Gene transfer to *Clostridium cellulolyticum* ATCC 35319

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Although much is known about the bacterial cellulosome and its various protein components, their contributions to bacterial growth on cellulose and the process of cellulolysis *in vivo* cannot currently be assessed. To remedy this, the authors have developed gene transfer techniques for *Clostridium cellulolyticum* ATCC 35319. Firstly, transfer of Tn1545 has been obtained using an *Enterococcus faecalis* donor. Secondly, IncP-mediated conjugative mobilization of plasmids from *Escherichia coli* donors has also been achieved. The yield of transconjugants in both cases was low and was probably limited by the suboptimal growth conditions that must of necessity be employed for the co-culture of oligotrophic *C. cellulolyticum* with copiotrophic donors. A restriction endonuclease was detected in crude extracts of *C. cellulolyticum*. This enzyme, named Ccel, is an isoschizomer of MspI (HpaII). Electro-transformation was employed to establish plasmids containing the replication functions of pAM β 1 (*En. faecalis*), pIM13 (*Bacillus subtilis*), pCB102 (*Clostridium butyricum*), pIP404 (*Clostridium perfringens*) and pWV01 (*Lactococcus lactis* subsp. *cremoris*) in *C. cellulolyticum*. Transformants were only obtained if the DNA was appropriately methylated on the external C of the sequence 5'-CCGG-3' using either BsuFI methylase *in vivo* or MspI methylase *in vitro*. Plasmids based on the pAM β 1 and pIM13 replicons were more stably maintained than one based on the pCB102 replicon. Selection of transformants on solid medium led to low apparent transformation efficiencies (approx. 10² transformants per μ g DNA) which might, in part, reflect the low plating efficiency of the organism. Selection of transformants in liquid medium led to a higher apparent yield of transformants (between 10⁵ and 10⁷ transformants per μ g DNA). The methods developed here will pave the way for functional analysis of the various cellulosome components *in vivo*.

Keywords: conjugation, electro-transformation, methylation, cellulosome

INTRODUCTION

The bacterial cellulosome is a multi-component cell-surface organelle found in several species of *Clostridium*, as well as in certain other organisms (Felix & Ljungdahl, 1993; Shoham *et al.*, 1999). This sophisticated complex of cellulolytic enzymes has been characterized most extensively in the thermophilic species *Clostridium thermocellum* (Bayer *et al.*, 1998; Shoham *et al.*, 1999). A considerable amount is also known about the cellulosomes of two mesophilic

species, *C. cellulovorans* (Doi *et al.*, 1998) and *C. cellulolyticum* (Bélaich *et al.*, 1997; Gal *et al.*, 1997; Pagès *et al.*, 1997, 1999; Reverbel-Leroy *et al.*, 1997). This last species has also been quite extensively characterized physiologically (Giallo *et al.*, 1985; Gelhaye *et al.*, 1993a, b; Guedon *et al.*, 1999a, b; Payot *et al.*, 1998). To date, molecular analysis of the bacterial cellulosome has been based exclusively on studies of recombinant proteins (Bayer *et al.*, 1998; Bélaich *et al.*, 1997). Functional analysis '*in clostridio*' still awaits the development of suitable laboratory protocols for effecting gene transfer.

Gene transfer to many species of *Clostridium* is now possible using a variety of methods, including protoplast

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transformation, electro-transformation and conjugation (reviewed by Young & Cole, 1993; Young *et al.*, 1999). Moreover, allelic replacement, which is essential for the analysis of gene function *in vivo*, has been achieved in three species, *C. beijerinckii* (Wilkinson & Young, 1994), *C. acetobutylicum* (Green *et al.*, 1996) and *C. perfringens* (Awad *et al.*, 1995).

An ever-growing range of clostridia has been electro-transformed (Young *et al.*, 1999 and references therein). The activity of host restriction enzymes remains a significant barrier to electro-transformation with double-stranded DNA. If the nature of the restriction system is known, transforming DNA may be protected by methylation *in vivo* or *in vitro* (Mermelstein & Papoutsakis, 1993; Davis *et al.*, 2000). As an alternative to electro-transformation, the extremely broad-host-range conjugation machinery of IncP plasmids may be employed to mobilize plasmids from *Escherichia coli* to a wide range of Gram-positive bacteria (Trieu-Cuot *et al.*, 1987). Vectors containing the *cis*-acting *oriT* site (origin of transfer) of the IncP plasmid RK2 are mobilized if all other conjugation functions are provided *in trans* on an IncP plasmid or as part of the donor chromosome. This method was initially optimized for *C. beijerinckii* (Williams *et al.*, 1990a, b) and it has since been employed for transferring DNA to other clostridia (Young *et al.*, 1999 and references therein).

Conjugative transposons are useful tools for insertional mutagenesis and mutational cloning (Clewell & Flannagan, 1993; Young, 1993a, b; Scott & Churchward, 1995). The most widely used representatives are the broad host-range streptococcal transposons, Tn916 (Franke & Clewell, 1981) and Tn1545 (Courvalin & Carlier, 1987). One or other of these elements has been transferred to a wide range of clostridia (Young *et al.*, 1999 and references therein).

