Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*

D. ROSS WILLIAMS, DANIELLE I. YOUNG and MICHAEL YOUNG*

Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK

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The conjugation mechanism of IncP plasmids may be employed to mobilize small non-conjugative plasmids from *Escherichia coli* to a wide range of different organisms. This strategy has been adapted for use with the Gram-positive anaerobe, *Clostridium acetobutylicum* NCIB 8052. Several shuttle vectors containing replicons from pAM β 1 (*Enterococcus faecalis*), pCB101 (*Clostridium butyricum*) or pWV01 (*Streptococcus cremoris*), together with the *cis*-acting *oriT* region of RK2, have been constructed, and transferred to and established in this organism. One of the vectors apparently contains a hot-spot for insertion of IS1. Conjugative mobilization of plasmids from *E. coli* will provide a useful alternative to electroporation for effecting gene transfer to this industrially important anaerobe.

Introduction

Clostridium acetobutylicum has long been employed for the industrial-scale production of acetone and butanol by fermentation of carbohydrates in low-grade agricultural wastes (reviewed by Jones & Woods, 1986). More recently, it has been recognized (Morris, 1983) that this organism is potentially useful for synthesis of high-value products via stereo-selective or stereo-specific reductions that are difficult to accomplish chemically. A significant drawback to the industrial use of *C. acetobutylicum* has been a lack of suitable methods for genetic manipulation of this organism. However, various procedures for introducing plasmids have now been developed (reviewed by Young *et al.*, 1989) and this should greatly improve prospects for future biotechnological exploitation of *C. acetobutylicum*.

These procedures include electroporation (Oultram *et al.*, 1988a) and the mobilization of plasmids from *Bacillus subtilis* as cointegrates with pAM β 1 (Oultram *et al.*, 1987, 1988b). Since pAM β 1 is structurally unstable in *B. subtilis* (Van der Lelie & Venema, 1987), transfer frequencies using the latter method are very low. An alternative procedure, that might be suitable for effecting plasmid transfer to *C. acetobutylicum*, has recently been described (Trieu-Cuot *et al.*, 1987). It is based on the extremely broad-host-range Gram-negative conjugation system encoded by IncP plasmids such as RK2 (Thomas & Smith, 1987). These plasmids are transferable to organisms representative of many genera,

although in some instances (e.g. in *Anabaena*) they cannot replicate after transfer (Krishnapillai, 1988).

The conjugation machinery of IncP plasmids is remarkably non-specific and small non-conjugative plasmids can be mobilized to a variety of Gram-positive organisms. Trieu-Cuot *et al.* (1987) constructed a mobilizable vector containing a selectable marker and replication functions operative in both *Escherichia coli* and Gram-positive bacteria, into which was incorporated the *cis*-acting *oriT* site of RK2. This last component is the origin of conjugative DNA transfer, corresponding to the site at which the DNA duplex is nicked in preparation for transfer of a single strand from donor to recipient (Willems & Wilkins, 1984; Guiney *et al.*, 1988). All other conjugation functions were provided *in trans* on an IncP plasmid.

This paper establishes the utility of the IncP conjugation mechanism for mobilizing small, non-conjugative, bifunctional plasmids directly from *E. coli* to the Gram-positive, obligate anaerobe, *C. acetobutylicum*.

Methods

Bacterial strains and plasmids. These are listed in Tables 1 and 2.

Culture media. Strains of *E. coli* were maintained on Luria-Bertani medium (which contains, per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g). Strains of *C. acetobutylicum* were maintained on clostridial basal medium (CBM: O'Brien & Morris, 1971). The media employed for filter mating experiments are described below.

