

## GENETIC MANIPULATION OF ACIDOPHILIC BACTERIA WHICH ARE POTENTIALLY APPLICABLE IN COAL BENEFICIATION

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Keywords: acidophilic bacteria; conjugation; coal desulfurization

### INTRODUCTION

The economic and practical aspects of a biological coal desulfurization process are the subject of increasing study (1-6). Depyritization of coal by the bacterium, *Thiobacillus ferrooxidans* has been known for some time (7-9), and pilot scale experiments are underway (3,6). A number of limitations have already been recognized for this process, foremost of which is the speed with which the microorganisms grow and attack the pyritic sulfur. Metal toxicity and mass transfer dynamics also present formidable hurdles.

Removal of organic sulfur substituents poses even more difficult problems at this time, not least of which is the lack of efficient candidate organisms. Potential candidates at this time resemble members of the *Pseudomonadaceae*, common environmental bacteria. These organisms enjoy moderate temperatures (28-37 °C) and neutral pH, while the organisms investigated for depyritization processes are typically acidophiles, some of which also happen to be thermophiles (*Sulfolobus*, for example).

These various limitations in the microorganisms being examined for a viable desulfurization process have led us to initiate studies on the extension of molecular genetic techniques to acidophilic bacteria, with an ultimate goal of introducing desirable characteristics for desulfurization (enhanced growth rate, metal resistance, biochemical capacity to degrade organic sulfur) either directly into *T. ferrooxidans*, or, alternatively, into a heterotrophic acidophile which can coexist in the same environment as *T. ferrooxidans*. We are focusing on members of the genus *Acidiphilium*, one such acidophilic heterotroph.

### EXPERIMENTAL

**Bacterial strains and plasmids.** Strains and plasmids used in this study are described in Table 1. *Acidiphilium* strains were grown in Modified Acidophile Salts (MAS) media, which is a modification of that used by Wichlacz and Unz (10). MAS medium contained 1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM KCl, 0.86 mM K<sub>2</sub>HPO<sub>4</sub>, 10 mM MgSO<sub>4</sub>, 6.6 mM CaCl<sub>2</sub>, 2.6 mM FeSO<sub>4</sub>, 0.01% yeast extract and 0.1% glycerol, pH 3.5. Solidified media were prepared with the addition of 0.4 % GelRite gellan gum (Kelco). Where indicated, tetracycline (Tc) was added to the media at 40 µg/ml.

**Electroporation.** Cells were electroporated as described previously (14-16). A BTX Transfector 100 electroporation device was used. Late log phase cells were washed and concentrated to cell densities of between 10<sup>9</sup>-10<sup>11</sup> cells/ml in 1 mM HEPES, pH 7.0. After electroporation, cells were diluted 20-fold into MAS medium to allow expression of the antibiotic resistance phenotype. Cells were

plated on 40 µg/ml Tc in MAS medium, and transformed colonies were clearly visible after three days. The experiments were performed at 32 °C unless otherwise indicated.

**Conjugation.** Spot matings were performed essentially as described by Miller (11). One milliliter exponential cultures of donor and recipient cells were centrifuged, washed twice and resuspended in 1 ml of 1 mM HEPES, pH 7.0. Donor cells were diluted 50-fold and a 25 µl aliquot was spotted onto a dry nutrient agar (Difco) plate. The liquid was allowed to absorb into the agar, at which time a 25 µl aliquot of the undiluted recipient cells was spotted directly onto the dried donor cells. Approximately  $1 \times 10^8$  recipient cells were used. Matings were allowed to proceed for three hours, after which the cells were recovered and resuspended in 200 µl MAS medium. The cells were then plated on solid MAS medium containing 40 µg/ml Tc. Transconjugants usually appeared within 3-4 days.

**Selection of rifampicin-resistant acidophiles.** Mutants resistant to rifampicin were selected by plating cells on MAS plates on which a sterile filter disk (1/2" diameter) impregnated with 200 µg/ml rifampicin was placed. Colonies growing up to the filter disk, within the zone of inhibition were picked and checked for stable resistance by repeated subculturing on liquid and solid media. Mutants were typically resistant to 50-200 µg/ml rifampicin.

**Conjugal transfer between acidophiles.** Spot matings between acidophiles were performed as described above, with the exception that matings were allowed to proceed overnight, and all recipients used were resistant to rifampicin. All selective plates contained 50 µg/ml each of tetracycline and rifampicin.

**Plasmid isolation, restriction digestion gel electrophoresis and ligation.** Plasmids were isolated using the alkaline lysis method of Birnboim and Doly (12), as described for small scale isolations in Maniatis (13).

Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim and Promega, and digestions and ligations were carried out in appropriate buffers according to the manufacturers' instructions. Agarose gel electrophoresis was typically performed using 0.4% agarose (FMC, LE grade) gels prepared in 0.5X TBE and run in the same buffer (13). After running, gels were stained in a 2µg/ml ethidium bromide solution for 30 minutes and DNA was visualized with a UV transilluminator (Spectroliner) at 310 nm.

## RESULTS AND DISCUSSION

RP4-based plasmids have previously been introduced into *Acidiphilium facitlis* by conjugation and electroporation (14-16). Plasmids based on RP4 belong to the incompatibility group, IncP1. In order to determine whether plasmids from other incompatibility groups were stably maintained in *A. facitlis*, we obtained the plasmids pSUP104 (IncQ) and pUCD615 (IncW). These plasmids were introduced into the mobilizing *Escherichia coli* strain, S17.1 (17). When compared with the mobilization of pRK415 (ca.  $1 \times 10^{-5}$  transfers/recipient), transfer of pSUP104 and pUCD615 are much less efficient, with frequencies of transfer on the order of  $10^{-9}$  and  $10^{-8}$  per recipient, respectively.

