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Construction of Conjugative Gene Transfer System Between *E. coli* and Moderately Thermophilic, Extremely Acidophilic *Acidithiobacillus caldus* MTH-04

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Abstract A genetic transfer system for introducing foreign genes to biomining microorganisms is urgently needed. Thus, a conjugative gene transfer system was investigated for a moderately thermophilic, extremely acidophilic biomining bacterium, *Acidithiobacillus caldus* MTH-04. The broad-host-range IncP plasmids RP4 and R68.45 were transferred directly into *A. caldus* MTH-04 from *Escherichia coli* by conjugation at relatively high frequencies. Additionally the broad-host-range IncQ plasmids pJRD215, pVLT33, and pVLT35 were also transferred into *A. caldus* MTH-04 with the help of plasmid RP4 or strains with plasmid RP4 integrated into their chromosome, such as *E. coli* SM10. The Km^r and Sm^r selectable markers from these plasmids were successfully expressed in *A. caldus* MTH-04. Futhermore, the IncP and IncQ plasmids were transferred back into *E. coli* cells from *A. caldus* MTH-04, thereby confirming the initial transfer of these plasmids from *E. coli* to *A. caldus* MTH-04. All the IncP and IncQ plasmids studied were stable in *A. caldus* MTH-04. Consequently, this development of a conjugational system for *A. caldus* MTH-04 will greatly facilitate its genetic study.

Key words: *Acidithiobacillus caldus*, moderately thermophilic, conjugative transfer, IncP plasmid, IncQ plasmid

Microbes have participated in the deposition and solubilization of heavy metals in the earth's crust since geologically ancient times. Now, the use of acidophilic, chemolithotrophic iron- and sulfur-oxidizing microbes in processes to recover metals from certain types of copper, uranium, and gold-bearing minerals or mineral concentrates is well established [2, 3, 21]. Furthermore the bacterial leaching of sulfur-containing minerals in bioreactors has been commercialized in many parts of the world, including Australia, South Africa, and South America [8, 29].

A variety of chemoautolithotrophic bacteria, which are capable of oxidizing iron- or sulfur-containing minerals, can be easily isolated from acidic mine drainage and mineral ores. It was also recently established that at higher temperatures (between 40 and 50°C), *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) and *Leptospirillum ferrooxidans* are the dominant organisms in oxidizing ores, and it has been well documented that these organisms play roles in the bioleaching process as bacterial consortia [8, 24, 27, 28, 30].

A. caldus is a Gram-negative, moderately thermophilic (40 to 50°C), acidophilic, sulfur-oxidizing, and chemolithotrophic bacterium [11, 14, 15]. It derives energy by oxidizing reduced or partially reduced sulfur compounds (sulfur, thiosulfate, tetrathionate, *etc.*) and fixes carbon dioxide from the atmosphere [10,18]. It is also the most common sulfur (S^0)-oxidizing bacterium isolated from continuous biooxidation reactors operating at temperatures between 40 and 50°C, and would seem to have a potential role in the recovery of gold from arsenopyrite ores when used together with iron-oxidizing bacteria from the genus *Leptospirillum* [8, 16, 27].

Although the physiology of *A. caldus* has been well studied, little information is available about its molecular genetics. Recently, a broad-host-range IncQ-like plasmid, pTC-F14, was isolated from *A. caldus* and its mobilization region analyzed [12, 35]. In addition, arsenical resistance, which is important for bioleaching microorganisms in the processing of arsenopyrite, was found in *A. caldus* via an inducible chromosomally encoded mechanism, although the level of arsenical resistance was very low [9]. Similar to other chemoautolithotrophic biomining bacteria, such as *A. ferrooxidans* and *A. thiooxidans*, certain characteristics of *A. caldus* (*e.g.*, its slow growth rate, low cell yield, and

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low level of arsenical resistance) limit its leaching efficiency. Therefore, genetic improvement of this organism is needed to facilitate more efficient industrial application. However, no data have yet been presented regarding a way to introduce foreign genes into this extremely acidophilic biomining bacterium, *A. caldus*.

