PERIODICO di MINERALOGIA Volume 88, No. 1, 2019

Biochemical and Genetic Characterization of *Lactobacillus Plantarum* cmgb – 1 Strain Used as Probiotic

TATIANA VASSU, DIANA SMARANDACHE, ILEANA STOICA, ELENA SASARMAN, D. FOLOGEA, F. MUSAT, ORTANSA CSUTAK, ANA – MARIA NOHIT, OANA IFTIME, RALUCA GHERASIM

MICROGEN - Center for Research in Genetics, Microbiology and Biotechnologies University of Bucharest, 1-3 Aleea Portocalilor, 77206 Bucharest, Romania.

Abstract

In this paper we present a biochemical, genetic characterization and a molecular improvement method of a lactic acid bacteria strain used as a probiotic. Morphophysiological, biochemical and genetic data confirmed that our strain belongs to the species Lactobacillus plantarum and was introduced in our culture collection under the name CMGB-1. Plasmid profile analysis revealed two distinct DNA molecular species.

In order to develop a molecular improvement technique of lactobacilli, we have performed an electrotransfer experiment (under optimized conditions) of two plasmid vectors – pC194 and pHV41, in the Lactobacillus strain.

The plasmid analysis of the pC194-transformants confirmed the vector's presence, along with the two natural plasmids. pHV41-transformants presented only one of the natural plasmids, and a different, hybrid molecule, presumed to have been generated by means of a genetic recombination event between pHV41 and the second natural plasmid.

Plasmid stability assays confirmed the presence of the two vectors on the electrotransformants even after 100 cell generations.

Keywords: *Lactobacillus*, probiotics, molar percentage G+C, electrotransformation, pC194, pHV41

Introduction

During the last decades, due to the beneficial impact of microorganisms used as probiotics on human and animal health, progressive attention has been focused on biological and molecular characterization and improvement of such microbes. Most data accumulated so far have revealed that lactic acid bacteria (LAB) are a major microbial group having beneficial effects on the health status of host organisms.

The beneficial effects of probiotics are based on many and mostly incompletely known mechanisms, out of which the most important are: inhibition of intestinal pathogenic bacteria by production of organic acids and by pH reduction, prevention of pathogens' adherence to the intestinal mucosa, production of bacteriocins [1], increase of food

assimilation and of detoxification processes, immune stimulation and decrease of heart failure and cancer incidence [2].

Modern techniques in molecular biology provide a series of possibilities for the improvement of commercially important lactobacilli. Of special interest are the methods of DNA transfer by electroporation. Due to the vast heterogenity within the *Lactobacillus* genus, electroporation protocols need to be optimized for each species and strain. Effects of different parameters (e.g. growth phase, cell density, cell wall weakening agent, DNA concentration, electric field parameters) on electrotransformation efficiency have been assessed [4, 5, 6, 7, 8, 9].

In recent years, application of recombinant DNA technology and DNA transfer techniques have allowed accumulation of molecular information on the LAB genome, as well as on the genetic improvement experiments on these bacteria [3]. As LAB proved to be very difficult to transform through commonly used techniques (conjugation, transduction, transformation), a lot of studies focused on optimizing electrotransfer techniques on such bacterial strains over the last decade [10, 21]. On the other hand, genome analysis in LAB, as well as genetic improvement studies, require sets of cloning and/or expression vectors [11, 12, 13].

Our study deals with biochemical and genetic characterization of a new LAB strain used as probiotic. We also report results of electromediated gene transfer experiments with plasmid cloning vectors pC194 and pHV41 on this strain (**Table1**).

Table 1. Characteristics of vectors used in electroporation.

Vector	Size (kbp)	Phenotype	Unique restriction sites
pC194	2.9	Cm^r (30 μg/ml)	*Bgl I, Hae III, Hind III si Hpa I* Ehrlich, 1977 (18)
pHV41	9	Cm^r (30 μg/ml) Km^r (20 μg/ml)	*Bgl II,Bam HI, Eco RI, Xba I* Michel, 1980 (19)

Materials and Methods

Bacterial Strains and Growth Media

The *Lactobacillus* sp. strain used in this study has been previously isolated from calf ruminal liquid on solid/soft Man-Rogosa-Sharpe media (MRS) (g/L, peptone 10.0, meat extract 8.0, yeast extract 4.0, D(+) glucose 20.0, di-potassium hydrogen phosphate 2.0, tween-80 1.0, di-ammonium hydrogen citrate 2.0, sodium acetate 5.0, magnesium sulphate 0.2, manganese sulphate 0.04 supplemented with 14.0 or 7.0g agar-agar for solid or, respectively, soft MRS) [14]. Strain purity has been verified by three successive subcloning passages from a single colony. The culture has been grown at 37^{o}C in microaerophilic conditions without shaking.