Although the number of clostridia that are amenable to genetic analysis is constantly increasing, there have been no reports so far of gene transfer to cellulolytic species. [Note, however, that Tn1545 has been transferred to *Eubacterium cellulosolvens* (Anderson *et al.*, 1998), which is a very close relative of the clostridia.] We describe herein the development of conjugation and electro-transformation procedures for *Clostridium cellulolyticum* ATCC 35319, which we have employed to establish a range of plasmids as well as a conjugative transposon in this organism. They represent a first step towards functional analysis of the cellulosome *in clostridio*.

METHODS

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

Culture media and bacterial growth. Strains of *E. coli* were maintained on Luria-Bertani medium (Sambrook *et al.*, 1989). *C. cellulolyticum* ATCC 35319 was routinely grown on GS medium, modified from that described by Garcia-Martinez *et al.* (1980), which contains: KH₂PO₄, 3.7 mM; K₂HPO₄, 3.5 mM; urea, 33 mM; MgCl₂, 2.5 mM; CaCl₂, 0.3 mM; FeSO₄, 0.004 mM; sodium β -glycerophosphate, 28 mM; trisodium

citrate, 10 mM; MOPS, 48 mM; D-cellobiose, 15 mM; cysteine.HCl, 6.3 mM; yeast extract, 0.5% (w/v); pH 7.2. VM medium was derived from GS medium by replacing yeast extract with: D-biotin, 0.08 μ M; pyridoxamine, 0.02 μ M; cyanocobalamin, 0.001 μ M; *p*-aminobenzoic acid, 0.15 μ M; thiamin, 0.9 μ M; L-alanine, 0.22 μ M. Bacterial growth was monitored by following the OD₆₀₀ with time. The generation time in this medium is approx. 6 h at 34 °C. GS and VM were solidified with 1.5% (w/v) agar, as appropriate.

Filter mating procedure. The optimized procedure was adapted from that of Williams *et al.* (1990a) as follows. Donor strains of *E. coli* and *Enterococcus faecalis* were grown overnight, at 37 °C in aerobic conditions, to stationary phase in brain heart infusion broth (BHIB, Oxoid) supplemented with 50 μ g ampicillin ml⁻¹ and 10 μ g erythromycin ml⁻¹, selective for their mobilizable plasmid or conjugative transposon, respectively. The following morning, donor strains were diluted back ten-fold and regrown to OD₆₀₀ > 1.0 (about 5×10^8 c.f.u. ml⁻¹). Alternatively, they were diluted with an equal volume of pre-warmed BHIB. Donor cells were washed twice in pre-warmed BHIB just before mating, to remove traces of the selective antibiotic. All manipulations involving the recipient, *C. cellulolyticum* ATCC 35319, were carried out at 34 °C in an anaerobic workstation (Don Whitley Scientific). The recipient was grown for 17–24 h in GS medium to late exponential phase at OD₆₀₀ 0.5–1.0 (about 5×10^6 c.f.u. ml⁻¹). The low number of c.f.u. recovered is due, in part, to a tendency to grow in filaments, but this organism also shows a low plating efficiency. Donor and recipient cells were mixed at a 10:1 ratio by pipetting 0.2 ml donor culture into 2 ml recipient culture. The bacteria were harvested by filtration through a nitrocellulose filter (Whatman WCN, pore size 0.45 μ m, 25 mm diameter). Filters were incubated overnight at 34 °C, bacteria uppermost, on plates spread with 2 mg catalase (Sigma). For matings with *E. coli* donors, GS medium was used; for matings with *En. faecalis* donors, VM medium was used. Bacteria were harvested from the filters the following morning by vortex mixing in 0.5 ml holding buffer (1 mM MgSO₄, 25 mM potassium phosphate, pH 7.0) and serial dilutions were prepared in this buffer. Viable counts of donor and recipient bacteria were obtained by plating on BHIB and either GS (for *E. coli* matings) or VM (for *En. faecalis* matings), respectively. Recipient bacteria were counter-selected by aerobic incubation whereas donor bacteria were counter-selected by incorporation of 10 μ g trimethoprim ml⁻¹ (*E. coli*) or the absence of a fermentable carbon source (*En. faecalis*). Transconjugants were selected anaerobically on the above media supplemented with 10 μ g erythromycin ml⁻¹. Transconjugants appeared 4–5 d after plating. Transfer frequencies were expressed as the number of transconjugants per recipient colony formed after the mating period.

E. coli donor strains were constructed by introducing the plasmids to be mobilized into a strain of HB101 in which the IncP β plasmid, R702 (Thomas & Smith, 1987), was already present.