Conjugation, one of the main gene transfer processes among bacteria, involves the transmission of genetic material from one bacterium to another and requires cell-to-cell contact. Although the transfer frequency of conjugation is lower when compared with transduction by phage and transformation, it is still a very useful method for introducing foreign genes to receptors, in the case of no phages or available electrotransformation method. Early in the 1980s, gene transfer by conjugation was reported from *E. coli* to certain *Thiobacillus* strains growing preferentially at a near-neutral pH, such as *T. novellas* [5], *T. neapolitanus* [19], and *T. versutus* [33]. In the 1990s, successful plasmid transfer by conjugation from *E. coli* to two extremely acidophilic obligately autotrophic bacteria, *T. thiooxidans* (now *A. thiooxidans*) [17] and *T. ferrooxidans* (now *A. ferrooxidans*) [26], was performed by the current authors, despite marked differences in the growth requirements between the two genera. As a result, the level of resistance to arsenic was greatly increased by the expression of arsenical-resistant genes constructed in the plasmid in *A. ferrooxidans* [25]. Accordingly, this study examined the transfer of plasmids by conjugation from *E. coli* to the extremely acidophilic chemolithotrophic *A. caldus* as a method of genetically engineering this organism for both biomining and basic research.

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were grown in a Luria broth or Luria agar at 37°C, whereas the strains of *A. caldus* MTH-04, isolated from the acidic drainage of a hot-spring in the Tengchong area, Yunnan Province of P. R. China, were grown at 40°C in a liquid Starkey-S^0 inorganic medium, as described previously [17]. Elemental sulfur (S^0) (boiling sterilized) was added after inoculation. The solid Starkey-$Na_2S_2O_3$ medium (pH 4.6–4.8.) for *A. caldus* was prepared by mixing components A (double-strength basal salts, pH 4.8–5.0) and B (2% [wt/vol] agar) in equal volumes after autoclaving and cooling to 50°C, and then component C (filter-sterilized concentrated solution of sodium thiosulfate) was added immediately to a final concentration of 1% (wt/vol) [17]. This solid medium was also supplemented with a 0.05% (wt/vol) yeast extract when used as the mating medium for *E. coli* and *A. caldus*. Ampicillin (Ap) (50–100 μg/ml), kanamycin (Km) (50–100 μg/ml), or streptomycin (Sm) (50–100 μg/ml) were added to the LB medium for *E. coli*, and Km (200 μg/ml) or Sm (200 μg/ml) added to the Starkey-S^0 liquid medium, whereas Km (50–100 μg/ml) or Sm (50–100 μg/ml) were added to the solid Starkey-$Na_2S_2O_3$ medium for the selection of the *A. caldus* transconjugants. The plasmids in *A. caldus* and *E. coli* were isolated using the method described by Birnboim and Doly [1] with a slight modification (lysozyme was omitted from solution I).

The conjugation between *E. coli* and *A. caldus* was conducted by filter mating. The donor cells were harvested by centrifugation in the late exponential growth phase, whereas the recipient cells were harvested in the stationary phase. The sulfur precipitates of *A. caldus* were removed from the liquid culture by low-speed centrifugation (800 ×*g*). Both the donor and the recipient cells were washed three times with a basal salt solution of the Starkey-$Na_2S_2O_3$ medium (pH 4.8–5.0), resuspended in the same solution, and mixed at a ratio of 1:1. Next, 0.1 ml of the cell suspension (about 2×10^{10} cells per ml) was transferred to a filter membrane (0.45 μm pore size, 25 mm diameter) that was placed on the mating medium. After incubation at 37°C for 48–72 h (*A. caldus* as recipient, *E. coli* as donor), 24 h (*E. coli* as recipient, *A. caldus* as donor), or 1 h (between *E. coli* cells in Luria agar), the

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Phenotype or genotype	Source or reference
E. coli		
C600	supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21	[22]
HB101	supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1	[22]
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ (lac-proAB) F'[traD36 proAB$^+$ lac I^q lacZΔM15]*	[22]
SM10	Thr leu hsd recA Km^r RP4-2-Tc::Mu integrated	[31]
A. caldus MTH-04	Wild-type	[20]
Plasmids		
RP4	Ap^r Tc^r Km^r IncP *tra*$^+$, 60 kb	[4]
R68.45	Ap^r Tc^r Km^r IncP *tra*$^+$, variant of R68 60 kb	[13]
pJRD215	Sm^r Km^r IncQ *mob*$^+$, 10.2 kb	[6]
pVLT33	Km^r IncQ *mob*$^+$, 10 kb	[7]
pVLT35	Sm^r IncQ *mob*$^+$, 9.8 kb	[7]

