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Electrotransformation of *Clostridium thermocellum*

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Electrotransformation of several strains of *Clostridium thermocellum* was achieved using plasmid pIKm1 with selection based on resistance to erythromycin and lincomycin. A custom-built pulse generator was used to apply a square 10-ms pulse to an electrotransformation cuvette consisting of a modified centrifuge tube. Transformation was verified by recovery of the shuttle plasmid pIKm1 from presumptive transformants of *C. thermocellum* with subsequent PCR specific to the *mls* gene on the plasmid, as well as by retransformation of *Escherichia coli*. Optimization carried out with strain DSM 1313 increased transformation efficiencies from <1 to $(2.2 \pm 0.5) \times 10^5$ transformants per μg of plasmid DNA. Factors conducive to achieving high transformation efficiencies included optimized periods of incubation both before and after electric pulse application, chilling during cell collection and washing, subculture in the presence of isoniacin prior to electric pulse application, a custom-built cuvette embedded in an ice block during pulse application, use of a high (25-kV/cm) field strength, and induction of the *mls* gene before plating the cells on selective medium. The protocol and preferred conditions developed for strain DSM 1313 resulted in transformation efficiencies of $(5.0 \pm 1.8) \times 10^4$ transformants per μg of plasmid DNA for strain ATCC 27405 and $\sim 1 \times 10^3$ transformants per μg of plasmid DNA for strains DSM 4150 and 7072. Cell viability under optimal conditions was ~50% of that of controls not exposed to an electrical pulse. Dam methylation had a beneficial but modest (7-fold for strain ATCC 27405; 40-fold for strain DSM 1313) effect on transformation efficiency. The effect of isoniacin was also strain specific. The results reported here provide for the first time a gene transfer method functional in *C. thermocellum* that is suitable for molecular manipulations involving either the introduction of genes associated with foreign gene products or knockout of native genes.

Clostridium thermocellum is a gram-positive, anaerobic, thermophilic, endospore-forming bacterium capable of fermenting cellulose and other β-glucan substrates to ethanol, acetic acid, lactic acid, CO_2, and H_2 (3). The cellulosome produced by this organism is an archetypical example of a complexed-type cellulase enzyme system (2, 33, 35). In addition, *C. thermocellum* exhibits one of the highest described growth rates on cellulose (12, 14) and is of biotechnological interest for making ethanol and perhaps other products (12, 13, 32). Although the genome sequence of *C. thermocellum* is nearly complete at the time of writing (http://genome.jgi-psf.org/draft_microbes/cloth/cloth.home.html) and a number of genes from *C. thermocellum* have been cloned in other bacteria (29, 33), methods are not established for the introduction of foreign genes into this organism. The absence of such methods has been a significant impediment to studies of *C. thermocellum* aimed at increasing both fundamental understanding and applied capability.

Described protocols for gene transfer in gram-positive bacteria are based on natural competence (in rare instances) (43), conjugation (conjugative mobilization) (1, 8, 24, 43, 45), transformation of partial or complete protoplasts (11, 31, 37, 46), or, most commonly, electrotransformation (ET) (5, 7, 10, 15, 16, 22, 23, 27, 34, 41, 43, 47). The presence of a restriction system(s) is an important property of recipient strains in the context of developing methods for gene transfer (9, 25), and specific methylation has been required to protect transforming DNA from attack by restriction endonucleases and to achieve high transformation frequencies in several *Clostridium* species (4, 8, 18). Enhancement of ET efficiency has also been achieved by adding glycine to growth media to weaken the cell wall (7), by mild treatment of cells with muralytic enzymes (27, 34, 46), or by growth in the presence of isonicotinic acid hydrazide (isoniacin) (15). Screening of multiple strains to obtain electrocompetent recipients has been useful in developing transformation methods (23, 26). Special care to maintain high cell viability is important when attempting to detect relatively rare transformation events, and this is a particular concern when working with obligate anaerobes (12).

The development and application of gene transfer methods has been pursued most extensively among gram-positive obligate anaerobes in the case of *Clostridium acetobutylicum* (11, 19, 22, 27, 41) and is also well established among several other mesophilic noncellulolytic *Clostridium* species (20, 27, 47). An ET protocol developed for the cellulolytic mesophile *Clostridium cellulolyticum* (8, 38) has been used to express pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* (6). Examples of gene transfer to gram-positive obligate anaerobic thermophiles are limited to *Thermoanaerobacterium* species (10, 15, 17) and a preliminary report of protoplast transformation of *C. thermocellum* (40). In these studies, the highest efficiencies of ET ($\sim 10^3$/μg of DNA) were obtained by Mai et al. (15–17) for *Thermoanaerobacterium* sp. strain JW/SL-YS485, subsequently named *Thermoanaerobacterium saccharolyticum* (15). The protocol developed by these investigators featured incubation in the presence of isoniacin and use of the shuttle vector pIKm1, with a ColE1 origin of

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TABLE 1. Bacterial strains used in this study

Microorganism	Particular phenotype	Source
E. coli		
DH5α	Dam^+ Dcm^+	Invitrogen[a]
GM272	Dam^- Dcm^-	*E. coli* Genetic Stock Center[b]
C. thermocellum		
ATCC 27405		ATCC[c]
DSM 1313		DSMZ[d]
DSM 4150		DSMZ

[a] Invitrogen, Grand Island, N.Y.
[b] Yale University, New Haven, Conn.
[c] American Type Culture Collection, Manassas, Va.
[d] Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

replication and *mls* and *aph* genes encoding resistance to erythromycin-lincomycin and kanamycin, respectively (15, 17, 21, 39).