Characterization of the restriction system. Crude extracts of *C. cellulolyticum* were prepared from a 20 ml late-exponential-phase culture, using the method described by Azeddoug & Reysset (1991). The soluble cellular extract was adjusted to 50% (v/v) glycerol and stored at –20 °C. For restriction assays, 350–400 ng DNA substrate was mixed with 2.5 μ l of crude extract (approx. 10 μ g protein) and incubated for 2 h at 37 °C in 25 μ l of 10 mM Tris/HCl, pH 7.5, buffer containing 50 mM NaCl, 10 mM MgCl₂, 1 mM dithioerythritol, 0.01% BSA. The products were analysed by electrophoresis through agarose (1.2% w/v). For methylation

Table 1. Bacterial strains

Strain	Genotype or phenotype*	Source/reference
<i>Clostridium cellulolyticum</i> H10 (ATCC 35319)	Wild-type	E. Petitdemange, Nancy/Petitdemange <i>et al.</i> (1984)
<i>Escherichia coli</i> DH5 α	λ^- <i>endA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 recA1 gyrA96</i> (Na ^R) <i>relA1</i> Δ (<i>argF-lacZYA</i>)U169 [ϕ 80 <i>lacZ</i> AM15]	Hanahan (1985)
<i>Escherichia coli</i> HB101	F ⁻ <i>hsdS20</i> ($r_B^- m_B^-$) <i>endoI recA13 supE44 ara-14 proA2 galK2 rpsL20</i> (Sm ^R) <i>xyl-5 mtl-1</i>	Laboratory strain/Boyer & Roulland-Dussoix (1969)
<i>Escherichia coli</i> TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>)[ϕ 80 <i>lacZ</i> AM15] Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7679 <i>galU galK rpsL</i> (Sm ^R) <i>endA1 nupG</i>	Appligene
<i>Enterococcus faecalis</i> BM4110::Tn1545	(Em ^R Tc ^R Km ^R Fus ^R Rif ^R)	P. Courvalin, Institut Pasteur/Courvalin & Carlier (1986)

* Antibiotic-resistance markers have been abbreviated as follows: Em, erythromycin; Fus, fusidic acid; Km, kanamycin; Na, nalidixic acid, Rif, Rifampicin; Sm, streptomycin; Tc, tetracycline.

Table 2. Plasmids

Plasmid	Relevant characteristics	Gram ⁺ replicon	Source or reference
R702	Tra ⁺ Mob ⁺ IncP Tc ^R Sm ^R Su ^R Hg ^R	–	Hedges & Jacob (1974)
pAT187	Tra ⁻ Mob ⁺ Km ^R	pAM β 1	Trieu-Cuot <i>et al.</i> (1987)
pM.BsuF1	Met ⁺ Cm ^R	–	Davis <i>et al.</i> (2000)
pMTL540	Ap ^R , Em ^R in Gram ⁺ hosts	pCB102	N. P. Minton, CAMR
pCTC1	Tra ⁻ Mob ⁺ Ap ^R , Em ^R in Gram ⁺ hosts	pAM β 1	Williams <i>et al.</i> (1990b)
pGK12	Tra ⁻ Mob ⁻ Cm ^R Em ^R	pWV01	Kok <i>et al.</i> , 1984
pJIR418	Tra ⁻ Mob ⁻ Cm ^R Em ^R	pIP404	Sloan <i>et al.</i> , 1992
pCTC501	Tra ⁻ Mob ⁺ Ap ^R , Em ^R in Gram ⁺ hosts	pCB101	Young <i>et al.</i> (1993)
pCTC511	Tra ⁻ Mob ⁺ Ap ^R , Em ^R in Gram ⁺ hosts	pCB101	Williams <i>et al.</i> (1990b)
pECII	Ap ^R , Em ^R in Gram ⁺ hosts	pIM13	Ph. Soucaille, INSA, Toulouse
pECN2*	Ap ^R , Em ^R in Gram ⁺ hosts, 5' <i>cipC</i>	pIM13	This paper

* Plasmid pECN2 is a derivative of pECII, containing a 1.6 kbp fragment from entirely within the coding sequences of the 5' end of *cipC*.

protection assays, *HpaII* methylase (*M.HpaII*) and *MspI* methylase (*M.MspI*) (New England Biolabs) were used according to the supplier's instructions. Methylated DNA was purified using QIAEX II (QIAGEN) before incubation with restriction endonucleases.

Electro-transformation procedures. *C. cellulolyticum* was grown for 17–24 h in 50 ml cultures in GS medium to late exponential phase, i.e. OD₆₀₀ 0.5–1.0 (about 5×10^6 c.f.u. ml⁻¹). Bacteria were harvested by centrifugation in sealed tubes for 10 min at 6000 g and 4 °C in a Hettich EBA

12 R centrifuge. Cells were washed twice with 10 ml ice-cold electroporation buffer (270 mM sucrose; 1 mM MgCl_2 ; 5 mM sodium phosphate buffer, pH 7.4) in the anaerobic chamber and resuspended in a final volume of 1 ml of this buffer. Plasmid DNA (1–2 μg) was added to pre-chilled electroporation cuvettes (Equibio, 0.2-cm inter-electrode distance) followed by 200 μl cell suspension, and the cuvettes were incubated on ice for 10 min.

