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Construction of Recombinant Bioleaching Bacteria *Thiobacillus ferrooxidans* with Fluoride-Resistant Activity

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Abstract: *Thiobacillus ferrooxidans* is an important bioleaching bacterium, widely used in leaching, recovery of low-grade metals and environmental bioremediation. In bio-metallurgy industry, fluoride existing in some hard rock uranium deposits in south China severely affects the bacteria viability and inhibit leaching reproduction. In this study, the *flr-4* gene that has strong resistance to fluorine ion in *Caenorhabditis elegans*, was cloned into pET30a vector and expressed in *E. coli* BL21. the ability of fluoride resistance in *E. coli* was increased obviously. To increase the fluoride-resistance of bioleaching bacterium *T. ferrooxidans* strain 1 (*T.f1*), the *flr-4* shuttle recombinant plasmid pJRD215-*flr-4* was constructed and transferred into *T.f1* by conjugation. pJRD215-*flr-4* was stable in *T.f1* with more than 70% retention after 50 generations. Recombinant *T.f1* was tolerant to fluorine ions and grew well under fluoride (F^-) stress especially at F^- concentrations ranging from 10 mg/L to 60 mg/L.

Keywords: Bioleaching Bacteria, *Thiobacillus ferrooxidans*, Conjugation Transfer, Fluoride Resistance, *flr-4* Gene, Genetic Modification

Introduction

Thiobacillus ferrooxidans (*T. ferrooxidans*), a Gram-negative, extremely acidophilic, obligately autotrophic bacterium, is firstly isolated in 1947 and majorly distributed in soil, seawater and sulfur spring. Now it is industrially widely used in bioleaching, recovery of low-grade copper, uranium and many other precious metals and environmental protection (Martínez-Bussenius *et al*., 2016; Xu *et al*., 2014; Jones *et al*., 2015; Zhang *et al*., 2016; Guven and Akinci, 2013). It is also a bioleaching microorganism that was well studied and was of most economic benefits in biological metallurgy. In the past, considerable works have been done to improve *T. ferrooxidans* genetically, including plasmid isolation (Chakravarty *et al*., 1995), gene cloning (Levicán *et al*., 2002; Guiliani and Jerez, 2000; Butcher *et al*., 2000), identification and isolation (Mahmoud *et al*., 2005; Zhou *et al*., 2003). However, the drawbacks of slow growth rate, low cell yield and sensitivity to uranium, mercury, arsenic, fluoride and other ions have limited its further applications. Therefore, genetic improvement of *T. ferrooxidans* is very urgent for efficient metallurgical function.

Fluorine ion was toxic to eukaryotic organisms (Yu *et al*., 2018; Narsimha, 2018) and prokaryotic cells at high concentrations, the bioleaching bacteria employed in bio-metallurgy are very sensitive to it. Fluoride affects the survival of bacteria and inhibits the cell growth and leaching reproduction. In south China, the hard rock uranium deposits contain large amounts of fluoride (CaF_2). In the process of bioleaching, the fluorine ions are often released from the dissolving ores and accumulated in reactors. In the end, the growth of bacteria is inhibited almost completely. Therefore, the leaching solution needs to be replaced frequently, which results in low production efficiency and the increased cost. So it is very important to increase the fluoride-resistance activity of bioleaching bacteria.

Chansley and Kral (1989) transformed DNA extracted from fluoride-resistant mutants of *Streptococcus mutans* GS-5 into fluoride-sensitive cells of the same strain. Transformation with DNA from first-step mutants produced transformants with resistance to either 600 or 1,000 μg/mL, both of which are within the first-step resistance range (400 to 1,000 μg/mL). In five of six of the transformation experiments, the transformant resistance levels were greater than those of

their respective DNA donors. When a second-step mutant resistant to 3,000 μg/mL was used as a DNA donor, four different levels of resistance were observed in the transformants (600, 1,000, 1,500 and 2,000 μg/mL). In 1994 Katsura *et al.* (1994) analyzed 13 fluoride-resistant mutants of the nematode *Caenorhabdities elegans* (*C. elegans*) and cloned five genes responsible for fluoride resistance (*flr-1*, *flr-2*, *flr-3*, *flr-4* and *flr-5*). Mutants in three of the genes (class 1 genes: *flr-1*, *flr-3* and *flr-4*) are resistant to 400 μg/mL NaF, but they grow twice as slowly as and have smaller brood size than wild-type worms even in the absence of fluoride ion. In contrast, mutants in the other two genes (class 2 genes: *flr-2* and *flr-5*) are only partially resistant to 400 μg/mL NaF, with almost normal growth rates and brood sizes in the absence of fluoride ion. Studies on the phenotypes of double mutants showed that class 2 mutations are epistatic to class 1 mutations concerning growth rate and brood size but hypostatic with respect to fluoride resistance.

In this study, *flr-4* gene was cloned from *C. elegans* and firstly expressed in *E. coli* to observe its effect on the fluoride-resistant activity of *E. coli*, then shuttle expression plasmid pJRD215-*flr-4* was constructed and transferred to *T. ferrooxidans* strain 1 *(T.f1)* in order to increase its ability of fluoride resistance.

