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Electrotransformation studies in *Clostridium cellulolyticum*

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Electropermeabilization of *Clostridium cellulolyticum* was optimized using ATP leakage assays. Electrotransformation was then performed under optimized conditions (6 to 7.5 kV cm^{-1} field strength applied during 5 ms to a mixture containing methylated plasmids and late exponential phase cell suspensions (10 molecules:1 cell) in a sucrose-containing buffer). Transformants were only obtained when 7 or 7.5 kV cm^{-1} pulses were applied. Transformation efficiencies evaluated from the growth curves of transformed cells were between 10^5 and 10^7 transformants per microgram of plasmid DNA for five different replicon-based plasmids. Restriction nuclease digestion patterns of pJIR418 purified from transformed *Clostridia* and *Escherichia coli* were indistinguishable, indicating that heterologous DNA was structurally stable in the *Clostridium* strain. Copy numbers of 130, 70 and 10 were estimated from purification yield for pCTC1, pKNT19 and pJIR418, respectively. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 271–274.

Keywords: cellulosome; *Clostridum cellulolyticum*; electropermeabilization; electrotransformation

Introduction

Cellulose is a major biological compound on earth. This insoluble biopolymer of glucose is resistant to degradation. Over the past few decades, there has been increasing scientific and applied interest in cellulolytic microorganisms, which are used in digesters to recycle the increasing amounts of cellulosic wastes produced. Cellulases can be used in various processes of the paper and textile industries.

In recent years, research has been focused on detailed studies of the enzymes secreted by cellulolytic microorganisms. Various anaerobic bacteria produce multienzymatic complexes called cellulosomes, the first example of which was described in *Clostridium thermocellum* [15]. *Clostridium cellulolyticum* is a mesophilic cellulosome-producing bacterium capable of degrading crystalline insoluble cellulose and of using monomers (glucose and cellobiose) as carbon and energy sources [11,12]. A large number of genes coding for cellulosome subunits have been cloned and the proteins have been studied *in vitro* in terms of enzymatic activities and mode of action [6–8,10,23], 3D structure [4,21], affinity to cellulosic substrates [9,20] and synergistic actions [10]. A detailed understanding of the composition and assembly system of these complexes has been gained [9,19].

Further functional studies of cellulosomes in *C. cellulolyticum* are hindered by the lack of gene transfer methods adapted to this strain. Jennert *et al* [13] recently succeeded in transferring both the mobilizable plasmid pCTC1 from *Escherichia coli* and the conjugative transposon Tn*1545* from *Enterococcus faecalis* to *C. cellulolyticum* by conjugation. In this last study, transfer frequencies of approximately 10^{-5} transconjugants per recipient were obtained using pCTC1, although results were unfortunately not reproducible.

The current paper presents the critical parameters of the electrotransformation procedure.

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Materials and methods and results

Electropermeabilization of C. cellulolyticum

Experimental conditions that allow efficient electropermeabilization, and thus subsequent electrotransformation, depend on the species and even on the strain examined, such as demonstrated for *Clostridium perfringens* [1,3,22]. Thus, these conditions must be carefully determined for each strain. Cell suspensions of live *C. cellulolyticum* (ATCC 35319) require manipulation under a nitrogen atmosphere containing less than 1 ppm O_2. In order to determine the optimal conditions of *C. cellulolyticum* electropermeabilization, ATP leakage was monitored using the technique described by Sixou *et al* [24], adapted to *C. cellulolyticum*. Measuring ATP leakage represents a reliable assay for measuring this permeabilization. This simple procedure allows a number of electropulsing conditions to be tested. The yield of electrotransformation of bacteria such as *E. coli*, *Salmonella typhimurium* and *Brevibacterium lactofermentum* is closely related to the extent of cell permeabilization induced by the electric field.

