Electrofusion of cells of Acetogen *Clostridium sp.* MT 351 with *erm*(B) or *cat* in the chromosome

Michael Tyurin[1, *], Michael Kiriukhin[2], Vel Berzin[1]

[1]Syngas Biofuels Energy, Inc., 2441 Del Monte, Houston, TX 77019, USA. [2]Ajinomoto Company, 1st Dorozny pr. 1-1, Moscow, Russia 117545

We report electrofusion (EF) of untreated cells of the Acetogen Clostridium sp. MT 351 as a step in developing tools for metabolic engineering in this group of microorganisms. This obligate anaerobe strain was isolated from the agricultural lagoon using the enrichment procedure in the presence of carbon monoxide and hydrogen. This strain reduced carbon monoxide to acetate, produces spores and stained Gram (+). Electrofusion was proven efficient for chromosomal recombination using this model system utilizing integration suicidal vectors comprising either erm(B) or cat. At first, we have obtained recombinants resistant to either Erythomycin or Chloramphenicol. Such recombinants were electrofused and selected as resistant to both Erytromycin and Chloramphenicol. The double antibiotic resistant clones were selected at the frequency 5.5 $\pm$ 0.3 x 10^{-5} calculated per the number of either cell partner at the cell viability 15 $\pm$ 2%. The erm(B) and cat were amplified from the total DNA preps of such recombinants using primers specific for each antibiotic resistance gene. The electrofusion technique is a fast and reliable alternative to the established procedures of protoplast fusion developed for both, Gram-negative and Gram-positive bacteria. This is the first report on electrofusion of Gram (+) microorganisms.

* Corresponding author: Syngas Biofuels Energy, Inc., 2441 Del Monte, Houston, TX 77019, USA. Email: michael@syngasbiofuelsenergy.com

Introduction

In metabolic engineering, it is essential to possess tools for gene insertion, removal, modification, and/or replacement via natural mechanism of recombination. Traditional approaches include multi-step genetic transformation process with selection of introduced genes using acquired antibiotic resistance phenotype. Each subsequent step adds a new antibiotic for the selection. Use of additional antibiotics for selection extends the process and increases energy demand by the transformed cell. The energy is spent for expressing the extra antibiotic resistance over selection and maintenance. Incorporated steps of removal of the introduced antibiotic resistance gene do not cure the problem of the excessive cumulative time to meet the objectives. Therefore it takes a substantial time to engineer genome using multiple subsequent recombination events.

Unipolar electroporation pulses cause significant polarization of electrodes in electroporation cuvette and thus create biofilm on positively charged surface(s) [1, 2]. Combination of non-uniform electric field with the pulse rising time of <1 μs led to electrofusion of bacterial cells in such biofilm at frequencies detectable within routine electro-transformation experiments. The process resembled "welding" of the cell membranes under low temperatures [1]. Using untreated *E. coli* cells it was demonstrated that cell electrofusion represents a common physical phenomenon during efficient electrotransfor-mation if electric treatment was performed in non-uniform electric field. The resulted electrofusion products were cell agglomerates

comprising the content of several parental cells. The type of agglomerates depended on the number of neighboring parental cells, their arrangement relative to each other and the dynamics of the sample temperature during the process. The agglomerates represented temporary structures which existed for several hours in optimal nutrient media [3]. Such agglomerates represented the living cell systems with increased entropy and thus are increasingly unstable. Natural decrease of such entropy caused the agglomerates to dissolve back to single cells with regular cytoplasm/membrane ratio.

During fusion of protoplasts or spheroplasts, the changes in the system entropy happen at the expense of the energy pools of individual cells making the process really lengthy. This makes industrial researchers look for alternative avenues of reaching the same objectives.

In contrary to the protoplast fusion, electrofusion uses the energy of electric pulses to form cell agglomerates. This saves the energy in each individual cell pool for the only post-pulse recovery and chromosomal re-arrangements. The specific benefit of such managing of the energy pool during electrofusion is that the resulted single recombinants utilize their energy only for the recovery from the pulse damage, genome repairs and rearrangements. Therefore electrofusion renders much faster recovery as compared to fusion of protoplasts or spheroplasts [3, 4, 5]. In the last case scenario, the energy pool is also used for maintenance of the fusion product during recombination event and following expression of the recombined DNA under selective conditions.

The mechanism of electrofusion in Gram (-) cells such as *E. coli* features formation of electropores in the areas of cell surface with the neighboring “lipid spots” on the cell surface [3]. “Lipid spots” correspond to the cell areas with no periplasmic space and the cell membranes face the outside. During the pulse application, two or more cells form a multicellular electrofusion product when lipid spots in neighboring cells are positioned against each other. The products of electrofusion have spherical structure and comprise the most of the intracellular contents of the fused cells-partners. Close proximity of chromosomes inside such cell agglomerates provides a unique opportunity for the recombination events. The agglomerate then resolves leading to recovery of single cells containing mixed cytoplasmic content and chromosome recombination products in all its variety (gene “shuffling”). While a variety of recombination events take place, only those are detected depending on the particular selection conditions. Chromosomal gene recombination was detected at the frequency of ~10^{-4} – 10^{-5} [3]. Total time required for engineering of combined chromosomal recombinants using electrofusion was significantly shorter compared to generation of the same recombinants in subsequent multiple steps or via fusion of proto- or spheroplasts [3, 4, 5].

