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Electrotransformation of *Clostridium thermosaccharolyticum*

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Transformation of the thermophile *Clostridium thermosaccharolyticum* ATCC 31960 was achieved using plasmid pCTC1 and electroporation. Evidence supporting transformation was provided by Southern blots, detection of the plasmid in 10 out of 10 erythromycin-resistant clones, retransformation of *E. coli* and *C. thermosaccharolyticum* with plasmid DNA isolated from *C. thermosaccharolyticum*, and a proportional relationship between the number of transformants and the amount of DNA added. Transformation efficiencies were very low for plasmid DNA prepared from *E. coli* (0.6 transformants mg^{-1} DNA), although somewhat higher for plasmid DNA prepared from *C. thermosaccharolyticum* (52 transformants mg^{-1} DNA). Transformation-dependent erythromycin resistance indicates that an adenosine methylase gene originating from *Enterococcus faecalis*, a mesophile, is expressed in *C. thermosaccharolyticum*. The plasmid pCTC1 appears to be replicated independently of the chromosome, as indicated by visualization of recovered plasmid on gels, and retransformation using recovered plasmid. pCTC1 is maintained in *C. thermosaccharolyticum* at both 45 and 60°C. Restriction analysis showed little or no rearrangement occurred upon passage through the thermophile.

Keywords: transformation; electroporation; *Clostridium thermosaccharolyticum*; thermophile

Thermophilic clostridia have been proposed for production of ethanol from cellulosic biomass [14,24]. In particular, the pentose-fermenting *C. thermosaccharolyticum* has been proposed as a partner in ethanol fermentation with the cellulolytic *Clostridium thermocellum* [22,26].

A major obstacle to using these and other clostridia for ethanol production is that the *Clostridia* have a branched fermentation pathway, resulting in a reduced ethanol yield due to formation of other fermentation products, notably acetate and lactate [11]. Pathway engineering to reduce or eliminate the formation of organic acids is a promising approach for improving ethanol yields, but is hampered by a lack of information regarding the molecular genetics of thermophilic *Clostridia*. Although many genes have been cloned from these thermophiles, notably those encoding cellulase components, no foreign genetic material has been reported to have been introduced into either *C. thermocellum* or *C. thermosaccharolyticum*. Transformation of a thermophilic *Clostridium*, *C. thermohydrosulfuricum* (syn. *Thermoanaerobacter thermohydrosulfuricus* [13]) has been reported using vector pUB110 [25].

We report here the electrotransformation of *C. thermosaccharolyticum* with plasmid pCTC1. pCTC1 is a 7.2-kb shuttle vector [28] based on the broad host range Gram-positive bacterial replicon pAMβ1 and the Gram-negative bacterial R2 replicon. The plasmid replicates by a unidirectional theta mechanism [5], which has been linked to stable plasmid maintenance and segregation [12]. Antibiotic resistance determinants encoded on pCTC1 are ampicillin resistance and erythromycin resistance, with the latter coded for by a constitutive adenine methylase [4].

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Materials and methods

Microorganisms, DNA and chemicals

C. thermosaccharolyticum strain HG8 (ATCC 31960) was provided by Arnold Demain, Massachusetts Institute of Technology, Cambridge, MA, USA. *Clostridium beijerinckii* NRRL B-592 and plasmid pHR106 [3] were gifts from R Zsigray, University of New Hampshire, Durham, NH, USA. Plasmid pCTC1 was a gift from M Young, University College of Wales, Aberystwyth, UK. All chemicals were from Sigma Chemical Company (St Louis, MO, USA) or Stratagene (La Jolla, CA, USA).

Electroporation

Electrocompetent cells of *C. thermosaccharolyticum* were prepared from cells grown in 100 ml of a complex medium (GBG medium) [15] in serum vials (Bellco, NJ, USA) at 60°C for approximately 48 h to an $OD_{600\ nm}$ of 1.05 ± 0.15. The cells were centrifuged without precautions to ensure anoxic conditions at 10000 × *g* for 10 min at 4°C and washed with ice-cold sterile distilled water three times. The pellet from 100 ml of culture was suspended in 500 μl ice-cold sterile distilled water, brought up to 20% glycerol (using autoclaved, 100% glycerol) and stored in 40-μl aliquots at −80°C or used immediately. Frozen cells were thawed on ice immediately prior to adding plasmid DNA. A 3-μl volume of plasmid DNA at the concentration noted was added to the ice-cold cell aliquot, and cells plus DNA were immediately transferred to an ice-cold 0.2-cm electroporation cuvette (BioRad, Hercules, CA, USA) and the pulse applied with settings: 2.0 kV potential, 800 Ω resistance and 25 μF capacitance resulting in a time constant of approximately 16 ms. Arcing was observed if either a higher voltage or resistance were used. To determine killing frequency, control cells were transferred to the cuvette without added plasmid DNA, either pulsed as above or using a 0-kV potential, serially diluted in and plated onto GBG medium. In addition to the above method, the proto-