Cell suspensions were prepared from cells grown at 34°C in 50-ml vials (Bellco,Vineland, NJ) of GS medium [13] supplemented with 2 g l^{-1} cellobiose, under argon atmosphere. The cells were first centrifuged in the culture vial at 2000×*g* for 30 min at 4°C and washed three times with ice-cold electropulsing buffer in an anaerobic chamber (Jacomex, Livry Gargan, France). The cells were then resuspended in the same buffer to obtain a 5×10^9 cells ml^{-1} suspension. Electropulsing of 30-μl aliquots of the cell suspension was performed in a gene pulser cuvette with a 0.1-cm electrode gap width (BioRad, Hercules, CA) using a Jouan PS15 electropulsator (Jouan, Saint Herblain, France). This apparatus delivers square wave pulses that provide a constant field of programmed duration and tension during the discharge. The sample was then removed from the anaerobic chamber. The amount of leaked ATP was measured under aerobic conditions, exactly 1 min after the pulse treatment, by mixing 20 μl of cell suspension with 280 μl of 100 mM Tris-acetate buffer, pH 7.7 (30°C), 4 μl of 1.5 mg ml^{-1} luciferin solution (Boehringer-Mannheim, Germany)

Table 1 Permeabilization of *C. cellulolyticum* in various buffers

Buffer	ATP leakage (%)
Glycerol 15%	11
Na phosphate buffer 5 mM pH 7.4, $MgCl_2$ 1 mM, sucrose 272 mM	20
Hepes 5 mM pH 7.4	5
Hepes 5 mM pH 7.4, sucrose 272 mM	18
Hepes 5 mM pH 7.4, sucrose 500 mM	57

Cells were collected at OD_{450}=0.4 (exponential growth phase). ATP leakage was measured as described in the text. Percent values represent the amount of released ATP measured after 1 min of incubation at room temperature after the electric pulse, relative to the total amount of cellular ATP measured from toluene-treated cells (approximately 160 pmol ATP per 10^8 cells).

and 10 μl of 1 mg ml^{-1} luciferase solution (Boehringer-Mannheim) in 100 mM Tris-acetate buffer, pH 7.7. Light emission by the luciferin/luciferase complex (proportional to the amount of ATP) was measured using a luminometer connected to a chart recorder. The basal level of detection was determined with untreated cells. Total ATP content was determined from cells treated with toluene.

Various buffers were assayed and, for each buffer, field values between 2 and 8.5 kV cm^{-1} were tested. ATP leakage increased with increasing tension up to a plateau when reached approximately 6 kV cm^{-1}, and this for all the buffers tested. Table 1 summarizes maximum percentages of ATP leakage observed at the plateau for cells collected in the early exponential phase of growth ($OD_{450\ nm}$=0.4). ATP leakage was higher for cells suspended in buffers containing sucrose, regardless of the buffer used (phosphate–$MgCl_2$ or Hepes). Cells collected at various stages of growth: early exponential phase ($OD_{450\ nm}$=0.04), late exponential phase ($OD_{450\ nm}$=0.75) and stationary phase ($OD_{450\ nm}$=0.84), were resuspended in Hepes buffer containing 0.5 M sucrose and subjected to the electropermeabilization test. The results clearly showed that electric pulses were more efficient on late exponential phase cells (95% of ATP leakage) than on early exponential (57%) or stationary phase cells (70%).

Electrotransformation of C. cellulolyticum

Electrotransformation of *C. cellulolyticum* was assayed using various shuttle vectors, namely pAT18 [26], pKNT19 [2] and pJIR418 [25], based on different replicons (pAMβ1, pIM13 and pIP404, respectively). Electrocompetent cells of *C. cellulolyticum* were prepared from late exponential phase cells (OD_{450} 0.7 to 0.9). Cells were collected by centrifugation and washed three times with ice-cold electrotransformation buffer containing sucrose. Finally, cells were resuspended in the same buffer to obtain a 10^{11} cells ml^{-1} suspension; 50 μl of suspension (5×10^9 cells) were mixed with 5 μl of plasmid solution (containing about 5×10^{10} molecules) and then subjected to a 5-ms square wave pulse at 6, 6.5, 7 or 7.5 kV cm^{-1}. Cells were immediately resuspended in 500 μl of GS medium, dispensed by 100-μl aliquots into Hungate tubes containing 10 ml of GS medium supplemented with 4 g l^{-1} of cellobiose, and incubated between 6 and 15 h at 34°C before selection of transformants on solid medium (GS supplemented with 4 g l^{-1} cellobiose, 15 g l^{-1} agar and erythromycin (Em) or thiamphenicol (Tm) at 10 μg ml^{-1}, depending on the plasmid used). Plates were incubated in anaerobic jars under 2 bar of 80% N_2, 20% CO_2 atmosphere.

