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GENETIC TRANSFER IN ACIDOPHILIC BACTERIA

FF ROBERTO
AW Glenn
D Bulmer
TE Ward
Biotechnology
Idaho National Engineering Laboratory
Idaho Falls, ID 83415-2203

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GENETIC TRANSFER IN ACIDOPHILIC BACTERIA

ABSTRACT

There is increasing interest in the use of microorganisms to recover metals from ores, as well as to remove sulfur from coal. These so-called bioleaching processes are mediated by a number of bacteria. The best-studied of these organisms are acidophiles including *Thiobacillus* and *Acidiphilium* species.

Our laboratory has focused on developing genetic strategies to allow the manipulation of acidophilic bacteria to improve and augment their utility in large scale operations. We have recently been successful in employing conjugation for interbacterial transfer of genetic information, as well as in directly transforming *Acidiphilium* by use of electroporation. We are now testing the properties of IncP1, IncW and IncQ plasmid vectors in *Acidiphilium* to determine their relative usefulness in routine manipulation of acidophiles and transfer between organisms. This study also allows us to determine the natural ability of these bacteria to transfer genetic material amongst themselves in their particular environment. Such information will be critical in obtaining permission for release of genetically-engineered microorganisms into the environment for industrial purposes.

INTRODUCTION

The use of microorganisms to remove pyritic and organic sulfur from coal is of interest from both economic and practical perspectives. Outside of the cost of crushing the coal to an appropriate size, initial capital investment in the bioreactor plant, and nutrient provisions, there is very little cost involved in a microbial desulfurization process. The use of microorganisms may also allow the attack and removal of sulfur which would otherwise not be feasible by other means. Pilot-scale processes using bacteria are already being examined (1-6). Depyritization of coal appears to be readily achievable, while the attack of the organic sulfur moieties is still an area of intense study. The organisms which accomplish depyritization require conditions (acid pH, CO_2, O_2) quite different from those demanded by the candidate microorganisms which might remove organic sulfur from coal (neutral pH, complex organic media), and bioreactor designs must currently incorporate two-stage processes to accomodate both types of microorganisms (6). Molecular genetic techniques could conceivably allow the manipulation of candidate microorganisms to produce a single microorganism, or set of microorganisms, which could achieve depyritization and organic desulfurization under common conditions. Enhancement of these biochemical processes could also greatly speed up the biological processing of coal.

Our laboratory has been developing genetic techniques to allow manipulation of acidophilic bacteria, such as *Thiobacillus ferrooxidans* and *Acidiphilium facilis*. These organisms are known to play an important role in biological coal depyritization, and we hope to introduce new characteristics into these organisms which will allow them to remove both organic and pyritic sulfur from coal efficiently.

MATERIALS AND METHODS

Bacterial strains and plasmids

Acidiphilium facilis type strain PW2 was acquired as ATCC strain 35904. Other *Acidiphilium* strains used, CM1, CM3A, CM4A, CM5, CM7, and CM9 were isolated from water collected at the Blackbird cobalt mine, 20 miles

southwest of Salmon, Idaho (D. Thompson and P. Wichlacz, unpub.), while PW1, AWB and BBW from acidic mine drainage sites in central Pennsylvania were obtained from P.L. Wichlacz (7,8). *Escherichia coli* strain S17.1 (*thi pro hsdR*$^-$ *hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7; 9) used in conjugation experiments was obtained from R.Simon. *Acidiphilium* strains were grown in Modified Acidophile Salts (MAS) media, which is a modification of that used by Wichlacz and Unz (7). MAS medium contained 1 mM $(NH_4)_2SO_4$, 2 mM KCl, 0.86 mM K_2HPO_4, 10 mM $MgSO_4$, 6.6 mM $CaCl_2$, 2.6 mM $FeSO_4$, 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. Plasmids used in this study included IncP1 group plasmids R68 and the RK2-based vectors, pRK415 (10) and pLAFR3 (11) which carry tetracycline resistance markers. IncQ plasmid pSUP104 (12), carrying tetracycline resistance (obtained from R. Simon) and IncW plasmid pUCD615 (13), carrying kanamycin and ampicillin resistance markers (obtained from C. Kado) were also examined. Plasmid TfA6 was isolated from *Thiobacillus ferrooxidans* strain A6. The mobilizable pBR322-derivative, pLVC18, was the kind gift of Dr. G. Warren.