col of Mermelstein *et al* [17] was also used for electroporation of either *C. thermosaccharolyticum* or *C. beijerinckii*. Electrocompetent cells of *C. beijerinckii* were grown at 30°C on George and Chen medium [8] in serum bottles to an $OD_{600\,nm}$ of 0.95.

DNA manipulations

Southern blot analysis was with a random labeled probe [16]. Total genomic DNA was isolated by centrifuging 5 ml of culture ($OD_{600\,nm}$ >0.7) and suspending the pellet in 500 μl of 10× TE (100 mM Tris, 10 mM EDTA, pH 7.5) with 30 mg ml^{-1} lysozyme plus 1000 U ml^{-1} mutanolysin and incubating the suspension at 37°C for 15 min. SDS was added to 1% and the solution was incubated at 65°C for 10 min. Proteinase K was then added to 200 μg ml^{-1} and incubated for 1 h at 65°C. The mixture was extracted twice with phenol/chloroform and once with chloroform. The DNA was precipitated with 2.5 volumes of ethanol and air dried. The pellet was dissolved in 200 μl of TE (10 mM Tris, 1 mM EDTA, pH 7.5) and RNase was added to 100 μg ml^{-1}. This mixture was incubated at 37°C for 30 min followed by one extraction with phenol and one with phenol/chloroform. The DNA was again ethanol-precipitated, dissolved in 25 μl distilled water and partially restriction digested with *Eco*R1 or *Hind*III or transformed by $CaCl_2$ precipitation into *E. coli* [16].

A plasmid maxi preparation of *C. thermosaccharolyticum* was by alkaline lysis using the reagents supplied in the 5 Prime → 3 Prime, Inc (Boulder, CO, USA) spin column plasmid prep kit with the following modification: 2 liters of cells ($OD_{600\,nm}$ = >0.8) grown on GBG medium plus 10 μg ml^{-1} erythromycin were pelleted and resuspended in 10 ml of 25% sucrose plus 30 mg ml^{-1} lysozyme and 1000 U ml^{-1} mutanolysin. This mixture was incubated for 15 min at 37°C and was then processed as described by the spin column kit using two spin columns for the sample generated from 2 liters of culture. Plasmid was also prepared from *C. thermosaccharolyticum* cells by cesium chloride gradient centrifugation [16] using the enzyme treatment described above followed by alkaline lysis of the cells. Miniprep plasmid purification for *C. thermosaccharolyticum* followed the procedure described for *Lactococcus lactis* [18] with the following change: lysozyme was used at 30 mg ml^{-1} and mutanolysin was added at 1000 U ml^{-1}. Plasmid DNA was prepared from *E. coli* using the 5 Prime → 3 Prime, Inc spin column plasmid prep kit as directed by the manufacturer.

Quantification of plasmid DNA isolated from *C. thermosaccharolyticum* was performed by measuring UV absorbance (at 260 nm) from cesium chloride gradient-prepared DNA [18], which resulted in much less background (presumably chromosomal fragments) than the spin column methods. Plasmid bands were of similar intensity for DNA prepared from both methods when viewed on agarose gels loaded with DNA prepared from the same amount of cells. Electroporation was carried out only with spin-column prepared DNA, as the density gradient-prepared DNA resulted in arcing and the low plasmid yield made desalting impractical.