This study was undertaken with the objective of developing a gene transfer method for *C. thermocellum.*

MATERIALS AND METHODS

Media, strains, and cultivation conditions. The strains used in this study are listed in Table 1. *C. thermocellum* cells were grown in DSM 122 broth as described elsewhere (http://www.dsmz.de/media/med122.htm) with the following modifications: the concentration of $K_2HPO_4 \cdot 3H_2O$ was reduced from 7.2 to 1.9 g/liter, and glutathione was replaced by cysteine-HCl at a final concentration of 0.5 g/liter. Either Avicel or cellobiose was included, as appropriate, at 5 g/liter. For growth on plates, 0.58% Difco agar (Becton Dickinson, Sparks, Md.) was added to modified DSM 122 cellobiose broth. For selection of transformants, or to grow transformants for genomic DNA isolation, the cellobiose broth additionally contained erythromycin and lincomycin, each at 20 μg/ml (Sigma, St. Louis, Mo.). All manipulations, associated with preparation of cultures and subsequent ET were carried out inside an anaerobic chamber equipped with a model 2000 incubator (Coy Laboratory Products, Inc., Grass Lake, Mich.). *Escherichia coli* cells were grown at 37°C in Columbia broth or on Columbia agar (Columbia broth plus 1.2% Difco agar) containing 200 μg of ampicillin/ml for selection of transformants when appropriate.

Source, preparation, and detection of plasmid. Plasmid pIKm1 (15) was a gift from Juergen Wiegel (University of Georgia, Athens). pIKm1 DNA was isolated from *E. coli* using the Qiagen (Valencia, Calif.) Plasmid Midi kit, and *C. thermocellum* total DNA was isolated using the Qiagen Genomic-tip 100/G kit, following directions provided by the supplier. Detection of the *mls* gene in Em^r Lm^r transformants of *C. thermocellum* was performed by PCR using total DNA preparations as templates and 5′-AAGAGGGTTATAATGAACGAGAAAA-3′ (primer 1) and 5′-AAAAATAGGTACACGAAAAACAAGTTA-3′ (primer 2), designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), with an expected PCR product size of ~800 bp. The presence of pIKm1 in Em^r Lm^r transformants of *C. thermocellum* was also verified by DNA rescue experiments using *E. coli* DH5α, with ET using standard procedures (30), followed by visualization of the recovered pIKm1 and its *Pst*I digests in agarose gels.

Pulse generation system. A custom-built pulse generation system (42) was used. The main components of the system are a signal generator and a high-voltage power supply, both custom built, as well as a high-voltage power tetrode and a 40-μF, 10-kV power capacitor (both from JCV Sphere, Saratov, Russian Federation). As shown in Fig. 1, and described in more detail elsewhere (44), these components are arranged in a circuit with the ET cuvette in series with the power tetrode and featuring TAP600 series heat-sinkable planar resistors (Ohmite Mfg. Co., Rolling Meadows, Ill.). The pulse generator was located outside the anaerobic chamber, with high-voltage cables from the generator (based on 22 AWG39X2215-9A HV wire; Dearborn CDT, Wheeling, Ill.) plugged into custom-built connectors which penetrated the rigid plastic wall of the chamber and were sealed with silicon in a manner similar to that described previously (41).

ET cuvettes. Custom-made cuvettes were based on 2-ml sterile disposable polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, Pa.). As shown in Fig. 2, a molded epoxy plug fitted to the top of the centrifuge tube encased soldered connections between high-voltage cables and flat parallel electrodes made of polished 305 stainless steel (Metalmen Sales, Inc., New York, N.Y.), with a 2-mm gap. The high-voltage cables were plugged into custom-built connectors penetrating the rigid plastic wall of the chamber. The electrodes extended to the bottom of the tube without touching the tube wall. Bio-Rad (Hercules, Calif.) electroporation tubes (0.2-cm gap) and a Bio-Rad shocking chamber were also used.

Preparation of cells for ET. One milliliter of an Avicel-grown stock culture was inoculated into 10 ml of cellobiose broth and incubated until growth was evident. One milliliter of this culture was then inoculated into 10 ml of the same broth

