J. Microbiol. Biotechnol. (2004), **14**(1), 56–61

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Expression of *E. coli* Phosphofructokinase Gene in an Autotrophic Bacterium *Acidithiobacillus thiooxidans*

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Received: March 14, 2003
Accepted: September 23, 2003

Abstract A plasmid pSDK-1 containing the *Escherichia coli* phosphofructokinase-1 gene (*pfkA*) was constructed, and transferred into extremely acidophilic *Acidithiobacillus thiooxidans* Tt-7 by conjugation with the aid of plasmid RP4 at a frequency of 10^{-5} per recipient. This plasmid was stable in *A. thiooxidans*. The *pfkA* gene from *E. coli* could be expressed in this obligately autotrophic bacterium, but the enzyme activity (21.6 U/g protein) was lower than that in *E. coli* (K12: 85.9 U/g protein; DF1010 carrying plasmid pSDK-1: 96.6 U/g protein). In the presence of glucose, the Tt-7 transconjugants consumed glucose, leading to a better growth yield.

Key words: *Acidithiobacillus thiooxidans*, phosphofructokinase, conjugation

Acidithiobacillus thiooxidans is a Gram-negative, acidophilic bacterium which derives its energy by oxidizing inorganic sulfur or its compounds and obtains its carbon by fixing CO_2. It has been used industrially in metal leaching from mineral ores and in the microbial desulfurization of coal in combination with *A. ferrooxidans*. However, the slow growth rate and the low cell yield of this organism have limited its further use. Studies showed that some genes, such as the isocitrate dehydrogenase (ICDH) gene (*icd*), from *A. thiooxidans* could function in *E. coli* [11]. Some plasmids have been transferred successfully from *E. coli* to *A. thiooxidans*, and genes originating from heterotrophic bacteria could be expressed as well [12, 13, 20]. It is, therefore, possible to construct new *A. thiooxidans* strains with better growth rates and increased leaching efficiencies.

Phosphofructokinase (EC 2.7.1.11) is the key enzyme in the control of glycolysis. Research has revealed that the activities of phosphofructokinase, as well as some other key enzymes of the EMP, ED pathways and Krebs cycle, such as 6-phosphogluconate dehydrase and α-ketoglutarate dehydrogenase, are lacking in *A. thiooxidans* [18]. These organisms, therefore, cannot respire organic substance and obtain energy from them. In this paper, plasmid pSDK-1 containing the *E. coli pfk*A gene encoding this enzyme was constructed and transferred into *A. thiooxidans* Tt-7 by conjugation. The *pfk*A gene could be expressed under the control of the promoter from *E. coli* in *A. thiooxidans*, and the effect of glucose on the growth of this new organism was also studied. This work would provide new insights into how to improve the growth rate and leaching performance of the obligately autotrophic bacteria.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1.

E. coli strains were grown in Luria broth or on Luria agar at 37°C. *E. coli* DF1010 lacking phosphofructokinase activity cannot grow on mannitol, but can grow gluconeogenically. But, *E. coli* DF1010 with plasmid carrying an active phosphofructokinase gene can be selected by genetic complementation on mannitol-selective plates [9]. *A. thiooxidans* was grown in Starkey inorganic medium at 30°C as described previously [13]. Elemental sulfur (S^0) was added before inoculation. The solid medium was prepared by mixing components A (double strength basal salts and agar, pH 4.8, autoclaved) and B (2% sodium thiosulfate, filter sterilized) in equal volumes before using. This medium was supplemented with 0.05% (w/v) yeast extract, when used as a mating medium for *E. coli* and *A. thiooxidans*.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Phenotype or genotype	Source or reference
E. coli strains		
K-12	Wild-type	Stock Center of China, Beijing
DF1010	*garB10, fhuA22, ompF627*(T2R), *ΔpfkB201, fadL701*(T2R), *recA56, relA1, pit-10, spoT1, Δ(rhaD-pfkA)*200, *rrnB-2, creC510*	*E. coli* Genetic Stock Center, Yale University
C600	*supE44, hsdR, thi-1, thr-1, leuB6, lacY1, tpnA21*	17
HB101	*supE44, hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1*	17
A. thiooxidans strain		
Tt-7	Wild-type	13
Plasmids		
RP4	Ap^r, Tc^r, Km^r, IncP, tra^+	6
pJRD215	Km^r, Sm^r, IncQ, mob^+	7
pSDK-1	Km^r, IncQ, mob^+, $pfkA^+$	This study

