and showing the different growth effects depending on the different energy substrates.

Identification of AfeI-synthesizing acyl-HSLs in different energy substrates

To avoid interference from another acyl-HSL synthetase (Act) in A. ferrooxidans (Rivas et al., 2007), the afeI-expression-only strain ΔafeI&act(ΔafeI) and the control strain ΔafeI&act(215) were constructed to identify the AfeI-synthesized acyl-HSLs on cell growth of A. ferrooxidans and showing the different growth effects depending on the different energy substrates.

Exhibited the characteristic protonation of homoserine lactone (m/z 102.05) (Morin et al., 2003), and the MS2 spectra of these five acyl-HSLs were the same as those of the standard compounds. Because 3-OH-C16 is not commercially available, it was identified using a previously reported method (Morin et al., 2003; Farah et al., 2005). The [M+H] + value of 356.2801 observed in the extracts was almost identical to the theoretical [M+H] + number of 3-OH-C16 (356.2790; Fig. S1F and I). And the LC-MS-MS experiments detected a value of m/z 102.0541 in the MS2 results (356.2801), which is characteristic of acyl-HSLs protonation. Thus, we concluded that afeI can synthesize six types of acyl-HSLs (C12, C14, 3-OH-C10, 3-OH-C12, 3-OH-C14 and 3-OH-C16) in A. ferrooxidans cells grown in S0-enriched media. Furthermore, the relative content of these six acyl-HSLs in the extracts was detected via the peak area normalization method (Ni et al., 2019); the acyl-HSL concentrations were found to occur in the following order: 3-OH-C14 > 3-OH-C12 > 3-OH-C16 > C12 > C14 > 3-OH-C10. In Fe2+-enriched media, three acyl-HSLs were identified with the following relative concentration order: 3-OH-C14 > 3-OH-C12 > 3-OH-C10. Thus, different signal molecules were synthesized by afeI depending on the presence of different energy substrates, among which 3-OH-

Fig 5. Growth of the A. ferrooxidans wild-type strain supplemented with the extracted acyl-HSLs. A, B. The addition of acyl-HSLs into A. ferrooxidans cultures grown in Fe2+-enriched media at the beginning of cultivation; D–F, addition of acyl-HSLs into A. ferrooxidans cultures grown in S0-enriched media at different cultivation stages. HSLs/S-ΔafeI and HSLs/S-ΔafeI indicate acyl-HSLs extracted from culture broths of the afeI overexpression and deletion strains respectively, of A. ferrooxidans grown in S0-enriched media; HSLs/F-ΔafeI and HSLs/F-ΔafeI indicate acyl-HSLs extracted from culture broths of the afeI overexpression and deletion strains respectively, of A. ferrooxidans grown in Fe2+-enriched media. CK indicates the blank control. [Color figure can be viewed at wileyonlinelibrary.com]
C14 was the most abundant and may play important roles in growth and metabolism regulation. Moreover, the relative quantification results showed that the content of acyl-HSLs detected in the overexpression strain cultures were 2.97 and 2.47 times higher than those of the control strain in S0- and Fe2+-enriched media respectively.

Identification of functional acyl-HSLs involved in A. ferrooxidans growth modulation

To identify the key acyl-HSLs involved in A. ferrooxidans growth regulation, five acyl-HSL standards, including C12, C14, 3-OH-C10, 3-OH-C12 and 3-OH-C14 were purchased to perform add-back assays. C12-HSL inhibited A. ferrooxidans growth in Fe2+-enriched media (Fig. 6A), while other acyl-HSLs had no obvious effect (Fig. 6C). Moreover, the addition of 3-OH-C14-HSL on the 4th day stimulated cell growth at the log growth phase (Fig. 6B) and promoted EPS synthesis in A. ferrooxidans grown in S0-enriched media (Fig. S2). However, adding other acyl-HSLs to S0-enriched A. ferrooxidans media did not have any statistically significant effect on cell growth (Fig. 6D).