Table 1. *Bacterial strains*

Strain	Genotype or phenotype*	Source/reference
<i>E. coli</i>		
HB101	F ⁻ <i>hsdS20</i> (r _B , m _B) <i>recA13 ara-14 lacY1 proA2 galK2 rpsL20</i> (Sm ^R) <i>xyl-5 mtl-1 supE44</i> (λ ⁻)	Laboratory strain/Boyer & Roulland-Dussoix (1969)
J53-1	F ⁻ <i>pro met gyrA96</i> (Na ^R) (λ)	C. M. Thomas, University of Birmingham/Clowes & Hayes (1968)
JM107	<i>endA1 gyrA96</i> (Na ^R) <i>thi-1 hsdR17</i> (r _K , m _K) <i>supE44 relA1</i> (λ ⁻) <i>Δ(lac-proAB)</i> (F ⁻ <i>traD36 proAB lacZΔM15</i>)	Laboratory strain/Yanisch-Perron <i>et al.</i> (1985)
SM10†	F ⁻ <i>thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44</i> Mu _c ⁺ (λ ⁻) [RP4-2(Tc::Mu)] Km ^R Tra ⁺	P. Trieu-Cuot, Institut Pasteur/Simon <i>et al.</i> (1983)
<i>C. acetobutylicum</i> NCIB 8052		Laboratory strain

* Antibiotic resistances: Sm, streptomycin; Na, nalidixic acid; Km, kanamycin.

† RP4-2(Tc::Mu) is integrated into the bacterial chromosome.

Table 2. *Plasmids*

Plasmid	Relevant characteristics*	Gram-positive replicon	Source or reference
pAT187	Tra ⁻ Mob ⁺ Km ^R	pAMβ1	Trieu-Cuot <i>et al.</i> (1987)
pRK24†	Tra ⁺ Mob ⁺ IncP Ap ^R Tc ^R <i>trpE</i>	-	Thomas & Smith (1987)
R702‡	Tra ⁺ Mob ⁺ IncP Km ^R Tc ^R Sm ^R Su ^R Hg ^R	-	Hedges & Jacob (1974)
pCB101	Tra ⁻ Mob ⁻ cryptic	pCB101	Collins <i>et al.</i> (1985)
pGK13	Tra ⁻ Mob ⁻ Cm ^R Em ^R	pWV01	Kok <i>et al.</i> (1984)
pUC8§	Tra ⁻ Mob ⁻ Ap ^R	-	Larkin <i>et al.</i> (1986)
pMTL30	Tra ⁻ Mob ⁺ Ap ^R Em ^R <i>lacZ'</i>	-	Williams <i>et al.</i> (1990)
pMTL31	Tra ⁻ Mob ⁺ Ap ^R Em ^R <i>lacZ'</i>	-	Williams <i>et al.</i> (1990)
pMTL500E	Tra ⁻ Mob ⁻ Ap ^R Em ^R <i>lacZ'</i>	pAMβ1	Oultram <i>et al.</i> (1988a)
pCTC1	Tra ⁻ Mob ⁺ Ap ^R Em ^R	pAMβ1	This paper
pCTC3	Tra ⁻ Mob ⁺ Cm ^R Em ^R	pWV01	This paper
pCTC40	Tra ⁻ Mob ⁺ Ap ^R Em ^R	pAMβ1	This paper
pCTC401	Tra ⁻ Mob ⁺ Ap ^R Em ^R	pAMβ1::IS1	This paper
pCTC41	Tra ⁻ Mob ⁺ Ap ^R Em ^R	pAMβ1	This paper
pCTC511	Tra ⁻ Mob ⁺ Ap ^R Em ^R	pCB101	This paper

* The Mob⁺ phenotype is conferred by *oriT*. Antibiotic resistances: Ap, ampicillin; Tc, tetracycline; Su, sulphonamide; Hg, merbromin; Cm, chloramphenicol; Em, erythromycin (MLS^R); others are as indicated in the footnotes to Table 1.† pRK24 is RK2 with the Km^R gene inactivated by insertion of a *Hind*III fragment bearing *trpE*.‡ R702 lacks *Tn1* and does not confer ampicillin resistance (Hedges & Jacob, 1974).§ pUC8β contains a 240 bp internal *Hae*III fragment of IS1 which has homology only with the larger *Pvu*II fragment of IS1.

|| Details of the construction of these plasmids are given in the legend to Fig. 1.