Materials and Methods

Bacterial and Plasmid

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

Construction of Expression Vector pET30a-flr-4

The *flr-4* cDNA was generated by reverse transcription-PCR (RT-PCR) from total RNA isolated from *C. elegans*. Briefly, total RNA was isolated from *C. elegans* using UNIQ-10 column total RNA isolation Kit (Sangon, Shanghai) and first strand cDNA was synthesized using PrimeScript™ RT-PCR Kit (TaKaRa), PCR was used to amplify *flr-4* cDNA using the forward primer (5′-CCCGGATCCATGCCAATAAATTAC-3′) and the reverse primer (5′-CTCGAGCTAGTTTTCT-TCATTTGCTGG-3′) which contains restriction sites *Bam*H I and *Xho*l I , respectively. The amplification conditions for PCR were 95°C, 30 s; 61°C 30 s; 72°C, 2 min for 30 cycles, followed by a final extension step at 72°C for 10 min and the PCR enzyme was LA *Taq* with GC Buffer (TaKaRa). The PCR products were cloned into pMD18-T Vector (TaKaRa) and transformed into DH5α. Positive colonies were selected by LB/Amp (100 μg/mL) plates and then 6 mL overnight culture grown. The recombinant plasmid was extracted using EasyPure Plasmid MiniPrep Kit (Trans), the cloned fragment was identified by PCR and double digested overnight with *Bam*H I and *Xho*l I at 37°C, then confirmed by DNA sequencing. The *flr-4* cDNA fragment was recovered after electrophoresis and subcloned into the *Bam*H I and *Xho*l I restriction sites of pET30a expression vector and transformed into *E. coli* BL21 (DE3) competent cells.

Expression Analysis of Flr-4 Gene in E. Coli BL21

The *E. coli* BL21 (DE3) cells transformed with pET30a-*flr-4* plasmid were cultured and then induced with IPTG. Briefly, the cells were inoculated into 6 mL of LB broth containing 50 μg/mL kanamycin (kana) and grown overnight (12 h) at 37°C with gentle shaking. Next day, 1 mL of overnight culture was inoculated into 100 mL of TB broth and incubated at 37°C for about 3 h to reach mid-log phase. Recombinant protein was induced by adding IPTG to a final concentration of 0.01 mmol/L, 0.04 mmol/L, 0.08 mmol/L, 0.1 mmol/L, 0.4 mmol/L, 0.8 mmol/L and 1.0 mmol/L, induction at 37°C for 4 h, then the cells were lysed in 1×SDS sample buffer for 5 min at 100°C and the lysates were analyzed by 10% SDS-PAGE. The proteins in gels were detected by Coomassie brilliant blue R250 staining. To confirm foreign protein Flr-4 expression in BL21 cells Western blotting analysis was conducted using anti-His antibody to the 6×His-tag.

Effect of Flr-4 Products on E. Coli

When the *E. coli* grew to OD_{600} to 0.6~1.0 in TB medium (Kana 50 μg/mL), IPTG was used to induce the expression of *flr-4* gene in recombinant *E. coli*, then the fluoride-resistant ability of *E. coli* was detected. BL21 (DE3)-pET30a-*flr-4* with induction, BL21 (DE3)-pET30a-*flr-4* with no induction and the control BL21 (DE3) were 1:100 inoculated to TB medium respectively, added with IPTG to a final concentration of 1.0 mmol/L and NaF 9.6 g/L. their OD_{600} values were observed every 2 h, compared to the control BL21 (DE3) growth condition in TB medium (not adding NaF).

Construction of pJRD215-flr-4 Shuttle Expression Plasmid

pJRD215 belongs to the broad-host-range IncQ group plasmids and was able to shuttle between *E. coli* and *T. ferrooxidans*. A fluoride-resistant plasmid of pJRD215-*flr-4* was constructed by inserting *flr-4* coding sequence into the IncQ plasmid of pJRD215. The *flr-4* fragment was amplified by PCR with forward primer (5′-GGCGGTACCATGCCAATAAATTACAATCG-3′) and reverse primer (5′-GCCGTCTAGACTAGTTTTCT-TCATTTGC-3′) which contains restriction sites *Kpn* I and *Xba* I , respectively. The *flr-4* amplification fragment and plasmid pJRD215 were digested overnight with *Kpn* I and *Xba* I at 37°C, the *flr-4* and pJRD215 fragments were recovered after gel electrophoresis, then *flr-4* coding sequence was subcloned into pJRD215 vector and the recombinants were transformed into *E. coli* SM10. The inserted fragment was identified by PCR, double enzyme digestion and DNA sequencing.