Table 1. Identification of the acyl-HSLs in S0 or Fe2+-containing medium by LC-MS/MS.

| Acyl-HSLs | Chemical formula | [M+H]+ ion detected (m/z) | Structural formula | S0 | Fe2+ |
|-----------|------------------|---------------------------|--------------------|----|------|
| 3-OH-C14  | C14H29O4N        | 328.2400                  | ![Structure](image) | *** | ***  |
| 3-OH-C12  | C14H29O4N        | 300.2100                  | ![Structure](image) | *** | **   |
| 3-OH-C16  | C16H35O4N        | 356.2790                  | ![Structure](image) | **** | *    |
| C12       | C16H29O3N        | 284.2220                  | ![Structure](image) | *** | ND   |
| C14       | C18H33O3N        | 312.2530                  | ![Structure](image) | **  | ND   |
| 3-OH-C10  | C14H25O4N        | 272.1850                  | ![Structure](image) | *   | ND   |

Asterisk indicates relative concentration in the extracts; ND indicates not detected.

C14 was the most abundant and may play important roles in growth and metabolism regulation. Moreover, the relative quantification results showed that the contents of acyl-HSLs detected in afel overexpression strain cultures were 2.97 and 2.47 times higher than those of the control strain in S0- and Fe2+-enriched media respectively.

Metabolic pathways regulated by Afel/R in different energy substrate

The differentially expressed genes (DEGs) were detected by RNA-seq (Table S1), and the DEGs of interest were verified by Real-time quantitative PCR (RT-qPCR).

Genes associated with energy metabolism were differentially expressed in the afel overexpression strains in different energy substrates. In Fe2+-enriched media, overexpression of afel led to downregulation of genes in the rus, cyo, pet, doxDA, and hdr operons as well as the dsrE, tusA and sqr genes (AFE_1792), and upregulation of petB1 in the petI operon as well as cydA, tetH and sdo (AFE_2644), suggesting inhibitory effects on ferrous iron- and sulfur-oxidizing pathways (Fig. 7). Notably, a hydrogenase gene cluster (AFE_0700-AFE_0719, AFE_0700 encodes a sigma54-dependent regulator (SDR), Fig. S3) exhibited significant downregulation in this condition (Fig. 7), implying the significance of Afel/R mediated hydrogen metabolism on the growth of A. ferrooxidans in Fe2+-media. When S0 was used as the energy substrate, overexpression of afel resulted in the obvious upregulation of genes in the doxDA, and hdr clusters, and downregulation of the sqr (AFE_0267) and
The high expression levels of the majority of sulfur-oxidizing genes indicated the sulfur-oxidizing system was stimulated by overexpression of \textit{afeI} in S\textsuperscript{0}-culture. Simultaneously, the iron-oxidizing system, \textit{rus} operon and \textit{pet} operon, was significantly inhibited in the S\textsuperscript{0}-cultivated \textit{afeI} overexpression strain (Fig. 7). Therefore, AfeI/R could effectively modulate the metabolisms of sulfur, iron and hydrogen to...

Fig 7. Effect of the AfeI/R QS system on the transcriptional profiles of \textit{A. ferrooxidans}. This is the valid mean value of fold changes (FC) determined by RT-qPCR. S-OE\textit{afeI} and Fe-OE\textit{afeI} represent the \textit{afeI} overexpression in S\textsuperscript{0}- and Fe\textsuperscript{2+}-enriched media respectively. FC \(\geq 2\), \(P \leq 0.05\), upregulated; \(FC \leq 0.5\), \(P \leq 0.05\), downregulated; \(0.5 \leq FC \leq 2\), \(P \geq 0.05\), no change (data are not shown in the figure).

sdo (AFE_0269) genes (Fig. 7). The high expression levels of the majority of sulfur-oxidizing genes indicated the sulfur-oxidizing system was stimulated by overexpression of \textit{afeI} in S\textsuperscript{0}-culture. Simultaneously, the iron-oxidizing system, \textit{rus} operon and \textit{pet} operon, was significantly inhibited in the S\textsuperscript{0}-cultivated \textit{afeI} overexpression strain (Fig. 7). Therefore, AfeI/R could effectively modulate the metabolisms of sulfur, iron and hydrogen to...
control cell growth and population size of *A. ferrooxidans* in different energy-substrates.

