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

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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 pAMβ1 (*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.

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 pAMβ1 (Oultram *et al.*, 1987, 1988b). Since pAMβ1 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 (Willets & 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*.

**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*.

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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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HB101</td>
<td>F⁻ hsdS20 (rK, mG) recA13 ara-l4 lacY1 proA2 galK2 rpsL20 (SmR)* xyl-5 mtl-1 supE44 (λ⁻)</td>
<td>Laboratory strain/Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>J53-1</td>
<td>F⁻ pro m7 gyrA96 (NalR) (λ)</td>
<td>C. M. Thomas, University of Birmingham/Clowes &amp; Hayes (1968)</td>
</tr>
<tr>
<td>JM107</td>
<td>endA1 gyrA96(NalR) thi-l hsdR17 (rK, mG) supE44 relA1 (λ⁻) Δlac-proAB (F traD36 proAB lacP2ZAM15) [RP4-2(Tc::Mu)] KmR (λ⁻)</td>
<td>Laboratory strain/Janisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>SM10†</td>
<td>F⁻ thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44 Mu+ (λ⁻)</td>
<td>P. Trieu-Cuot, Institut Pasteur/Simon et al. (1983)</td>
</tr>
</tbody>
</table>

C. acetobutylicum NCIB 8052

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics*</th>
<th>Gram-positive replicon</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAT187</td>
<td>Tra⁻ Mob⁺ KmR</td>
<td>pAMβ1</td>
<td>Trieu-Cuot et al. (1987)</td>
</tr>
<tr>
<td>R702‡</td>
<td>Tra⁺ Mob⁺ IncP KmR TcR SmR SuR HpR</td>
<td></td>
<td>Hedges &amp; Jacob (1974)</td>
</tr>
<tr>
<td>pCB101</td>
<td>Tra⁻ Mob⁺ cryptic</td>
<td>pCB101</td>
<td>Collins et al. (1985)</td>
</tr>
<tr>
<td>pGK13</td>
<td>Tra⁻ Mob⁺ CmR EmR</td>
<td>pWV01</td>
<td>Kok et al. (1984)</td>
</tr>
<tr>
<td>pUC88β‡</td>
<td>Tra⁻ Mob⁺ ApRβ</td>
<td></td>
<td>Larkin et al. (1986)</td>
</tr>
<tr>
<td>pMTL30</td>
<td>Tra⁺ Mob⁺ ApRβ SmR lacZ</td>
<td></td>
<td>Williams et al. (1990)</td>
</tr>
<tr>
<td>pMTL31</td>
<td>Tra⁺ Mob⁺ ApRβ lacZ</td>
<td></td>
<td>Williams et al. (1990)</td>
</tr>
<tr>
<td>pMTL500E</td>
<td>Tra⁺ Mob⁺ ApRβ lacZ</td>
<td>pAMβ1</td>
<td>Oultram et al. (1988a)</td>
</tr>
<tr>
<td>pCCT1†</td>
<td>Tra⁻ Mob⁺ ApR β EmR</td>
<td>pAMβ1</td>
<td>This paper</td>
</tr>
<tr>
<td>pCCT3†</td>
<td>Tra⁻ Mob⁺ CmR EmR</td>
<td>pWV01</td>
<td>This paper</td>
</tr>
<tr>
<td>pCCT40†</td>
<td>Tra⁻ Mob⁺ ApR β EmR</td>
<td>pAMβ1</td>
<td>This paper</td>
</tr>
<tr>
<td>pCCT40†</td>
<td>Tra⁻ Mob⁺ ApR β EmR</td>
<td>pAMβ1:ISI</td>
<td>This paper</td>
</tr>
<tr>
<td>pCCT41†</td>
<td>Tra⁻ Mob⁺ ApR β EmR</td>
<td>pAMβ1</td>
<td>This paper</td>
</tr>
<tr>
<td>pCCT51†</td>
<td>Tra⁻ Mob⁺ ApR β EmR</td>
<td>pCB101</td>
<td>This paper</td>
</tr>
</tbody>
</table>

* Antibiotic resistances: Sm, streptomycin; Na, nalidixic acid; Km, kanamycin.
† pRK24-2(Tc::Mu) is integrated into the bacterial chromosome.
‡ pUC8β contains a 240 bp internal HaeIII fragment of IS1.
§ pRK24 is RK2 with the KmR gene inactivated by insertion of a HindIII fragment bearing trpE.
¶ R702 lacks Tn1 and does not confer ampicillin resistance (Hedges & Jacob, 1974).