No transformants were obtained using the described procedure. This was thought to be due to the presence of the restriction enzyme *Cce*I, which is synthesized by *C. cellulolyticum* [13]. Plasmid DNA is protected against *Cce*I by *in vitro* methylation. Therefore, *Msp*I methylase (Biolabs, Beverly, MA) was used here according to the supplier's instructions. Methylated DNA was then purified by the QiaexII (Qiagen, Hilden, Germany) procedure before use in electrotransformation assays. As previously described [13], transformants were selected on solid medium following a pulse on a cell–plasmid mixture at 7 or 7.5 kV cm^{-1} in buffer containing sucrose. However, estimated transformation efficiencies did not exceed 10^2 transformants per microgram of DNA, regardless of the plasmid used.

Alternatively, transformant growth kinetics in selective liquid medium were monitored by following OD_{450}. Electrotransformed bacteria were first incubated in GS medium supplemented with cellobiose at 4 g l^{-1}, in the same manner as for solid selection; cells immediately started logarithmic growth (Figure 1). Initial OD_{450} was reduced by around 60% compared to nonpulsed cells. This might be explained by cell lysis induced by the pulse shock. The transformant fraction of the cells was then selected after an overnight culture at 34°C by subculturing the suspension into 100 ml of fresh GS medium supplemented with 4 g l^{-1} cellobiose and 10 μg ml^{-1} of the appropriate antibiotic. An example of growth kinetics of the pCTC1 transformed cells is shown in Figure 1. The theoretical initial number of transformed cells (at t_0) was deduced from cell density at t by using the growth formula $X=X_0e^{\mu t}$ (μ=0.092 h^{-1}, X=biomass at t, and X_0=biomass at t_0). The correlation between optical densities and bacterial densities was established by light-microscopy cell count. In the exponential phase, the number of cells was 3×10^8 cells ml^{-1} per

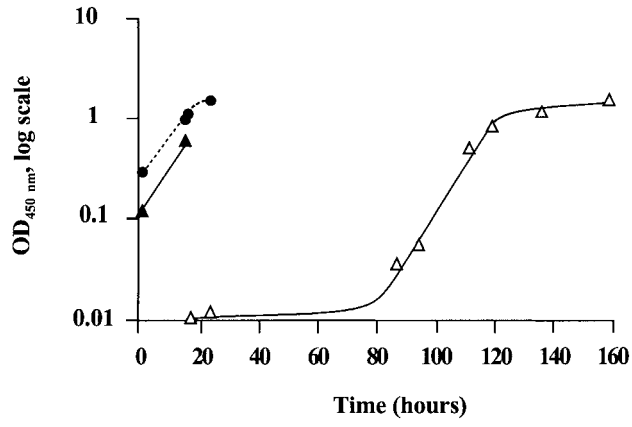

Figure 1 Selection of transformants in liquid medium. Ten microliters of cell suspension (around 10^9 cells) in Hepes 5 mM, pH 7.4, sucrose 0.5 M (●) or 100 μl of transformation mixture diluted 10-fold in GS medium (around 10^9 pulsed cells) (▲) were added to 10 ml of sterile GS medium supplemented with cellobiose at 4 g l^{-1} and incubated overnight at 34°C. Transformants were then selected by subculturing the overnight culture in 100 ml of sterile GS medium supplemented with cellobiose at 4 g l^{-1} and erythromycin at 10 μg l^{-1} (△). Electrotransformation was performed by applying a 5-ms pulse at 7 kV cm^{-1} to a mixture containing 50 μl of cells (around 5×10^9) in Hepes 5 mM, pH 7.4, sucrose 0.5 mM plus 5 μl of methylated pCTC1 (0.4 μg).