Cell cultivation

C. thermosaccharolyticum was grown either on liquid medium (GBG) in anaerobic Hungate tubes or in serum bottles (Bellco), or on plates prepared using GBG plus 40 g L^{-1} agar [15]. Selection for transformants and determination of killing frequency was at 45°C. The plates were incubated in BBL (Cockeysville, MD, USA) H_2 plus CO_2 anaerobic jars for 5 days for cells on non-selective media or for at least 10 days for cells on selective media. Although *C. thermosaccharolyticum* transformants were selected on plates containing 1 μg ml^{-1} erythromycin, plasmid-containing cells were resistant to at least 5 μg ml^{-1} erythromycin on plates; higher concentrations were not tested. Care was exercised when using erythromycin to avoid acidic conditions, as antimicrobial activity decreases at low pH [19,20]. The survival frequency of cells following electroporation was 0.95 ± 0.5 (95% survived) based on four independent experiments and a total of sixteen cell counts for both electroporated and non-electroporated cells. *C. beijerinckii* was grown on George and Chen medium at 30°C as described above for 4 days, and was selected in liquid culture at 60 μg ml^{-1} chloramphenicol and on plates at 30 μg ml^{-1} chloramphenicol.

Antibiotic sensitivity of C. thermosaccharolyticum

Growth of *C. thermosaccharolyticum* at 45°C in the presence of antibiotics was tested using GBG medium. In liquid medium, freshly grown cells at an OD of 0.90 ± 0.10 were inoculated at 5% vol/vol into Hungate tubes containing antibiotics at concentrations from 0 to 125 μg ml^{-1}. The extent of growth was determined by OD measurements, taken after 3, 7, and 14 days. Optical densities <0.05 at all incubation times are reported as no growth. For solid medium, sensitivities are reported for antibiotic levels at which no growth was observed after 14 days. Sensitivity to erythromycin, the resistance marker carried on plasmid pCTC1, was determined to be 1 μg ml^{-1} and 10 μg ml^{-1} for solid and liquid GBG medium, respectively. Thiamphenicol was effective at preventing growth at 20 μg ml^{-1} in solid medium and 25 μg ml^{-1} in liquid medium. Both chloramphenicol and kanamycin were not effective up to 125 μg ml^{-1}.

Results

Transformation of C. thermosaccharolyticum with pCTC1

Transformation of *C. thermosaccharolyticum* ATCC 31960 was achieved using electroporation and plasmid pCTC1. For plasmid DNA prepared from *E. coli*, the transformation frequency (transformants/viable cell) was about 3×10^{-10} (Table 1). For DNA prepared from *C. thermosaccharolyticum*, the frequency was about an order of magnitude higher. The transformation efficiency (transformants μg^{-1} DNA) was higher by two orders of magnitude for DNA prepared in *C. thermosaccharolyticum* as compared with DNA prepared in *E. coli*. The observed survival frequency did not differ from 1 at a 0.05 level of significance. Despite numerous attempts, we were unable to transform *C. thermosaccharolyticum* using the conditions (including a sucrose/phosphate buffer, 0.4-cm gap, and fresh cells) of

Table 1 Transformation frequency and efficiency for *C. thermosaccharolyticum* in relation to experimental conditions

DNA source[a]	*C. thermosaccharolyticum*	Electrotransformation frequency[c] (N^b)	Transformants μg^{-1} DNA
Glycerol buffer, 0.2-cm gap[d]			
pCTC1 (*C. thermosacch.*)	frozen cells	$3.2 \pm 3.3 \times 10^{-9}$ (8)	51 ± 10
pCTC1 (*C. thermosacch.*)	fresh cells	$2.2 \pm 0.3 \times 10^{-9}$ (6)	52 ± 6
pCTC1 (*E. coli*)	frozen cells	$2.6 \pm 0.6 \times 10^{-10}$ (6)	0.6 ± 0.4
pCTC1 (*E. coli*)	fresh cells	$3.9 \pm 0.7 \times 10^{-10}$ (6)	0.5 ± 0.5
Sucrose/phosphate buffer, 0.4-cm gap[d]			
pCTC1 (*C. thermosacch. or E. coli*)	fresh cells	$<1.0 \times 10^{-11}$ (10)	none recovered

[a]The plasmid used for the transformation is indicated, followed by the host organism from which the plasmid was prepared.
[b]N = number of independent electroporation experiments, each performed in a separate cuvette. Transformation using pCTC1 from *E. coli* was observed a total of nine times in these experiments, corresponding to an average of about one transformant per electroporation event. Transformation using pCTC1 from *C. thermosaccharolyticum* was observed a total of 55 times, corresponding to about four transformants per electroporation event.
[c]Number of transformants per viable cell following electroporation.
[d]pCTC1 from *C. thermosaccharolyticum* (grown at 45°C) at a concentration of 30 μg DNA ml^{-1}; pCTC1 prepared from *E. coli* HB101 at a stock concentration of 420 μg DNA ml^{-1}. See Materials and methods for a complete description of methods.