Electroporation

A BTX Transfector 100 electroporation device was used. Late log phase cells were washed and concentrated to cell densities of between 10^9-10^{11} cells/ml in 1 mM HEPES, pH 7.0. Pulse durations and field strengths were varied as reported. After electroporation, cells were diluted 20-fold into MAS medium and incubated 17-20 h to allow expression of the antibiotic resistance phenotype. Where Tc selection was used, 1 µg/ml Tc was added to expression medium to induce the Tc resistance genes. Cell numbers approximately doubled during the expression period. 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 from *E.coli* to Acidophiles

Spot matings were performed essentially as described by Miller (14). 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 (approximately 1 x 10^8 recipient cells were used) was spotted directly onto the dried donor cells. 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 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 were resistant to rifampicin. All selective plates contained 40 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 (15), as described for small scale isolations in Maniatis (16).

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 (16). After running, gels were stained in a 2µg/ml ethidium bromide solution for 30 minutes and DNA was visualized with a UV transilluminator (Spectroline) at 310 nm.

RESULTS AND DISCUSSION

Maintenance of different incompatibility group plasmids in *Acidiphilium*

We previously described the successful introduction of RP4-based plasmids into *Acidiphilium facilis* by conjugation and electroporation (17-19). The mobilizable plasmid used, pRK415, is a derivative of RP4, a member of the IncP1 plasmid incompatibility group. In order to determine whether plasmids from other incompatibility groups were stably maintained in *A. facilis*, we obtained the plasmids pSUP104 (IncQ) and pUCD615 (IncW). Circular maps of these plasmids are shown in Figure 1. The frequency with which these plasmids are transferred from *E.coli* strain S17.1 (chromosomal transfer functions) to strain PW2 is presented in Table 1. In general, it appears that pRK415 produces transconjugants much more efficiently than pSUP104 or pUCD615. This may be a result of poor replication of these plasmids in *A.facilis*, inefficient expression of the antibiotic resistance genes carried by these plasmids, or alternatively, that the transfer functions carried by *E. coli* S17.1 may not be able to mobilize IncQ or IncW plasmids effectively.

Conjugal transfer between acidophiles

Acidiphilium spp. contain numerous plasmids of varying sizes, whose functions are unknown (17-19). Endogenous conjugation functions could be assayed for by monitoring the ability of these strains to mobilize broad-host range plasmids to other acidophiles. The identification of plasmid(s) carrying such functions 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*. 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. A schematic diagram of our approach to find such functions is shown in Figure 2.

Matings were performed between 9 *Acidiphilium* strains which had been mated with *E.coli* or electroporated to introduce pRK415 or pLAFR3 and rifampicin-resistant mutants of PW1, PW2 and AWB. In general, very low frequencies of plasmid mobilization were observed (Table 2). Consistent with what was observed with conjugation from *E. coli* (17-19), and electroporation, strain PW1 acted as a successful recipient in the largest number of cases (five), and yielded ten-fold greater transconjugant frequencies, in some cases . Matings with strain AWB as recipient were successful in only one case.

Subsequent analysis of some of the putative transconjugants revealed the presence of the mobilized plasmid. We are currently performing genomic fingerprinting experiments to further verify that the transconjugants are derived from the recipient strains used, and are not some other class of donor mutant. However, in no case did control platings of any of the donors give rise to spontaneous Rf/Tc mutants.

