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Detection and validation of a small broad-host-range plasmid pBBR1MCS-2 for use in genetic manipulation of the extremely acidophilic *Acidithiobacillus* sp.

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ABSTRACT

An efficient genetic system for introducing genes into biomining microorganisms is essential not only to experimentally determine the functions of genes predicted based on bioinformatic analysis, but also for their genetic breeding. In this study, a small broad-host-range vector named pBBR1MCS-2, which does not belong to the IncQ, IncW, or IncP groups, was studied for the feasibility of its use in conjugative gene transfer into extremely acidophilic strains of *Acidithiobacillus*. To do this, a recombinant plasmid pBBR-tac-Sm, a derivative of pBBR1MCS-2, was constructed and the streptomycin resistant gene (Sm^r) was used as the reporter gene. Using conjugation, pBBR-tac-Sm was successfully transferred into three tested strains of *Acidithiobacillus*. Then we measured its transfer frequency, its stability in *Acidithiobacillus* cells, and the level of resistance to streptomycin of the transconjugants and compared this with the IncQ plasmid pJRD215 control. Our results indicate that pBBR1MCS-2 provides a new and useful tool in the genetic manipulation of *Acidithiobacillus* strains.

1. Introduction

The extremely acidophilic, obligatory chemolithoautotrophic thiobacilli, such as *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus caldus*, are widely spread in sulfide deposits, acid mine water and soil. They can obtain energy from the oxidation of reduced sulfur compounds or ferrous iron to support their autotrophic growth on carbon dioxide or carbonate (Rawlings, 2002, 2005; Dopson and Lindstrom, 1999; You et al., 2011). Since they possess the unique physiological characteristics and outstanding capacity to grow under pH 2.0, these bacteria do not only have widespread industrial applications in mineral leaching, and desulphurization of coal and oil, but also are interesting to study from a fundamental biological point of view (Rawlings, 2005) .

Now the entire or partial genome sequences of some strains of the acidophilic thiobacilli are available in GenBank, such as *At. ferrooxidans* ATCC 23270, *At. ferrooxidans* ATCC 53993, *At. caldus* ATCC 51756 and *At. caldus* SM-1 (Valdes et al., 2008a, 2009; You et al., 2011). Bioinformatic-based analysis coupled with studies on genomics, metagenomics, comparative genomics, transcriptomics, etc., provides a valuable platform to search for genome-wide candidate genes encoding proteins involved in important metabolic pathways and many predicted regulatory models for iron and sulfur energy metabolism and central carbon metabolism have been constructed (Quantrini et al., 2006, 2009; Valdes et al., 2008b; Bonnefoy and Holmes, 2011; Bird et al., 2011). This knowledge greatly helps us to understand the physiological functions and roles of these microorganisms in bioleaching. However, there are still many hypothetical reactions and missing steps in these metabolic pathways. So, a convenient genetic system would be helpful not only for elucidating the functions of candidate genes involved in these pathways but also to facilitate the genetic breeding of *Acidithiobacillus* strains for industrial applications.

The extremely acidophilic bioleaching microorganisms of *Acidithiobacillus* are difficult to handle experimentally that their genetic system has been particularly challenging. Before there has been only one report each about introducing plasmids into strains *At. ferrooxidans* and *At. caldus* by electrotransformation (Kusano et al., 1992; Chen et al., 2010). The method of conjugation has been successfully developed in three species of *Acidithiobacillus* (Jin et al., 1992; Peng et al., 1994; Liu et al., 2007). With this method some plasmids from different incompatibility broad-host-range (bhr) groups, such as IncQ, IncP, and IncW, have been transferred from *E. coli* cells into different *Acidithiobacillus* strains. So far, the IncQ plasmids have been shown to be the best cloning vectors in *Acidithiobacillus* (Liu et al., 2000, 2007). By introducing and expressing heterologous genes on plasmids in *Acidithiobacillus*, the characteristics of the engineered strains have been improved, such as increased mercury resistance, or improved capacity for Fe^{2+} or glucose metabolism (Chen et al., 2011; Liu et al., 2011; Tian et al., 2004). However, the

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plasmids are typically large in size and difficulty in genetic manipulation makes using the currently available IncQ vectors, such as plasmid pJRD215, extremely discommodious and laborious. So, more efficient vectors are undoubtedly essential and important to satisfy the increasing need for genetic studies of *Acidithiobacillus*.

In this study, a small bhr plasmid pBBR1MCS-2, which does not belong to the IncQ, IncW, or IncP groups (Kovach et al., 1995), was studied for the feasibility of using the conjugative gene transfer system from *E. coli* into the extremely acidophilic *At. caldus* and *At. thiooxidans*. To characterize the functions of the new plasmid, the streptomycin resistant gene (*Sm*^{r}) was used as a reporter gene and all experiments were carried out simultaneously and compared with an IncQ plasmid pJRD215 control.