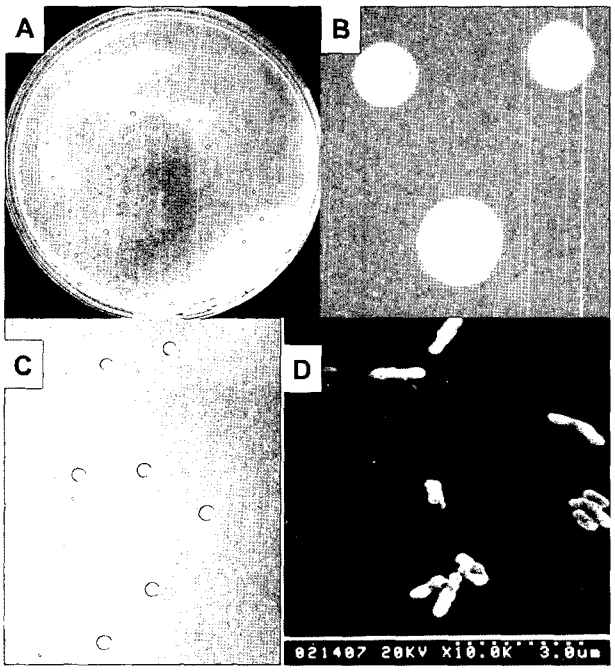

Fig. 1. Morphology of *A. caldus* MTH-04 (RP4).
Typical colonies of *A. caldus* MTH-04 (RP4) on solid sodium thiosulfate media: **A**. Showing whole plate; **B**. Showing part of plate; **C**. Showing single colonies under dissecting microscope; **D**. *A. caldus* MTH-04 (RP4) cells under scanning electron microscopy.

cultures were washed off the filter with 2.0 ml of saline, diluted, and then plated on appropriate selective and nonselective plates (Luria agar for *E. coli* cells and solid Starkey-$Na_2S_2O_3$ medium for *A. caldus* cells). As a control for spontaneous mutation, the recipient strain was also plated on the same selective plates. After an appropriate period of cultivation (24 h for *E. coli* at 37°C and 10–14 days for *A. caldus* at 40°C), the colonies were counted and the plasmid transfer frequency was calculated based on the number of transconjugants on selective plates divided by the number of recipients on nonselective plates.

First, the *E. coli* self-transmissible, incompatibility group P plasmids RP4 and R68.45 were tested. Using Km^r as the selective marker, the RP4 and R68.45 plasmids were both transferred from *E. coli* C600 donors to *A. caldus* MTH-04 recipients by conjugation. The colonies of *A. caldus* MTH-04 (RP4) transconjugants that formed on the solid Starkey-$Na_2S_2O_3$ medium with added Km (100 μg/ml) are shown in Fig. 1. The *A. caldus* MTH-04 control was only able to grow on this medium without Km. Several types of colonies were found on the medium as a result of different incubation times at 40°C. The most commonly observed type after 6–7 days of incubation at 40°C was generally circular, convex with a regular margin, and transparent with a smooth surface, whereas the size of the colonies varied from 0.5–2.0 mm (Figs. 1B, 1C). Although visible to the human eye, the colonies were more easily viewed under a dissecting microscope. The *A. caldus* transconjugant was a short, rod-shaped, motile, G^- organism, approximately (0.6–0.8) μm× (1.0–2.0) μm (Fig. 1D), the same as *A. caldus* MTH-04 [20], and the transfer frequencies are shown in Table 2. However, no Ap-resistant *A. caldus* MTH-04 transconjugant was detected, suggesting that the Ap^r marker from the IncP plasmids was not expressed in MTH-04. Furthermore no spontaneous Km^r mutation occurred in the *A. caldus* MTH-04 control. Therefore, this is the first report of the successful transfer of the IncP plasmids from *E. coli* into *A. caldus* and the expression of a selectable marker (Km^r) from the IncP plasmid in *A. caldus*.

To confirm the transfer of the IncP plasmids into *A. caldus* MTH-04, reverse-conjugation was conducted, where *A. caldus* MTH-04 transconjugants containing plasmids RP4 or R68.45 were used as the donors to cross with *E.*

Table 2. Transfer frequencies of IncP and IncQ plasmids between *E. coli* strains and *A. caldus* MTH-04[a].