Filter mating procedure. The optimized procedure (Williams *et al.*, 1990) was as follows. Donor strains of *E. coli* were grown overnight aerobically to OD₆₀₀ > 4.0 (about 5 × 10⁹ c.f.u. ml⁻¹) in brain heart infusion broth (BHIB) supplemented with antibiotics selective for the mobilizable plasmids that they contained. The recipient strain, *C. acetobutylicum* NCIB 8052, was grown overnight anaerobically in TYG medium (which contains, per litre: tryptone, 30 g; yeast extract, 20 g; glucose 5 g; sodium mercaptoacetate, 1 g). Exponentially growing cultures, at an OD₆₀₀ of 0.6–1.2 (about 5 × 10⁷ c.f.u. ml⁻¹), were used for matings. Donor and recipient bacteria were mixed in a 1000:1 ratio, by pipetting 0.2 ml of the recipient culture into 2 ml of the donor culture. Bacteria from this mating mixture were deposited by filtration on a cellulose nitrate membrane (Whatman WCN, 0.45 µm pore size, 2.5 cm diam.). Filters were incubated overnight, bacteria uppermost, on plates containing reinforced clostridial medium (RCM) solidified with 1.5% (w/v) agar and spread with 2 mg catalase (Sigma). Bacteria

were harvested from the filter the following morning by vortex mixing in 0.5 ml holding buffer (HB: 25 mM-potassium phosphate pH 7.0, 1 mM-MgSO₄) and serial dilutions were prepared in this buffer. Viable counts of donor and recipient bacteria were obtained by plating on BHIB and CBM, respectively; recipient and donor bacteria were counter-selected by aerobic incubation and by incorporation of trimethoprim (10 µg ml⁻¹), respectively. Transconjugants were selected anaerobically on CBM supplemented with trimethoprim (10 µg ml⁻¹) and either kanamycin (80 µg ml⁻¹) or erythromycin (10 µg ml⁻¹), as appropriate. All manipulations were carried out at 37 °C; those involving *C. acetobutylicum* were carried out in an anaerobic cabinet (Don Whitley Scientific). Frequencies of plasmid transfer are expressed as the number of transconjugants per donor or recipient colony formed after the mating period.

Two attempts were made to effect plasmid transfer to *C. acetobutylicum* strains P262, DSM 1731 and ATCC 824 using the above

procedure, which had been optimized for strain NCIB 8052 (Williams *et al.*, 1990). They were unsuccessful. As has been noted previously (Woolley *et al.*, 1989), the last two strains are not closely related to strain NCIB 8052. Strain P262, which is closely related to strain NCIB 8052, produces copious amounts of extracellular polysaccharide material, which may interfere with filter mating. It would therefore appear that the technique will require modification for use with these organisms.

E. coli donor strains containing R702 were constructed in two steps. The plasmid to be mobilized was introduced into strain HB101 by transformation, after which the IncP helper plasmid was introduced by conjugation from strain J53-1. Matings between *E. coli* strains were carried out according to the method of Trieu-Cuot & Courvalin (1985).

The presence of authentic plasmid DNA in transconjugants was routinely verified by electrophoretic analysis of the products obtained after digestion with a variety of restriction endonucleases.

Molecular methods. The methods employed for DNA extraction and manipulation were those described by Maniatis *et al.* (1982). For small-scale plasmid extraction from *C. acetobutylicum*, the bacterial pellet from 3 ml of an overnight CBM culture containing appropriate antibiotics was suspended in 100 µl 25% (w/v) sucrose, 25 mM-Tris/HCl pH 8.0, 10 mM-EDTA containing 10 mg lysozyme ml⁻¹ and incubated for 1 h at 37 °C before proceeding with the standard alkaline lysis method (Maniatis *et al.*, 1982). This initial step was carried out anaerobically.

Restriction endonucleases, T4 DNA ligase, Klenow polymerase and T4 polymerase were used according to the manufacturers' instructions (variously: Northumbria Biologicals, Boehringer, Pharmacia, Gibco-BRL and Stratagene). DNA fragments were purified using 'GeneClean' (Stratagene). Transformation of *E. coli* strains JM107 and HB101 was done by the Simanis method (Hanahan, 1985).