optical density unit at 450 nm. Estimated transformation efficiency values were between 10^5 and 10^7 transformants per microgram of DNA (4×10^6 in the example presented) when cells were pulsed in sucrose-containing buffer and under an electric field of 7 and 7.5 kV cm^{-1}. Many different replicon-based plasmids were tested: pCTC1 [27], pAT18 [26] and pMTL500E [18], based on the *E. faecalis* pAMβ1 replicon, and pCTC511 [27], pMTL540E (NP Minton, CAMR), pKNT19 [2], pJIR418 [25] and pGK12 [14] based on the pCB101 and pCB102 replicons from *Clostridium butyricum*, the *Bacillus subtilis* pIM13, the *C. perfringens* pIP404 and the *Lactococcus lactis* subsp. *cremoris* pWV01 replicons, respectively. All plasmids were established with approximately equal efficiencies, with the exception of the pCB101-based plasmid (no transformant obtained). The erythromycin and chloramphenicol resistance markers from various origins were all functional in this strain (Cm^R was selected using thiamphenicol).

Electrotransformation of *C. cellulolyticum* was successfully optimized. Electropermeabilization was shown to depend on the growth phase of cells and the field strength applied. As already observed for *E. coli* [5] and *Vibrio cholerae* [16], the osmolarity of the pulsing buffer greatly influenced the transformation efficiency of *C. cellulolyticum*. Indeed, no transformants were obtained in Hepes buffer lacking sucrose. Finally, site-specific methylation of transforming DNA was shown to be essential for efficient transformation, as has been described for *C. acetobutylicum* [17]. The study of growth kinetics of transformants led to transformation efficiency values much higher than those obtained from plating, suggesting that the latter values have been underestimated because of a low plating efficiency [13].

Plasmid purification from C. cellulolyticum transformants and analysis

Plasmids were extracted from cells in 10 ml of culture ($OD_{450\ nm}$=0.8, approximately 2.4×10^9 cells) using the procedure described by Williams *et al* [27] and solubilized in 20 μl of water. Four microliters of the various plasmid solutions were analyzed by agarose gel electrophoresis. Plasmids were all visualized and resembled corresponding plasmids purified from *E. coli*, with the exception of the pWV01-based plasmid pGK12, which seemed to be present in low copy numbers and thus could not be purified in sufficient amount using standard small-scale purification procedures (data not shown). DNA preparations from transformation negative control cultures (nonshocked cell–plasmid mixtures) did not exhibit any plasmid DNA. pJIR418 had to be overloaded (30 μl) to be visualized (Figure 2, part B, lane 3). Rather high minimum copy numbers of 70 and 130 were estimated from the purification yield for pIM13- and pAMβ1-based plasmids, respectively. This last value is of the same order of magnitude as that previously found by Trieu-Cuot *et al* [26], for pAT18 in various bacterial strains (80 to 200 copies per chromosome). The *C. perfringens* pIP404-based plasmid pJIR418 was maintained at a lower copy number (about 10 copies/cell). Up to now, pJIR418 had only been used in *C. perfringens*.

Identical restriction patterns were obtained for pJIR418 purified from both *E. coli* (Figure 2, lane 1) and *C. cellulolyticum* (lane 2); plasmids were digested with *Hin*dII in part A and *Bst*YI in part B. As the complete sequence of pJIR418 is known, additional restriction analyses using various enzymes were performed (data not shown). The plasmid pJIR418 purified from *C. cellulolyticum* was indistinguishable from that purified from *E. coli*. This plasmid