Table 1: The strains and plasmids used in this study

Strain or plasmid	Phenotype or genotype	Source or reference
E. coli		
DH5α	F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1	This laboratory
BL21 (DE3)	F- ompT hsdSB(rB-mB-) dcm gal（DE3）	CGMCC
SM10	thr leu hsd recA Km^r RP4-Tc::Mu	CGMCC
T. ferrooxidans		
T.f1	Wide type, private strain	isolated
Plasmid		
RP4	Ap^r Tc^r KM^r IncP Tra^+	Pansegrau *et al.* (1994)
pMD18-T	Ap^r	Taraka
pET30a	$Kana^r$	invitorgen
pJRD215	$Kana^r$ Sm^r IncQ Mob^+	invitorgen

Conjugation Transfer of pJRD215-flr-4 into T.f1

The successful conjugation transfer between *E. coli* and *T. ferrooxidans* has been reported by Peng *et al*. (1994b). According to them, we tried to transfer the pJRD215-*flr-4* into *T.f1* by conjugation transfer. The kanamycin resistance was chosen as the selection marker.

The donor *E. coli* SM10 (pJRD215-*flr-4*) was grown in 2:2 basal salt medium supplemented with 0.5% yeast extract and Kana for the plasmid at 37°C. The recipient *T.f1* was grown in 9K sulfur liquid medium (pH 3.5) at 30°C. Donor cells were harvested by centrifugation at late exponential growth phase, recipient cells were harvested at the stationary phase. Iron or sulfur precipitates were removed by gentle centrifugation (100×g). Both the donor and recipient cells were washed three times with basal salt solution of mating and then mixed at ratio of 1:1. Then 0.1 mL of cell suspension (approximate 4×10^{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 60 h, the filter was transferred to 3.0 mL of basal salt solution of solid 2:2 medium, diluted and plated on 2:2 solid medium with Kanamycin. Then positive colonies were selected and identified by PCR.

Analysis of The Stability of pJRD215-flr-4 in T.f1

In accordance with Peng *et al.* (1994b), the single colonies of *T.f1* transconjugants on the selective plates were transferred into 9K liquid medium (20 mL), 1:1000 of the fully grown culture was transferred to 20 ml of fresh 9K liquid medium and cultured at 30°C with vigorous shaking for 5 days. After five transfers (more than 50 generations) had been made, samples were diluted and plated on solid 2:2 medium with or without kanamycin (300 μg/mL) and cultured at 30°C for two weeks. The colony numbers on the plates were counted, plasmid stability was calculated as the ratio between the number of colonies observed in the presence and absence of kanamycin.

Measurement of Fluoride–Resistant Activity of T.f1 Transconjugants

Wild-type *T.f1* and *T.f1* transconjugants were inoculated into 9K medium containing NaF of various final concentrations from 0 to 80 mg/L, cell numbers was counted by microscopic counting every day using blood cell counting board. The growth curves were made according to log cell numbers per milliliter culture versus culture time.

Results

Construction of pMD18-T-flr-4 and pET30a-flr-4

From *C. elegans,* total RNA was isolated and the coding sequence of *flr-4* was obtained by RT-PCR with the use of the gene specific primers, then was inserted into cloning vector pMD18-T. The amplified *flr-4* fragment was about 1750 bp (as shown in Fig. 1). After flr-4 coding sequence was confirmed by DNA sequencing, it was cloned into expression vector pET30a. As shown in Fig. 2, the molecular sizes of both *flr-4* fragment and pET30a plasmid are correct, indicating that the *flr-4* coding sequence was inserted successfully into pET30a vector.

Expression of the Protein Flr-4 in E. Coli

The *E. coli* BL21 (DE3) cells transformed with plasmid of pET30a-*flr-4* produced recombinant 6×his fusion protein. SDS-PAGE analysis revealed that recombinant Flr-4 accumulated up to 30% of the total proteins of *E. coli*, with an expected molecular weight of 65kD. Recombinant protein Flr-4 in *E. coli* (DE3) was detected by Western blotting analysis with an anti-His antibody (as shown in Fig. 3).

Effect of flr-4 Expression on E. Coli

To investigate the role of Flr-4 in *E. coli* recombinants, we cultured recombinant BL21 carrying pET30a-*flr-4* in TB medium with or without NaF. As shown in Fig. 4, wild-type BL21 (DE3) grew well without NaF and grew slower with 9.6 g/L NaF.

Whereas, BL21-pET30a-*flr-4* grew well with the pressure of 9.6 g/L NaF after induction of IPTG, its growth curve was similar to that of wild-type BL21 cultured in normal TB medium (not adding NaF), meanwhile, BL21-pET30a-*flr-4* after induction of IPTG displayed a marked growth advantage over wild-type BL21 in the presence of NaF. These results suggested that the *flr-4* expression products in *E. coli* BL21 were effective and functional and that Flr-4 protein conferred the fluoride-resistant activity of host cells.