Results

Transfer of plasmid pAT187 to C. acetobutylicum

Trieu-Cuot *et al.* (1987) have shown that plasmid pAT187 (Fig. 1), can be mobilized by an IncP plasmid from an *E. coli* donor strain into a wide range of Gram-positive bacteria. pAT187 is a derivative of pBR322, into which has been incorporated the *oriT* segment of RK2, together with a 5.1 kb *EcoRI* fragment of pAMβ1, which assures replication in Gram-positive bacteria. It also contains the *aphA-3* gene from Tn1545, which encodes resistance to kanamycin and is selectable in a wide range of organisms, both Gram-positive and Gram-negative. Both the replication origin of pAMβ1 and the Tn1545 *aphA-3* gene are expressed in *C. acetobutylicum* (Oultram & Young, 1985; Reyssset & Sebald, 1985; Yu & Pearce, 1986; Woolley *et al.*, 1989).

Initial experiments were carried out to determine whether pAT187 could be mobilized into *C. acetobutylicum* from an *E. coli* donor strain (HB101) harbouring the IncP plasmid pRK24. Although kanamycin is only weakly selective in anaerobic organisms, judicious adjustment of the antibiotic concentration in the selective medium enabled plasmid transfer to be detected (Table 3). The presence of pAT187 in transconjugants obtained from several independent matings was con-

firmed by analysis of plasmid DNA with a variety of restriction endonucleases. Representative data are shown in Fig. 2.

Construction and mobilization of a high-copy-number vector

The weak staining intensity of plasmid DNA in lysates of *C. acetobutylicum* (Fig. 2) suggests that pAT187 replicates at low copy number in this organism. A high-copy-number vector, pCTC1 (Fig. 1), was constructed by inserting a small restriction fragment carrying *oriT* from RK2 into pMTL500E (Oultram *et al.*, 1988a). The vector, which is based on the pMTL20 plasmid backbone (Chambers *et al.*, 1988 – see also below) contains a 2.8 kb sub-fragment of the pAMβ1 replication region, which promotes replication at high copy number in *C. acetobutylicum* (Swinfield *et al.*, 1990). It also contains the Em^R gene of pAMβ1 (Brehm *et al.*, 1987), which is more readily selectable in *C. acetobutylicum* than is the Km^R gene of pAT187. Plasmid pCTC1 was transferred to *C. acetobutylicum* from two *E. coli* donors, strain HB101 harbouring the autonomous IncP plasmid R702 and strain SM10 (Simon *et al.*, 1983), in which the *tra* functions are present on the bacterial chromosome as an integrated copy of RP4-2(Tc::Mu) (Table 3). Plasmid DNA was much more prominent in lysates of *C. acetobutylicum* strains harbouring pCTC1 than in those of strains harbouring pAT187 (Fig. 2), suggesting that pCTC1 is indeed maintained at a higher copy number in this organism than is pAT187.

Screening of replicons for ability to function in C. acetobutylicum

A pair of vectors, pMTL30 and pMTL31 (Fig. 1), were constructed, containing *oriT* inserted in either orientation in the pMTL20E backbone (Minton *et al.*, 1988). The ColE1-derived replication origin of the pMTL plasmids is the same as that of the pUC plasmids (Vieira & Messing, 1982; Yanisch-Perron *et al.*, 1985). As a result of a single base pair change in the RNAI/RNII region, the pMTL and pUC plasmids replicate at a three-fold higher copy number than pBR322 in *E. coli* (Chambers *et al.*, 1988). Both pMTL30 and pMTL31 were equally efficiently mobilized in matings between *E. coli* strains (data not shown). They lack a replication origin active in Gram-positive bacteria and hence they cannot become established in *C. acetobutylicum* (Table 3). Replication origins from plasmids found in Gram-positive bacteria were inserted into the polylinker regions of pMTL30 and pMTL31 to test their ability to function in *C. acetobutylicum*.