In some experiments, bacteria were pulsed once at 1.5 kV, 25 μF and 100 Ω using a Bio-Rad Gene Pulser electroporation apparatus. The resulting pulse duration was 1.9–2.0 ms. Fresh GS medium (1 ml) was added to the cuvette immediately after electroporation and the cell suspension was transferred to a further 1 ml GS for overnight incubation. The following morning, bacteria were harvested by centrifugation, resuspended in 200 μl GS and spread on two GS agar plates supplemented with 10 μg erythromycin ml^{-1} . Transformation efficiencies were expressed as the number of transformants per μg DNA. Since an 18 h period was allowed for phenotypic expression, during which two to three bacterial generations may have occurred, the transformation frequencies are probably overestimated by between four- and eightfold.

In some experiments, a JOUAN PS15 electropulsator (JOUAN, France) was used. This apparatus delivers square wave pulses, which provide a constant electric field of pre-programmed duration and voltage. The mixture containing 10^9 cells and 10^{10} plasmid molecules (e.g. 80 ng pCTC1) was subjected to a 5-ms pulse at 6.5, 7, or 7.5 kV cm^{-1} . The electroporated cells were incubated overnight in 10 ml fresh GS medium before plating on solid medium and transformation efficiencies were evaluated as described above. Alternatively, after an overnight phenotypic expression period without antibiotic, the electroporated cells were subcultured in 100 ml GS liquid medium supplemented with 10 μg erythromycin ml^{-1} . Growth of the transformed bacteria was monitored by following the OD_{600} . Apparent transformation efficiencies were derived by extrapolating the growth curves to zero time (assuming exponential growth throughout). This gave a theoretical initial OD_{600} for the transformed fraction of the bacterial population. This value was converted into the theoretical number of transformants at t_0 (1 OD_{600} unit corresponds to a total count of 2.3×10^8 cells ml^{-1} culture), from which the apparent number of transformants per μg DNA was derived. (Note that apparent transformation efficiencies will be underestimated if there is a lag before growth commences.)

Before electroporation, plasmid DNA was purified from *E. coli* DH5 α and then subjected to methylation *in vitro* using *MspI* methylase. In some experiments DNA was methylated *in vivo* using the *Bacillus subtilis* site-specific *BsuFI* methylase (Walter *et al.*, 1990; Davis *et al.*, 2000). Both of these enzymes methylate the sequence CCGG on the external cytosine. The gene encoding this enzyme was cloned in plasmid pACYC184 by T. Davis (CAMR, Porton Down), who kindly provided the resulting plasmid, pM.BsuFI, in the TOP10 strain of *E. coli*. This strain was transformed with the target plasmids. Total plasmid DNA was then extracted and used directly for electroporation experiments.

Molecular methods. The methods employed for DNA extraction and manipulation were those described by Sambrook *et al.* (1989). Large-scale plasmid extraction from *E. coli* was achieved using the QIAGEN Midi Prep Kit according to the manufacturer's instructions. For small-scale plasmid extraction from *C. cellulolyticum*, 6 ml of an overnight culture was subjected to the extraction procedure described previously by

Williams *et al.* (1990a) for *Clostridium beijerinckii* NCIMB 8052. Total DNA was extracted using a method adapted from that of Noirot *et al.* (1987).

Methylases (New England Biolabs), restriction endonucleases (Promega, New England Biolabs) and T4 DNA ligase (Promega) were used according to the manufacturers' instructions. DNA fragments were purified using the QIAEX agarose extraction kit (QIAGEN). Transformation of *E. coli* strains DH5 α , HB101 and TOP10 was carried out as described by Hanahan (1985).

Standard procedures were employed for Southern hybridizations (Southern, 1975). DNA fragments were transferred to a positively charged nylon membrane (Boehringer) and hybridized with digoxigenin-labelled probes (Boehringer). Plasmid pAT187 (Trieu-Cuot *et al.*, 1987), which contains an *aphA-3* gene very similar to that of Tn1545 (Caillaud *et al.*, 1987), was employed to detect Tn1545.

RESULTS

Transfer of pCTC1 to *C. cellulolyticum* by conjugation

In some preliminary experiments (data not shown) the plasmid pCTC1, containing the replication machinery of the *En. faecalis* plasmid pAM β 1 (LeBlanc & Lee, 1984), was transferred at a low frequency to *C. cellulolyticum*. The mating procedure was then optimized with respect to the following parameters: growth phase of the recipient, ratio of donor to recipient c.f.u. in the mating mixture, mating period and mating medium. This resulted in enhanced pCTC1 transfer frequencies, with values as high as 1.2×10^{-5} transconjugants per recipient being obtained in some experiments. The most crucial parameters appeared to be the ratio (10:1) of donor to recipient c.f.u. in the mating mixture and the physiological state (mid-exponential) of the recipient. However, the optimized procedure was not always reproducible in our hands; even when all the above conditions were met, experiments sometimes failed or gave unacceptably low transfer frequencies of the order of 10^{-7} – 10^{-8} per recipient. Plasmid DNA was isolated from four *C. cellulolyticum* transconjugants obtained in independent experiments. The presence of authentic pCTC1 was verified by looking for the characteristic pattern of restriction fragments obtained after *EcoRI* digestion (data not shown). Conjugative transfer and establishment of the plasmid pCTC501 (Young *et al.*, 1993) could not be demonstrated using the optimized protocol.