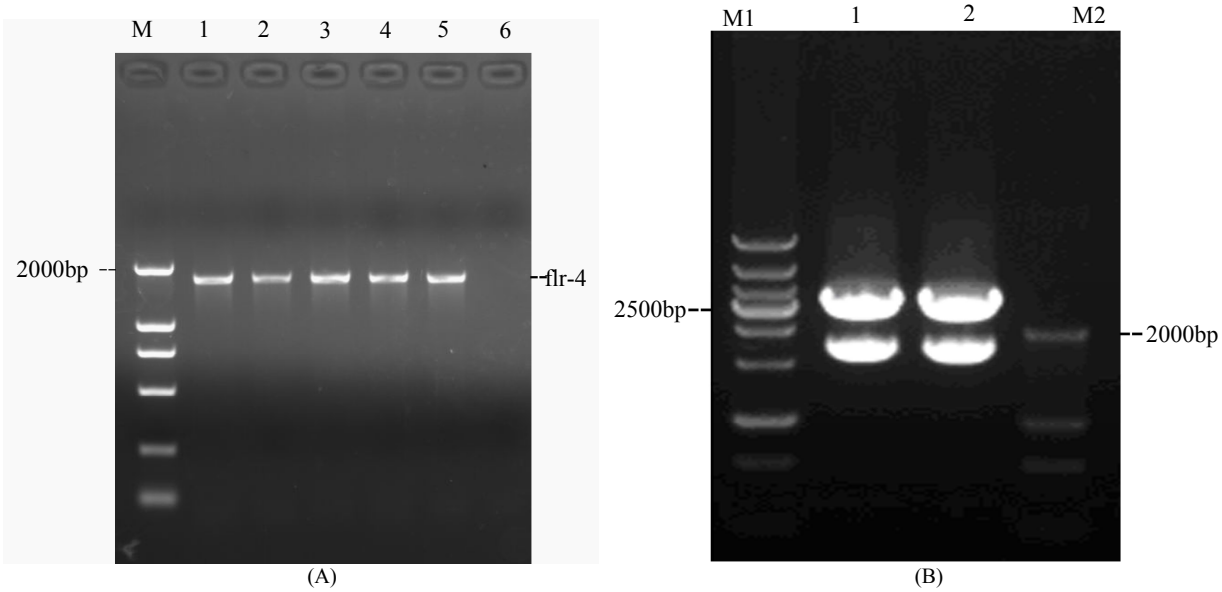

Fig. 1: Agarose gel electrophoresis of PCR analysis (A) and double enzyme digestion by BamH Ⅰ and Xhol Ⅰ (B) of recombinant plasmid pMD18-T-italic type (A. M: DL2000 DNA Marker, Lane 1~4: the fragment of flr-4, Lane 5: positive control, 6: negative control; B. M1: DL6000 DNA Marker, Lane 1~2: pMD18-T-italic type(BamH Ⅰ/Xhol Ⅰ), M2: DL2000 DNA Marker)

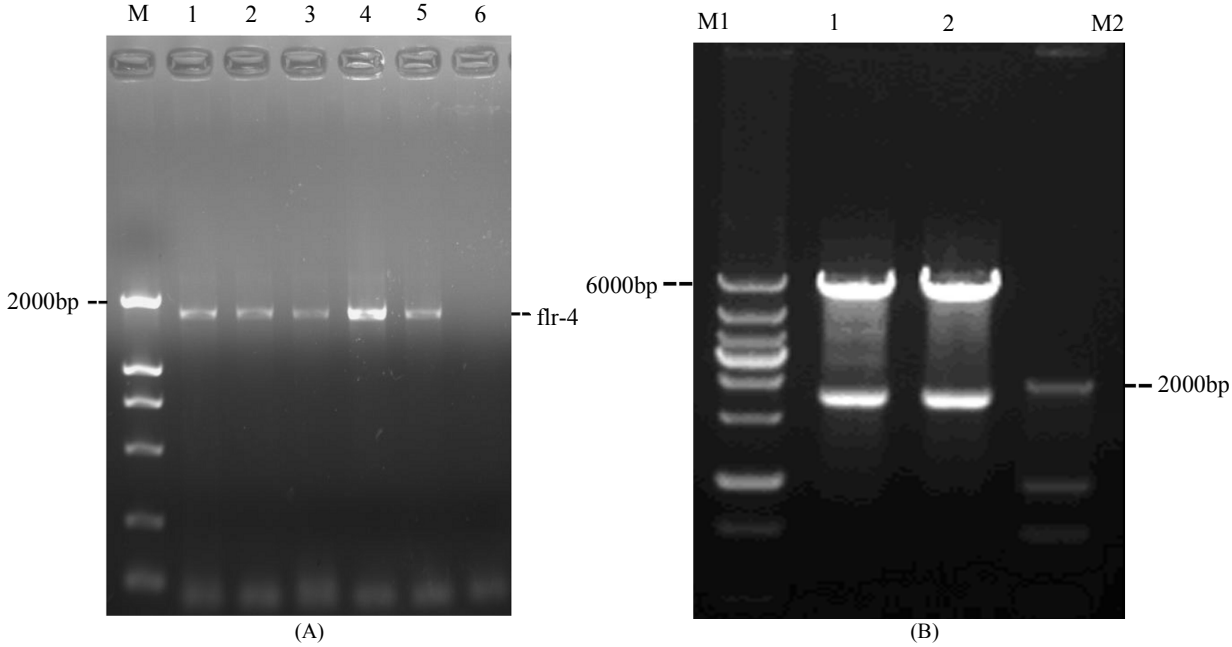

Fig. 2: Agarose gel electrophoresis of PCR analysis (A) and double enzyme digestion by BamH Ⅰ and Xhol Ⅰ (B) of recombinant plasmid pET30a- italic type; (A. M: DL2000 DNA Marker, Lane 1~4: the fragment of flr-4, Lane 5: positive control, 6: negative control; B. M1: DL6000 DNA Marker, Lane 1~2: pET30a- italic type (BamH Ⅰ/Xhol Ⅰ), M2: DL2000 DNA Marker)