Construction of Plasmid pSDK-1 Containing *pfk*A Gene
Plasmid pSDK-1 containing the *pfk*A gene was constructed by standard protocols for DNA manipulation [17]. The genomic DNA of *E. coli* K-12 was isolated and used as the template for PCR of the *pfk*A gene. The following two oligonucleotide primers for the *pfk*A gene were designed according to the published sequence information [4]: forward primer: 5'-GTACGGATCCTTGGCCTGACCTG-AATCAAT-3' (*Bam*HI), and reverse primer: 5'-GCGCAA-GCTTCCGACTCTCTTATGTTGTGT-3' (*Hin*dIII). PCR reactions were performed as follows: an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 sec, and elongation at 72°C for 2 min 30 sec, then followed by a final extension for 10 min at 72°C. The plasmid pJRD215 can be efficiently transferred between species by conjugal transfer in the presence of a conjugation-proficient plasmid, such as RP4, and stable in *A. thiooxidans* [12, 13]. The 1.4 kb PCR amplified fragments, which contain the promoter and coding sequence of the *pfk*A gene, were purified using the Agarose Gel DNA Extraction Kit from Roche and cloned into the pJRD215 vector after being digested with *Bam*HI and *Hin*dIII to construct the plasmid pSDK-1. pSDK-1 was then transformed into *E. coli* DF1010 and screened by deficient complementation on mannitol-selective medium. The 1.4 kb DNA fragment inserted in pSDK-1 was sequenced by the dideoxy chain termination method [19], which was performed with Beckman CEQ2000XL DNA Analysis System.

Conjugation
Crosses between strains were conducted by filter mating. Donor cells were harvested by centrifugation at the late exponential growth phase; recipient cells were harvested at the stationary phase. Sulfur precipitates were removed from the liquid culture by low speed centrifugation (800 ×g). Both the donor and recipient cells were washed three times with basal salt solution of mating medium (pH 4.6- pH 4.8), resuspended in the same medium, and then mixed at a ratio of 1:1. Then, 0.1 ml of cell suspension (about 2×10^{10} cells per ml) was transferred to a filter membrane (0.45 μm pore size, 25 mm diameter) placed on mating medium. After incubation at 30°C for 72 h, the cultures were washed from the filter to 2.0 ml of saline, diluted, and then plated on appropriate selective plates and nonselective plates and incubated at 30°C for 7 days. As a control for spontaneous mutation, both parental strains were plated on the same selective plates. The frequency of conjugation was calculated by the number of transconjugants divided by that of recipients. Plasmids in *A. thiooxidans* were isolated by the method described by Birnboim and Doly [3], with slight modification (lysozyme was omitted in solution I). *A. thiooxidans* cells were washed with solution I at least twice before isolation of plasmid.

Stability of pSDK-1 in *A. thiooxidans*
Single colonies of *A. thiooxidans* transconjugants on the selective plates were transferred into antibiotic-free Starkey-S^0 medium (20 ml). After incubating at 30°C for 7 days, 1% of the fully grown cultures was transferred to 20 ml of fresh Starkey-S^0 liquid medium and incubated at 30°C for 5 days. After five times of transfer (more than 50 generations), the cultures were diluted and plated on Starkey-$Na_2S_2O_3$ solid media with or without antibiotic, and incubated at 30°C. Ten days later, the percentage of plasmid maintenance was calculated by the number of colonies formed on media with antibiotic, divided by that formed on media without antibiotic. The presence of the *pfkA* gene insert was analyzed by PCR with the *pfkA* oligonucleotides on isolated transconjugants.

Phosphofructokinase Assays
Cells were collected by centrifugation, washed twice with deionized water, and suspended in 50 mmol/l Tris (pH 7.4), 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 1 mmol/l PMSF (1:4, w/v). The resuspended cells were frozen, thawed,

treated with a ultrasonic oscillator (20 kHz, 350 watts) for 30 min at 0°C, and then centrifuged at 15,000 ×g for 20 min at 4°C. After appropriate dilutions, the supernatants were used to assay for phosphofructokinase activity, as previously described [14]. The concentration of protein was determined by the Coomassie brilliant blue dye-binding assay [5] using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed by the method of Laemmli [16].