The transcription of eight SDRs was clearly influenced by the *afeI* (Fig. 7). Upon growth on Fe$^{2+}$-containing media, there were four SDR genes (AFE_0693, 0700, 0957 and 2597) as well as the sigma 54 gene (AFE_3025) downregulated in the *afeI* overexpression strain (Fig. 7). Upon growth on S$^{0}$-containing media, there were three SDR genes (AFE_0693, 0957 and 2696) downregulated in the *afeI* overexpression strain. Thus, the differential expression of SDR genes indicated the strong and extensive impacts of the AfeI/R QS system on the sigma54-regulated pathways in different energy-substrates.

Genes associated with membrane permeability were differentially expressed in the *afeI* overexpression *A. ferrooxidans* strains (Fig. 7). The oprD (AFE_1497) and *tonB* (AFE_1991) genes involved in EPS transport (Abbas et al., 2007; Zhang et al., 2018) were markedly downregulated in the *afeI* overexpression strain grown on Fe$^{2+}$-media, while both of these genes were upregulated in the *afeI* overexpression strain grown on S$^{0}$-media (Fig. 7). Conjugal transfer related genes were markedly downregulated in the *afeI* overexpression strain grown on S$^{0}$-media, while both of these genes were no changed in the *afeI* overexpression strain grown on Fe$^{2+}$-media (Fig. 7).

The transcription levels caused by *afeI* overexpression were different under different energy substrates. This indicated that the versatile regulation of AfeI/R QS system in *A. ferrooxidans* was dependent on energy substrates.

**Discussion**

In this study, we revealed that AfeI/R-like QS system could not only function in the S$^{0}$-cultivating process through the use of key acyl-HSLs but also play an important regulatory role in bacterial ferrous iron oxidation, cell growth and quorum size in Fe$^{2+}$-enriched media. Sulfur and ferrous iron are the two crucial energy substrates for acidophiles, which could affect population development and community formation in the natural habitats of AMD sites and terrestrial hot springs and in the industrial bioleaching processes (Rawlings, 2002; Rohwerder et al., 2003). The prevalence of AfeI/R system in the sulfur- and ferrous iron-oxidizing species of *Acidithiobacillus* (Fig. 1), together with the inhibiting effect caused by the overexpression of *afeI* or the addition of exogenous acyl-HSLs (Figs 3A, C, 5A, B and 6A), implied that the AfeI/R system in *Acidithiobacillus* have evolved the regulatory capacity on bacterial ferrous metabolism. To our knowledge, this is the first report that QS system is involved in the regulation of bacterial ferrous metabolism, cell growth and population density in Fe$^{2+}$-cultivation. Moreover, our results manifested that the key acyl-HSL-inducible EPS synthesis could influence the sulfur oxidation and cell growth of *A. ferrooxidans* in the S$^{0}$-enriched media (Figs 3E, G and 4B, D, Fig. S2).

An AfeI/R-mediated energy-substrate-dependent regulation model in *A. ferrooxidans* was proposed on the basis of the extensive influences of energy substrates on the synthesis of acyl-HSLs, the regulatory effects of signal molecules and the AfeI/R-regulated pathways/systems (Fig. 8). According to the energy substrates, AfeI could synthesize different kinds of acyl-HSLs, and some of the signal molecules could function as the ‘stimulator’ or ‘inhibitor’ with the prerequisite of specific energy substrate to regulate the expression of genes involved in metabolic pathways and regulatory systems. Thus, the AfeI/R-mediated versatile regulation could offer varied strategies for *A. ferrooxidans* to modulate its genes expression and phenotypes in sulfur- and ferrous iron-contained extremely acidic environments.