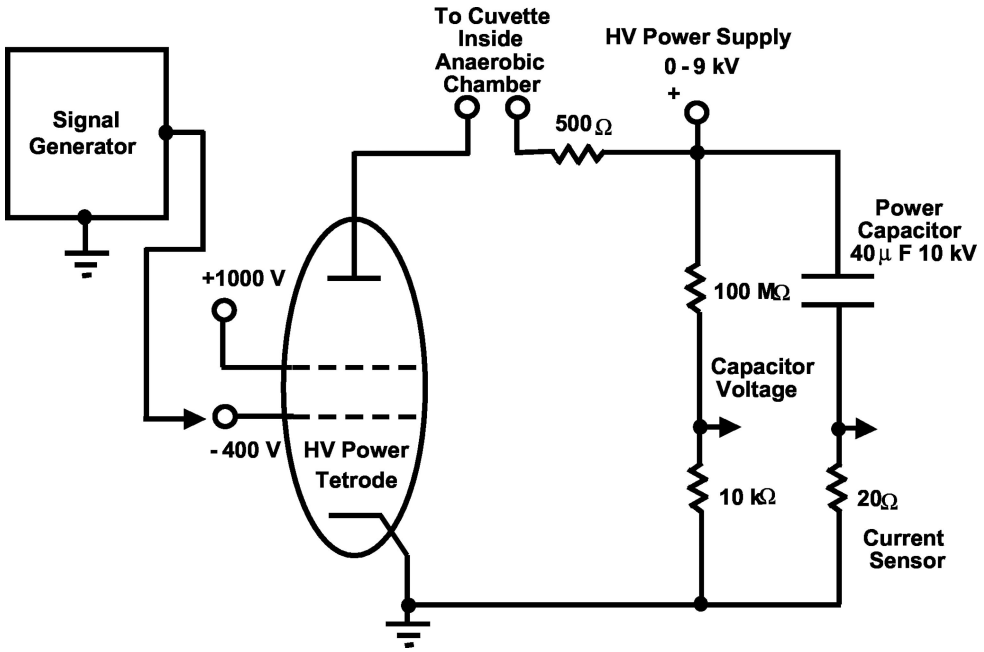

FIG. 1. Schematic diagram of custom-built generator.

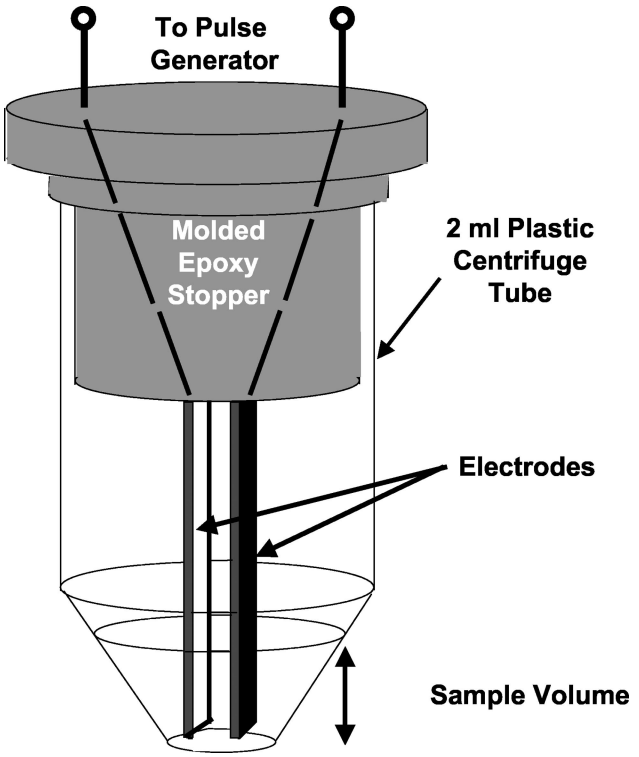

FIG. 2. Custom cuvette design.

and incubated for 9 h. One milliliter of the resulting culture was inoculated into 60 ml of cellobiose broth and incubated overnight. The overnight culture was then diluted 1:3 in prewarmed cellobiose broth and subcultured for 3 h (or as indicated in the text) in a 500-ml bottle with stirring in the presence or absence of isoniacin (Sigma), as indicated in the text. The culture was chilled on ice, and cells were collected by centrifugation in 15-ml capped polypropylene tubes (Becton Dickinson & Co, Franklin Lakes, N.J.) at maximum speed in a Fisher model 225 centrifuge for 10 min. The resulting cell pellets were washed twice with an equal volume of prechilled 0.2 M cellobiose, with chilling on ice for 5 min between washes. After the supernatant was decanted by inverting the tubes, the cell pellets were resuspended in the remaining volume of the supernatant (approximately equal to the volume of the cell pellets) and collected in a single 15-ml capped polypropylene tube. The resulting cell suspension contained ~9×10^{10} cells/ml and was kept on ice until it was used.

Pulse application and subsequent cell cultivation. Ninety microliters of cell suspension was added to cuvettes, either modified centrifuge tubes or from Bio-Rad, and kept on ice prior to use. For custom cuvettes based on centrifuge tubes (see above), an ice block was prepared with water-filled centrifuge tubes embedded in it. These tubes were then removed just before electric-pulse application, leaving behind tube-shaped holes into which cuvettes with cell samples were inserted. The entire ET procedure, including incubation after electric-pulse application, was performed with cuvettes placed in holes in the ice block. In the case of 0.2-cm-gap Bio-Rad cuvettes, placing them in an ice block was not possible because of the shocking-chamber design. Therefore, the cuvettes were chilled on ice and carefully dried just before pulse application. Two microliters (or other volume as indicated) of a plasmid DNA preparation containing ~2.5 μg of DNA was added and gently mixed with the cell suspension just before pulse application.