We tentatively take this data (Table 2) to indicate that strains CM9, PW2, CM5, CM7 and possibly BBW contain conjugative functions, with PW2 being most efficient at transferring RP4-derived mobilizable plasmids.

Construction of a mobilizable Thiobacillus ferrooxidans plasmid

A component of our primary goal of establishing techniques for genetic manipulation of *T. ferrooxidans* and *Acidiphilium* is the construction of suitable vector plasmids using native plasmids. A 2.1 kilobase (kb) plasmid was isolated from *T. ferrooxidans* strain A6. This plasmid, designated pTfA6, was originally cloned in pBR322, and subsequently, transferred into pUC128, taking advantage of a unique Hind III site. The orientation of the unique Hind III and Pst I restriction sites in

this plasmid are shown in Figure 3. The plasmid was cloned as either a Hind III or Pst I fragment *in toto* in pLVC18, a mobilizable pBR322 derivative, to generate the chimeric plasmids, pIRC4 and 5 (strategy shown in Figure 3). We have used this construct 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 (Ap resistant) arose from this mating. Since pLVC18 does not possess a broad-host range origin of replication, and electroporation results had led to the conclusion that the pBR322 origin does not function in *Acidiphilium*, we must assume that the origin of replication resident on the A6 plasmid is functioning in *Acidiphilium*. Experiments with pIRC5 are underway.

Rawlings has previously described derivatives of three *T. ferrooxidans* plasmids which are capable of being mobilized by IncP1 or IncW conjugative plasmids, but not by IncN plasmids (20, 21). Furthermore, at least one of these plasmids (a 12 kb plasmid from *T. ferrooxidans* strain FC) possessed a broad-host range origin of replication which functioned in *E.coli*, *Pseudomonas aeruginosa* and *T. novellus*. It would therefore not be unexpected to find that pTfA6 might also possess a broad-host range origin of replication, although this plasmid is considerably smaller than the plasmids previously described.

Experiments are underway to determine the entire DNA sequence of pTfA6.