Initially, plasmids pCTC40 and pCTC41 were con-

Fig. 1. Plasmids. These were constructed as follows: A 760 bp *Pst*I fragment from pAT187, containing *oriT*, was inserted into the *Pst*I site of pMTL500E to give pCTC1. The *oriT* segment was excised from pCTC1 on an 850 bp *Eco*RI–*Hind*III fragment and inserted into *Eco*RI- and *Hind*III-digested pGK13 (Kok *et al.*, 1984) to generate pCTC3. pCTC40 and pCTC41 were constructed by inserting a 2.8 kb *Eco*RI–*Bam*HI fragment of pMTL500E, containing the pAM β 1 replication origin, into *Eco*RI- and *Bam*HI-digested pMTL30 and pMTL31, respectively. pCTC401 arose by spontaneous insertion of *IS1* into pCTC40. A 3.5 kb *Sau*3A fragment of pCB101, bearing the replication origin, was inserted into the *Bam*HI site of pMTL31 to give pCTC511. For all plasmids except pCTC3 (see text), the replication origin allowing plasmid maintenance in *E. coli* (denoted pUC) is that of pUC8 (Vieira & Messing, 1982). All plasmids except pCTC3 are based on the pMTL20 backbone (Chambers *et al.*, 1988; Minton *et al.*, 1988). Antibiotic resistances are denoted as in Table 1 and 2. In pCTC3, the gene conferring resistance to Em is from pE194; in all other plasmids it is derived from pAM β 1. The genes conferring resistance to Cm and Km are from pC194 and Tn1545 (*aphA-3*), respectively. Restriction sites are denoted as follows: $\circ$, *Ava*I; $\bullet$, *Bal*I; $\square$, *Bam*HI; $\blacksquare$, *Bst*EII; $\triangle$, *Eco*RI; $\blacktriangle$, *Hind*III; ∇ , *Pst*I; $\blacktriangledown$, *Pvu*II; $\diamond$, *Mbo*I; $\blacklozenge$, *Bgl*II; $\triangleleft$, *Sau*3A; $\blacktriangleleft$, *Bcl*II. Note that plasmid pAT187 is drawn to a different scale.

Fig. 2. Detection of plasmids in transconjugants. Plasmids extracted from *E. coli* HB101 (E) and *C. acetobutylicum* NCIB 8052 (C) were digested with the restriction endonucleases indicated. In the lane containing pCTC40 from *E. coli* a faint band resulting from insertion of IS1 into the smaller *EcoRI*-*Bam*HI fragment is visible between the two brighter bands. This band is not observed with pCTC40 extracted from *C. acetobutylicum* (the topmost band in this case results from partial digestion).

Table 3. Frequencies of plasmid transfer to *C. acetobutylicum*

Plasmid	Mobilization*	Frequency per donor†	Frequency per recipient‡
pAT187	pRK24	$2.9 (\pm 4.0) \times 10^{-7}$ [3]	$1.4 (\pm 1.4) \times 10^{-6}$ [3]
pCTC1	RP4-2(Tc::Mu)	$6.0 (\pm 4.4) \times 10^{-7}$ [5]	$1.1 (\pm 1.2) \times 10^{-5}$ [5]
pCTC1	R702	$3.8 (\pm 6.0) \times 10^{-6}$ [10]	$2.8 (\pm 3.4) \times 10^{-5}$ [10]
pCTC40	R702	$3.4 (\pm 0.5) \times 10^{-6}$ [3]	$1.5 (\pm 0.3) \times 10^{-5}$ [3]
pCTC401	R702	$2.6 (\pm 1.9) \times 10^{-6}$ [4]	$3.6 (\pm 2.4) \times 10^{-5}$ [4]
pCTC41	R702	$6.1 (\pm 6.0) \times 10^{-6}$ [3]	$4.7 (\pm 2.8) \times 10^{-5}$ [3]
pCTC511	R702	$2.7 (\pm 5.2) \times 10^{-7}$ [8]	$8.1 (\pm 13.7) \times 10^{-7}$ [8]
pCTC3	RP4-2(Tc::Mu)	$2.0 (\pm 1.2) \times 10^{-6}$ [3]	$1.0 (\pm 0.3) \times 10^{-5}$ [3]
pMTL30‡	RP4-2(Tc::Mu)	$< 3.7 \times 10^{-9}$ [1]	$< 3.5 \times 10^{-9}$ [1]
pMTL31‡	RP4-2(Tc::Mu)	$< 2.1 \times 10^{-9}$ [1]	$< 3.3 \times 10^{-9}$ [1]
pMTL500E‡	RP4-2(Tc::Mu)	$< 7.4 \times 10^{-10}$ [1]	$< 4.4 \times 10^{-9}$ [1]