A 10-ms square pulse (field strength as appropriate) was applied to the samples prepared as described above. After pulse application, the cell suspensions were diluted with 500 μl of chilled cellobiose broth, kept on ice for 10 min, and incubated in the absence of antibiotic selection at 58°C for a postelectropulsing recovery period of 4 h (or as indicated). Then, 500 μl of prewarmed (58°C) cellobiose broth containing 0.5 μg of erythromycin/ml was added to a final antibiotic concentration of 0.25 μg/ml, and incubation continued for 6 h. The sample, or a fraction of it, was then transferred into a 50-ml capped polypropylene tube (Becton Dickinson & Co., Franklin Lakes, N.J.), 50 ml of melted (58°C) cellobiose agar with 20 μg each of erythromycin and lincomycin/ml was added to each tube, and two 100-mm-diameter plates were poured from each tube and allowed to solidify at room temperature for 30 to 40 min. The solidified plates were incubated for 5 to 8 days upside down at 58°C in GasPak 100 jars (Becton Dickinson & Co., Sparks, Md.) with a few milliliters of sterile water added to the bottom of the jar to prevent drying of the agar surface.

Determination of cell viability and statistical analysis. Cell viability controls were treated identically to the samples subjected to electric pulses, including successive dilutions with 500 μl of cellobiose broth followed by incubation as described above, except that the control samples were not exposed to an electrical pulse and the agar medium did not include selective antibiotics. Thereafter, portions of the samples of the electrically treated cells and the cell viability controls were diluted as necessary and mixed with melted (58°C) cellobiose agar. The percent viability was calculated based on the number of CFU observed for electrically treated samples relative to control samples.

All mean values are averages of at least three replicate measurements. The significance of differences between means was evaluated based on a one-sided *t* test as described elsewhere (28).

RESULTS

Verification of transformation. Transformation of *C. thermocellum* was carried out using a custom-built pulse generator and plasmid pIKm1, with selection based on combined resistance to erythromycin and lincomycin as described in Materials and Methods. Antibiotic-resistant colonies were observed following the ET of strains ATCC 27405 and DMS 1313 and subsequently of strains DSM 4150 and DSM 7072 (when subcultured in the presence of isoniacin). The incidence of such colonies was initially detected at low efficiency (~1 to 10/μg of DNA), with higher efficiencies observed subsequently after optimization as described below. In control samples treated in the absence of pIKm1, we did not observe spontaneous mutants exhibiting simultaneous resistance to erythromycin and lincomycin at the concentrations used.

PCR was carried out using primers specific to the *mls* gene (21) with total DNA from Em^r Lm^r clones used as templates. As shown in Fig. 3, fragments with the expected size of ~800 bp were obtained for presumptive transformants of strains ATCC 27405 (lane 8), DSM1313 (lane 9), and DSM 4150 (lane 10), as well as for a positive control (lane 7), but not for negative controls (lanes 2 through 6).

To further confirm transformation, plasmid rescue experiments were carried out in which total DNA isolated from Em^r Lm^r clones of strains ATCC 27405, DSM 1313, and DSM 4150 was used to transform *E. coli* DH5α. For all three strains, plasmid DNA was repeatedly recovered from *E. coli* transformants (Fig. 4). The similarity of the restriction digest patterns for pIKm1 used to transform *C. thermocellum* strains and recovered from *E. coli* transformants suggests that neither deletions nor significant rearrangement occurred.

Partial optimization of ET conditions for strain DSM 1313. Partial optimization of ET conditions was performed using strain DSM 1313 (Table 2). The variables considered included the duration of subculturing prior to electric-pulse application, whether the cells were chilled during manipulations prior to exposure to an electrical pulse, the choice of cuvette design, the duration of the postelectropulsing recovery period (consisting of incubation without selection), and whether the cells were subcultured with isoniacin. It may be noted that the different cuvette designs used, either a modified centrifuge tube or a Bio-Rad cuvette, provide different extents of cooling (see Materials and Methods).

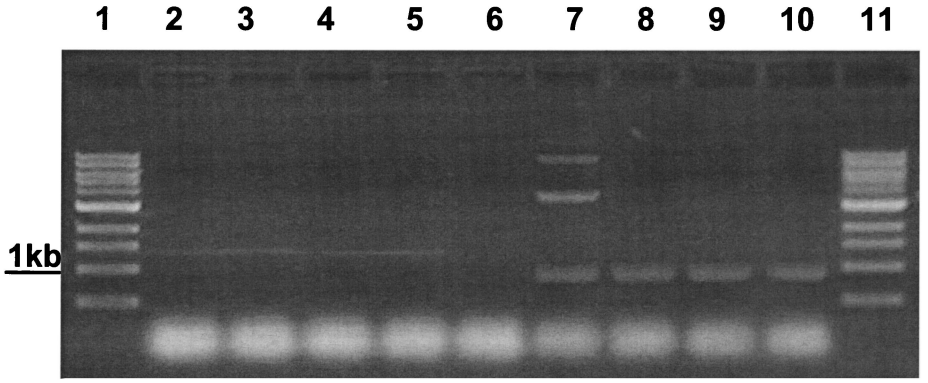

FIG. 3. Detection of *mls* gene in Em^r Lm^r clones of *C. thermocellum* strains by using PCR with various DNA templates. Lanes 1 and 11, 1-kb ladder; lanes 2 to 4, total DNAs from nontransformed cells of *C. thermocellum* ATCC 27405 (lane 2), DSM 1313 (lane 3), and DSM 4150 (lane 4); lane 5, pUC18; lane 6, no template; lane 7, pIKm1; lanes 8 to 10, total DNAs of Em^r Lm^r clones of *C. thermocellum* ATCC 27405 (lane 8), DSM 1313 (lane 9), and DSM 4150 (lane 10).