2. Materials and methods

2.1. Strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria broth or Luria agar at 37 °C. *At. caldus* MTH-04 (Liu et al., 2004), isolated from the acidic drainage of a hot-spring in the Tengchong area, Yunnan Province of P. R. China, was grown at 40 °C in modified inorganic liquid Starkey-S^0 or solid Starkey-$Na_2S_2O_3$ medium (Starkey, 1925) as described previously (Jin et al., 1992). Sulfur, previously sterilized by intermittent steaming, was added aseptically at about 10 g/L at the time of inoculation. The solid Starkey-$Na_2S_2O_3$ medium was prepared in two parts, the double-strength basal salts (pH 4.8) and agar, which were separately autoclaved and combined after cooling to 50 °C. Sodium thiosulfate, previously sterilized by passage through a sterile Millipore filter, was added at the same time to a final concentration of 1% (wt/vol). *At. thiooxidans* ATCC 19377 was grown in the above media at 30 °C. The solid Starkey-$Na_2S_2O_3$ medium was supplemented with 0.05% (wt/vol) yeast extract, when used as a mating medium for *E. coli* and *Acidithiobacillus* sp. Ampicillin (Ap) (50–100 μg/mL), kanamycin (Km) (50–100 μg/mL) or streptomycin (Sm) (50–100 μg/mL) was added to the LB medium for *E. coli*, and Km (300 μg/mL) or Sm (300 μg/mL) was added to the Starkey-S^0 liquid medium, whereas Km (50–100 μg/mL) or Sm (50–100 μg/mL) was added to the solid Starkey-$Na_2S_2O_3$ medium for selection of *Acidithiobacillus* transconjugants.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
E. coli SM10	*Thr leu hsd recA Km*^{r} *RP4-2-Tc::Mu*	Simon et al., 1983
E. coli JM109	*RecA1, supE44, endA1, hsdR17, gryA96, relA1, thi(lac-proAB), lacZ, laclq, traD36*	Laboratory stored
E.coli C600	*Integrated thr, leu, hsd*	Laboratory stored
At. caldus MTH-04	Wild type *At. caldus*	Liu et al., 2004
At. thiooxidans 19377	Standard ATCC wild type *At. thiooxidans*	ATCC[a]
At. thiooxidans 12	Wild type *At. thiooxidans*	Laboratory stored
Plasmids		
pBBR1MCS-2	*Km*^{r}, pBBR1 replicon, *mob*$^+$	Kovach et al., 1995
pUC19	*Ap*^{r}, ColE1 replicon, cloning vector	Laboratory stored
pMMB6	*Ap*^{r}, *Sm*^{r}, IncQ, *mob*$^+$	Bagdasarian et al., 1983
pJRD215	*Km*^{r}, *Sm*^{r}, IncQ, *mob*$^+$	Davison et al., 1987
RP4	*Ap*^{r}, *Tc*^{r}, *Km*^{r}, IncP, *tra*$^+$	Datta et al., 1971
pBBR-tac-Sm	pBBR1MCS-2 containing *Sm*^{r} gene with P_{tac}	This study
pUC-tac-Sm	pUC19 containing *Sm*^{r} gene with P_{tac}	This study

[a] ATCC, American Type Culture Collection.

2.2. Conjugation

The conjugation experiments between *E. coli* cells and *At. caldus* or *At. thiooxidans* were performed by filter mating as described previously (Liu et al., 2007). The mating temperature for the cross was 37 °C between *E. coli* and *At. caldus*, and 30 °C between *E. coli* and *At. thiooxidans*. The incubations for the selection of transconjugants of *At. caldus* and *At. thiooxidans* were performed at 40 °C and 30 °C, respectively. The frequencies of plasmid transfer were calculated based on the number of transconjugants on selective plates divided by the number of recipients on nonselective plates (Tian et al., 2004).

2.3. Chemicals, enzymes, and DNA manipulations

Ampicillin, kanamycin, and streptomycin were purchased from Sangon (Shanghai, China). Restriction enzymes, T4 DNA ligase, λ DNA/*Hin*d III and DL2000^{TM} DNA markers were purchased from TaKaRa (Dalian, China). Plasmid DNA was prepared using a Plasmid Mini Kit I (OMEGA Bio-tek, USA). DNA was separated on agarose gels and purified using a Gel Extraction Kit (OMEGA Bio-tek, USA). Restriction endonuclease digestion, ligation, transformation, agarose gel electrophoresis, and other standard recombinant DNA techniques were performed according to standard procedures (Sambrook et al., 1989).

2.4. PCR

PCR was performed using PrimeSTAR^{TM} HS DNA polymerase from TaKaRa (Dalian, China) according to the manufacturer's recommendations. The primers used for PCR amplification were synthesized by Invitrogen Biotechnology Co. Ltd (Shanghai, China). In general, 50–100 ng of DNA was used in a 50 μL reaction volume containing 10 μL 5×PrimeSTAR buffer (Mg^{2+} plus), 4 μL dNTP mixture (2.5 mM each), 1 μM of each primer, and 0.5 μL of PrimeSTAR^{TM} HS DNA polymerase (2.5 U/μL). Reactions were carried out in a DNA Thermal Cycler 480 from PERKIN ELMER with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60.4 °C for 30 s, and elongation at 72 °C for 2.5 min, and then a 10-min extension incubation at 72 °C.

2.5. Construction of plasmid pBBR-tac-Sm

First, the 2088-bp DNA fragment carrying a *Sm* resistance gene with the *tac* promoter was amplified by PCR from plasmid pMMB6 (Bagdasarian et al., 1983) using primers F1 (5′-CCACAAGCTTATCGACTGCACGGT-3′) and F2 (5′-TAGTGGATCCTGTTTGGGGTCGTTTG-3′), based on the sequence of *tac* promoter (GenBank ID: K01728) and *Sm* resistance gene of plasmid RSF1010 (GenBank ID: NC001740), respectively. *Hin*d III and *Bam*H I sites were added to the primers. The amplified fragments were double digested with *Hin*d III and *Bam*H I, and then inserted into the *Hin*d III/*Bam*H I cloning sites of pBBR1MCS-2. The resulting plasmid was 7188 bps in size and designated as pBBR-tac-Sm.

2.6. DNA sequencing

The inserted *Hin*d III–*Bam*H I fragment of pBBR-tac-Sm was subcloned into the *Hin*d III/*Bam*H I cloning sites of pUC19 to generate pUC19-tac-Sm, which was used for sequencing. Sequencing reactions were carried out using a 3730 DNA analyzer by Invitrogen Biotechnology Co. Ltd (Shanghai, China). The sequencing result of the