Donor	Recipient	Selective marker	Transfer frequency[b]
E. coli	*A. caldus*		
C600 (RP4)	MTH-04	Km^r	$(3.47\pm1.35)\times10^{-6}$
C600 (R68.45)	MTH-04	Km^r	$(1.97\pm0.64)\times10^{-6}$
SM10 (pJRD215)	MTH-04	Sm^r	$(3.46\pm0.36)\times10^{-5}$
C600 (pJRD215, RP4)	MTH-04	Km^r, Sm^r	$(5.03\pm0.83)\times10^{-4}$
		Km^r	$(6.18\pm1.18)\times10^{-4}$
C600 (pVLT33, RP4)	MTH-04	Km^r	$(3.85\pm1.63)\times10^{-5}$
C600 (pVLT35, RP4)	MTH-04	Sm^r	$(6.28\pm2.13)\times10^{-5}$
		Km^r, Sm^r	$(8.49\pm1.18)\times10^{-7}$ (co-transfer)
A. caldus	*E. coli*		
MTH-04 (RP4, pVLT35)	C600	Sm^r	$(4.71\pm0.65)\times10^{-6}$
MTH-04 (pJRD215) and *E. coli* C600 (RP4)[c]	C600	Sm^r	$(2.60\pm1.20)\times10^{-6}$

[a]Data are averages of at least three independent experiments.
[b]Mean±Standard deviation.
[c]*E. coli* C600 (RP4) was used as a helper strain.

coli JM109 recipients on the mating medium. After mating for 24 h, the *E. coli* JM109 reverse-transconjugants were screened on Luria agar plates supplemented with the corresponding antibiotic. As expected, *E. coli* reverse-transconjugants containing plasmids RP4 or R68.45 were successfully obtained. It was also interesting that all the selective markers of RP4 and R68.45, including Ap^r, were recovered and expressed in the *E. coli*. The presence of the RP4 and R68.45 plasmids in the *A. caldus* MTH-04 transconjugants and *E. coli* JM109 reverse-transconjugants was also verified using plasmid isolation (Fig. 2), and the same bands corresponding to plasmids RP4 and R68.45 were detected in the strains. Since both IncP plasmids are quite large in size (>50 kb), the bands emerged farther up the smeared linear chromosome DNA (~23 kb).

Thereafter, the conjugative transfer of three *E. coli* incompatibility group Q plasmids, pJRD215, pVLT33, and pVLT35, was studied. The IncQ and related IncQ-like plasmids have a very broad host range, and are capable of replication in a wide range of G^- and several G^+ bacteria [35]. Although not self-transmissible, they are efficiently mobilized by helper IncP plasmids or strains containing integrated RP4, such as *E. coli* SM10. Yet, few reports are available on the transfer mechanism, as it is a very complicated process involving a lot of genes, among which the products of *tra* genes have proven to be essential [32]. Thus, *E. coli* cells harboring plasmid RP4 or *E. coli* SM10 could be considered as donor cells. First, the conjugation between *E. coli* SM10 (pJRD215) and *A. caldus* MTH-04 was conducted by filter mating, as described above. Plasmid pJRD215 was mobilized from *E. coli* SM10 into MTH-04 with the aid of plasmid RP4 integrated in the chromosome of SM10, using Sm^r as the selective marker. The transfer frequency of plasmid pJRD215 was $3.46\pm0.36\times10^{-5}$ (Table 2). Next, crosses between *E. coli* C600 (pJRD215, RP4), C600 (pVLT33, RP4), C600 (pVLT35, RP4), and *A. caldus* MTH-04 were performed. The donors were obtained by mating *E. coli* HB101 (RP4) with C600 (pJRD215), C600 (pVLT33), or C600 (pVLT35) and subsequent screening on Luria agar plates supplemented with the corresponding antibiotics, followed by verification using plasmid isolation (Fig. 3). All three IncQ plasmids were transferred into *A. caldus* MTH-04 with the help of plasmid RP4 in the same donor strain, and little difference was found between the transfer frequencies (Table 2). The transfer frequency of plasmid pJRD215 with helper plasmid RP4 in the same *E. coli* strain was higher than when using *E. coli* SM10 with RP4 integrated in the chromosome (Table 2). The Km^r and Sm^r markers from pJRD215 were both expressed in *A. caldus* MTH-04. The transfer of the plasmids was confirmed by their isolation from the *A. caldus* MTH-04 transconjugants (Fig. 3). The same bands corresponding to plasmids pJRD215 (10.2 kb), pVLT33 (10.0 kb), and pVLT35 (9.8 kb) were detected in both the *E. coli* donor and the *A. caldus* MTH-04 transconjugant strains.