Transformation efficiency exhibited a strong dependence (at least several hundredfold and generally several thousandfold) on all variables tested, with the exception of isoniacin addition, which had a relatively small but significant positive effect for strain DSM 1313. Preferred conditions were identified and used for subsequent experiments as follows: subculturing period, 3 h; subculturing in the presence of isoniacin; chilling during cell collection and washing prior to electric treatment; use of a modified centrifuge tube with cooling in an ice block; postelectropulsing recovery period, 4 h. These conditions resulted in $>10^5$ transformants/μg of DNA for strain DSM 1313.

Transformation of other strains and effect of subculturing in the presence of isoniacin. Transformation using the preferred conditions found as described above was investigated for strains ATCC 27405, DSM 4150, and DSM 7072, as well as DSM 1313, and the effect of subculturing in the presence of isoniacin was also investigated (Table 3). Using conditions optimized for strain DSM1313, the transformation efficiency obtained for strain ATCC 27405 subcultured in the presence of 20 μg of isoniacin/ml (6.5×10^4 transformants μg^{-1}) was not as high as that obtained for strain DSM 1313 ($\sim 2.8 \times 10^5$ transformants μg^{-1}), and efficiencies were much lower ($< 2 \times 10^3$ transformants μg^{-1}) for strains DSM 4150 and DSM 7072. Subculture in the presence of isoniacin resulted in an increase in transformation efficiency for all four strains investigated, but the extent of the increase appeared to be strain specific. Subculture in the presence of isoniacin at 20 μg/ml improved transformation efficiency by ~4-fold for strain DSM 1313 and ~100-fold for ATCC 27405 and DSM 4150. Transformation of strain DSM 7072, albeit at an efficiency of only $\sim 1.1 \times 10^3$ transformants μg^{-1}, was observed only for cells subcultured in the presence of isoniacin.

Effect of DNA loading. The yield of transformants for strain DSM1313 was investigated in relation to DNA loading (micro-

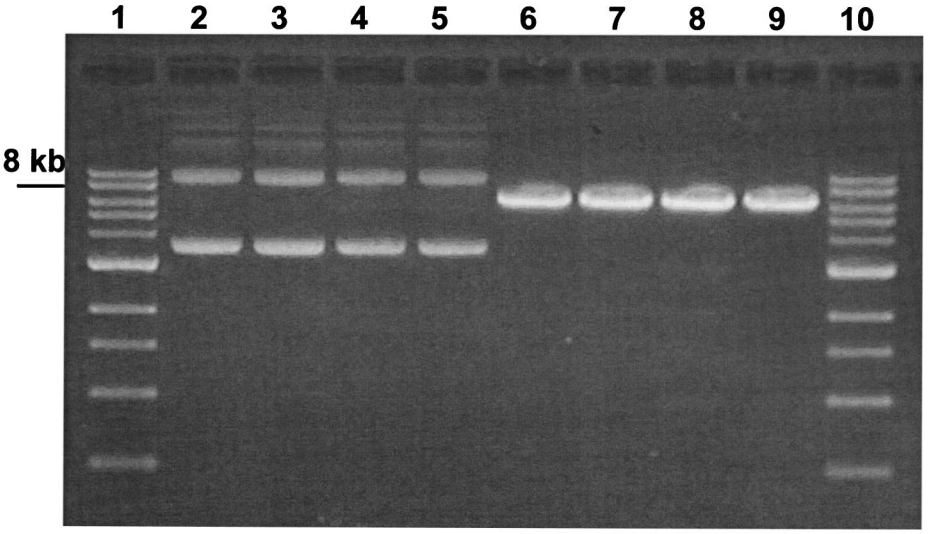

FIG. 4. pIKm1 in *E. coli* DH5α after rescue from total DNA of Em^r Lm^r clones of *C. thermocellum.* Lanes 1 and 10, 1-kb ladder; lane 2, pIKm1 used for ET of *C. thermocellum*; lanes 3 to 5, pIKm1 isolated from *E. coli* electrotransformed with total DNAs of *C. thermocellum* ATCC 27405 (lane 3), DSM 1313 (lane 4), and DSM 4150 (lane 5); lanes 6 to 9, *Pst*I digests of the DNA preparations shown in lanes 2 to 5.