Cell wall in Gram (+) bacteria [1, 6] and similar functional makeup in Archaebacteria present mechanical barrier which prevents cell membranes from close contact essential for electrofusion event [3]. We reported earlier the engineering of double *ldh*$^-$ and *pta/ack*$^-$ recombinants of Gram (+) - like thermophilic microorganism *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 producing only ethanol as the result of such recombinations Such double lactate and acetate knock-outs resulted also from electrofusion of untreated cells of respective initial single knock-outs under the conditions optimal for electrotransformation to generate *ldh*$^-$ or *pta/ack*$^-$ strains using respective suicidal knock-out vector constructs [7].

In this report, we describe the electrofusion of antibiotics resistant recombinants of our newly isolated Acetogen strain *Clostridium sp.* MT 351. The recombinants were resistant to Em and Cm,

which resulted from the chromosomal integration of both *erm*(B) and *cat* under selection in the presence of both antibiotics. The process took us only 96 hours from the moment of the electrofusion event to obtain single purified cultures of such double chromosomal recombinants of *Clostridium sp.* MT 351. This is the first report on electrofusion of untreated cells of Gram (+) microorganisms.

Materials and methods

Bacterial Strain, Antibiotics and DNA preparations

A spore forming Gram (+)-staining mesophilic microorganism *Clostridium sp.* MT 351 was isolated from the agricultural lagoon sediment using enrichment procedure in sealed serum bottles under 50 psi of the mixture of carbon monoxide and hydrogen (60% and 40 %, respectively) with the culture transfers to a fresh bottle every three to four days for five consecutive transfer cycles. The bottles were filled with reduced liquid medium Syngas Fermentation Broth (SFB), containing g/L: NaCl 0.8; NH_4Cl 1.0; KCl 0.1; $MgCl_2 \times 6H_2O$ 0.2; $CaCl_2 \times 2H_2O$ 0.03; KH_2PO_4 0.1; $NaHCO_3$ 1.0; Nitrilotriacetic acid 0.02; $MnSO_4 \times 2H_2O$ 0.01; $(NH_4)_2Fe\ (SO_4)_2 \times 6H_2O$ 0.008; $CoCl_2 \times 6H_2O$ 0.004; $ZnCl_2$ 0.0003; $NiCl_2 \times 6H_2O$ 0.00025; Na_2SeO_4 0.0003; $CuSO_4 \times 5H_2O$ 0.0003; $KAl(SO_4)_2 \times 12H_2O$ 0.0004; H_3BO_3 0.0001; $Na_2MoO_4 \times 2H_2O$ 0.0002; $Na_2WO_4 \times 2H_2O$ 0.0002; Cysteine-HCl x H_2O 0.25; $Na_2S \times 9H_2O$ 0.25; Nicotinic acid 0.00025; Cyancobalamin 0.00025; p-Aminobenzoic acid 0.00025; D-Ca-Pantothenate 0.00025; Thiamine-HCl 0.00025; Riboflavin 0.00025; Lipoic acid 0.0003; Folic acid 0.0001; Biotin 0.0001; Pyridoxine-HCl 0.05; Yeast Extract 1.0; and Resazurin (1.0% solution) 0.2 ml. Filter sterilized stock solutions of sodium sulfide, microelements and vitamins were added aseptically to the autoclaved medium to make the composition indicated above. For plating SFB additionally contained Dextrose 5 g/L and Agar powder 12 g/L to solidify SFB for making agar (SFA) plates.

Media reduction and all manipulations with the cells were performed in modified Anaerobe Chamber AS-580 (Anaerobe Systems, CA) with neoprene sleeves and gloves for strictly anaerobic conditions (<1 ppm of Oxygen after the atmosphere was balanced by the internal catalyst). The synthetic synthesis gas contained mixture of 40% hydrogen and 60% carbon monoxide. Such syngas composition ensured elimination of oxygen traces quickly with no need to monitor oxygen separately from the Hydrogen content. Petri dishes were poured, left solidify, inoculated and incubated in anaerobic Vacu-Quick Jars (Almore International, EA) [8] at the syngas pressure inside maintained at ~6 - 7 psi with complete change of the atmosphere every 6 hours. All incubations were performed at 36^o C. The pH of the media fluctuated from 6.5 to 6.0 due to the changes in the syngas composition inside the vessels due to microbial production of carbon dioxide.

For selection and maintenance of recombinants SFB and SFA were used. The media contained Erythromycin (Em) and/or Chloramphenicol (Cm) (Sigma, St. Louis, MO) at concentrations 40 and 50 µg/ml respectively. Plasmid DNAs from *E. coli* JM109 (New England BioLabs, MA) grown at 100 µg/ml Ampicillin (Sigma, St. Louis, MO) in BHI or BHI with 12 g/L of Agar was extracted using Qiagen Miniprep Kit (Valencia, CA). Genomic DNA from recombinants of *Clostridium* sp. MT 351 was isolated using our quick miniprep procedure below and further purified with Qiagen Gel Extraction Kits (Valencia, CA) for Gram (+) microorganisms if needed. Genomic DNAs from *Clostridium thermocellum* ATCC 27405 and *Clostridium acetobutylicum* ATCC 824 were purchased from ATCC (Manassas, VA).