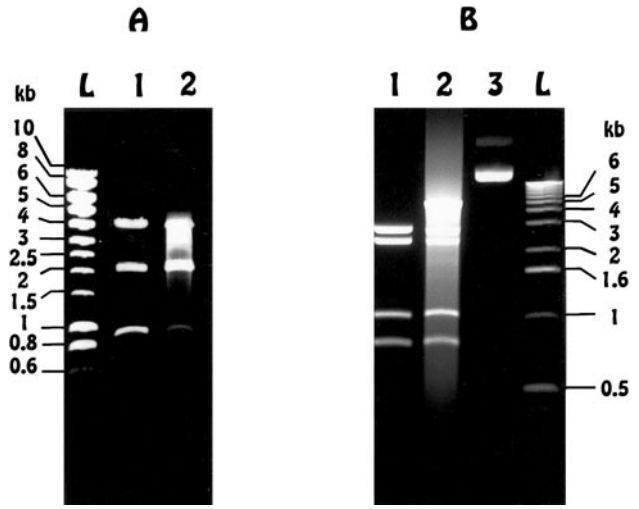

Figure 2 Restriction nuclease analysis of pJIR418 purified from *C. cellulolyticum*. Plasmids purified from *E. coli* (lane 1) and *C. cellulolyticum* (lane 2) were digested with *Hin*dII (part A) or *Bst*YI (part B). L: ladder. Lane 3: uncut pJIR418 plasmid. The sizes are expressed in kilobases.

is structurally stable in *C. cellulolyticum*. Certain replicons, such as pAMβ1 and pIP404, are theta-replicating replicons, and others, such as pIM13 and pWV01, are rolling circle replicating plasmids. Jennert *et al* [13] evaluated the segregational instability of three of these plasmids. They found pAMβ1- and pIM13-based plasmids to be moderately stable, with frequencies of plasmid loss evaluated to be in the order of 7.8×10^{-3} and 4.7×10^{-3} per generation, respectively. The third plasmid, pMTL540E, exhibited a rather high segregational instability, with a frequency of plasmid loss around 3.6×10^{-2} per generation [13].

Electrotransformation thus appears as the method of choice (reliability, efficiency) for transferring DNA in *C. cellulolyticum*. This technique should make it possible to work at the genetic level in this strain. Future studies will involve the use of this approach to study the cellulolytic system in *C. cellulolyticum*. Suicide or ts replicative vectors will be tentatively transferred in order to construct knock-out mutants of various *cel* or *cip* genes. The various stable replicative vectors could potentially be used to clone genes and subsequently modify the composition of the cellulosomes.

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References

1 Allen SP and HP Blaschek. 1990. Factors involved in the electroporation of *Clostridium perfringens*. *FEMS Microbiol Lett* 70: 217–220.
2 Azeddoug H, J Hubert and G Reysset. 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. *J Gen Microbiol* 138: 1371–1378.