TABLE 2. Partial optimization of ET conditions for strain DSM 1313 subcultured in the presence of 20 μg of isoniacin/ml[a]

Condition	Value	Transformation efficiency (μg^{-1})
Subculture period (h)[b]	0	0.4 ± 0.56
	1	6.4 ± 6.0
	2	$4.1 \times 10^2 \pm 3.8 \times 10^2$
	3	$2.2 \times 10^5 \pm 1.0 \times 10^5$[g]
	4	$8.7 \times 10^2 \pm 1.5 \times 10^2$
Chilling during cell collection and washing prior to electric pulse application[c]	Without chilling	2.3 ± 3.5
	With chilling	$2.5 \times 10^5 \pm 0.5 \times 10^5$[g]
Cuvette design and cooling protocol[d]	Custom cuvettee (centrifuge tube) to an ice block	$1.7 \times 10^5 \pm 0.3 \times 10^5$[g]
	Bio-Rad cuvette with prechilling	$3.9 \times 10^2 \pm 3.7 \times 10^2$
Postelectropulsing recovery period (h)[e]	0.25	55 ± 32
	1	$3.4 \times 10^2 \pm 2.7 \times 10^2$
	2	$6.4 \times 10^2 \pm 3.1 \times 10^2$
	4	$1.9 \times 10^5 \pm 0.8 \times 10^5$[g]
	9	$3.8 \times 10^4 \pm 4.3 \times 10^4$
Additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection[f]	Yes	$2.3 \times 10^5 \pm 1.0 \times 10^5$[g]
	No	$1.6 \times 10^4 \pm 0.76 \times 10^4$

[a] All experiments were carried out with 2.5 μg of pIKml DNA isolated from *Dam*$^+$ *E. coli.* A 10-ms square pulse at 25 kV/cm was used.
[b] Tested with chilling, in a custom cuvette (centrifuge tube) in an ice block, with a postelectropulsing recovery period of 4 h, and with additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection.
[c] Tested with a subculture period of 3 h, in a custom cuvette (centrifuge tube) in an ice block, with a postelectropulsing recovery period of 4 h, and with additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection.
[d] Tested with a subculture period of 3 h, with chilling, and with additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection.
[e] Tested with a subculture period of 3 h, with chilling, in a custom cuvette (centrifuge tube) in an ice block, and with additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection.
[f] Tested with a subculture period of 3 h, with chilling, in a custom cuvette (centrifuge tube) in an ice block, and with additional 6-h incubation with erythromycin at 0.25 μg/ml prior to selection.
[g] Significantly different from the next-closest value in the particular experimantal series ($P < 0.001$).

grams of DNA per 90-μl cell suspension). As shown in Fig. 5, the transformation yield increased linearly with increasing DNA at low loading (≤~2 μg of DNA), reached a maximum at ~8 μg of DNA, and declined at yet higher DNA loads. About 300 transformants were detected in the presence of 0.1 μg of DNA. Cell viability for samples with 0.1 μg of plasmid DNA was ~50% of that for controls not exposed to electrical pulses, declined slightly to ~40% at the optimal DNA load of 8 μg, and declined further to ~20% at 16 μg of DNA, the maximum load tested.

Effect of field strength. Transformation efficiency and viability were examined in relation to field strength for strains DSM 1313 (20 μg of isoniacin/ml) and strain 27405 (9 μg of isoniacin/ml) (Fig. 6). For both strains, transformation efficiencies increased monotonically with increasing field strength over the range tested, while cell viability decreased by ~50%. Transformation efficiencies in the absence of isoniacin were lower than in the presence of isoniacin for all voltages tested, with a more pronounced response to isoniacin addition observed for strain ATCC 27405 than for strain DSM 1313 (data not shown).

Effect of plasmid methylation. Transformation of strains ATCC 27405 and DSM 1313 was carried out with pIKm1 DNA isolated from *E. coli* strain DH5α (Dam$^+$) or GM272 (Dam$^-$). As shown in Table 4, Dam methylation of pIKm1 DNA resulted in ~7-fold increase in the ET efficiency for strain ATCC 27405 and ~40-fold for strain DSM1313.

DISCUSSION

Several strains of *C. thermocellum* have been successfully transformed with plasmid pIKm1, with selection based on resistance to erythromycin and lincomycin. It may be inferred from our results that both the ColE1 origin of replication and

TABLE 3. Transformation of various strains subcultured with and without isoniacin[a]

Isoniacin (μg/ml)	Transformation efficiency (transformants/μg) for *C. thermocellum* strain:			
	DSM 1313	ATCC 27405	DSM 4150	DSM 7072
None	$(6.3 \pm 1.3) \times 10^4$	$(1.4 \pm 0.99) \times 10^3$	4.2 ± 3.9	0
19	$(1.4 \pm 0.1) \times 10^5$[c]	$(6.4 \pm 0.14) \times 10^4$[c]	ND[b]	ND
20	$(2.9 \pm 0.47) \times 10^5$[c]	$(6.4 \pm 0.23) \times 10^4$	$(1.2 \pm 0.1) \times 10^3$[c]	$(1.1 \pm 0.1) \times 10^3$[c]

[a] All experiments were carried out with 2.5 μg of pIKml DNA isolated from *Dam*$^+$ *E. coli.* Electric treatment was carried out using a single 10-ms square pulse and an electric-field strength of 25 kV/cm. The values of other variables corresponded to the preferred conditions identified in Table 2.
[b] ND, not determined.
[c] Value statistically different from the preceding value in the same column at a P value of <0.002.