Total DNA isolation

Total DNA isolation was performed after enzymatic treatment with lysozyme followed by the SDS lysis, RNA digestion, Proteinase K treatment to decrease the viscosity of the aqueous phase and deactivate endo- and exo-

nuclease activities, deproteinization by Tris-equilibrated (pH 8.0) Phenol:Chloroform: Isoamyl Alcohol mixture (25:24:1 vol/vol/vol) in the presence of 1 M NaCl to enhance the Phenol extraction, and precipitation of the DNA with 3 volumes of ethanol in 0.1 M Sodium Acetate (pH 5.2). The protocol was used as described [9] in application to the extraction of total DNA from Corynebacteria [10]. All enzymes and reagents were purchased from Sigma (St. Louis, MO).

Microscopy, growth temperature, sensitivity to oxygen, catalase reaction and detection of spores

Microscopy was performed using Gram-stained cells of 24 h-old cultures of *Clostridium sp.* MT 351 grown in SFB at 36^{o}C. Production of spores was tested as described [8] and also using the procedure recommended to separate spores from vegetative cell in *Clostridium acetobutylicum* ATCC 824 for storage purposes [11]. For *Clostridium sp.* MT 351, cells from 10 ml of 120 h - old culture grown in SFB were collected by centrifugation, resuspended in 0.5 ml of the same medium in Hungate tubes and incubated in boiling water for 10 min. After cooling, each sample was transferred to 25 ml of SFB and incubated in shakers under CO as described above for 72 – 96 h with recovery of single colonies of *Clostridium sp*. MT 351 as it was verified by the rep-PCR. Germination of spores was attempted under aerobic conditions as well to make sure we deal with obligate anaerobic microorganism. Growth temperatures were checked in the anaerobic chamber in the range of 26, 36, and 40^{o}C to determine the best suited temperature for spore germination. Cells from single colonies grown on SFA were tested for catalase production using 15% Hydrogen Peroxide.

Conditions for electrotransformation

Electrotransformation was performed on ice using Syngas Biofuels Energy, Inc. electroporation generator [10] with custom-made electrodes shown in figure 1a providing electric field distribution pattern shown in figure 1b. We optimized the duration and voltage for a single square pulse [10] with some modifications [12]. The optimal electric field strength of 20 kV/cm at the pulse duration of 10 ms was used for all the experiments leading to selection of electrotransformants resistant to Cm or Em and in electrofusion experiments. The resulted Em and Cm resistant recombinants were then mixed together at the ratio 1:1 to prepare samples as for the electro-transformation and treated with the identical electric pulses. The recombinant products of electrofusion were selected on SFA containing both Cm and Em.

Pulses were recorded in real time at the cell sample via precision voltage divider 1:1,000 (pulse voltage) and at the pulse current sensor as described in [10, 13]. A twenty Ω precision thing film low inductance resistor served as the current sensor. It connected the negatively charged plate of the power capacitor with the Ground. Tektronix TDS220 digital storage oscilloscope (Agilent Technologies, CA) was used to record the real time images of pulse voltage and current. Oscilloscope triggering was performed from the channel recording the pulse current since the pulse current was shifted slightly ahead of the pulse voltage in the real time due to significant electric capacitance of the cell samples. Images on the oscilloscope screen were photographed to document the authenticity of the recordings.

For comparison purposes we used standard BTX-Harvard Apparatus ECM 830 Square Wave Electroporator and standard shock chamber (Bio-Rad, Hercules, CA) along with the standard 0.1 mm plastic disposable cuvettes (Bio-Rad, Hercules, CA).

Antibiotic resistance genes and suicidal vectors for integration

Homology region for chromosomal integration:

We have designed a nucleotide sequence (HR) sharing homology with genomes of *Clostridium acetobutylicum* ATCC 824 (NC_003030), *Clostridium thermocellum* ATCC 27405 (NC_009012) and *Eubacterium limosum* KIST612