The regulatory effects of AfeI/R on sulfur metabolism and cell growth of *A. ferrooxidans* in S$^{0}$-enriched media could be attributed to its regulation of EPS synthesis. EPS can enhance the adhesion of cells to solid energy substrates, and provide an active reaction space between the cells and the surface of the substrates (Gehrke et al., 1998; Harneit et al., 2006). Overexpression of *afeI* stimulated the EPS synthesis (Fig. 4B), which in turn enhanced the attachment and bioerosion of cells on elemental sulfur (Fig. 4D). This process could accelerate the activation and oxidation of extracellular elemental sulfur (Gehrke et al., 1998; Harneit et al., 2006), resulting in the increase of sulfur-oxidizing capacity (upregulation of sulfur-oxidizing genes in Fig. 7). Thus, we confirmed that the AfeI/R-mediated regulation on EPS synthesis could influence the sulfur metabolism and cell growth of *A. ferrooxidans* in S$^{0}$-enriched media. The add-back assays suggested that 3-OH-C$_{14}$-HSL could function as a ‘stimulator’ to regulate EPS synthesis and cell growth of *A. ferrooxidans* in S$^{0}$-enriched media (Fig. 6B and Fig. S2). Therefore, the 3-OH-C$_{14}$-HSL-inducible EPS synthesis could be the regulatory strategy of AfeI/R for *A. ferrooxidans* to modulate its sulfur metabolism and cell growth in S$^{0}$-enriched media. The increase in the levels of signal molecules upon overexpression of *afeI* or addition of exogenous acyl-HSLs could promote sulfur metabolism and cell growth in favorable growth environments (lag and log phases) but did not change the final population density in the S$^{0}$-enriched media (Figs 3E, G, 5C–E and 6B). Thus, the role of AfeI/R could be defined as an ‘accelerator’ for the development of the population, but not a ‘quorum maker’ for *A. ferrooxidans* in S$^{0}$-enriched media.

The strong inhibitory effect of either overexpression of *afeI* or addition of acyl-HSLs on the ferrous iron oxidation,
cell growth and quorum size of \( A. \) \textit{ferrooxidans} in \( Fe^{2+} \)-enriched media (Figs 3A, C, 5A, B and 6A) suggested that AfeI/R could be considered as an efficient ‘inhibitor’ for \( A. \) \textit{ferrooxidans} cultivated in \( Fe^{2+} \)-enriched media. Neither overexpression nor deletion of \( afeI \) was observed to have an effect on EPS synthesis for cells grown in \( Fe^{2+} \)-enriched media (Fig. 4A). These results provide two evidence: first, AfeI/R could not regulate the synthesis of EPS in the \( Fe^{2+} \)-enriched media; second, the QS-mediated regulation was not caused by EPS in this condition. The obvious downregulation of genes encoding electron transporter and respiratory chain (\( rus, pet \) and \( cyo \) operon) may be the reason for the decrease of ferrous oxidation capacity and cell density of the \( afeI \) overexpression stain in \( Fe^{2+} \)-enriched media (Figs 3A, C and 7). The striking down-regulation of a hydrogenase gene cluster in \( afeI \) overexpression strain provides another clue for understanding the AfeI/R-mediated regulation in \( Fe^{2+} \)-media (Fig. 7 and Fig. S3). The HupR-containing hydrogenase genes cluster was suggested to catalyze the conversion of dihydrogen to protons and electrons in \( A. \) \textit{ferrooxidans} (Schröder \textit{et al.}, 2007; Kalms \textit{et al.}, 2018). The significant down-regulation of the hydrogenase cluster probably reduced the generation of intracellular protons, which likely altered the pH homeostasis that is important for ATP biosynthesis (Lubitz \textit{et al.}, 2014; Hansen and Perner, 2016; Kalms \textit{et al.}, 2018). Thus, AfeI/R may participate in the regulation of hydrogen metabolism, which could be another reason for the decrease of cell growth and population density of the \( afeI \) overexpression stain in \( Fe^{2+} \)-enriched media.