* The donor strain containing pRK24 or R702 was HB101. RP4-2(Tc::Mu) is present as a chromosomal insertion in strain SM10.

† Values in parentheses are population standard deviations of the means (σ_{n-1}); the number of determinations, n , is shown in square brackets.

‡ Plasmids pMTL30, pMTL31 and pMTL500E did not produce any transconjugants.

structed by inserting the replication origin of pAM β 1 into pMTL30 and pMTL31, respectively (Fig. 1). These plasmids contain the same elements as pCTC1, except that the positions of the pAM β 1 replication origin and the *oriT* segment are exchanged. The frequencies of transfer of pCTC40 and pCTC41 from an *E. coli* donor strain harbouring R702 to *C. acetobutylicum* were similar

(Table 3). This indicates that mobilization was equally efficient irrespective of the orientation of *oriT*. The frequency of mobilization of both plasmids was also similar to that observed with pCTC1, indicating that the relative positions of the pAM β 1 replication origin and *oriT* have no pronounced effect on plasmid mobilization frequency.

Fig. 3. Insertion of *IS1* into pCTC40. Comparison of the sensitivity of pCTC40 and pCTC401 to a variety of restriction endonucleases. Lanes marked with an asterisk are DNA samples hybridized with the fragment of pUC8 β that detects *IS1* (see Table 2). Note that this probe contained small amounts of contaminating pUC8 sequences that resulted in weak hybridization with the pMTL20 backbone of pCTC40 and pCTC401.

Plasmid pCTC511 (Fig. 1) contains the replication functions of plasmid pCB101 from *Clostridium butyricum* NCIB 7423 (Minton & Morris, 1981; Collins *et al.*, 1985). pCB101 belongs to the extensive family of plasmids found in Gram-positive bacteria that replicate via a single-stranded DNA intermediate (Minton & Oultram, 1988; Young *et al.*, 1989; Gruss & Ehrlich, 1989). pCTC511 was transferred to *C. acetobutylicum* from *E. coli* strain HB101 harbouring R702 at a frequency slightly lower than those obtained for the various pCTC plasmids containing the pAM β 1 replication functions (Table 3). It appears to replicate at high copy number in *C. acetobutylicum* as indicated by the staining intensity of plasmid DNA in lysates (Fig. 2).

Lastly, plasmid pCTC3 (Fig. 1), which contains the replication origin of the *Streptococcus cremoris* plasmid pWV01 (Vosman & Venema, 1983), was tested for ability to replicate in *C. acetobutylicum*. Like pCB101, pWV01 belongs to the extensive family of plasmids that replicate via a single-stranded DNA intermediate (Peijnenburg *et al.*, 1989; A. Gruss & S. D. Ehrlich, personal communication). Since the pWV01 replication functions are active in both Gram-positive and Gram-negative hosts (Kok *et al.*, 1984), pCTC3 was constructed without incorporating the pMTL plasmid backbone. In consequence, it is the smallest of the vectors constructed here (Fig. 1). Plasmid pCTC3 was mobilized from *E. coli* to *C. acetobutylicum* at a frequency similar to that observed for plasmids containing the replication origin of plasmid

pAM β 1 (Table 3). The weak staining intensity of plasmid DNA in lysates of *C. acetobutylicum* strains harbouring pCTC3 (Fig. 2) suggests that this plasmid replicates at low copy number in this organism.