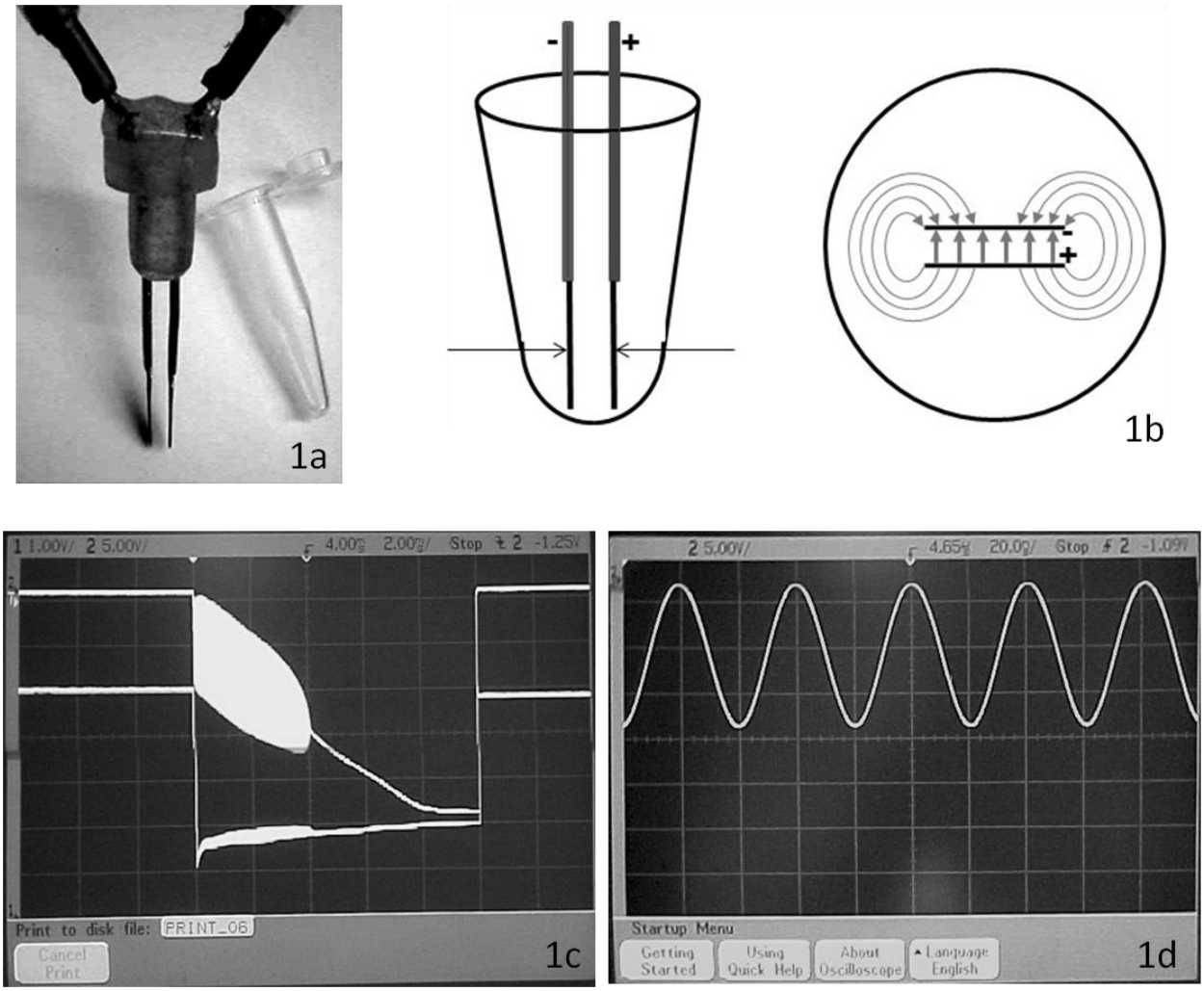

Figure 1. 1a - plate-parallel flat electrodes made from titanium alloy VK-2 fit disposable 0.5-0.6 ml PCR tube used as electroporation cuvette; 1b - schematic diagram of the electric field distribution shown for the cross section of the electric treatment volume; 1c - typical real time records of the pulse current at the pulse current sensor (top) and the pulse voltage (bottom) at 1:1,000 precision voltage divider tool; 1d - pulse current oscillations as in 1c recorded with horizontal deflection 20 ns per division. The calculated frequency is 23.8 MHz (the same as in 1c).

(NC_014624) and plasmid pIM13 (NC_001376) as based on the revealed homology of the DNA for use as the component for the integration suicidal vectors for transformation of *Clostridium sp.* MT 351: 5'-AAAATTGATT TTTTGAAATA AAATTATTAA GTAGAAAAAA AATTGATTTT TTGAAATAAA ATTATTAAGT AGAAAAAATT TTGCGTATTC TTTCTATATA TAATTTTACA AAAAATTTTG CGTATTCTTT CTATATATAA TTTTACAAAA AAGGAGGAAA AAATGAAATT TTTTTCTTTT AAATACAACG AGAAATAGAT ATCGGATTAA AAAAGGAGGA AAAAATGAAA TTAACAAGTT GATTTTTTGA AATAATATAA CAAGTTGATT TTTTGAAATA ATATTTTTTC TTTTAAATAC AAGGCAAAAT AGATATTAGA TTAA-3'.

If templates containing ORF DNA of *erm*B (AY334073) or *cat* (FM201786) and the pair of primers: 5'-AAAAATTTTG CGTATTCTTT CT-3' and 5'-AATCCGATAT CTATTTCTCG TTG-3' were used, we did not obtain PCR products with the sequence homologous with the HR.

Suicidal vectors for integration: Two suicidal vectors for integration of antibiotic resistance

gene into the chromosome of *Clostridium sp.* MT 351 were engineered by ligation of the inserts into MCS of pUC19 (New England BioLabs, MA) used as a backbone for DNA accumulation in *E. coli* JM109 (New England BioLabs, MA). Vector pMVT*erm*(B) had the insert comprising a synthetic Em-resistance gene with the promoter and terminator sequences for the DNA of *Moorella thermoacetica* ATCC 39073 (CP000232), region 2626906…2627526, upstream and downstream of *erm*(B) (AY334073), respectively. Promoter and terminator sequences were identified using Softberry Bacterial Promoter, Operon and Gene Finding tool (http://linux1.softberry.com/). The resulted synthetic *erm*B operon was flanked with the HFs upstream from the promoter and downstream from the terminator region for integration to the chromosome of *Clostridium sp.* MT 361. Vector pMVT*cat* was engineered with the insert comprising *cat* gene (FM201786) used instead of the *erm*(B) as above. Detection of *erm*(B) was performed by using 5'-ATTTAGGCTT CCGACAAGCA-3' (primer I) and 5'-ATCTGTGGTA TGGCGGGTAA-3' (primer II) primers. Detection of *cat* was confirmed with 5'-TCCTGCATGA TAACCATCAC A-3' (primer III) and 5'-CAATAGCGAC GGAGAGTTAG G-3' (primer IV) primers.