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 x 10⁸ 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 x 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/His pH 8.0, 10 mM-EDTA containing 10 μg 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 polynucleotide were used according to the manufacturers’ instructions (variously: Northumbria Biologica, 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; Reysset & Sebal, 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 confirmed 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 EmR gene of pAMβ1 (Brehm et al., 1987), which is more readily selectable in *C. acetobutylicum* than is the KmR 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>s::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/RNAII 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 PstI fragment from pAT187, containing oriT, was inserted into the PstI site of pMTL500E to give pCTC1. The oriT segment was excised from pCTC1 on an 850 bp EcoRI-HindIII fragment and inserted into EcoRI- and HindIII-digested pGK13 (Kok et al., 1984) to generate pCTC3. pCTC40 and pCTC41 were constructed by inserting a 2.8 kb EcoRI-BamHI fragment of pMTL500E, containing the pAMβ1 replication origin, into EcoRI- and BamHI-digested pMTL30 and pMTL31, respectively. pCTC401 arose by spontaneous insertion of IS1 into pCTC40. A 3.5 kb Sau3A fragment of pCB101, bearing the replication origin, was inserted into the BamHI 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 pAMβ1. The genes conferring resistance to Cm and Km are from pC194 and Tn5 (aphA-3), respectively. Restriction sites are denoted as follows: ○, AccI; ■, BsuF1; □, BamHI; △, BsrEI1; △, EcoRI; ▲, HindIII; ▽, PstI; ▼, PvuII; ○, MboI; ●, BglII; ○, Sau3A; ▲, BclI. Note that plasmid pAT187 is drawn to a different scale.
Plasmid transfer to C. acetobutylicum

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-BamHI 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mobilization*</th>
<th>Frequency per donor†</th>
<th>Frequency per recipient‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAT187</td>
<td>pRK24</td>
<td>2.9 (±4.0) x 10^-3</td>
<td>1.4 (±1.4) x 10^-6 [3]</td>
</tr>
<tr>
<td>pCTC1</td>
<td>RP4-2(Tc::Mu)</td>
<td>6.0 (±4.4) x 10^-7</td>
<td>1.1 (±1.2) x 10^-5 [5]</td>
</tr>
<tr>
<td>pCTC1</td>
<td>R702</td>
<td>3.8 (±6.0) x 10^-8 [10]</td>
<td>2.8 (±3.4) x 10^-9 [10]</td>
</tr>
<tr>
<td>pCTC40</td>
<td>R702</td>
<td>3.4 (±0.5) x 10^-6</td>
<td>1.5 (±0.3) x 10^-5 [3]</td>
</tr>
<tr>
<td>pCTC401</td>
<td>R702</td>
<td>2.6 (±1.9) x 10^-6</td>
<td>3.6 (±2.4) x 10^-5 [4]</td>
</tr>
<tr>
<td>pCTC41</td>
<td>R702</td>
<td>6.1 (±6.0) x 10^-8</td>
<td>4.7 (±2.8) x 10^-5 [3]</td>
</tr>
<tr>
<td>pCTC511</td>
<td>R702</td>
<td>2.7 (±5.2) x 10^-7</td>
<td>8.1 (±13.7) x 10^-7 [8]</td>
</tr>
<tr>
<td>pCTC3</td>
<td>RP4-2(Tc::Mu)</td>
<td>2.0 (±1.2) x 10^-6</td>
<td>1.0 (±0.3) x 10^-5 [3]</td>
</tr>
<tr>
<td>pMTL30†</td>
<td>RP4-2(Tc::Mu)</td>
<td>&lt;3.7 x 10^-9 [1]</td>
<td>&lt;3.5 x 10^-9 [1]</td>
</tr>
<tr>
<td>pMTL31†</td>
<td>RP4-2(Tc::Mu)</td>
<td>&lt;2.1 x 10^-9 [1]</td>
<td>&lt;3.3 x 10^-9 [1]</td>
</tr>
<tr>
<td>pMTL500E‡</td>
<td>RP4-2(Tc::Mu)</td>
<td>&lt;7.4 x 10^-10 [1]</td>
<td>&lt;4.4 x 10^-9 [1]</td>
</tr>
</tbody>
</table>

* 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); the number of determinations, n, is shown in square brackets.
‡ Plasmids pMTL30, pMTL31 and pMTL500E did not produce any transconjugants.

Plasmids pCTC1, except that the positions of the pAMβ1 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 pAMβ1 replication origin and oriT have no pronounced effect on plasmid mobilization frequency.
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 pAMβ1 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 pAMβ1 (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 *PstI* and *PvuII* and new unique sites for *BstEII* and *Ban* 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 pAMβ1 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 pAMß1 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 EcoRI fragment of pAMß1 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. ceremoris, 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 ISI 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 ISI transposition in pCTC40, but not in pCTC41, is not known. It is also of interest that insertion of ISI 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 pAMß1 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 pAMß1 replication region and the nature of the apparent hotspot for ISI 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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References