Bacillus subtilis strains carrying the two plasmid vectors (pC194 and pHV41) were grown in Luria-Bertani (LB) broth, pH 7.2 (g/L sodium chloride 10.0, tryptone 10.0, yeast extract 5.0) supplemented with cloramphenicol (Cm) 30 μg/mL for pC194 and, respectively, Cm 30 μg/mL and kanamycin (Km) 20 μg/mL for pHV41, at 37^{o}C in aerobic conditions, with shaking.

Escherichia coli K-12 V517, carrying several plasmid DNA species used as molecular weight markers in agarose gel electrophoresis (**Table 2**), was grown in LB broth under the same conditions as *B.subtilis* strains, but with no antibiotic added (as this strain has no antibioresistance gene, neither on the chromosome, nor on the plasmids).

For long-term preservation, at –70^{o}C, bacterial strains were stored on MRS broth (for LAB strain) or LB broth (for *B. subtilis* and *E. coli* strains) supplemented with 20% glycerol.

Table 2. Size and weight of the eight distinct molecular species of plasmid DNA from *E.coli* V517.

Name	Size [kbp]	Molecular weight [Md]
pVA517A	53.7	35.8
pVA517B	7.215	4.81
pVA517C	5.46	3.64
pVA517D	5.07	3.38
pVA517E	4.005	2.67
pVA517F	3.03	2.02
pVA517G	2.64	1.76
pVA517H	2.055	1.37

Morphological, Physiological and Biochemical Tests

Gram staining, colony morphology, catalase activity, spore formation, cell motility, nitrate reduction and gas production from glucose were determined according to methods for LAB [15]. Other tests included starch hydrolysis, tetrazolium salts reduction, exopolysaccharides production, protease activity, lypolitic activity, resistance to biliary salts and to several antibiotics. Beside all these tests, for the taxonomic identification of the LAB strain, we also used API 50 CHL galleries (API Biomerieux), according to the manufacturer's recommendations and with *Lactobacillus plantarum* ATCC 8014 as reference strain.

Growth in MRS was tested at different temperatures between 5 – 60^{o}C and different pH values (3.0 – 9.6). Tolerance to salt concentrations was determined by monitoring cell growth on thiamin-supplemented media (APT) (g/L peptone from casein 12.5; yeast extract 7.5; D (+) glucose 10.0; sodium chloride 5.0; tri-sodium citrate 5.0; di-potassium hydrogen phosphate 5.0; Tween 80 0.2; magnesium sulphate 0.8; manganese chloride 0.14; iron (II) sulphate 0.04; thiaminium dichloride 0.001, pH 6-7) (*Deibel, 1957; Evans, 1951*), containing various sodium chloride concentrations (0.5% -12%).

Isolation and Purification of Genomic DNA

Genomic DNA of the LAB strain was isolated from 3 mL of overnight cultures (37^{o}C in liquid MRS). Harvested cells has been washed in TEG (Tris 25mM, EDTA 10mM, glucose 50mM, pH 8.0) and resuspended in 500 μL TEG supplemented with 30mg/mL lysozyme. Cell lysis was obtained after adding 50 μl SDS 10 % and incubation for at least 2h at 37^{o}C. The cell lysate was treated (for 20 min at –20^{o}C) with 40 μL KCl 2.5M. After centrifugation the supernatant was extracted once with an equal volume of phenol:clorophorm:isoamyl alcohol (25:25:1) and then with chlorophorm:isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol at –20^{0}C [16, 17].

Plasmid DNA Analysis

The two vectors used in the electrotransformation experiments - pC194 (2.9 Kbp Cm^R) and, respectively, pHV41 (9.0 Kbp Cm^R Km^R) were isolated from the two harboring *Bacillus subtilis* strains, grown on LB broth supplemented with the required antibiotic (18).

For the LAB strain we performed plasmid DNA analysis, both before (to determine the plasmid profile of the recipient LAB strain) and after the electrotransfer experiment (to verify the vector's presence and, respectively, vectors' stability in the transformants), grown in MRS broth.

In all the cases, plasmid DNA was isolated and purified using a modified alkaline-lysis technique [*Birnboim (19); Ish-Horowicz (20*)]. The lysozyme concentration in TEG buffer (Tris 25mM, EDTA 10mM, glucose 50mM pH 8.5) was 30 mg/mL for the LAB strain and, 20 mg/mL for the *B.subtilis* strains respectively. Cell lysis was achieved using NaOH/SDS solution (pH 12.5) and incubation 20 min at 37^{o}C followed by 10 min on ice. Protein removal was carried out with phenol followed by chloroform: isoamyl alcohol (24:1) extraction. Plasmid DNA was precipitated with two volumes of 95% cold ethanol, and the pellet was then resuspended in 20-30 μL of TE (Tris 10 mM, EDTA 1 mM pH 8.0).