Validation of recombinants and products of electrofusion using repPCR

We verified the identify of recombinants of *Clostridium sp.* MT 351 and the products of their electrofusion via using repPCR and also confirming the production of only acetic acid from CO during growth in SFB using HPLC analysis of the culture broths. We used primers for amplification of multiple repetitive sequences in the chromosome of *Clostridium sp.* MT 351 based on modification of primers for the CDS of the carbon-monoxide dehydrogenase catalytic subunit gene *coo*SV of *Carboxydothermus hydrogenoformans* Z-2901 (CP000141). The primers were 5'-TTATAACCAT TTCCAAGA-3' (primer V) and 5'-AAATCCATTA AACCATT-3' (primer VI).

DNA sequencing and synthesis as well as synthesis of all primers were performed by Invitrogen (Carlsbad, CA).

Phusion® High-Fidelity PCR Kit (New England BioLabs, MA) was used for all PCR reactions.

HPLC Analysis

For HPLC analysis we used standard mixture containing formate, acetate, ethanol, ethylacetate, butyrate and n-butanol along with the major organic compounds of the SFB (glucose and yeast extract) on Aminex 87H column (Bio-Rad Laboratories, Inc., Hercules, CA) at 55^{o}C. The mobile phase consisted of the 5 mM sulfuric acid at a flow rate 0.6 ml/min. Detections were performed via refractive index using Waters 410 IR detector (Milford, MA). The minimal detection level was 0.3 µM.

Results

The cells of 24- hour old culture of *Clostridium sp*. MT 351 stained Gram(+) and were catalase negative. The cells had spores located terminally. The spores did not germinate under aerobic conditions confirming that *Clostridium sp*. MT 351 was an obligate anaerobic microorganism. Growth temperature of 36^{o}C provided the fastest spore germination and the shortest cell duplication time as it was checked in serial dilution tests described [8].

We detected the carbon monoxide-supported cell growth in SFB after 60 min exposure of the cell pellets to 50% ethanol at 37^{o}C. The same was observed after exposing similarly prepared cell pellets for 10 min to the boiling temperature of water. Both treatments were performed under anaerobic conditions.

Acetic acid was the only product of carbon monoxide supported growth of *Clostridium sp*. MT 351. Liquid cultures accumulated up to 140 mM of acetic acid after 7 days of incubation in the presence of syngas mixture at 50 PSI.

Antibiotic sensitivity

Strain *Clostridium sp.* MT 351 was tested for the sensitivity to Em or Cm at the concentrations of each antibiotic 2, 4, 8, 16, 20, 32, 40, 50, 64, 84 and 100 μg/ml. At concentrations of Em 4 μg/ml or 8 μg/ml of Cm in either SFB or SFA the frequency of detection of spontaneously resistant mutants was ~10^{-8} per the number of cells inoculated as it was confirmed in the sample dilution plating tests [8]. For the electrotransformation and electrofusion experiments we chose 20 and 32 μg/ml for Em and Cm respectively. We found that no detectable mutants were revealed even at ~2 x 10^9 cell inoculums at these concentrations of the antibiotic. Such concentration still provided growth rates of the recombinant colonies similar to those of the non-transformed cells. The Em^r or Cm^r recombinant clones were detected at the frequencies of about ~10^{-3} per the number of sensitive recipient cells used for electrotransformation with suicidal integration vectors.

Equipment design and electric treatment

We devoted a special attention to maintaining the constant temperature during the electric treatment. We performed preliminary testing using micro thermo couple with the response time 5 μs coupled with a digital storage oscilloscope. The thermo couple was immersed to the cell samples of *E. coli* in water placed in disposable polypropylene tubes (cuvettes) imbedded in ice block to precisely fit the wells for the tubes prepared as described [1, 3, 8, 12]. The samples were subjected to electric treatment with square 10 ms pulses each at the range of voltages from 1,500 V to 7,500 V with 200 V increment. The sample volume 0.2 ml maintained constant temperature of about 4^{o}C in the electric field range of 12 – 25 kV/cm for the inter-electrode distance of 2.0 mm and the electrode setup shown in figure 1a. We used 0.2 ml cell sample volumes for all electrotransformation and electrofusion experiments throughout this report. The schematic diagram of the electric field distribution is shown in figure 1b.

For comparison purposes we used standard BTX-Harvard Apparatus ECM 830 Square Wave Electroporator and standard shock chamber (Bio-Rad, Hercules, CA) along with the standard 0.1 mm plastic disposable cuvettes (Bio-Rad, Hercules, CA). We used chromosomal recombinants obtained from the described above experiments using our custom-build equipment for these comparative experiments. We did not obtain any colonies resistant to both Em and Cm. At the output voltage set at 3kV, we sometimes observed electric sparks in the cuvette areas right above the samples at least partially destroying our samples due to reaching limit of the electric field strength of the gas atmosphere surrounding the electrodes with applied high voltage.

Pulse forms and specific pulse components for successful ET or EF

Figure 1c shows real time records of the pulse current and voltage which led to reproducible selection of recombinants. The top curve represents the pulse current. The start pulse current was approximately 0.5A then increasing to about 2.5A at the cutoff pulse edge. It appears from figure 1c that the functions of the pulse voltage and pulse current were in a reverse proportion as expected for the partial power capacitor discharge conditions.