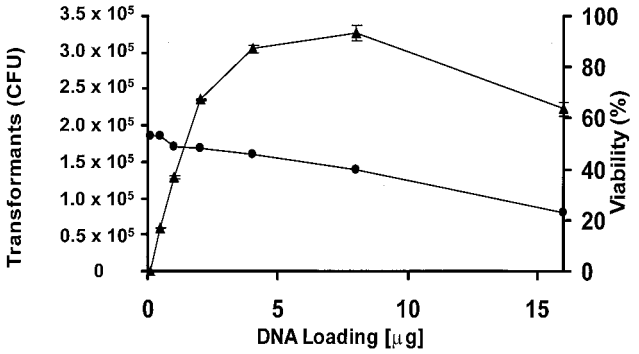

FIG. 5. ET yield (▲) and cell viability (●) in relation to DNA load for *C. thermocellum* DSM 1313. All experiments were carried out with pIKm1 DNA isolated from *Dam*$^+$ *E. coli* cells. A square 10-ms pulse and an electric-field strength of 25 kV/cm were used. The values of other variables corresponded to the preferred conditions identified in Table 2. The cells were subcultured in the presence of 9 μg of isoniacin/ml. The error bars indicate standard deviations.

TABLE 4. Influence of Dam methylation on electrotransformation efficiency of *C. thermocellum*[a]

E. coli strain as source of DNA	Transformation efficiency (transformants/μg)	
	ATCC 27405	DSM 1313
DH5α (Dam$^+$)	$(2.5 \pm 1.5) \times 10^4$	$(2.8 \pm 0.7) \times 10^5$
GM272 (Dam$^-$)	$(3.5 \pm 1.8) \times 10^{3b}$	$(6.5 \pm 2.6) \times 10^{3c}$

[a] Cells were subcultured in the presence of isoniacin at 9 and 20 μg/ml for strains ATCC 27405 and DSM 1313, respectively. Experiments were carried out at an electric-field strength of 25 kV/cm with 2.5 μg of DNA/sample and a single 10-ms square pulse, and the values of other variables corresponded to the preferred conditions identified in Table 2.

[b] Value statistically different from the value immediately preceding in the same column at a P value of <0.04.

[c] Value statistically different from the value immediately preceding in the same column at a P value of <0.002.

the *mls* antibiotic resistance gene (gene product, macrolide-lincosamide-streptogramin B resistance protein) originating from *Bacillus subtilis* (21) are functional in *C. thermocellum.*

After optimization using strain DSM 1313, preferred conditions were determined, including a 3-h subculture period in the presence of 20 μg of isoniacin/ml, chilling throughout the cell preparation and electric-pulse application, use of a custom cuvette in an ice block, a 4-h postelectropulsing incubation, inclusion of an induction period, 2.5 μg of DNA per 90-μl cell suspension, a field strength of 25 V/cm, and a Dam$^+$ strain of *E. coli* as the source of plasmid DNA. For the eight independent experiments under these conditions reported here (five from Table 2 and one each from Table 3, Table 4, and Fig. 6), the mean transformation efficiency was 2.2×10^5 transformants · μg of DNA^{-1} with a standard deviation of 0.51×10^5. The protocol and preferred conditions developed for strain DSM 1313 also resulted in high transformation efficiencies for strain ATCC 27405. Based on three independent determinations under these conditions (Table 3, Table 4, and Fig. 6), except with isoniacin at 9 rather than 20 μg/ml, a transformation efficiency of $5.0 \times 10^4 \pm 1.8 \times 10^4$ was obtained. Since ~2.5 μg of DNA was used per transformation experiment, these efficiencies correspond to 5.3×10^5 transformants per electroporation event for strain DSM 1313 and 1.3×10^5 transformants per event for strain ATCC 27405.

For the other two strains investigated, DSM 4150 and DSM 7072, transformation under conditions optimized for strain DSM 1313 resulted in efficiencies of $<2 \times 10^3$ μg^{-1}. Optimization studies directed at strains other than DSM 1313 were not undertaken and might well result in higher transformation efficiencies. Strain-to-strain variation was observed for several characteristics in addition to transformation efficiency. For example, subculturing in the presence of isoniacin had a larger beneficial effect on the transformation efficiency for strain ATCC 27405, while Dam methylation of plasmid DNA had a more pronounced benefit for strain DSM 1313 than for strain ATCC 27405.