Mermelstein *et al* [17] for transformation of *Clostridium acetobutylicum*. We were however successful at using this method to reproduce the transformation of *C. beijerinckii* NRRL B-592 with plasmid pHR106 as described by Birrer *et al* [3].

Plasmid DNA prepared from pCTC1-transformed *C. thermosaccharolyticum* grown at 60°C in the presence of 10 μg ml^{-1} erythromycin was added in increasing amounts to cells until arcing caused by increased osmotic strength was observed. The observed relationship between number of transformants and DNA added (Figure 1) was essentially linear, indicating that DNA (as opposed to cells) was limiting in this experiment. The expected zero *y*-intercept was not observed, for which we are not able to offer an explanation at this time.

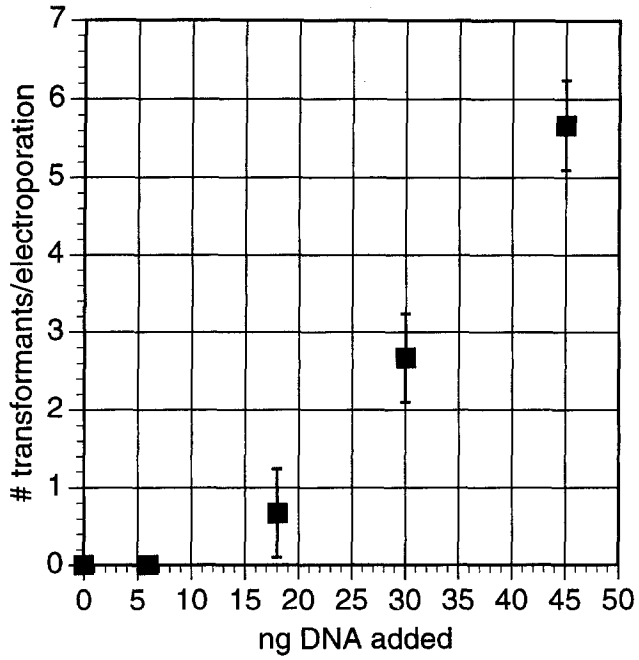

Figure 1 Number of transformants of *C. thermosaccharolyticum* with increasing amounts of DNA. Each point represents the mean from three experiments with ± one standard deviation indicated.

Recovery of plasmid from transformed C. thermosaccharolyticum

The maxi-preparation yielded about 30 μg of pCTC1 from a 2-liter culture of *C. thermosaccharolyticum* grown at 45°C. A faint plasmid band was observed when DNA from pCTC1-transformed cells of *C. thermosaccharolyticum* grown at 45°C in the presence of 10 μg ml^{-1} erythromycin was run on agarose gels, and the pattern of *Bam*HI digestion was as expected for pCTC1 (data not shown). Plasmid DNA prepared from cells incubated at 60°C was not observed on agarose gels. However, DNA obtained from cells incubated at 60°C could be used to transform *E. coli*.

The presence of plasmid pCTC1 DNA in *C. thermosaccharolyticum* following electroporation was confirmed by Southern blot (Figure 2). Total cellular DNA was isolated from erythromycin-resistant cells grown in the liquid culture and the DNA was partially digested with restriction endonucleases and probed with linearized pCTC1 produced in *E. coli*. A positive signal was observed in each of the lanes with DNA prepared from a transformant but not in the lane with genomic DNA prepared from an untransformed control (Lane 2).

Retransformation with recovered plasmid

Genomic DNA recovered from liquid cultures derived from ten erythromycin-resistant colonies was $CaCl_2$ transformed into *E. coli*. Transformants were observed (approximately 10^1–10^2 per electroporation event) with each of ten genomic DNA isolates from the erythromycin-resistant *C. thermosaccharolyticum* (data not shown). No spontaneously resistant *C. thermosaccharolyticum* mutants were seen on erythromycin plates after plating $>10^{10}$ cells on each of ten plates.

pCTC1 was recovered from transformed *C. thermosaccharolyticum* grown at either 45°C or 60°C, $CaCl_2$ transformed into *E. coli*, reisolated, and digested with three different restriction enzymes. For both temperatures and all three restriction enzymes, electrophoretic banding patterns were essentially identical for DNA which had been maintained in *C. thermosaccharolyticum* as compared to DNA which had not (Figure 3).