Transfer of Tn1545 to *C. cellulolyticum* by conjugation

In mating experiments with the *En. faecalis* BM4110 donor, which contains multiple copies of Tn1545 (Woolley *et al.*, 1989), Em^R transconjugants were obtained at frequencies of between 1.7×10^{-6} and 3.8×10^{-8} per recipient. DNA was extracted from two independent transconjugants, digested with *HindIII*, *EcoRI* and *PstI* and the presence of Tn1545 was verified by Southern hybridization. A Tn1545-specific probe hybridized with multiple *HindIII* fragments in DNA

Fig. 1. Detection of Tn1545 in *C. cellulolyticum* transconjugants. DNA was isolated from two transconjugants (1 and 2) obtained in independent mating experiments between *S. faecalis* BM4110 and *C. cellulolyticum*. After digestion and blotting, DNA samples were hybridized with an *aphA-3*-specific probe, which detects junction fragments at the left end of Tn1545. Lanes 1 and 14 contain bacteriophage λ DNA. Lanes 2 and 3 are controls containing DNA isolated from *Clostridium beijerinckii* strain A10 and *C. cellulolyticum*, respectively. The former harbours a single copy of Tn1545 (Evans *et al.*, 1998), whereas the latter lacks the element. DNA from strain 1 is in lanes 4, 6, 8, 10 and 12; DNA from strain 2 is in lanes 5, 7, 9, 11 and 13. DNA samples were digested with *Hind*III (lanes 1–5), *Eco*RI (lanes 6 and 7), *Pst*I (lanes 8 and 9), *Hind*III + *Eco*RI (lanes 10 and 11), or *Hind*III + *Pst*I (lanes 12 and 13). The bands marked with asterisks in lane 14 (2586 and 11497 bp fragments) associate during electrophoresis via their single-stranded cohesive ends to generate the band at 14.1 kbp.

isolated from both transconjugants, whereas no signal was detected with wild-type DNA (Fig. 1). Since *Hind*III does not cleave Tn1545 between the *aphA-3* gene detected by hybridization and the left end of the element (Caillaud *et al.*, 1987), hybridizing bands should correspond to junction fragments containing the transposon end and adjacent clostridial DNA. The presence of multiple bands normally reflects multiple sites of transposon insertion into the bacterial chromosome. However, the various fragments in Fig. 1 are not all of equivalent intensity, suggesting that some of them may be partial digestion products. The digestions with *Eco*RI and *Pst*I alone are not informative in this regard, but the samples digested with *Pst*I plus *Hind*III show simplified hybridization patterns, indicating that Tn1545 has probably inserted into at least three different sites in the two strains (strain 2 probably harbours two copies of Tn1545).

Characterization of a restriction system in *C. cellulolyticum* ATCC 35319

Attempts to electro-transform *C. cellulolyticum* with various shuttle vectors were generally unsuccessful, suggesting that the organism has an active restriction system, responsible for degrading the incoming DNA. Many clostridia produce type II restriction enzymes (<http://rebase.neb.com>), which in some cases have

Fig. 2. The restriction system of *C. cellulolyticum*. (a) Determination of the restriction specificity using pJIR418. DNA Plasmid pJIR418 (lane 1) was incubated with *C. cellulolyticum* cell extract in lane 2 and with *Msp*I in lane 3. Lane 4 contains a 1 kb DNA extension ladder (Life Technologies). (b) Protection of DNA by methylation. 1 kb ladder (BRL) (lane 1); pJIR418 (lane 2), incubated with *C. cellulolyticum* cell extract (lane 3), methylated by *M.Hpa*II and then incubated with *Hpa*II (lane 4) or clostridial cell extract (lane 5), methylated by *M.Msp*I and then incubated with *Msp*I (lane 6) or *C. cellulolyticum* cell extract (lane 7).

proved to be responsible for low transformation efficiencies (Azeddoug *et al.*, 1989; Mermelstein & Papoutsakis, 1993). Incubation of plasmid pJIR418 (Fig. 2a, lane 1) with a crude extract of *C. cellulolyticum* resulted in a pattern of discrete DNA fragments (Fig. 2a, lane 2). Comparison of the number and sizes of the DNA fragments with the published restriction map of pJIR418 (<http://www.infobiogen.fr>) indicated that the crude extract contained an isoschizomer of *Msp*I (*Hpa*II) [5'-CCGG-3'], which we have named *Cce*I. The recognition specificity of *Cce*I was verified by comparing the patterns of restriction fragments obtained after incubating pJIR418 DNA with the crude extract and with *Msp*I (Fig. 2a, lanes 2 and 3).