The energy substrates could influence both the types of acyl-HSLs produced by AfeI and the regulatory function of these acyl-HSLs. Due to the presence of other potential acyl-HSL-synthase genes (\( act \)) in \( A. \) \textit{ferrooxidans}, the specific acyl-HSLs produced by AfeI remained unclear. Therefore, we constructed the \( afeI \)-expression-only strain \( \Delta act(afeI) \) and the \( afeI \) and \( act \) double knockout strain \( \Delta afeI&act(215) \) to determine which acyl-HSLs were generated by AfeI. Our results demonstrated that acyl-HSLs with C-3 hydrogen and hydroxyl substituents were synthesized by AfeI in \( S^{0} \)-enriched media, whereas only 3-hydroxyl-HSLs were found in the \( Fe^{2+} \)-enriched media (Table 1). Interestingly, previously reported acyl-HSLs in \( A. \) \textit{ferrooxidans} (i.e., 3-oxo-HSLs and 3-hydroxy-C\textsubscript{8}-HSL) were not detected in this study (Farah \textit{et al.}, 2005). Synthesis of acyl-HSLs requires S-adenosylmethionine substrates and an acylated acyl carrier protein (acyl-ACP) from the fatty acid synthesis pathway, and growth conditions could influence the availability of acyl-ACP substrates (Parsek and Greenberg, 2000; Teplitski \textit{et al.}, 2003). Thus, the differences between the acyl-HSLs observed herein and in previous reports could be due to the influence of other potential QS systems (Act), as well as differences in
cultivation methods and environments. Abundant 3-OH-
C_{14}^+\text{-HSL} was detected in both Fe^{2+} and S^{0}-enriched
media (Table 1), but this compound was functional only
in the S^{0}-enriched media (Fig. 6B and C). Although C_{12}^-
HSL showed an inhibitory effect on the growth of A.
ferrooxidans in Fe^{3+}-enriched media (Fig. 6A), it was
detected only in the S^{0}-media but not in Fe^{2+}-media
(Table 1). Due to the lack of a 3-OH-C_{16} standard, an
add-back assay for this acyl-HSL was not performed in
this study. The role of 3-OH-C_{16}^+HSL and other uniden-
tified acyl-HSLs produced by Afel in the regulation of
A. ferrooxidans in Fe^{2+}-enriched media remains an open
question for future studies. These results indicated that
different signal molecules were used by Afel/R to modu-
late specific regulation pathways in different substrates.
Therefore, the substrate-dependent synthesis and regula-
tion of the acyl-HSLs is a key characteristic of the Afel/R
QS system, which probably allow A. ferrooxidans to
effectively cope with the different energy substrates in
the growth environments.

The significant changes in the transcriptomes of afel
knockout and overexpression strains both in S^{0} and Fe^{2+}
-enriched media (Fig. 7 and Table S1) suggested that
Afel/R is an important means for A. ferrooxidans regu-
lating its genes transcription in different energy substrates.
In the QS-mediated gene regulation system, the receptor
reacts to a signal molecule and then binds to the lux-box
sequence to control genes transcription (An et al., 2006;
Schaefer et al., 2008). The lux-box sequences in A.
ferrooxidans were predicted via the bioinformatic
approach (Banderas and Guiliani, 2013), and the lux-box
region upstream of the afel gene was determined via gel
mobility shift assays (Mamani et al., 2016). Based on
these results, DEGs containing lux-box sequences were
found in this study, including sulfur metabolism gene
(AFE_0269), pet operon (AFE_3107-3111), sigma-
54-dependent transcriptional regulator gene (AFE_0693
and 0957) and conjugal transfer gene (AFE_1694).
These results implied the direct regulation of AfelR on
genes, highlighting the control of the Afel/R QS
system on these pathways in A. ferrooxidans.