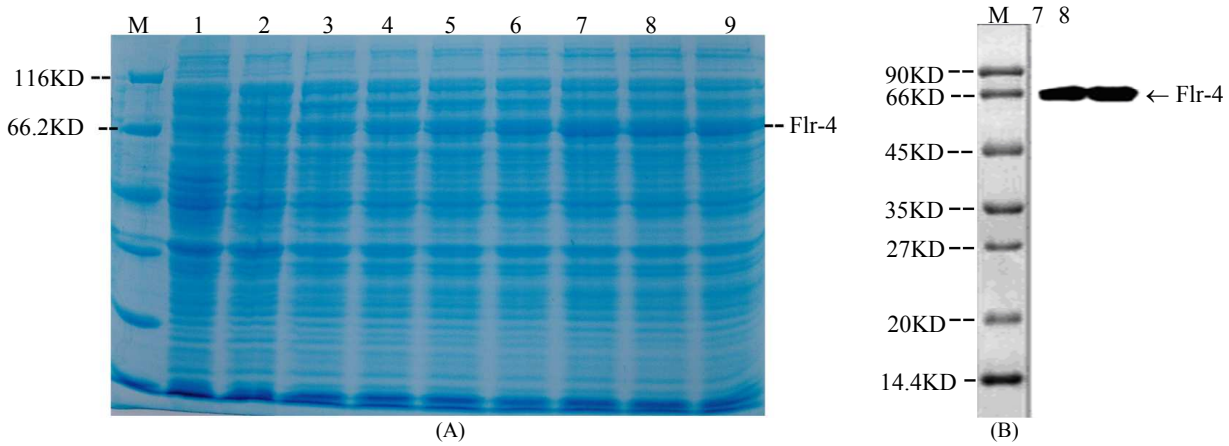

Fig. 3: The flr-4 expression in BL21 cells analysed by SDS-PAGE (A) and Western blotting analysis (B) (A. M: protein Marker, Lane 1: the total protein of BL21 (DE3), Lane 2: the total protein of recombinant BL21, Lane 3~9: Flr-4 expression in BL21 induced by IPTG, 0.01, 0.04, 0.08, 0.1, 0.4, 0.8, 1.0 mmol/L for 4 h, respectively; B. M: protein Marker, Lane 7 and Lane 8: Flr-4 expression in BL21 induced by 0.4 mmol/L and 0.8 mmol/L IPTG for 4 h, respectively)

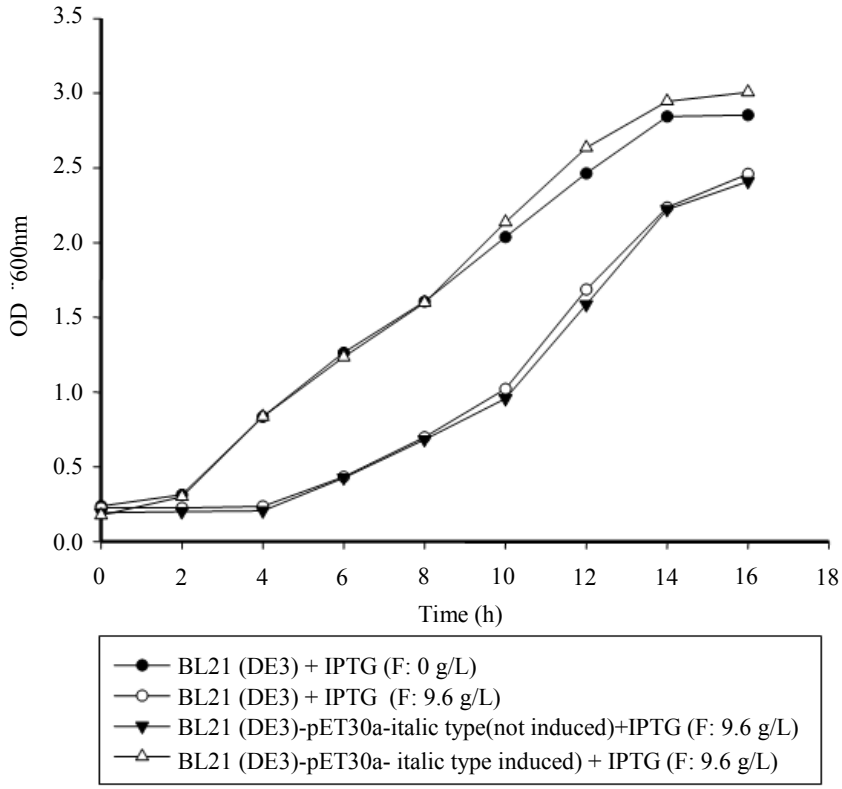

Fig. 4: The growth curves of *E. coli* and recombinant italic type which carried pET30a- italic type in different conditions