Protection of plasmids by methylation

The recognition sequence 5'-CCGG-3' may be modified by two commercially available cytosine-specific methylases, *M.Hpa*II and *M.Msp*I (New England Biolabs). *M.Hpa*II methylates the internal cytosine and *M.Msp*I the external cytosine. These two enzymes were used to methylate the test plasmid pJIR418. Methylated DNA was then digested with a cell-free extract from *C. cellulolyticum*. The products were analysed by agarose gel electrophoresis (Fig. 2b) along with control samples of undigested (lane 2) and non-methylated, digested DNA (lane 3). Methylation of pJIR418 by *M.Hpa*II (5'-C^mCCGG-3') did not protect the DNA from *Cce*I restriction (lane 5) although cleavage by the restriction

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

pECII, pMTL540E and pCTC511 were methylated *in vivo*, electro-transformed using the Bio-Rad Gene Pulser and transformation frequencies were derived by plating. pJIR418 and pGK12 were methylated *in vitro*, electro-transformed using the Bio-Rad Gene Pulser and transformation frequencies were not determined. pCTC1 was transferred using both methods.

Plasmid	Size (kbp)	Replicon	Origin	Antibiotic resistance marker*	Transformation efficiency‡ (µg DNA) ⁻¹
pCTC1	7.2	pAMβ1	<i>Enterococcus faecalis</i>	Em ^R	10 ²
pECII	4.5	pIM13	<i>Bacillus subtilis</i>	Em ^R	2 × 10 ²
pMTL540E	5.2	pCB102	<i>Clostridium butyricum</i>	Em ^R	0.5 × 10 ²
pJIR418	7.4	pIP404	<i>Clostridium perfringens</i>	Em ^R Cm ^R †	ND
pGK12	4.5	pWV01	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Em ^R Cm ^R †	ND
pCTC511	7.8	pCB101	<i>Clostridium butyricum</i>	Em ^R	no transformants

* All plasmids also encode resistance to ampicillin, selectable in *E. coli*.

† Cm^R is present on these plasmids, but it was not used as a selective marker in this investigation.

‡ Transformation efficiencies were derived from colony counts on solid medium.

ND, Not determined.

enzyme *Hpa*II was prevented (lane 4). On the other hand, methylation of the external cytosine by *M.Msp*I (5'-m⁵CCGG-3') protected the DNA against *Cce*I restriction (lane 7).

Transfer of plasmids to *C. cellulolyticum* by electro-transformation

The ability to protect DNA from *C. cellulolyticum* endonuclease activity by methylation opened up the possibility of using electro-transformation as a method for transferring DNA to *C. cellulolyticum*. Electro-transformation of appropriately modified DNA (see Methods) was employed to introduce plasmids from several different Gram-positive bacteria into *C. cellulolyticum* (Table 3). As expected from the conjugation experiments (see above), pCTC1 containing the pAMβ1 replicon was successfully transferred. In addition, several other plasmids containing the replicons of the *Bacillus subtilis* plasmid, pIM13 (Monod *et al.*, 1986; Azeddoug *et al.*, 1992), the *C. butyricum* plasmid, pCB102 (Minton & Morris, 1981; Collins *et al.*, 1985), the *C. perfringens* plasmid, pIP404 (Garnier & Cole, 1988) and the *Lactococcus lactis* subsp. *cremoris* plasmid, pWV01 (Leenhouts *et al.*, 1991) also generated transformants. No transformants were obtained with plasmid pCTC511 containing the replicon of the *C. butyricum* plasmid, pCB101 (Collins *et al.*, 1985; Brehm *et al.*, 1992), nor were there any antibiotic-resistant colonies in controls lacking plasmid DNA.

The apparent transformation efficiencies obtained (approx. 10² transformants per µg DNA, see Table 3) were low when transformants were selected on solid medium after an overnight expression period. This value is nevertheless an overestimate, because cells potentially grew during the time allowed for phenotypic

expression (two to three generations). To optimize the electro-transformation procedure and enhance transformation efficiencies, a JOUAN electropulsator was used. This delivers square wave pulses with constant electric field parameters over a programmed discharge duration (see Methods). The cell/plasmid (pCTC1) mixture was resuspended in a buffer containing either no sucrose, 270 mM sucrose, or 500 mM sucrose and was subjected to an electric field of 6.5, 7, or 7.5 kV cm⁻¹ for 5 ms. Transformants were only obtained with buffer containing 270 mM or 500 mM sucrose and at 7 or 7.5 kV cm⁻¹. Under successful electro-transformation conditions, transformation efficiencies evaluated on solid medium (as above) were approx. 10² transformants per µg DNA. However, when plating was avoided using the liquid culture technique (see Methods), estimated values of between 10⁵ and 10⁷ transformants per µg DNA were obtained. Cells pulsed in the absence of plasmid DNA were incubated in selective liquid medium as a negative control. No visible growth was detected, showing that the selection of spontaneous antibiotic-resistant mutants did not interfere with these experiments. The large differences between apparent transformation efficiencies using solid versus liquid medium might be explained by the low plating efficiency of the bacteria, leading to a gross underestimation of transformation efficiency based on the appearance of colonies on solid medium.