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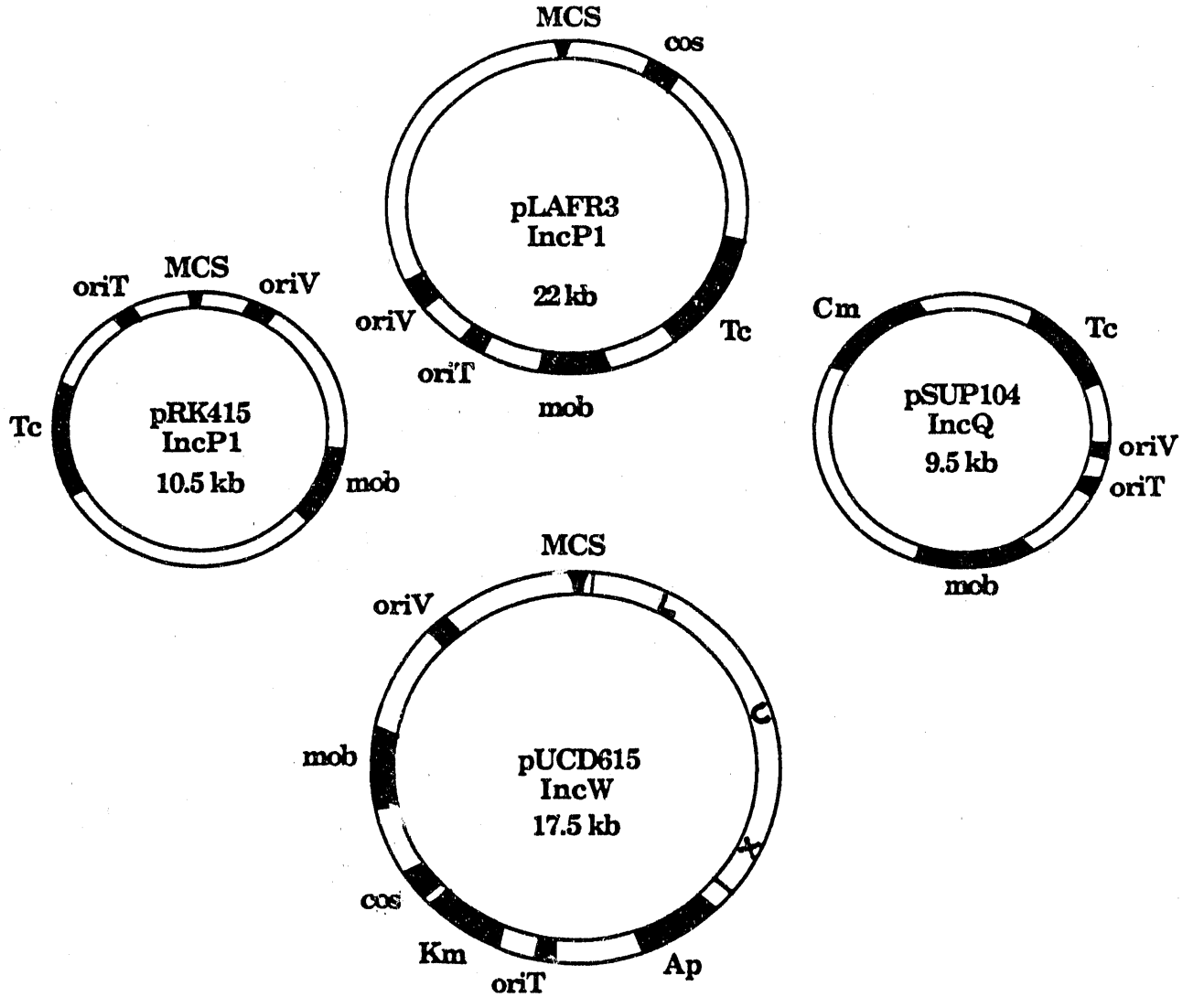

Figure 1. Mobilizable plasmids used in this study. Ap--ampicillin resistance gene; Tc--tetracycline resistance gene; mob--mobilization region; oriT--origin of conjugal transfer; Cm--chloramphenicol resistance gene; Km--kanamycin resistance gene; MCS--multicloning site (various);oriV--origin of vegetative replication; cos--cohesive end region of phage lambda.