Figure 1d shows the part of the pulse current with oscillations recorded at a different horizontal deflection rate to measure the actual frequency of the induced oscillations. The distance between peaks for this oscillating part is approximately 42 ns, which corresponds to 23.8 MHz. As we demonstrated earlier with the electric treatment of *C. thermocellum* ATCC 27405 such frequency reflects spontaneously generated oscillations of the square input pulse by the cell sample placed in our authentic circuit to actually achieve the efficient electric breakthrough of the cell membranes with high cell recovery rates after electric shock. The generation of oscillations becomes really visible only when it is significantly amplified by the power tetrode powered with the power

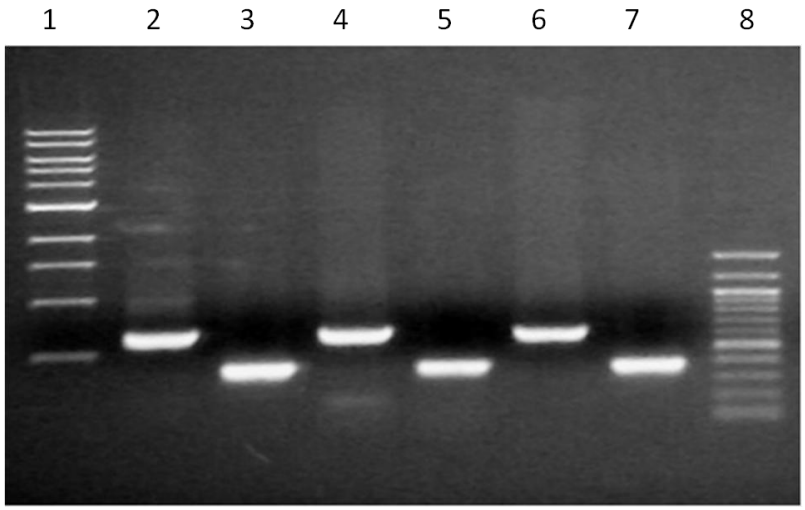

Figure 2. PCR products corresponding to CDS of *erm*(B) (AY334073.1) with templates isolated from *E. coli* (pMVT*erm*(B)) (lane 2), *Clostridium sp.* MT 351 (pMVT*erm*(B)) (lane 4), and *Clostridium sp.* MT 351 (pMVT*erm*(B), pMVT*cat*) (lane 6); PCR products corresponding to CDS of *cat* (FM201786) with templates isolated from *E. coli* (pMVT*cat*) (lane 3), *Clostridium sp.* MT 351 (pMVT*cat*) (lane 5), and *Clostridium sp.* MT 351 (pMVT*erm*(B), pMVT*cat*) (lane 7); 1 kb ladder (lane 1) and 100 bp ladder (lane 8) (New England BioLabs, MA)

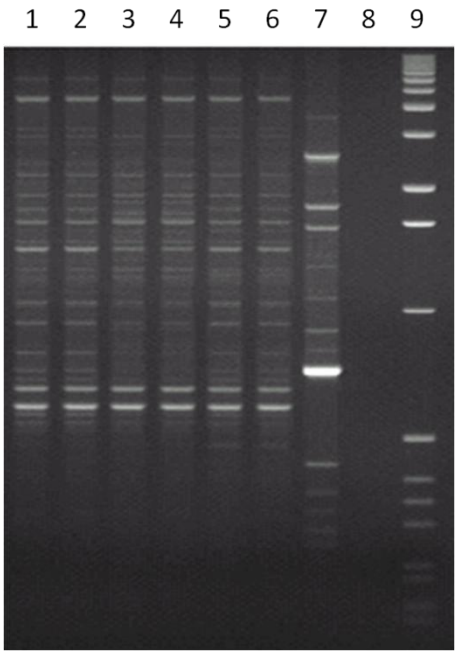

Figure 3. Rep-PCR fingerprint patterns of the recombinants of *Clostridium sp*. MT 351 with insertion of *erm*(B) (lanes 1, 2), *cat* (lanes 3, 4), electrofusants with both *erm*(B) and *cat* (lanes 5, 6), positive control for primers/PCR using DNA of *Clostridium thermocellum* ATCC 27405 as a template (lane 7), negative control (lane 8) and molecular weight marker (1 kb DNA ladder; Invitrogen, CA) (lane 9)

capacitor used in our apparatus design under the optimal conditions for the electric membrane breakdown [1, 3, 10, 13].

Chromosomal integration of the suicidal vectors

The single chromosomal recombinants of *Clostridium sp.* MT 351 which acquired ether

erm(B) or *cat* were detected at frequencies 6.0 $\pm$ 0.25 x 10^{-3} per the number of the untreated cells in the sample at the cell viability rate of 15 $\pm$ 1.5% after the treatment. In electrofusion experiments the frequency of selection of clones with both genes of antibiotic resistance was about 5.5 $\pm$ 0.3 x 10^{-5} calculated per the number of the parental clones with integrated *erm*(B) or *cat* and the cell viability rate of about 15 $\pm$ 2.0% calculated per each fusion partner. The presence of *erm*(B) or *cat* was detected using primers I, II, III and IV, respectively. The products with sizes of 649 bp for the *erm*(B) ORF and 382 bp for the *cat* ORF are shown in figure 2. No PCR products were detected if any of these primers were used for the PCR using DNA of the original antibiotic-sensitive clones of *Clostririum sp.* MT 351 as a template.