Reverse Transcription PCR (RT-PCR) Amplification

Total RNA was reverse-transcribed using a ThermoScript™ RT-PCR kit from Invitrogen. PCR amplification was carried out using the reverse-transcribed RNA with primer 1 (5'-CAGCAGCAGATCGATAGCGT-3') and primer 2 (5'-CAGCAGCAGATCGATAGCGT-3'). The PCR conditions were: 2 min at 94°C, 35 cycles of 40 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and finally 7 min at 72°C.

Effect of Glucose on the Growth of *A. thiooxidans*

A. thiooxidans were grown in Starkey-S^0 liquid media in the absence or presence of 5 g/l glucose separately. The cell growth rate was determined by measuring the cell densities of the culture at 660 nm. The concentration of glucose in culture supernatants was measured with SBA-40C Biosensor.

RESULTS AND DISCUSSION

Construction of Plasmid pSDK-1

The agarose gel electrophoresis of plasmid pJRD215 and pSDK-1 digested by *Bam*HI and *Hin*dIII showed that pSDK-1 carried the 1.4 kb heterogeneous fragment (Fig. 1). Sequence data confirmed that the 1.4 kb fragment insert in pSDK-1 had a sequence corresponding to that of the *pfkA* gene [4].

Mobilization of Plasmid pSDK-1 to *A. thiooxidans*

A. thiooxidans is an extremely acidophilic obligately autotrophic bacterium, whose optimal pH for growth is 1.5-2.5, whereas *E. coli* is a heterotrophic bacterium whose optimal pH for growth is 7.0-7.5. Although there are marked differences in their growth conditions, the mating medium described above could provide energy sources for either *E. coli* or *A. thiooxidans*. Using *E. coli* C600 (RP4, pSDK-1) as the donor and *A. thiooxidans* Tt-7 as the recipient, the plasmid pSDK-1 could be mobilized into *A. thiooxidans* strain with the aid of plasmid RP4. The

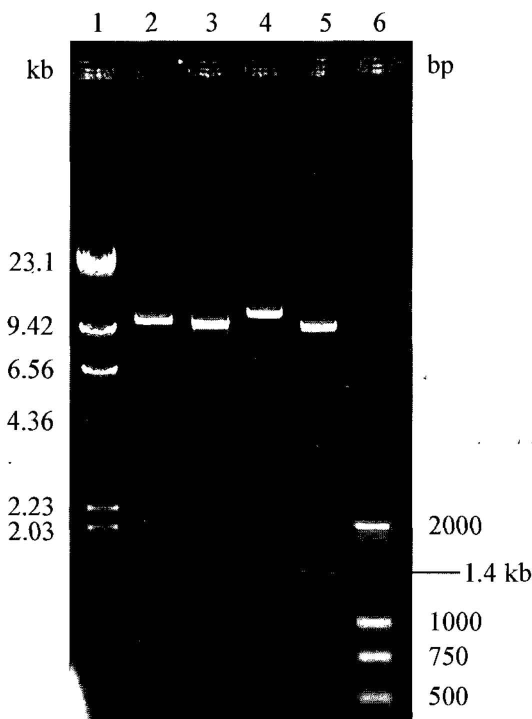

Fig. 1. Agarose gel electrophoresis of plasmids digested by *Bam*HI and *Hin*dIII.
Lane 1, DNA/*Hin*dIII marker; lane 2, pJRD215/*Hin*dIII; lane 3, pJRD215/*Bam*HI+*Hin*dIII; lane 4, pSDK-1/*Hin*dIII; lane 5, pSDK-1/*Bam*HI+*Hin*dIII; lane 6, DL2000 DNA marker.