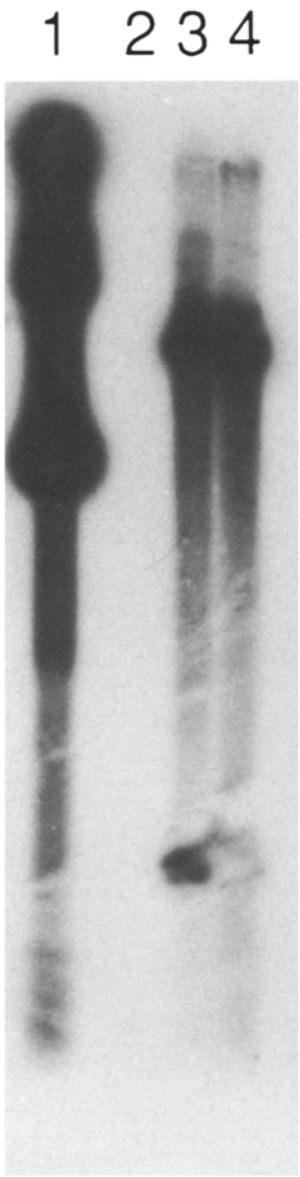

Figure 2 Southern blot of a 1.2% agarose gel probed with radiolabeled pCTC1. Lane 1, pCTC1 uncut from *E. coli*. Total cellular DNA prepared from: Lane 2, control cells of *C. thermosaccharolyticum* partially digested with *Eco*RI; Lane 3, transformed cells of *C. thermosaccharolyticum* partially digested with *Eco*RI; Lane 4, transformed cells of *C. thermosaccharolyticum* partially digested with *Hind*III.

Discussion

We report here transformation of *C. thermosaccharolyticum* by electroporation. Evidence in support of this includes a positive hybridization signal on a Southern blot of total cellular DNA from transformed *C. thermosaccharolyticum* when probed with pCTC1, visualization on agarose gels of pCTC1 plasmid DNA prepared from transformed *C. thermosaccharolyticum*, resistance to erythromycin correlated with the presence of the plasmid, transformation of *E. coli* with pCTC1 isolated from *C. thermosaccharolyticum* and re-isolation of the plasmid from *E. coli*, and an increasing number of *C. thermosaccharolyticum* transformants with increasing amounts of DNA.

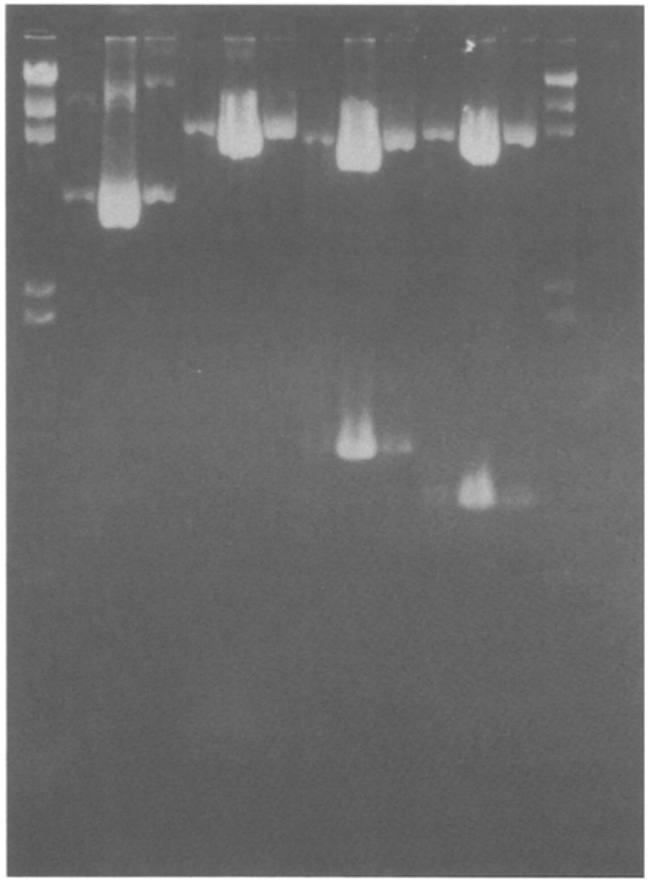

Figure 3 pCTC1 isolated from *C. thermosaccharolyticum* grown at either 45°C or 60°C and re-transformed into *E. coli* DH5α as compared to pCTC1 stock plasmid from *E. coli*. Lanes 1 and 14 marker *Hind*III digest of lambda DNA; Lanes 2–4 uncut (stock, 45°C, 60°C DNA); Lanes 5–7 *Ava*I; Lanes 8–10 *Eco*RI; Lanes 11–13 *Pst*I.