The Afel/R-mediated substrate-dependent versatile
regulation could be a noteworthy characteristic of QS reg-
ulation in these chemosynthetic sulfur- and ferrous
iron-oxidizing bacteria, differentiating to other reported
LuxR/R-like QS regulation in bacteria. AfelR, for its im-
portant roles in discriminating different acyl-HSLs and modu-
lating genes transcription, maybe a key factor for the
formation of the Afel/R-mediated versatile regulation in
different energy-substrates. The homology model
suggested that AfelR has a receptor domain that binds to
signal molecules and a regulatory domain that interacts
with DNA to regulate gene transcription (Zhang et al.,
2002; Soulère et al., 2008). The receptor domain of the
LuxR family has evolved differently to suit its hosts
(Bottomley et al., 2007). Alignment of AfelR and LuxR
family protein sequences revealed that the acyl-HSLs
receptor domain has highly conserved key amino acid
residues (Tyr58, Trp63, Asp75, Trp90, Ala105 and
Gly113), as well as some amino acid residues that are
less conservative than other strains (amino acids colored
in cyan in Fig. S4). Therefore, the receptor domain of the
AfelR may evolve some unique features, which leads to
the different recognition ability of AfelR on acyl-HSLs in
different energy substrates. In addition, it was reported
that the conformations of an acyl-HSL are diverse, such
as the linear and curved-shape alkyl chain (Soulère et al.,
2008). Acyl-HSLs with different conformations showed
different affinities to the AfelR receptor (Soulère et al.,
2008). Besides, it has been reported that the pat-
tern of acyl-HSLs produced by a single strain depends
largely on the media (Teplitski et al., 2003). Therefore,
it is speculated that different energy substrates may affect
the conformations of the acyl-HSLs, which in turn affects
the binding of the acyl-HSLs to the AfelR receptor, and
ultimately leads to the differences in signal recognition
and gene regulation of Afel/R system in different energy
substrates. Therefore, the structural differences between
AfelR and other LuxR family proteins as well as the
change of the conformations of signal molecules in differ-
ent substrates could contribute to the formation of Afel/R-
mediated substrate-dependent versatile regulation in the
sulfur- and ferrous iron-oxidizing bacteria.

In summary, Afel/R have evolved distinct regulatory
strategies specific to the energy substrates, and the Afel/
R-mediated substrate-dependent regulation could be an
important mechanism employed by these sulfur- and fer-
rous iron-oxidizing bacteria to maintain the balance
between their energy metabolisms and population develop-
ment in the sulfur- and ferrous iron-containing
extremely acidic environments. This study would be a
basis for further studies on the ecological functions of
Afel/R-like QS systems in the natural habitats and pro-
vide new insights in the synthetic biological research of
the chemoautotrophic bacteria.

Experimental procedures

Bacteria and growth conditions

The bacteria and plasmids used in this study are listed in
Table 2. Escherichia coli was cultivated at 37 °C in LB
media (Sambrook et al., 1982). The A. ferrooxidans
ATCC 23270 strain was grown in 9K inorganic salt media
with Fe^{2+} (10 g/L) or S^{0} (0.8% w/v) as energy sources,
and the pH was adjusted to 2.0 using H_{2}SO_{4}. Starkey-
Na_{2}S_{2}O_{3} agar media was used for A. ferrooxidans plate
cultures (Wang et al., 2012). Cell growth in the 9K-S^{0}

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Table 2. Bacteria and plasmids used in this study.