The large (≥100,000-fold) increases in transformation fre-

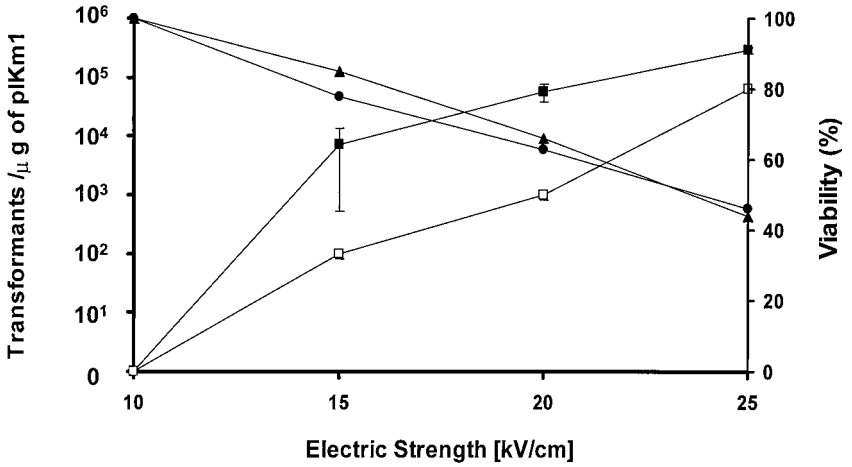

FIG. 6. ET of *C. thermocellum* DSM 1313 and ATCC 27405 subcultured in the presence of isoniacin as a function of field strength. Shown are the ET efficiencies for the cells of DSM 1313 (■) (20 μg of isoniacin/ml) and ATCC 27405 (□) (9 μg of isoniacin/ml) and the viabilities for the same cells of DSM 1313 (▲) and ATCC 27405 (●). The experiments were carried out with pIKm1 isolated from *Dam*$^+$ *E. coli* cells (2.5 μg/sample; 10-ms single square pulse; the values of other variables corresponded to the preferred conditions identified in Table 2). The error bars indicate standard deviations.

quency obtained after optimization, together with results from experiments aimed at characterizing the effects of several variables, offer insights into factors important in order to obtain high transformation frequencies. Our results clearly indicate that such factors include protocols for handling cells before and after electric pulse application and the conditions and equipment used for pulse application, as well as the identity of the recipient strain.

With respect to cell-handling protocols, successive transfers of liquid cultures ensured that cells were rapidly dividing and had high viability prior to electric pulse application. Transformation efficiencies with stationary-phase cultures were both much lower and less reproducible than those with cultures that had gone through the multistep subculturing procedure described above (data not shown). Inclusion of a 6-h incubation with erythromycin at 0.25 μg/ml following a recovery period in the absence of antibiotic but prior to incubation on plates with erythromycin and lincomycin each present at 20 μg/ml resulted in a 14-fold increase in transformation efficiency. Such an "induction period" has been shown to be essential in the case of some genes encoding MLS resistance (43), although the *mls* gene in pIKm1 does not normally require induction for expression in bacilli (21). Chilling during cell collection and washing prior to electric-pulse application and the duration of the postelectropulsing recovery time had the largest effects of any variables examined, although the underlying basis for these effects is speculative.

A dependence of transformation efficiency on electric-pulse parameters (e.g., field strength) such as observed here for *C. thermocellum* has routinely been reported, although seldom explained, in the literature on bacterial transformation (18, 27, 31, 41, 44). Variation of transformation conditions depending on the equipment used has been reported less frequently. The substantial (350-fold) increase in transformation efficiency observed for custom cuvettes consisting of modified centrifuge tubes in an ice block compared to prechilled Bio-Rad cuvettes may be due to more effective cooling for the former, different electric field geometries, or a combination of both. Our efforts to carry out transformation with commercially available pulse generators have met with very limited success (data not shown), providing a further indication of the importance of ET equipment, as well as conditions.

An *Mbo*I-like restriction system that recognizes the sequence 3′-GATC-5′ has previously been identified in *C. thermocellum* strain ATCC 27405 (9), as well as in several other thermophilic, anaerobic, cellulolytic isolates (25). The significant increase in efficiency observed here for strain DSM 1313 provides strong indirect evidence that this strain also has an *Mbo*I-like restriction system. We observed increased transformation efficiency accompanying the use of *dam*-methylated plasmid DNA (7-fold for strain ATCC 27405 and 40-fold in DSM 1313). Mermelstein and Papoutsakis (18) observed that plasmid methylation resulted in an ~10,000-fold increase in the transformation efficiency of *C. acetobutylicum*, and Jennert et al. (8) observed transformation of *C. cellulolyticum* only when plasmid DNA was methylated. Chen et al. (4) observed an absolute dependence on methylation in the presence of "preshock incubation" of a *Clostridium botulinum* strain but not in the absence of such incubation, suggesting that apparent sensitivity to methylation can be dependent upon the conditions used. The relatively modest effect of *dam* methylation observed here for *C. thermocellum* is not the result of a low number of restriction sites, since there are 23 *Mbo*I sites in pIKm1, and awaits explanation.

Isoniacin, first used in electroporation of anaerobic thermophiles by Mai et al. (15), was associated with increased transformation efficiency for all of the strains we tested. Isoniacin is known to interfere with fatty acid synthesis in *Mycobacterium tuberculosis* (36), consistent with the hypothesis that the isoniacin-mediated benefits are associated with changes in membrane composition and properties. The mechanism of enhanced transformation by isoniacin remains to be definitively elucidated.

For strains DSM 1313 and ATCC 27405, the results reported here provide for the first time a gene transfer method functional in *C. thermocellum* that is suitable for molecular manipulations involving either the introduction of foreign gene products or knockout of native gene products. Our work represents a significant step forward relative to the only prior report of transformation in *C. thermocellum* (40).

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