*Acidiphilium spp.* contain numerous plasmids of varying sizes, whose functions are unknown (14-16). Conjugation functions could be readily assayed for by monitoring the ability of these strains to mobilize broad-host range plasmids to other acidophiles. The identification of such plasmid(s) would be of great interest, since it would be expected that such transfer could occur at acid pH,

allowing the horizontal transfer of genetic information, not only between acidiphilia, but between other acidophiles as well, including *Thiobacillus ferrooxidans*, which has been demonstrated to possess mobilizable, broad-host range plasmids (18, 19). The discovery of such a genetic transfer mechanism in acidophilic bacteria might allow the exploitation of such a process for genetic manipulation of these bacteria.

*Acidiphilium* strains PW2, CM9, and CM9A harboring the mobilizable plasmid, pRK415 (20, 14-16) were mated with rifampicin-resistant mutants PW1, PW2 and AWB. Very low frequencies of plasmid mobilization were observed, although PW2(pRK415) and CM9A(pRK415) donors gave rise to some 50 colonies in two instances (Table 2). Owing to the number of spontaneous Rf/Tc double mutants arising from CM9A (data not shown), there is some question as to whether these are legitimate transconjugants. However, in the case of the PW2 donor, no spontaneous Rf/Tc mutants were observed, and subsequent analysis of the putative transconjugants revealed the presence of the mobilized plasmid. Genomic fingerprinting experiments are underway to further verify that the transconjugants are derived from the recipient strains used, and not some other class of donor mutant.

As part of our goal to establish techniques for genetic manipulation of *T. ferrooxidans* and *Acidiphilium*, we are constructing vector plasmids using native plasmids. A 2.1 kilobase (kb) plasmid was isolated from *T. ferrooxidans* strain A6. This plasmid, designated pTYA6, was originally cloned in pBR322, and subsequently, subcloned into pUC128, taking advantage of a unique Hind III site. The orientation of restriction sites in this plasmid on a circular map is shown in Figure 1. The Hind III fragment containing the A6 plasmid DNA was clone *in toto* into pLVC18, a mobilizable pBR322 derivative (G. Warren, unpublished results). This construct is being used to examine the ability of a *Thiobacillus* origin of replication to function in other acidophiles, namely, *Acidiphilium*. The chimeric plasmid, pIRC4, was introduced into *E. coli* strain S17.1 and subsequently mobilized into *A. facilis* PW2. 19 putative transconjugants arose from this mating. Since pLVC18 does not possess a broad-host range origin of replication, we must assume that the origin of replication resident on the A6 plasmid is functioning in *Acidiphilium*. It should be noted that several *T. ferrooxidans* plasmids have previously been shown to replicate in *E. coli*, *Pseudomonas aeruginosa* and *T. novellus* (18, 19).

## CONCLUSIONS

In our continuing efforts to develop genetic methodologies for manipulating acidophilic bacteria which are useful in the biological desulfurization of coal, we have discovered evidence for conjugative plasmids in *Acidiphilium*. The presence of such plasmids in acidophiles suggests that horizontal transfer of genetic information occurs naturally, and these plasmids may provide a suitable vehicle for the introduction of desirable traits into these bacteria.

In addition, an examination of the relative transfer frequencies of various plasmids of different incompatibility groups appears to indicate that IncP1-based vectors are the vehicles of choice when introducing exogenous genetic material into *Acidiphilium*.

## ACKNOWLEDGEMENTS

This work was supported under contract No. DE-AC07-76IDO1570 from the U.S. Department of Energy to the Idaho National Engineering Laboratory/EG&G Idaho, Inc.

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**TABLE 1. Bacterial strains and plasmids used in this study**

<u>Strain or plasmid</u>	<u>Genotype</u>	<u>Source or reference</u>
<i>Acidiphilium</i> strains		
PW2		10
PW2-Rf	Rf	This study
CM9		D. Thompson
CM9A		D. Thompson
AWB-Rf	Rf	10, This study
PW1-Rf	Rf	10, This study
<i>E. coli</i> strains		
S17.1	<i>pro res<sup>-</sup> mod<sup>+</sup> recA</i> RP4-2-Tc::Mu-Km::Tn7	17
Plasmids		
pRK415	<i>oriRK2 Tc<sup>r</sup> 10.5 kb</i>	20
pLVC18	<i>oripBR322 RSF1010 mob bom</i> <i>Tc<sup>r</sup> Ap<sup>r</sup> 5.9 kb</i>	G. Warren
pSUP104	<i>ori15A oriRSF1010 Tc<sup>r</sup> Cm<sup>r</sup> 9.5 kb</i>	21
pUCD615	<i>lux promoter probe, oripSa</i>	22
pTfA6	<i>oripBR322 Ap<sup>r</sup> Km<sup>r</sup> 17.55 kb</i> Cryptic <i>T. ferrooxidans</i> plasmid, 2.1 kb	This study

**TABLE 2. Transfer frequency of pRK415 between acidophilic heterotrophs (per recipient)**

<u>Donor (pRK415)</u>	<u>Recipient (Rf-resistant)</u>		
	<u>AWB</u>	<u>PW1</u>	<u>PW2</u>
CM9	0	8.55 x 10 <sup>-9</sup>	1.57 x 10 <sup>-8</sup>
CM9A	3.65 x 10 <sup>-7</sup>	0	5.51 x 10 <sup>-8</sup>
PW2	6.41 x 10 <sup>-9</sup>	4.27 x 10 <sup>-7</sup>	1.57 x 10 <sup>-8</sup>

**Figure 1. Restriction map of *T. ferrooxidans* plasmid TfA6**