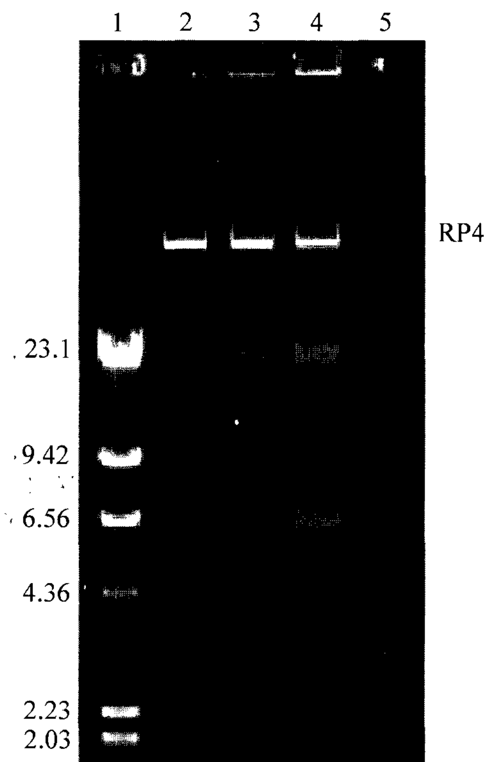

Fig. 2. Agarose gel electrophoresis of plasmid donor and tranconjugant strains.
Lane 1, DNA/*Hin*dIII marker; lane 2, C600 (RP4); lane 3, Tt-7 (RP4, pSDK-1); lane 4, C600 (RP4, pSDK-1); lane 5, Tt-7.

presence of RP4 and pSDK-1 in *A. thiooxidans* transconjugants were verified by plasmid isolation (Fig. 2). The transfer frequency of plasmid RP4 and pSDK-1 from *E. coli* C600 to *A. thiooxidans* Tt-7 was 2.6×10^{-5} using kanamycin resistance as the selective marker, and the spontaneous mutation rate was lower than 10^{-8}.

Stability of pSDK-1 in *A. thiooxidans*

The stability of plasmid pSDK-1 in Tt-7 was determined by checking for kanamycin resistance as described above. More than 68% of *A. thiooxidans* cells carried the recombinant plasmids after being cultured for 50 generations without selective pressure, which showed that pSDK-1 was maintained consistently in *A. thiooxidans*. The PCR analysis of isolated transconjugants showed the presence of the *pfkA* gene in *A. thiooxidans* (Fig. 3).

Expression of *pfkA* Gene in *A. thiooxidans*

Although *A. thiooxidans* can grow in an acidic environment as low as pH 1.0, the internal pH of the cell is close to neutral pH. Many *Acidithiobacillus* genes could be expressed in *E. coli*, and the corresponding protein could function, suggesting that the internal pH and cytoplasmic milieu of these two genera are similar. Therefore, it is possible that heterogeneous genes from other bacteria could function in *A. thiooxidans*. The expression of the *pfk*A gene was investigated by examining the enzyme activity in the crude cell-free extract of the transconjugants (Table 2). The specific activity of the phosphofructokinase-1 was slightly increased in *E. coli* DF1010 (pSDK-1) transformants, but decreased in *A. thiooxidans* Tt-7 (RP4, pSDK-1), compared to the wild-type *E. coli* K-12 cells. The result of SDS-PAGE showed that a major M_r 34,000 polypeptide band was detected in *E. coli* K-12, DF1010 (pSDK-1) and Tt-7 transconjugants, respectively (Fig. 4, lanes 2, 4, and 5), whereas no polypeptide bands of the corresponding size were detectable in *E. coli* DF1010 and Tt-7 (Fig. 4, lanes 3 and 6). Agarose gel electrophoresis of RT-PCR products confirmed that the *pfkA* gene was expressed in Tt-7 transconjugants (Fig. 5), thus revealing that the *E. coli*

Table 2. Comparison of enzyme activity in different strains.

Plasmid	Host	Specific activity of phosphofructokinase-1[a] U/g protein	
--	*E. coli* K-12	85.9±1.9	---
--	DF1010	00.0	---
--	Tt-7	00.0[b]	00.0[c]
pJRD215	DF1010	00.0	---
pSDK-1	DF1010	96.6±1.6	---
RP4, pSDK-1	Tt-7	21.6±0.5[b]	20.9±0.3[c]

[a]Values were obtained from three independent experiments and are shown as means±SD.
[b]Growth on Starkey-S^0.
[c]Growth on Starkey-S^0+Glucose (5 g/l).

Fig. 3. Analysis of PCR products on isolated transconjugants.
Lane 1, DL2000 DNA marker; lane 2, PCR amplification on Tt-7 (RP4, pSDK-1); lane 3, PCR amplification on Tt-7.