Construction of pJRD215-flr-4 Vector and Fluoride-Resistant T. Ferrooxidans

As shown in Fig. 5, the PCR products were clear and specific and the sizes of both the target fragment *flr-4* and vector pJRD215 are right, confirming that the *flr-4* fragment was inserted into pJRD215 successfully. Then *flr-4* fragment was identified in recombinant plasmid pJRD215-*flr-4* by DNA sequencing. The fluoride-resistant plasmid pJRD215-*flr-4* was transferred from *E. coli* SM10 to *T. fl* through conjugation with the help of the chromosome

integrated plasmid RP4 in *E. coli* SM10, kanamycin were selected using 2:2 solid selective plates. In order to confirm that conjugational transfer of pJRD215-*flr-4* from SM10 to *T.f1* was successful, PCR was performed. The results demonstrated that pJRD215-*flr-4* was indeed transferred into *T. f1* (not shown).

The Stability of pJRD215-flr-4 in T.f1

The stability of pJRD215-*flr-4* in *T.f1* was determined by checking for kanamycin resistance as described in Materials and Methods section. The results showed that plasmid pJRD215-*flr-4* carrying a heterogenous fluoride-resistant gene was quite stable in *T.f1*, more than 70% of *T.f1* cells remained to maintain pJRD215-*flr-4* plasmid after 50 generations without selective pressures of antibiotics and NaF (as shown in Fig. 6).

The Fluoride Resistance of T.f1 Transconjugants

The fluoride resistance ability of recombinant strain *T.f1* was evaluated by comparing its cell growth with the wild-type *T.f1*. The *T.f1* strains were culture in 9K liquid medium, with a 1:40 inoculation. Cell numbers were counted under microscope. The cell growth rates of *T.f1* transconjugants and wild-type *T.f1* in the presence of different concentration of NaF were shown as Fig. 7. The wild-type *T.f1* was quite sensitive to fluoride ion. The cell growth of wild-type *T.f1* started to be inhibited from 10 mg/L, its growth was influenced severely and it took 8 days to reach stable phase under 20 mg/L NaF, in contrast to 4 days under NaF-free medium. When fluoride levels achieved to 40 mg/L, the wild-type *T.f1* could not grow. In contrast, the recombinant *T.f1* improved the viability, it could grow at fluoride levels of 60 mg/L and it took 7 days to reach stable phase. Its growth was inhibited completely until fluoride ion concentration rose to 80 mg/L. The results showed that the fluoride resistance ability of recombinant *T.f1* was increased obviously, which gives the strain potential in applications of bioleaching in fluoride-containing ores.

Discussion

Compared to the traditional processes in leaching, microbial metallurgy becomes increasingly important, bioleaching is a conversion of an insoluble valuable metal into a soluble form by means of microorganisms (Schippers *et al.*, 2014). It has bright prospects in industry with the advantages of low cost, low energy consumption and environment-friendly, especially in the field of leaching of low-grade ores. Up to today many environmental microorganisms of importance in biomining and bioremediation have been isolated and domesticated from nature (Navarro *et al.*, 2013; Gumulya *et al.*, 2018; Ng *et al.*, 2016), such as *Acidithiobacillus caldus* (*A. caldus*), *Letospirillum ferriphilum* and *Acidithiobacillus thiooxidans*, *Thermophillic thiobacilli, T. ferrooxidans and* so on. Among them, *T. ferrooxidans* is one of the microorganisms which were studied and applied most widely and deeply (Brierley and Brierley, 2013; Latorre *et al.*, 2016).