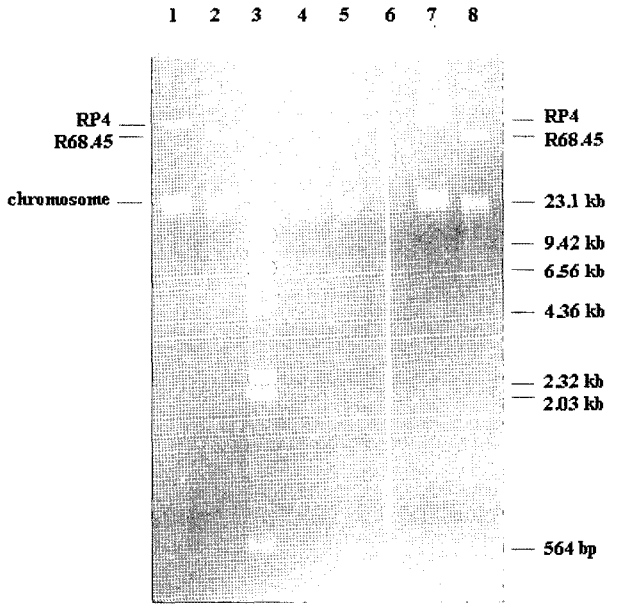

Fig. 2. Agarose gel electrophoresis (0.8% w/v) showing IncP plasmids RP4 and R68.45 in *E. coli* donor, *A. caldus* MTH-04 transconjugant, and *E. coli* reverse-transconjugant strains.
Lane 1, *E. coli* C600 (RP4); lane 2, *E. coli* C600 (R68.45); lane 3, λ DNA/HindIII marker; lane 4, *A. caldus* MTH-04 (RP4); lane 5, *A. caldus* MTH-04 (R68.45); lane 6, *A. caldus* MTH-04; lane 7, *E. coli* JM109 (RP4); lane 8, *E. coli* JM109 (R68.45).

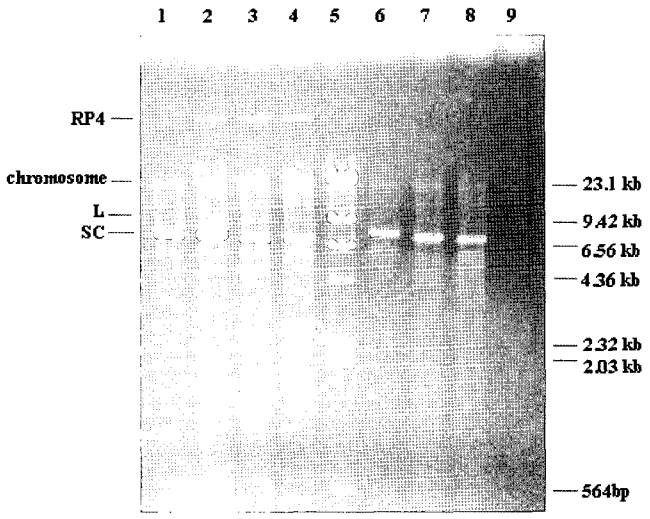

Fig. 3. Agarose gel electrophoresis (0.8% w/v) showing plasmids in *E. coli* donor and *A. caldus* MTH-04 transconjugant strains.
Lane 1, *E. coli* SM10 (pJRD215); lane 2, *E. coli* C600 (RP4, pJRD215); lane 3, *E. coli* C600 (RP4, pVLT33); lane 4, *E. coli* C600 (RP4, pVLT35); lane 5, λ DNA/HindIII marker; lane 6, *A. caldus* MTH-04 (pJRD215); lane 7, *A. caldus* MTH-04 (pVLT33); lane 8, *A. caldus* MTH-04 (pVLT35); lane 9, *A. caldus* MTH-04 control. SC: supercoiled plasmid DNA; L: linear plasmid DNA.