Instability of plasmid pCTC40 in *E. coli*

On several separate occasions, it was noted that strains of *E. coli* independently transformed with pCTC40 contained an enlarged derivative. The insertions (about 750 bp) introduced extra sites for *Pst*I and *Pvu*II and new unique sites for *Bst*EII and *Bal*I into the plasmid (Fig. 3). In order to keep pCTC40 intact in *E. coli*, it was necessary to maintain strains at 37 °C; when left on the bench and then subcultured, they were invariably found to harbour enlarged derivatives. This behaviour, and the properties outlined above, suggested that the instability of plasmid pCTC40 arose from the insertion of a copy of *IS1*. This was confirmed by Southern hybridization (Fig. 3), using an internal segment of *IS1* as probe (Larkin *et al.*, 1986). Analysis of several independent isolates with a variety of restriction endonucleases suggested that *IS1* always inserted into the same position (± 50 bp), within the DNA segment encoding the pAM β 1 replication functions. Nevertheless, the enlarged derivative, denoted pCTC401, was transferred from *E. coli* to *C. acetobutylicum* at a frequency comparable to that of the parental plasmid, pCTC40 (Table 3).

Discussion

The results reported here establish a convenient alternative to electroporation (Oultram *et al.*, 1988a), or conjugative mobilization from *B. subtilis* (Oultram *et al.*, 1987), for introducing plasmids into *C. acetobutylicum*. Using this new method for plasmid transfer, it has been established that replication functions of plasmids originating in three different heterologous hosts can function in *C. acetobutylicum*. Derivatives containing the replication region of plasmid pAM β 1 from *E. faecalis* have previously been transferred to this organism by conjugation from *B. subtilis* (Oultram & Young, 1985) and also by electroporation (Oultram *et al.*, 1988a). The apparent difference in copy number in *C. acetobutylicum* between the plasmid (pAT187) which contains a 5.1 kb *Eco*RI fragment of pAM β 1 and replicates at low copy number, and several others (pCTC1, pCTC40 and pCTC41) which contain a 2.8 kb sub-fragment and replicate at high copy number, is in agreement with the results of Swinfield *et al.* (1990), and suggests that the 2.3 kb segment missing from the sub-fragment contains an element that negatively controls plasmid copy number. pCTC511, containing the replication functions of pCB101 from *C. butyricum*, replicated at high copy number in *C. acetobutylicum*. In agreement with this finding, another plasmid containing the pCB101 replication functions has recently been transferred to this organism by electroporation (reviewed by Young *et al.*, 1989). Lastly, the finding that pCTC3, containing the replication functions of plasmid pWV01 from *S. cremoris*, was able to replicate at low copy number in *C. acetobutylicum* further extends the very wide range of organisms, both Gram-positive and Gram-negative, in which the replication functions of this plasmid are active (Kok *et al.*, 1984).

As far as is known, the only difference between pCTC40 and pCTC41 is the orientation of the *oriT* segment. Yet the former, but not the latter, tended to act as a target for *IS1* insertion; the element always (six independent isolates have been analysed) inserted into the same region (± 50 bp). Why this region acts as an apparent hotspot for *IS1* transposition in pCTC40, but not in pCTC41, is not known. It is also of interest that insertion of *IS1* did not impair the ability of pCTC401 to replicate in either *C. acetobutylicum* or *B. subtilis* (data not shown for the latter). Swinfield *et al.* (1990) have shown that a deletion extending into this region of the 2.8 kb sub-fragment of the pAM β 1 replication region abolishes its ability to function in *B. subtilis*. Nucleotide sequencing, to determine the precise insertion points in several independent isolates, might prove informative in relation to both the pAM β 1 replication region and the nature of the apparent hotspot for *IS1* insertion.

Finally, the results of this investigation further extend the extraordinary range of organisms into which plasmids may be transferred from *E. coli* by conjugative mobilization (Buchanan-Wollaston *et al.*, 1987; Trieu-Cuot *et al.*, 1987; Mazodier *et al.*, 1989; Heinemann & Sprague, 1989). They also establish that transfer occurs under strictly anaerobic conditions and hence, IncP plasmids may prove useful for effecting plasmid transfer from *E. coli* to other recalcitrant anaerobes, such as methanogens, for which conventional methods of gene transfer have so far proved ineffective.

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