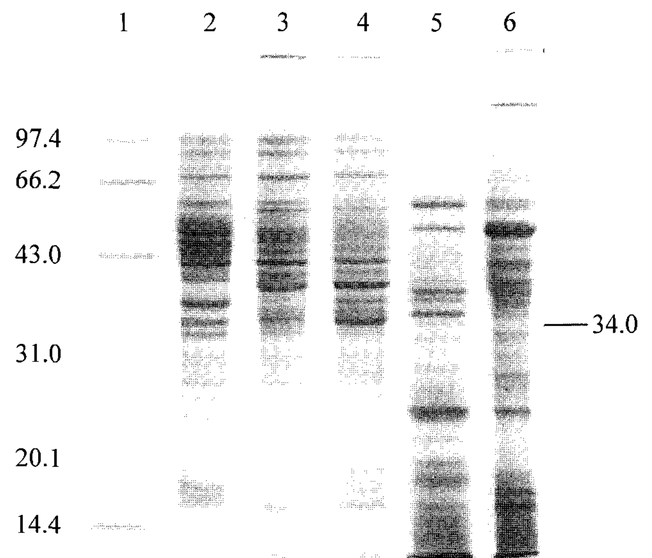

Fig. 4. SDS-PAGE analysis of the cell-free crude extracts of various strains.
Lane 1, Protein molecular weight marker; lane 2, *E. coli* K-12; lane 3, *E. coli* DF1010; lane 4, *E. coli* DF1010 (pSDK-1); lane 5, Tt-7 (RP4, pSDK-1); lane 6, Tt-7.

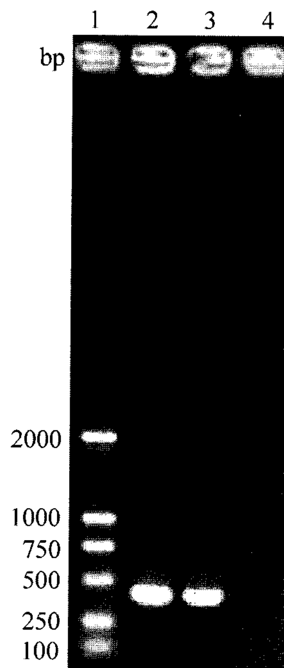

Fig. 5. Analysis of RT-PCR products of the *pfkA* gene in different strains.
Lane 1, DL2000 DNA marker; lane 2, *E. coli* DF1010 (pSDK-1); lane 3, Tt-7 (RP4, pSDK-1); lane 4, Tt-7.

promoter sequences could be recognized by the autotrophic bacterium. The expression of the *glnA* gene of other members of the *Acidithiobacillus* genus *A. ferrooxidans*, which is closely related to *A. thiooxidans* based on 16S rRNA sequence data, in *E. coli* is the first evidence that a promoter from an obligate autotroph is functional in a heterotroph [1]. Some *A. ferrooxidans* promoters are very similar to the promoters commonly found in typical heterotrophic bacteria [10]. A large number of *A. ferrooxidans* genes have been isolated by functional complementation of *E. coli* mutants. In addition, some genes originating from heterotrophic bacteria, including the kanamycin, streptomycin, tetracycline resistance genes, the *tra* gene of RP4, and the arsenic resistance gene [12, 13, 20], have been cloned and expressed in *A. thiooxidans*. All of these studies implied that the gene expression system might be similar in *E. coli* and *Acidithiobacillus* bacteria, even though there are great physiological differences between them.

However, the enzyme activity measured in Tt-7 (RP4, pSDK-1) was unexpectedly low. The possible explanation might be that the promoter cloned from *E. coli* could not be recognized efficiently in *A. thiooxidans*. Previous studies showed that the *rbcLS* genes as well as the *recA* and *alaS* genes from *A. ferrooxidans* were not expressed in *E. coli*, unless placed under the control of an *E. coli* promoter [8, 15]. In another research, the *A. ferrooxidans* *ntrA* gene product was able to promote σ^{54}-dependent transcription in *E. coli ntrA* mutants, but complementation was only partial and insufficient to permit growth of *E. coli ntrA* mutants on minimal medium plus arginine [2]. These indicated that some differences might exist in the gene expression systems between *Acidithiobacillus* and *E. coli*. The specific activity of phosphofructokinase-1 was also measured in different strains grown in the presence or absence of glucose (Table 2). The results showed that the expression of the *pfkA* gene in Tt-7 transconjugants was not influenced by glucose, indicating that the expression of the *pfkA* gene could not be controlled by the host strain.