RepPCR and HPLC validation of recombinant clones

We used primers V and VI for amplification of certain repetitive sequences in the chromosomal DNA of *Clostridium sp*. MT 351. The products of PCR with such primers served as distinctive markers to prove we have the right clones in addition to the data based on the morphology, antibiotic sensitivity patterns and metabolic profiling. The PCR products of two clones comprising *erm*(B), two clones comprising *cat* and two clones comprising both *erm*(B) and *cat* are shown in figure 3 along with specific positive (DNA of *Clostridium thermocellum* ATCC 27405) and negative (no template) controls. All the antibiotic resistant clones maintained the cell and colony morphology of the original antibiotic sensitive strain *Clostridium sp.* MT 351 and produced only acetic acid during growth under carbon monoxide atmosphere in SFB as supported by the HPLC data.

Discussion

The electroporation mechanism in some Acetogens and the advantages of the use of our electroporation generator

Cells of many microorganisms generate spontaneous oscillations of the electroporation pulse current if subjected to electric pulses generated by the SBE, Inc. electroporation generators. SBE, Inc. electroporation generators comprise high current high pulse high frequency pulse networks capable of delivering thousands of amperes within a fraction of a microsecond via pulse modulating power tetrodes [1, 3, 10]. We successfully used such generators in projects with *Corynebacterium glutamicum* ATCC 13032 [10], *Clostridium acetobutylicum* ATCC 824 [11], for the electrotransformation of *Clostridium thermocellum* ATCC 27405 and *Clostridium thermocellum* DSM 1313 [12, 13]. All the above mentioned references report generation of pulse current oscillations by the cell samples of various solventogenic Clostridia at different frequencies probably somewhat specific for the particular strains tested. The mechanism of such oscillations had been described [13]. We would like to reiterate some specific details which we believe are of great interest for the experts working on the biofuels of the next generation.

The pulse has to be long enough to accommodate the generation and then the decay of the pulse current oscillations. The pulse voltage has to be in the range ensuring survival of at least 1% of the initial recipient population for a given cell sample or better. The temperature in the sample has to be around 4.0 - 5.0^{o}C during and after the pulse application if monitored with a thermocouple at the 5 - 15 µs sampling rate.

Solventogenic microorganisms are stuffed with multi-layer membrane structures. Such structures serve as mechanical barriers preventing transforming DNA molecules from entering the cytosol. To eliminate the barrier function, each of the membrane layers has to be electrically broken. This process is somewhat similar to that of the capacitor insulator breakdown caused by excessive voltage. Transforming DNA molecules are then transported by the pulse electrophoretic force

to the cytosol allowing for the DNA replication and transcription [1, 2]. In the circuit design used by Syngas Biofuels Energy, Inc., the pulse electric field penetrates a single membrane layer and creates electric pores in it with the extra pulse current going through such pores. The tetrode amplifies this added current flux. Then next membrane layer is subsequently penetrated by the electric field in a similar way via the same mechanism and effect, and so on. The process of subsequent electric breakdown of membrane pockets located next to each other inside the cells happens again and again until all the membrane layers in the way of DNA to cytosol are punctured by the electric breakdown process. This moment is characterized by decay of the amplitude of the oscillations and subsequent flattening of the pulse current as shown in figure 1c.

Then the transforming DNA ends up in cytosol and attaches to cell membrane via the origin of replication (a replicating plasmid) or integrates to the chromosome if the homology is sufficient. It is possible to monitor this process in real time using digital storage oscilloscope function imbedded into the electroporation generator in some recent models. We found that pulses optimal for the electroporation of particular sample have pulse current and voltage shapes like it is shown in figure 1c where the part of the pulse with the oscillations corresponds to the accomplished electric breakdown of the intracellular membranes, and then there is a flat portion of the pulse current when the membranes are already broken and the DNA has entered the cells. The last pulse portion has the current higher as compared to the pulse current at the leading front edge since the generated electric pores in cell membranes add positive current flux to the initial pulse current.

We have to note that there are no commercial generators, except the design we have used for this project, enabling the real time monitoring and recording of the actual pulse current and voltage. Such monitoring is essential for identifying the optimal electrotransformation conditions for each particular new strain. The methodology of identifying is simple and quick not requiring the selection of the transformants and thus any plating. It does not require adding transforming DNA to the cell samples. The methodology is based on the recording of dynamics of the pulse current at the pulse current [1, 2, 10, 13]. Each positive pulse current flux recorded at the subsequent increase of the pulse voltage corresponds to a new cell membrane electroporation/electric membrane breakdown event for the cells with complex cellular morphology. If cells with simple morphology like *E. coli*, or Lactococci are used, then there is a non-linear sharp pulse current increase at a certain pulse voltage and further increase of the pulse voltage leads to the destroying of such samples with the over current.

The records collected in pulse current monitoring at different voltages are then used in the experiments with the transforming DNA added to the samples and the respective selection/plating procedures to select the desired recombinants. This is the first report on electrofusion of untreated cells of Gram (+) bacteria.

Chromosomal recombination

At low transformation frequencies a small proportion of naturally antibiotic resistant clones can be revealed along with the real antibiotic resistant transformants thus complicating and elongating the identification and purification of the transformants. Also, under selective conditions of culture transfers, the resistant mutants take advantage due to their relatively faster growth. The transformants have greater energy needs expressing antibiotic resistance genes and thus partially or even completely vanish from the samples with time. Therefore, the use of a single bacteriostatic antibiotic for the selection of transformants is complicated and time consuming for multi-step recombination experiments.