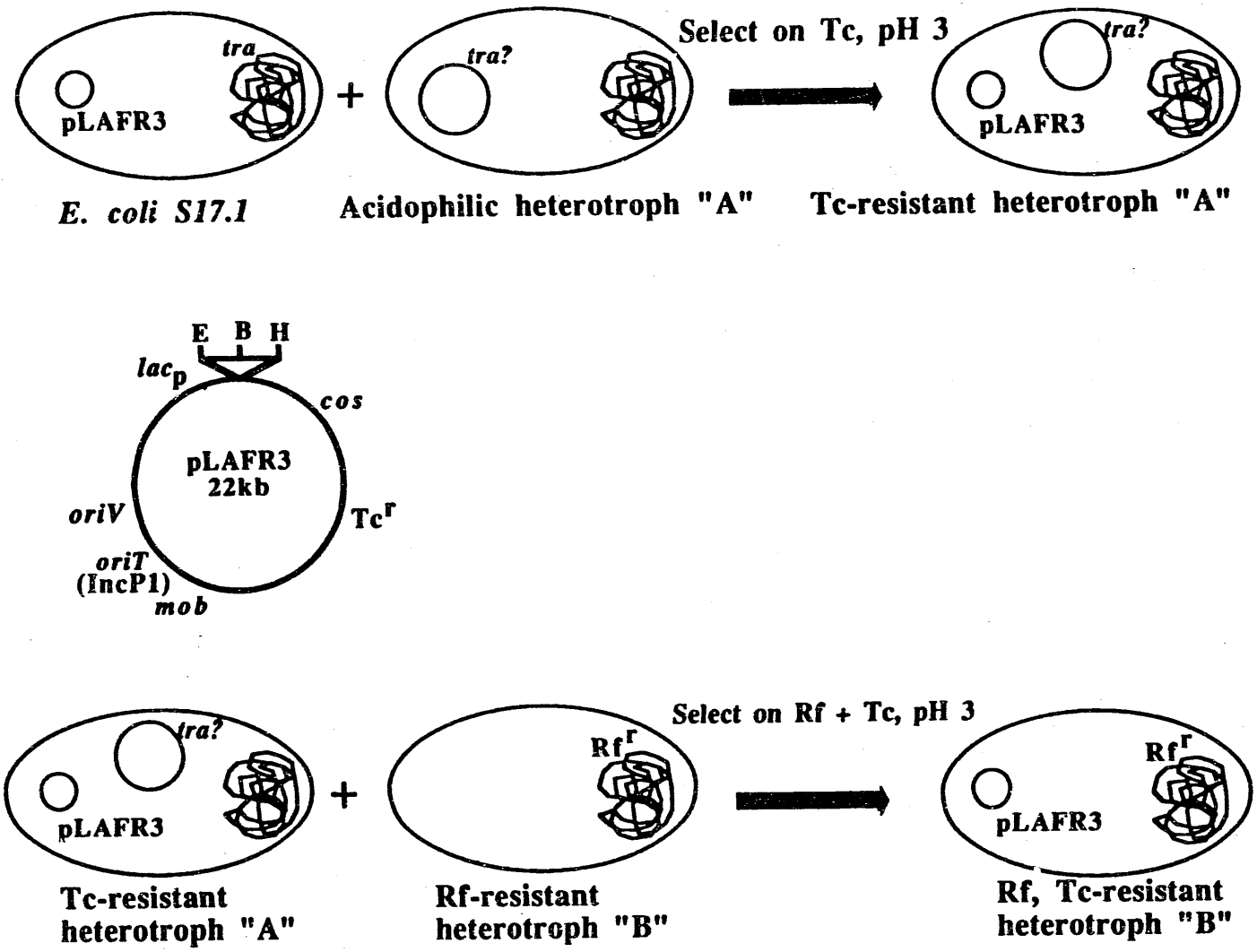

Figure 2. Strategy for identifying conjugative plasmids or functions in acidophilic heterotrophs.