| Strain and plasmids | Description | Source |
|---------------------|-------------|--------|
| *Acidithiobacillus ferrooxidans* ATCC 23270 | Type strain | ATCC |
| WT (pJRD215) | Wild type | ATCC |
| ΔafeI | αfeI gene deletion | This study |
| OEafeI | Overexpress αfeI gene | This study |
| Δact(αfeI) | act gene deletion and αfeI gene overexpression | This study |
| ΔafeI&act | Both αfeI and act genes deletion | This study |
| Escherichia coli DH5α | F′<sub>80</sub>lacZΔM15lacZYA-argF)U169 end A1 recA1 hsdR17(kdr<sup>+</sup>)supE44 thi-1 gyr96 relA1 phoA | TransGen Biotech |
| S17-1.pir | T<sub>p</sub> Sm<sup>s</sup> recA thii pro<sub>r</sub> Stramp<sub>r</sub> RP4-2-Tc:MuKmTn<sub>7</sub> pir | Bilecen and Yildz (2009) |
| SM10 | Km<sup>r</sup> thr leu tona lacY sup<sub>E</sub> recARP4-2-Tc::Mu | Simon et al. (1983) |
| Plasmids | | |
| pSUDII | Suicide plasmid; Ap<sup>r</sup> Km<sup>r</sup> oriTRP4 multi-cloning sites | Wang et al. (2016) |
| pSUDII::afeI (UHA + DHA) | Suicide plasmid for ΔafeI construction | This study |
| pSUDII::act(UHA + DHA) | Suicide plasmid for Δact construction | This study |
| pMSD1-l-Secl | pMSD containing the l-Secl gene | Wang et al. (2012) |
| pJRD215 | Sm<sup>r</sup> Km<sup>r</sup> IncQ Mob<sup>+</sup> | Davison et al. (1987) |
| pJRD215-ΔαfeI | Sm<sup>r</sup> Km<sup>r</sup> IncQ Mob<sup>+</sup> ΔαfeI gene | This study |

and 9K-Fe<sup>2+</sup> media was monitored via OD<sub>600</sub> nm measurements and the microscopic counting method respectively.

Mutant strain construction

The sequences for all primers used in this section are listed in Table S2. The markerless deletion of the αfeI gene (AFE_1999) in the *A. ferrooxidans* ATCC 23270 was performed as described previously (Wang et al., 2012). Two homologous arms were first amplified using the IUPF/R and IDWF/R primer pairs and ligated to the pSUDII plasmid. The generated suicide pSUDII-Homafel plasmid was then transferred into *A. ferrooxidans* via conjugation (Peng et al., 1994). Single-crossover recombinant strains were then selected. Then, the pMSD1-l-Secl plasmid was conjugated into the single-crossover recombinants, resulting in a second homologous recombination to generate the gene knockout and wild-type (WT) reversion. The ΔafeI was then identified via PCR using three primer pairs: P1F/P1R, P2F/P2R and P3F/P3R; the purified P1F/P1R-amplified PCR fragments were sequenced to confirm the mutation.

The αfeI gene and the tac promoter were amplified via PCR using the PtacF/PtacR and PIF/PIR primer pairs respectively. The two fragments were digested and ligated into the pJRD215 plasmid. The pJRD215-ΔαfeI plasmid was conjugated into the *A. ferrooxidans* ATCC 23270 strain to construct the αfeI overexpression strain. PCR amplification using plasmid-specific P4F/R primers was performed to confirm the overexpression strain.

An act gene-specific suicide plasmid was produced with the ACTUPF/R, ACTDWF/R primer pairs. The generated plasmid was then conjugated into WT and ΔafeI. Δact was identified using primers PA1F/R, PA2F/R and PA3F/R. ΔafeI&act was identified using primers P1F/R, P2F/R, P3F/R, PA1F/R, PA2F/R and PA3F/R. Then, the pJRD215-ΔαfeI and pJRD215 plasmids were conjugatively transferred into Δact and ΔafeI&act generating strains Δact(αfeI) and ΔafeI&act (pJRD215) respectively.

Determination of Fe<sup>2+</sup> and SO<sub>4</sub><sup>2-</sup> concentrations in culture media

The concentration of Fe<sup>2+</sup> in the liquid media was determined via the o-phenanthroline method as described previously (Herrera et al., 1989), whereas the concentration of SO<sub>4</sub><sup>2-</sup> was measured via ion chromatography (ICS-1100AR, DIONEX, USA) (Miura and Kawaoi, 2000).