Transformants obtained using pIM13- and pAMβ1-based plasmids were verified by extracting plasmid DNA from selected transformants and digesting it with diagnostic restriction endonucleases (Fig. 3). These data showed unequivocally that the pIM13 and pAMβ1 replicons function in *C. cellulolyticum*. Extraction yields of pJIR418 (pIP404-based plasmid) and pGK12 (pWV01-based plasmid) from *C. cellulolyticum* were

Fig. 3. Verification of plasmids isolated from *C. cellulolyticum* transformants. Plasmids were isolated from transformants obtained in independent experiments, as indicated in the Methods. (a) *EcoRI*-digested samples of pCTC1 (pAM β 1 replicon) from *E. coli* (lane 1) and *C. cellulolyticum* (lane 2); λ BstEII marker (lane 3). (b) *SacI*-digested samples of pECII (pIM13 replicon) from *E. coli* (lane 1) and *C. cellulolyticum* (lane 2); *SacI*-digested samples of pECN2 from *E. coli* (lane 3) and *C. cellulolyticum* (lane 4); λ HindIII (lane 5). Some incomplete digestion products are visible in lanes 1–4.

much lower than those of pIM13- and pAM β 1-based plasmids, suggesting that these are maintained at lower copy numbers. This was not surprising, especially in the case of pGK12 which was maintained in *Lactococcus lactis* (formerly *Streptococcus lactis*) and *Bacillus subtilis* at three and five copies per cell, respectively (Kok *et al.*, 1984).

Plasmid stability in *C. cellulolyticum*

To test the comparative stability of the various plasmids in *C. cellulolyticum*, strains carrying pCTC1, pECN2 and pMTL540E (pAM β 1, pIM13 and pCB102 replicons, respectively) were grown for 20 generations in the absence of selective antibiotic. Bacteria were then plated on GS agar and colonies were screened for plasmid loss by replica plating on selective and non-selective media. Frequencies of plasmid loss were approximated (Young & Ehrlich, 1989) by dividing the proportion of antibiotic-sensitive colonies by the number of generations of bacterial growth. Similarly low values were obtained for pCTC1 and pECN2, containing the pAM β 1 and pIM13 replicons, namely 7.8×10^{-3} and 4.7×10^{-3} per generation, respectively. The plasmid

pMTL540E, containing the pCB102 replicon, was rather less stable; the frequency of plasmid loss was 3.6×10^{-2} per generation.

DISCUSSION

The roles and functions of many of the components of the bacterial cellulosome have been extensively explored by analysis of recombinant gene products *in vitro* (see Bayer *et al.*, 1998 for a review). Unfortunately, it has not so far been possible to undertake functional analysis of any of the genes or their products *in vivo*, since none of the cellulosome-producing organisms is amenable to genetic analysis. However, Anderson *et al.* (1998) have very recently reported the low-frequency transfer of Tn1545 to the relatively poorly characterized organism *Eubacterium cellosolvens*. The physiology (Giallo *et al.*, 1985; Gelhaye *et al.*, 1993a, b; Guedon *et al.*, 1999a, b; Payot *et al.*, 1998) and the cellulolytic machinery (Bélaich *et al.*, 1997; Gal *et al.*, 1997; Pagès *et al.*, 1997, 1999; Reverbel-Leroy *et al.*, 1997) of *C. cellulolyticum* ATCC 35319 are comparatively well characterized. Therefore we developed procedures for transferring Tn1545, as well as several different plasmids, to this organism using conjugation and/or electrotransformation.

Although the frequencies of conjugative plasmid transfer to *C. cellulolyticum* reported here were sometimes as high as 10^{-5} per recipient, reproducibility was poor. Previous experience (Williams *et al.*, 1990a) indicated that high-efficiency, reproducible conjugation only occurs under conditions permitting good growth of both donor and recipient as they are co-cultured during the mating period. Since *E. coli* is a copiotroph and *C. cellulolyticum* is essentially an oligotroph, co-culture was always likely to prove problematic. Moreover, the low plating efficiency of *C. cellulolyticum* effectively meant that many potential transconjugants were lost in each experiment (although this is unlikely to affect the conjugation frequency). Similar problems pertained to the conjugative transfer of Tn1545 from *En. faecalis* to *C. cellulolyticum*, where even fewer transconjugants were often recovered. They may also account for the low frequencies of transfer of Tn1545 to *Eu. cellosolvens* that were reported recently (Anderson *et al.*, 1998). A single strand of DNA appears to be transferred during intergeneric conjugation (Young *et al.*, 1993). Therefore, DNA that is transferred by conjugation does not normally have to run the gauntlet of the restriction endonucleases that may be present in the recipient (see below). If an alternative oligotrophic donor in which IncP plasmids can replicate is identified in the future, higher numbers of recombinants and elevated transfer frequencies may be obtained. This may ultimately permit the use of conjugative transfer of non-replicative plasmids to effect allelic exchange.

Insertional mutagenesis using Tn1545 is similarly limited by low transfer frequencies from the copiotrophic *En. faecalis* donor. The element apparently inserted into three different sites in the *C. cellulolyticum* chromosome

(Fig. 1). The possibility that there is a preferred site for insertion in this organism (cf. the behaviour of the related element, Tn916, in *C. difficile*: Mullany *et al.*, 1991) cannot be excluded until larger numbers of transconjugants have been analysed.