Our results show that pCTC1 was replicated in *C. thermosaccharolyticum* at both 45 and 60°C, although the apparent plasmid yield was lower at the higher temperature. Increasing segregational instability of pAMβ1 replicons with increasing temperature has been reported [12]. It appears that the plasmid is replicated independently of the chromosome, as indicated by visualization on electrophoretic gels of plasmid recovered from transformed *C. thermosaccharolyticum* and retransformation of *C. thermosaccharolyticum* and *E. coli* with recovered plasmid. The observed transformation-dependent erythromycin resistance indicates that the adenine methylase determinant originating from *Enterococcus faecalis*, a mesophile, is expressed in *C. thermosaccharolyticum* at 45 and 60°C.

Rearrangements and deletions accompanying plasmid passage through Gram-positive microorganisms have been reported [9]. In particular, deletions have been reported to occur in pAMβ1 when it was transformed into non-native hosts [12]. Isolation of pCTC1 from *E. coli* following passage through *C. thermosaccharolyticum* did not produce any changes in the observed restriction pattern (Figure 3), indicating that little if any rearrangement occurred.

Electroporation conditions supporting transformation of *C. thermosaccharolyticum* were similar to those used for *Bradyrhizobium japonicum*, an aerobic, Gram-negative, soil

bacterium refractory to transformation [10]. A glycerol electroporation buffer such as that used here has also been utilized previously for transformation of the mesophilic *C. perfringens*, although phosphate-buffered sucrose, which did not support transformation of *C. thermosaccharolyticum*, has been used more commonly [1,3,17,21,23,29]. We observed no sensitivity of transformation frequency to the growth stage of *C. thermosaccharolyticum* cells over the range of $OD_{600\,nm} = 1.0 \pm 0.2$, and fresh and frozen cells showed the same transformation frequency. These observations contrast with reports of electroporation with fresh cells only for *C. acetobutylicum*, and growth phase being an important determinant of transformation frequency in both *C. acetobutylicum* (K Walters, Northwestern Univ, Evanston, IL, USA, personal communication) and *C. perfringens* [2,21].

Transformation frequencies observed for *C. thermosaccharolyticum* were low but reproducible at 2.7×10^{-9} per cell using DNA prepared from *C. thermosaccharolyticum* and 3.3×10^{-10} using DNA prepared from *E. coli*. When working at such frequencies, transformation could easily go undetected if care were not taken to ensure that a sufficient number of cells are plated. *C. beijerinckii* has also shown a low frequency of transformation by electroporation (1×10^{-7}) [3] while that for *C. acetobutylicum* is much higher (1.7×10^{8}) [17]. Although studies with *C. acetobutylicum* indicate that methylation to protect DNA from restriction digestion is the main factor responsible for high transformation efficiency [17], *C. thermosaccharolyticum* HG8 does not exhibit restriction endonuclease activity (Klapatch *et al*, submitted). A possible alternative explanation for the increased transformation frequency using plasmid DNA prepared from *C. thermosaccharolyticum* is that the topology of DNA affects gene expression [7,27] and may affect replicon recognition. Thermophilic bacteria have been reported to contain unique topoisomerase activity which may give rise to increased recognition of the replicon and increased transformation efficiency [6].

Following a report of polyethylene glycol-mediated transformation of the thermophile *C. thermohydrosulfuricum* (syn. *Thermoanaerobacter thermohydrosulfuricus* [13]) with plasmid pUB110 [25], no other report of transformation of a thermophilic *Clostridium* has appeared to our knowledge. Electrotransformation of *C. thermosaccharolyticum* with plasmid pCTC1 as described provides a key tool for further development of this microorganism, including metabolic pathway engineering to reduce or eliminate the diversion of substrate carbon from ethanol formation to the production of organic acids.

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