During mating, the IncQ and IncP plasmids were co-transferred into the *A. caldus* MTH-04 transconjugants, such as *A. caldus* MTH-04 (RP4, pVLT35), when both Km^r and Sm^r were used as the selectable markers. Although the co-transfer frequency was much lower (Table 2), no spontaneous Km^r and Sm^r mutation occurred in the *A. caldus* MTH-04 control. Furthermore, the *A. caldus* MTH-04 co-transconjugants were used as donors for further identification of the successful transfer of the IncQ plasmids from *E. coli* into *A. caldus*.

In addition to the isolation of plasmid DNA from the *A. caldus* MTH-04 transconjugants, reverse-mobilization was used to identify the plasmids in *A. caldus* MTH-04. Since the IncQ plasmids are not self-transmissible, the cross was carried out using the co-transconjugants, such as *A. caldus* MTH-04 (RP4, pVLT35), as the donor strains, and *E. coli* C600 as the recipient strain. However, in the absence of *A. caldus* MTH-04 co-transconjugants, the IncQ plasmids in *A. caldus* can be transferred back into *E. coli* with the helper IncP plasmids in the *E. coli* strain. Thus, the reverse cross was also performed using *A. caldus* MTH-04 (pJRD215) as the donor, *E. coli* C600 as the recipient, and *E. coli* C600 (RP4) as the helper strain. Similar plasmid transfer frequencies were obtained with the two reverse-transfer methods (Table 2), and *E. coli* reverse-transconjugants containing plasmid pJRD215 or pVLT35 successfully obtained. Although the transfer frequencies were not high, no plasmid transformation occurred from lysed cells, as no Km^r and Sm^r *E. coli* reverse-transconjugants were produced in the control without *E. coli* C600 (RP4) under the same conditions.

Finally, the stability of the IncP and IncQ plasmids in *A. caldus* MTH-04 was tested. Single colonies of *A. caldus* MTH-04 transconjugants containing different kinds of plasmid were transferred from selective plates into an antibiotic-free Starkey-S^0 liquid medium (20 ml) and cultured at 40°C for 6–7 days. One % (v/v) of the fully grown cultures was then transferred to 20 ml of a fresh Starkey-S^0 medium and incubated at 40°C for 6 days. After five transfers (more than 50 generations), the cultures were diluted and plated on a Starkey-$Na_2S_2O_3$ solid medium with and without an antibiotic, respectively, and incubated at 40°C for ten days. The colonies were then counted and the percentage of bacteria that retained the plasmid was calculated by dividing the number of colonies on the medium with the antibiotic by the number of colonies on the medium without the antibiotic (Table 3). All the IncP and IncQ plasmids studied were stable in *A. caldus* MTH-04.

In conclusion, IncP plasmids were transferred to *A. caldus* MTH-04 with relatively high frequencies (Table 2) and stably maintained (Table 3). IncQ plasmids, which are not self-transmissible, were also transferred with the help of IncP plasmids (Table 2, 3). When compared with the results of corresponding work with two other extreme acidiphiles from the genus *Acidithiobacillus*, *A. ferrooxidans* [26], and *A. thiooxidans* [17], the present data were quite similar, suggesting that, in the absence of alternative methods, plasmid transfer by conjugation is an effective method for the genetic manipulation of strains from this genus. Furthermore, the method of conjugation is applicable to more than one strain, as its effectiveness was also validated with two other strains of *A. caldus* (data not shown). The reverse transfer of the IncP and IncQ plasmids from *A. caldus* back to *E. coli* also confirmed the successful construction of a gene transfer system in an *A. caldus* strain.

Table 3. Stability of different plasmids in *A. caldus* MTH-04 transconjugants[a].

Plasmid	% of plasmid maintenance[b]
RP4	71±5
R68.45	67±4
pJRD215	85±4
pVLT33	79±5
pVLT35	81±6

[a]Data are averages of at least three independent experiments.
[b]Mean±Standard deviation.

The development of a conjugational system for *A. caldus* MTH-04 will greatly facilitate its genetic study, as the IncQ plasmids used in this study are broad-host-range, and can serve as vectors for introducing foreign genes into *A. caldus*. Moreover, the expression of resistance markers from both the IncP and IncQ plasmids in *A. caldus* suggests a similarity in the gene expression systems of heterotrophic *E. coli* and autotrophic *A. caldus*, despite their significant physiological differences. Finally, the plasmid transfer by conjugation introduced arsenic resistance genes derived from the *E. coli* plasmid R773 [23] into *A. caldus* MTH-04, and its resistance to sodium arsenite increased significantly [34].

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