Working on the development of the efficient electrotransformation procedure for *Clostridium thermocellum* strains we initially used plasmid replicons comprising *mls* gene from pIM13 (M13761) for selection using two antibiotics, Em and Lincomycin (Lm). Selection using two antibiotics regardless the mechanism of antimicrobial action is the solution to ensure that the population of the transformants would not undergo change if the introduced construct is stable. The probability of a double mutation event leading to the development of a double antibiotic resistance phenotype is equal to ~10^{-13} - 10^{-16}. Practically, it is impossible to have more than 10^{10} cells in a single sample for transformation. Therefore the probability to select at least one spontaneous antibiotic resistant mutant is less than at least 10^{-3} - 10^{-6}. The problem we have faced later on was that the DNA fragment of the above *mls* resistance gene shared some level of homology with the chromosomal DNAs of antibiotic-sensitive recipient cells of both ATCC 27405 and DSM 1313. That made this gene useless for vectors intended for targeted integration in gene knock-out experiments.

Therefore we had to develop an approach to choose the right antibiotic resistance genes for engineering recombinants with selection using antibiotics. The approach was to first check a possible homology of the elements of the vector other than its sequences designated for integration with the chromosomal DNA of the proposed recipient. Both *erm*(B) and *cat* we used in this report do not have detectable homology with the chromosome of *Clostridium sp*. MT 351, which makes such genes promising candidates in other projects as related to targeted gene integration, knock-out, replacement and/or removal using this strain of Acetogens. Instead, the 324 bp fragment with the revealed homology to the chromosomal DNA of *Clostridium sp*. MT 351 was incorporated into our integration vectors to ensure the integration events in our electrofusion experiments.

Electrofusion

We have shown earlier that electric pulses causing efficient cell electrotransformation with plasmid DNA may also produce electrofusants when particular electroporation equipment is used. The intimate proximity of the genetic elements inside such cell products of electrofusion causes gene contact resulting in genetic recombination events. While a variety of recombination events take place, only those are detected depending on the particular selection conditions, for instance clones resistant to both Em and Cm at the same time. The overall time necessary to obtain such products of chromosomal recombination is significantly shorter as compared to performing subsequent integration events. This is the first report on electrofusion of untreated cells of Gram(+) bacteria.

Acknowledgments

The research was supported by the funds of Syngas Biofuels Energy, Inc. Syngas Biofuels Energy, Inc. is the sole manufacturer and distributor of the electroporation and electrofusion equipment. Please visit our website for more information: www.syngasbiofuelsenergy.com.

Reference

1. Tyurin MV. 1992. Electrotransformation of bacteria. Antibiotiki i Khimiotherapiya (Russ). 37(5):51-55.
2. Tyurin MV, Livshits VA. 1994. Some practical aspects of bacterial electrotransformation. Biologicheskiye Membrany (Russ.) 11:117–139
3. Tyurin MV, Doroshenko VG, Oparina NYu. 1997. Electrofusion of *Escherichia coli* cells. Membrane and Cell Biology 11: 121–129.
4. Imada C, Okanishi M, Okami Y. 1999. Intergenus protoplast fusion between *Streptomyces* and *Micromonospora* with reference to the distribution of parental characteristics in the fusants. J. Biosci. Bioeng. 88: 143-147.
5. Dai M, Ziesman S, Ratcliff T, Gill RT, Copley SD. 2005. Visualization of protoplast fusion and quantitation of recombination in fused protoplasts of auxotrophic strains of *Escherichia coli*. Metab. Eng. 7: 45-52.
6. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology. 2(5): a000414. Doi: 10.1101/cshperspect.a000414

7. Desai SG, Shaw AJ, Lynd LR, Tyurin MV. 2007. Thermophilic organisms for conversion of lingo-cellulocis biomass to ethanol. WO2007053600, PCT/US2006/042442**.** http://www.wipo.int/patentscope/search/en/detail.jsf?docId=WO2007053600&recNum=1&docAn=US2006042442&queryString=FP:(WO07053600)&maxRec=1
8. Tyurin MV, Lynd L, Wiegel J. 2006. Gene transfer systems for obligately anaerobic thermophilic bacteria. In: Eds: Rainey F. and Oren A. Extremophilic Microorganisms. Methods in Microbiology. Elsevier. 35, 307-328
9. Tyurin MV. 1990. Optimization of the method of isolation of microamounts of plasmid DNA from lactobacilli. Mol. Gen. Microbiol. Virusol. 3:29-31.
10. Tyurin MV, Voroshilova EB, Rostova IuG, Oparina NYu, Gusiatiner MM. 1998. Electrical response of inner membrane structures of Corynebacteria during electrotransformation. Mikrobiologia. 67(3):338-44.
11. Tyurin MV, Padda R, Huang KX, Wardwell S, Caprette D, Bennett GN. 2000. Electrotransformation of *Clostridium acetobutylicum* ATCC 824 using high voltage radio frequency modulated square pulses. J. Appl. Microbiol. 88(2):220-227.
12. Tyurin MV, Desai SG, Lynd LR. 2004. Electrotransformation of *Clostridium thermocellum*. Appl. Envir. Microbiol. 70: 883-890.
13. Tyurin MV, Sullivan CR, Lynd LR. 2005. Role of spontaneous current oscillations during high-efficiency electro-transformation of thermophilic anaerobes. Appl. Envir. Microbiol. 71: 8069-8076.