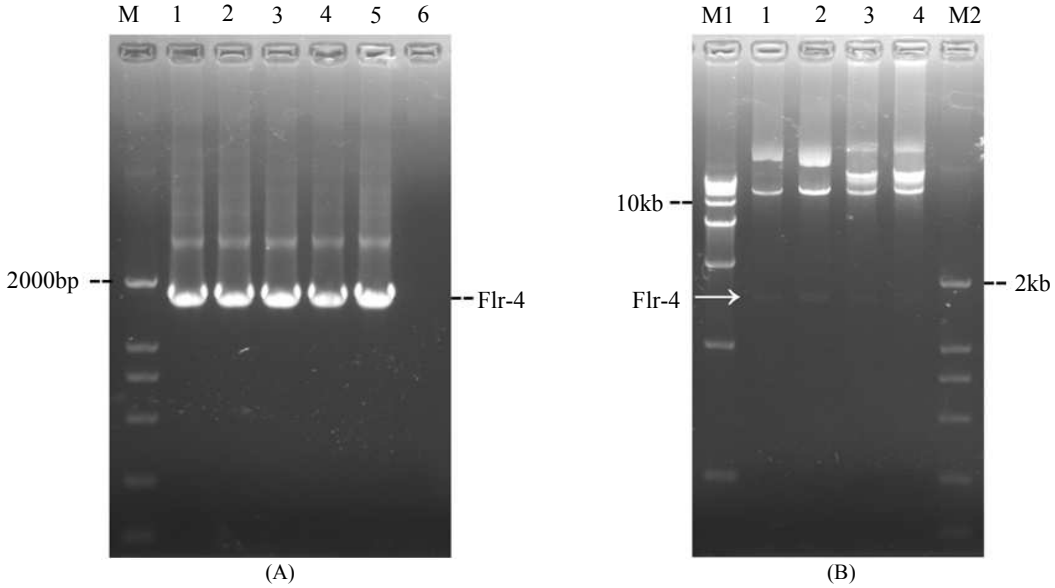

Fig. 5: Agarose gel electrophoresis of PCR analysis (A) and double enzyme digestion by *Kpn* I /*Xba*l I (B) of pJRD215-*flr-4* recombinants; (A. M: DL2000 DNA Marker, Lane 1~4: the fragment of *flr-4*, Lane 5: positive control, 6: negative control; B. M1: DL15000 DNA Marker, Lane 1~4: pJRD215-*flr-4* (*Kpn* I /*Xba*l I), M2: DL2000 DNA Marker)

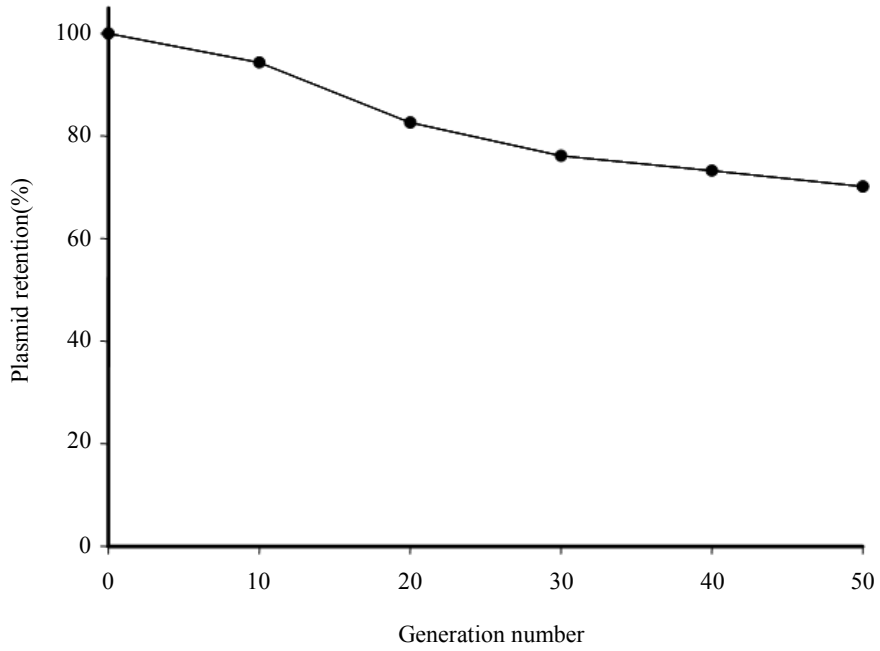

Fig. 6: The maintenance of the recombinant plasmid pJRD215- italic type in italic type

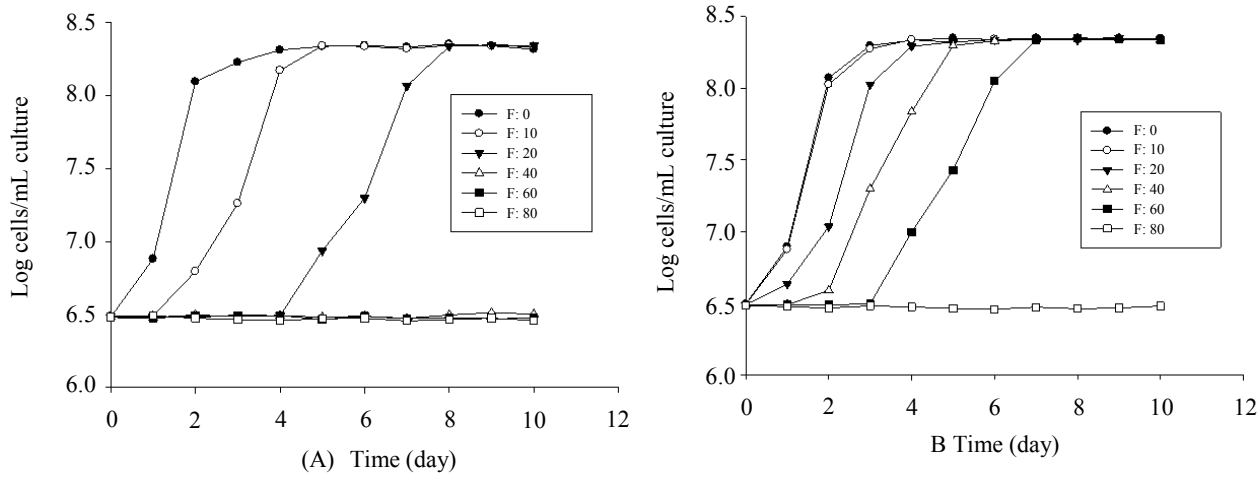

Fig. 7: Growth curves of wild-type *T.f1* (A) and recombinant *T.f1* (pJRD215-*flr-4*) (B) under various NaF concentrations