EPS extraction and analysis

EPS extraction was performed as described previously (More et al., 2014; Xiao et al., 2017). Cells were collected by centrifugation and adjusted to their final concentration (OD<sub>600</sub> nm = 1). Then, 1 ml of these cell suspensions were centrifuged at 12 000 g for 1 min at 4°C. The cells were then resuspended in 4 ml of TNE buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA, pH = 7.5) and centrifuged at 12 000 g for 10 min. The pellet was then resuspended in 4 ml TNE + SDS (0.1%). After a 5-min reaction period at room temperature, the samples were centrifuged at 12 000 g for 10 min to obtain EPS extracts. Afterward, the extracts were washed three times with TNE buffer and eluted in 50 mM Tris (pH 7.5). The total carbohydrate content in the EPS extracts was determined using the anthrone-sulfuric acid method (Ding et al., 2019). The protein concentration in the EPS extracts was measured using the Modified Bradford Protein Assay Kit (Sangon Biotech). The experiments were performed three times and each sample was set three biological replications. Statistical analysis was conducted via Student’s t-test using the GraphPad Prism software (version 7.0; GraphPad).
Sulfur coupon preparation and SEM

Sulfur coupons were prepared by melting sulfur powder and then pouring the liquid sulfur on a glass coverslip to cool and solidify (Bellenberg et al., 2014). The cell solutions were added to the sulfur-coupon-contained medium, and the cell density was adjusted to OD_{600} = 0.1. After 8 days of cultivation, the sulfur coupons were taken out, fixed with 2.5% glutaraldehyde, dehydrated in a series of graded ethanol solutions and critical point-dried. After gold sputtering, the sulfur coupons were observed by SEM (Quanta 250 FEG, FEI) (Liu et al., 2003).

Crude acyl-HSL extraction and identification

The cells were cultivated until they reached the stationary peak area from the LC-MS-MS results (Ni et al., 2005). C14, 3-OH-C10, 3-OH-C12 and 3-OH-C14 standards were purchased from Sigma (USA). Relative acyl-HSL quantification in the extracts was achieved by calculating the peak area from the LC-MS-MS results (Ni et al., 2019).

Add-back experiments

300 μl and 600 μl of the acyl-HSLs extracts from the S0- and Fe2+-enriched media were respectively added to 150 ml of media. The extract add-back assays in S0-enriched media were performed at 4th, 9th and 12th day. When Fe^{2+} was used as an energy substrate, the extracts were only added at the beginning of cultivation. The concentration used for each acyl-HSL standard was 10 μM. The acyl-HSLs were added on the 4th day in S0-enriched media and on the 9th day in Fe^{2+}-enriched media. The experiments were performed three times with three biological replications.

RNA extraction, real-time quantitative PCR and RNA sequencing

RNA was extracted from cell samples in the mid-log growth phase. The RNAprep Pure Cell/Bacteria Kit (Tiangen, China) was used for all RNA extractions according to the manufacturer’s instructions. The extracted RNA was then visualized via formaldehyde degeneration electrophoresis (Rivas et al., 2005). The A260 value and A260/A280 ratio were measured to determine RNA concentration and purity respectively. Reverse transcription was performed using the PrimeScript™ RT Reagent Kit (TaKaRa, China). RT-qPCR reactions were performed in a Roche LightCycler480 (Roche, USA) using the SYBR® Premix Ex Taq™ (TaKaRa) enzyme; alaS was used as a reference gene (Nieto et al., 2009). The 2^{ΔΔCt} method was used to analyse relative changes in gene expression (Livak and Schmittgen, 2001). RT-qPCR primers are listed in Table S2.

Statistical analysis

All experiments were performed three times with three biological replications. One-way analysis of variance coupled with Bonferroni’s multiple comparison test was used to compare. Statistical analysis was conducted via the Student’s t-test. All statistical analyses were performed using the GraphPad Prism software (version 7.0). Statistical significance is indicated with asterisks (***) indicates P < 0.0001, **** indicates P < 0.001, ** indicates P < 0.01 and * indicates P < 0.05) in the results section.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1**: Supporting Information