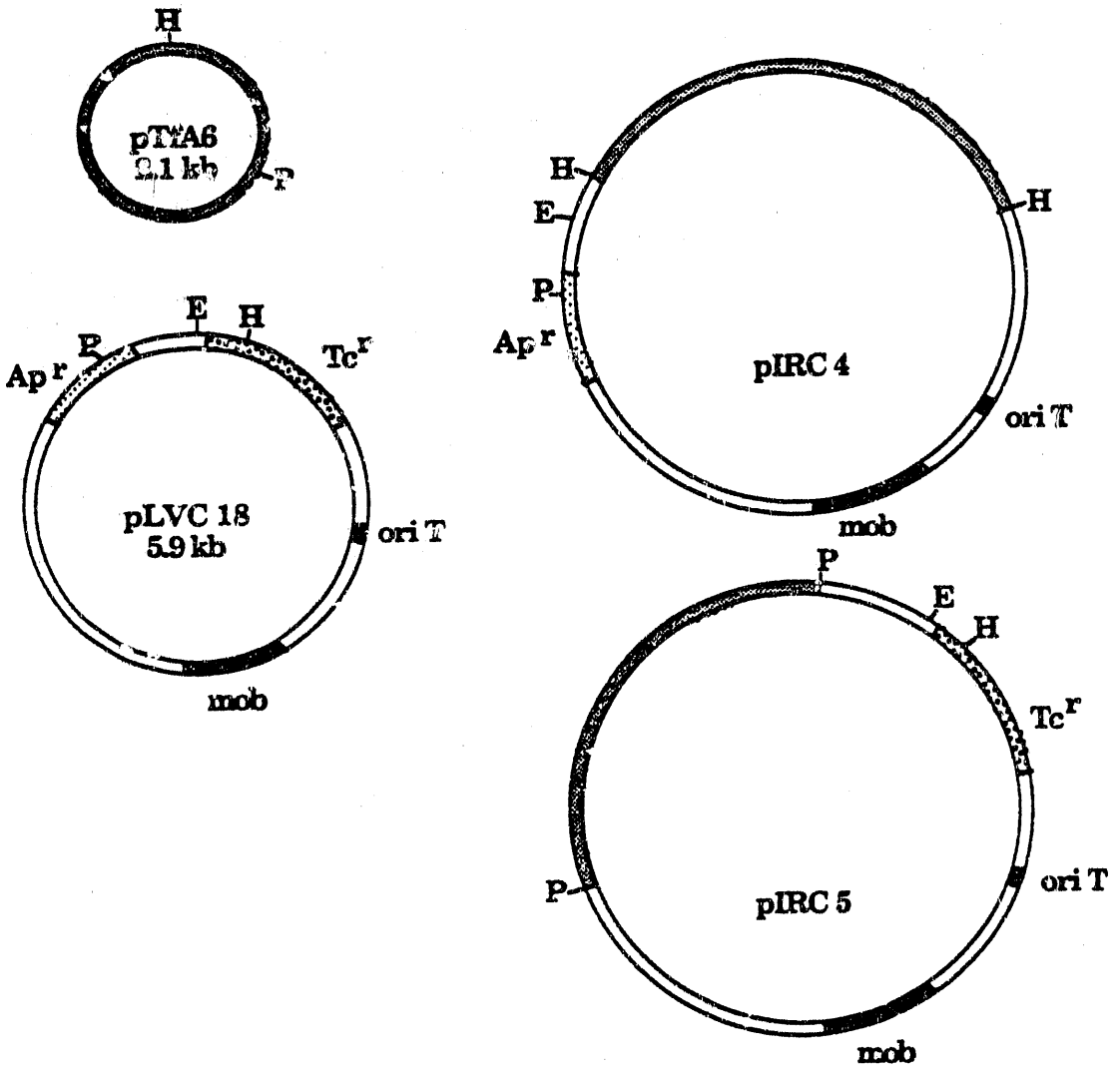

Figure 3. Construction of pIRC4 and 5. Restriction enzyme abbreviations: H--HindIII; E--EcoRI; P--PstI

Table 1

TRANSFER FREQUENCY OF IncP1, IncQ and IncW PLASMIDS FROM *E. coli* TO *A. facilis* STRAIN PW2
(per recipient)

Donor	Number of transconjugants
S17.1 (pRK415)	1.7×10^{-6}
(pSUP104)	5.2×10^{-8}
(pUCD615)	1.0×10^{-8}

Table 2

TRANSFER FREQUENCY OF pRK415 and pLAFR3 BETWEEN ACIDOPHILIC HETEROTROPHS
(per recipient)

	Recipient (Rf-resistant)		
Donor	AWB	PW1	PW2
BBW (pRK415)	0	3.2×10^{-7}	0
CM3A (pRK415)	0	0	0
CM9 (pRK415)	0	8.6×10^{-9}	1.6×10^{-8}
PW2 (pRK415)	6.4×10^{-9}	4.3×10^{-7}	1.6×10^{-8}
CM5 (pLAFR3)	n.d.*	1.5×10^{-7}	n.d.
CM7 (pLAFR3)	n.d.	4.4×10^{-8}	n.d.
CM1 (pLAFR3)	n.d.	0	n.d.
CM3A (pLAFR3)	n.d.	0	n.d.
CM4A (pLAFR3)	n.d.	0	n.d.

*n.d.--not determined

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