Effect of Glucose on the Growth of *A. thiooxidans*

The growth of *A. thiooxidans* transconjugants was studied in Starkey-S^0 liquid media in the absence or presence of 5 g/l glucose (Fig. 6A). Of great significance was that glucose significantly stimulated the cell growth of transconjugants, but had no effect on Tt-7 cells. The concentration of glucose in the growth medium did not change for the Tt-7 strain but decreased gradually for Tt-7 (RP4, pSDK-1) during the growth; however, the level of glucose decreased very slowly (Fig. 6B). The results revealed that the growth of *A. thiooxidans* was not influenced by glucose, whereas the growth of transconjugants

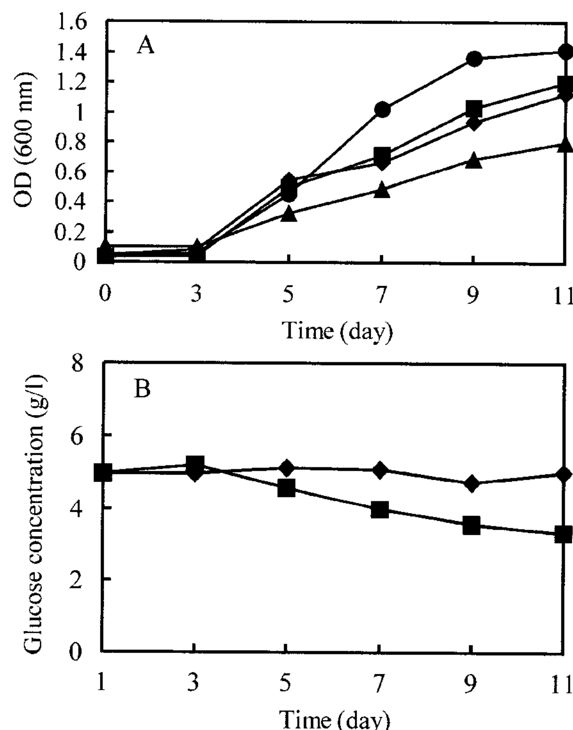

Fig. 6. A: Growth of *A. thiooxidans* transconjugants and the original strains in Starkey-S^0 medium in the absence or presence of 5 g/l glucose. Tt-7 (◆); Tt-7, growth in the presence of glucose (■); Tt-7 (RP4, pSDK-1) (▲); Tt-7 (RP4, pSDK-1), growth in the presence of glucose (●). **B:** Glucose concentration in cultures of Tt-7 (◆) and Tt-7 (RP4, pSDK-1) (■). Each data point represents the mean value of three independent experiments.

was stimulated by the consumption of glucose but its ability to consume the glucose was limited. The expression of the *pfk*A gene in the transconjugant caused assimilation of glucose to the synthesized cell carbon, but only to a limited extent and in a restricted pattern. Since the fixation of CO_2 has a high energy requirement, synthesis of a part of the cell material from glucose instead of CO_2 should have an energy sparing effect, which should lead to an increase in cell yield. It is conceivable that the low activities of enzymes might prevent the metabolism of glucose at a rate fast enough to satisfy major energetic and biosynthetic needs. At the same time, the lack of a complete Krebs cycle and low activities of key enzymes of the cycle undoubtedly limit the capacity to metabolize the cycle intermediates and probably accounts for failure to use them as energy or major carbon sources.

Since the industrial utilization of *A. thiooxidans* has been limited by the slow growth rate and the low cell yield of this organism, the faster growth strains would be useful in the metal leaching from mineral ores. For example, the genetically modified transconjugants can be applied in the accumulate of bacteria cells in the preprocessing of metal leaching by promoting the growth of transconjugants in the presence of glucose or other organic substrates. Our work may provide a possibility to develop new strains with better growth rates and increased leaching efficiencies. However, the activities of several enzymes, especially the key enzyme in the TCA cycle, α-ketoglutarate dehydrogenase, are lacking in *A. thiooxidans*; hence, the growth performance of this organism might not be markedly changed by cloning only one of the enzymes.

Acknowledgments

This work was supported by the National Natural Science Foundation of the People's Republic of China and the Special foundation of Chinese Ministry of Education.

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