In south China, large quantities of fluoride in the hard rock uranium deposits and some mineral ores will be released continuously during leaching process and limited the performance of microbial leaching. Therefore it is very urgent to do the genetic modification of *T. ferrooxidans* or other leaching microorganisms to increase their fluoride resistance. However, Due to the lack of interest genes and transfer tools the genetic improvement of this bacterium progressed slowly.

Introduction of plasmids into *T. ferrooxidans* strains has been first reported by electrotransformation, but the efficiency of electrotransformation was low (Kusano *et al*., 1992). Then Peng *et al*. (1994b) reported that the broad-host-range IncQ vector pJRD215 was mobilized to *T. ferrooxidans* with the aid of plasmid RP4 integrated in the chromosome of *E. coli* SM10 by conjugation transfer, pJRD215 was rather stable and all genetic markers were expressed in *T. ferrooxidans*. Liu *et al*. (2001) transferred IncQ (pJRD215), IncP (pJB3Kml) and IncW (pUFR034) group plasmids from *E. coli* to four *T. ferrooxidans* strains by conjugation, IncQ plasmid pJRD215 had the higher transfer frequency and was stable in these four strains. We concern about

whether an exogenous gene can be expressed in *T. ferrooxidans* strains. Peng *et al.* (1994a) constructed two arsenic-resistant plasmids based on pJRD215 and transferred them into *T. ferrooxidans* strains by conjugation, the arsenic resistance genes originating from a heterotrophic bacterium were expressed in this obligately autotrophic bacterium and arsenic-resistant abilities of *T. ferrooxidans* transconjugants were increased obviously. Chen *et al.* (2011) also used the IncQ plasmid of pJRD215 to construct a mercury-resistant plasmid of pTMJ212 and then transferred it from *E. coli* into *A. caldus* through conjugation. They observed that the cell growth of the recombinant *A. caldus* increased markedly under mercury stress. Together, it suggested that conjugation was a reliable genetic transfer tool from *E. coli* to *T. ferrooxidans*. Meanwhile, mobilizable plasmid pJRD215 was stable in many hosts besides *T. ferrooxidans* and could effectively carry and express exogenous genes (Meng *et al*., 2013). Therefore in this study we chose conjugation transfer and IncQ vector pJRD215 to introduce foreign genes into *T. ferrooxidans*.

Despite fluoride-resistant gene *flr-4* is originated from *C. elegans* which completely differs from *E. coli*, it was expressed and functionally effective in *E. coli* BL21 (DE3). The cell growth of wild-type BL21 (DE3) was inhibited in TB medium containing 9.6 g/L NaF, but that of the recombinant BL21 (DE3) expressing fusion protein Flr-4 was not influenced in this medium, it implied that the heterogenous protein Flr-4 could function properly in fluoride-resistance in BL21. After recombinant plasmid pJRD215-*flr-4* was conjugated into *T.f1*, compared with wild-type *T.f*1, recombinant *T.f*1 could grow under higher NaF pressure and reached stable phase in shorter time.

This study investigated the feasibility to increase the fluoride resistance for *T. ferrooxidans* and other bio-leaching bacterium by genetic improvement. In further researches, the more efficient plasmids and the genes with some special features need to be developed.

Conclusion

Fluoride existing in the hard rock uranium deposits and some mineral ores restricted the performance of microbial leaching, genetic modification provided a desired tool for bioleaching microorganisms to improve their anti-fluoride activity. In this study, the fluoride-resistant recombinant plasmid pJRD215-*flr-4* was constructed and transferred into *T. ferrooxidans* successfully by conjugation. The recombinant plasmid of pJRD215-*flr-4* was maintained stably in *T.f1* and recombinant *T.f1* grew very well under fluoride (F^-) stress especially at F^- concentrations ranging from 10 to 60 mg/L.

Acknowledgment

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Author's Contributions

Yun-Chang Cao and Hong-Bo Wen: Designed and performed the experiments, analyzed the data and prepared the paper.

Nan Hu and Yong-Dong Wang: Performed gene cloning experiments and constructed the recombinant plasmids.

Guang-Yue Li and De-Xin Ding: Designed the experiments and revised the manuscript.

Ethics

The authors declare their responsibility for any ethical issues that may arise after the publication of this manuscript.

Conflict of Interest

The authors declare that they have no competing interests. The corresponding author affirms that all of the authors have read and approved the manuscript.

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