3 Chen CK, CM Boucle and HP Blaschek. 1996. Factors involved in the transformation of previously non-transformable *Clostridium perfringens* type B. *FEMS Microbiol Lett* 140: 185–191.
4 Ducros V, M Czjzek, A Belaich, C Gaudin, HP Fierobe, JP Belaich, GJ Davies and R Haser. 1995. Crystal structure of the catalytic domain of a bacterial cellulase belonging to family 5. *Structure* 3: 939–949.
5 Eynard N, S Sixou, N Duran and J. Teissié. 1992. Fast kinetics studies of *Escherichia coli* electrotransformation. *Eur J Biochem* 209: 431–436.
6 Fierobe HP, C Gaudin, A Belaich, M Loufti, E Faure, C Bagnara and JP Belaich. 1991. Characterization of endoglucanase-A from *Clostridium cellulolyticum*. *J Bacteriol* 173: 7956–7962.
7 Fierobe HP, C Bagnara-Tardif, C Gaudin, F Guerlesquin, P Sauve, A Belaich and JP Belaich. 1993. Purification and characterization of endoglucanase C from *Clostridium cellulolyticum* — Catalytic comparison with endoglucanase A. *Eur J Biochem* 217: 557–565.
8 Gal L, C Gaudin, A Bélaich, S Pages, C Tardif and JP Bélaich. 1997. CelG from *Clostridium cellulolyticum*: a multidomain endoglucanase acting efficiently on crystalline cellulose. *J Bacteriol* 179: 6595–6601.
9 Gal L, S Pagès, C Gaudin, A Bélaich, C Reverbel-Leroy, C Tardif and JP Bélaich. 1997. Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum*. *Appl Environ Microbiol* 63: 903–909.
10 Gaudin C, A Bélaich, S Champ and JP Bélaich. 2000. CelE, a multidomain cellulase from *Clostridium cellulolyticum*: a key enzyme in the cellulosome? *J Bacteriol* 182: 1910–1915.
11 Giallo J, C Gaudin and JP Bélaich. 1985. Metabolism and solubilization of cellulose by *Clostridium cellulolyticum* H10. *Appl Environ Microbiol* 49: 1216–1221.
12 Giallo J, C Gaudin, JP Bélaich, E Petitdemange and F Caillet. 1983. Metabolism of glucose and cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. *Appl Environ Microbiol* 45: 843–849.
13 Jennert KCB, C Tardif, DI Young and M Young. 2000. Gene transfer to *Clostridium cellulolyticum* ATCC 35319. *Microbiology* 146: 3071–3080.
14 Kok J, JMBM van der Vossen and G Venema. 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* 48: 726–731.
15 Lamed R, E Setter and EA Bayer. 1983. Characterization of a cellulose-binding cellulase-containing complex in *Clostridium thermocellum*. *J Bacteriol* 156: 828–836.
16 Marcus H, JM Ketley, JB Kaper and RK Holmes. 1990. Effects of DNAse production, plasmid size and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock. *FEMS Microbiol Lett* 56: 149–154.
17 Mermelstein LD, NE Welker, GN Bennett and ET Papoutsakis. 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *Bio/Technology* 10: 190–195.
18 Oultram JD, M Loughlin, TJ Swinfield, JK Brehm, DE Thompson and NP Minton. 1988. Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. *FEMS Microbiol Lett* 56: 83–88.
19 Pagès S, A Belaich, C Tardif, C Reverbel-Leroy, C Gaudin and JP Belaich. 1996. Interaction between the endoglucanase CelA and the scaffolding protein CipC of the *Clostridium cellulolyticum* cellulosome. *J Bacteriol* 178: 2279–2286.
20 Pagès S, L Gal, A Bélaich, C Gaudin, C Tardif and JP Bélaich. 1997. Role of scaffolding protein CipC of *Clostridium cellulolyticum* in cellulose degradation. *J Bacteriol* 179: 2810–2816.
21 Parsiegla G, M Juy, C Reverbel-Leroy, C Tardif, JP Bélaich, H Driguez and R Haser. 1998. The crystal structure of the processive endocellulase CelF of *Clostridium cellulolyticum* in complex with a thiooligosaccharide inhibitor at 2.0 A resolution. *EMBO J* 17: 5551–5562.
22 Phillips-Jones MK. 1990. Plasmid transformation of *Clostridium perfringens* by electroporation methods. *FEMS Microbiol Lett* 66: 221–226.
23 Reverbel-Leroy C, S Pagès, A Bélaich, JP Bélaich and C Tardif. 1997. The processive endocellulase CelF, a major component of the *Clostridium cellulolyticum* cellulosome: purification and characterization of the recombinant form. *J Bacteriol* 179: 46–52.
24 Sixou S, N Eynard, JM Escoubas, E Werner and J Teissié. 1991. Optimized conditions for electrotransformation of bacteria are related to the extend of electropermeabilization. *BBA* 1088: 135–138.
25 Sloan J, TA Warner, P Scott, TL Bannan, DI Berryman and JI Rood. 1992. Construction of a sequenced *Clostridium perfringens–Escherichia coli* shuttle plasmid. *Plasmid* 27: 207–219.
26 Trieu-Cuot P, C Carlier, C Poyart-Salmeron and P Courvalin. 1991. Shuttle vectors containing a multiple cloning site and a *lacZ*α gene for conjugal transfer of DNA from *Escherichia coli* to Gram-positive bacteria. *Gene* 102: 99–104.
27 Williams DR, DI Young and M Young. 1990. Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. *J Gen Microbiol* 136: 819–826.