Electroporation proved to be a more reliable method for obtaining gene transfer, but only once the incoming DNA was protected from restriction. *C. cellulolyticum* ATCC 35319 protects its own DNA from restriction by methylation, and donor DNA that had been modified using an appropriate DNA methylase (*M.BsuF1* or *M.MspI* [5'-m⁵CCGG-3']) gave reproducible electrotransformation. Vectors containing the replication functions of five different plasmids, originating from five different organisms, were able to function in *C. cellulolyticum*. Plasmids containing the replication regions of the *B. subtilis* ssDNA plasmid pIM13, the *L. lactis* ssDNA plasmid pWV01, the streptococcal theta-replicating plasmid pAM β 1 (Bruand *et al.*, 1993), the *C. perfringens* theta-replicating plasmid pIP404 (Garner & Cole, 1988) and pCB102 from *C. butyricum*, with its unknown mode of replication (Minton *et al.*, 1993), were established with approximately equal efficiency. Although pCB101 is a ssDNA plasmid like pIM13 and, like pCB102, it originates from *C. butyricum*, we were unable to establish vectors (pCTC501 and pCTC511) containing this replicon in *C. cellulolyticum*, using either conjugation or electrotransformation.

The apparent transformation efficiencies for the various plasmids were rather low (approx. 10² transformants per μ g DNA, see Table 3) when transformants were selected on solid medium. The reason for this seems to be the low plating efficiency of this strain. Substantially higher apparent efficiencies were obtained when a non-conventional method was employed to derive the number of transformants originally present. Essentially, the growth kinetics of transformed bacteria in liquid medium were measured and used to extrapolate the number of transformants initially present. These results open up the possibility of effecting allelic replacement and insertional inactivation of genes in *C. cellulolyticum*, using suicide plasmids. Stepwise gene replacement using replicative, but segregationally unstable vectors (Maguin *et al.*, 1992; Biswas *et al.*, 1993) could also provide a useful alternative. We therefore explored the segregational stability of three representative plasmids in *C. cellulolyticum*.

Plasmids that have been transferred to an organism in which they do not normally reside are frequently unstable (Gruss & Ehrlich, 1988; Ehrlich *et al.*, 1991; Minton *et al.*, 1993). This instability may result from structural changes or from segregation of plasmid-less cells owing to inefficient plasmid replication and/or partitioning at cell division (Gruss & Ehrlich, 1989). Instability of plasmids containing two of the replicons employed here has previously been reported in other clostridia (Oultram *et al.*, 1988; Azeddoug *et al.*, 1992). Frequencies of segregation of plasmid-less cells were 4.1×10^{-2} per generation for a pAM β 1-based plasmid in

C. beijerinckii (Oultram *et al.*, 1988) and 2.3×10^{-2} per generation for a pIM13-based plasmid in *Clostridium saccharoperbutylacetonicum* NI-4081 (Azeddoug *et al.*, 1992). In *C. cellulolyticum* however, both the pAM β 1 (theta-replication) and the pIM13 (rolling circle, ssDNA plasmid) replicons were moderately stable in the absence of selection and segregated plasmid-less cells at frequencies of about 5×10^{-3} per generation. In view of the behaviour of pIM13-based plasmids in other organisms (Azeddoug *et al.*, 1992), the comparative stability of plasmids containing this replicon was unexpected. To explore the phenotypic consequences of disruption of *cipC*, which encodes the scaffoldin component of the *C. cellulolyticum* cellulosome, several derivatives of the pIM13-based plasmid, pECII, (e.g. pECN2) were constructed with the intention of obtaining *cipC* disruption mutants of *C. cellulolyticum*. The comparative stability of these plasmids in this organism prevented us from pursuing this line of enquiry. In view of their reduced stability, it might be provident to employ pCB102-based plasmids for this purpose in the future.

The results we have obtained demonstrate that *C. cellulolyticum* ATCC 35319 is amenable to genetic analysis. Significant difficulties still to be circumvented are the low frequency of conjugative gene transfer, which is probably related to the oligotrophic nature of this organism, and the low plating efficiency. This makes it difficult to recover cells stressed by conjugative transfer, electroporation, or possibly even the presence of a plasmid *per se* and we strongly suspect that most potential transformants/transconjugants are lost when bacteria are plated on agar-solidified medium. Reliable electrotransformation, in combination with a segregationally unstable replicon, such as that of pCB102, may yet provide a way of obtaining detectable homologous recombination and thus facilitate functional analysis of the bacterial cellulosome *in vivo*. It has recently been shown that UV irradiation of plasmid DNA enhances homologous recombination with the *Mycobacterium tuberculosis* chromosome (Hinds *et al.*, 1999). It will be of interest to determine whether a similar effect is observed with *C. cellulolyticum*.

ACKNOWLEDGEMENTS

We thank T. Davies for plasmid pM.BsuF1 and J. I. Rood for plasmid pJIR418. We are grateful to H.-P. Fierobe and A. Bélaich for reading the manuscript. This work was supported by the Commission of European Communities FAIR programme (contract CT 95-0191 [DG 12 SSMA]) and by grants from the Centre National de la Recherche Scientifique and the Université de Provence.

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Received 22 June 2000; revised 3 August 2000; accepted 30 August 2000.