Nucleic Acids Electrophoresis

Electrophoretic analysis of genomic and, respectively, plasmid DNA samples was performed using horizontal submerse agarose gel 0.8-1 % in TBE buffer (Tris 89mM, boric acid 89mM, EDTA 5mM, pH 8.5). *Eco*RI-digested DNA was electrophoresed in 1% agarose gel. In both cases, electrophoresis was run at 2.5 V/cm, and DNA was stained with ethidium bromide 0.5 μg/mL [17].

Spectrophotometric Analysis of Nucleic Acids

Spectrophotometric analysis of chromosomal and plasmid DNA was performed with an UV-VIS ULTROSPEC 3000 (Pharmacia-LKB) spectrophotometer. Absorption spectra were obtained for wavelength ranging between 200 and 320 nm. Samples' purity was estimated from A_{260} (nucleic acids), A_{280} (proteins) and A_{230} (polysaccharides). Contamination was considered to be minimum for A_{260}/A_{280} values ranging between 1.8 and 2.0 and, respectively, greater than 2.0 for A_{260}/A_{230}.

Determination of Molar Percentage of Guanine Plus Cytosine

Estimation of the molar percentage of guanine plus cytosine (mol% GC) in the chromosomal DNA of the LAB strain and, the reference strain *Lactobacillus plantarum* ATCC 8014 was accomplished by the thermal denaturation technique respectively. The same spectrophotometer was used, but equipped with specially designed cuvettes holder with Peltier system. The temperature was increased from 20^{o}C to 100^{o}C with an increment of 3^{o}C / min and the DNA absorbance values at λ = 260 nm have been continuously monitored. Molar percentage of GC was determined using Owen's formula :

% mol GC = 2,08 x Tm - 106,4 , (Owen, 1985)

The Electroporation Method

Electroporation was performed with a "home made" electroporation device, having an output as an exponential decay waveform. The recipient LAB strain was grown in MRS broth with 20 mM DL-threonine up to OD_{600} = 0.6, harvested and washed twice in the electroporation buffer (sucrose 952 mM, $MgCl_2 \cdot 6H_2O$ 3.5 mM) and resuspended in the same buffer (1 tenth of the original volume). A volume of 200 μL cell suspension (about 10^8 cells/mL) was transferred into BioRad cuvettes (2 mm aperture), chilled on ice for 5 min and mixed with 20 μL ice chilled vector containing 8.4 μg DNA of pHV41 and, respectively, 13.8 μg DNA of pC194. Subsequently, electrical discharge was performed (external resistance 600-ohms and capacity 30μF). Electrical field was varied between 0-8 kV cm^{-1}.

After electroporation, in order to accomplish cell recovery, the [cells + DNA] mixture was incubated for 2 h at 37°C in MRS broth supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$. For the electrotransformants' screening, cells were then plated on MRS supplemented with low concentration of antibiotics (i.e. 10μg/mL Cm and 6.6 μg/mL Km) [7]. Subsequent passages have been made on regular antibiotic concentrations, e.g. 30 μg/mL Cm and 20 μg/mL Km.

Appropriate negative controls have been used, e.g. "no DNA" non-electroporated samples. Cell viability after electroporation was estimated and the whole electroporation experiment was repeated several times.

Digestion with Restriction Endonucleases

For molecular confirmation of the electrotransformation event, we reisolated plasmid DNA from the electrotransformants. Restriction digestion was performed with 3U *Eco*RI (SIGMA)/μg DNA for 4h at 37°C [17] and electrophoretic patterns of the original vectors and the reisolated ones have been compared.

Estimation of Plasmid Stability

In order to estimate vectors' stability into the transformed LAB strain we used an adapted method [22, 23]. Transformants were grown to stationary phase on MRS with antibiotics, then diluted into nonselective MRS (no antibiotics) and grown to saturation at 37°C, in repeated passages, up to 100 cell generations. Culture samples were taken for each generation and spread on nonselective medium, after suitable dilution to give approximately 100 colonies per plate. Incubation at 37°C was continued for 1-2 days and replica plating was performed to test for Cm^R and Km^R.

Persistence of transformant DNA in the electrotransformants was checked both phenotypically (Cm^R and, respectively, Cm^R Km^R) and genetically by plasmid DNA analysis and agarose gel electrophoresis.

Results and Discussions

Morphological, Physiological and Biochemical Characterization of the LAB Isolate

Isolated LAB strain proved to be a microaerophylic, Gram-positive, catalase – negative, non-spore-forming rod. It produces lactic acid as a major fermentation product from glucose. As it is microaerophylic, when cultivated in liquid media (MRS), this strain forms turbidity and sediments. Microscopically, cells consist of short to long rods that appear as single cells, in pairs and in short chains. Surface colonies on agar plates are 0.5 to 2.0 mm in diameter, circular, lenticular, creamy-white. It grows at temperatures ranging from 37°C to 42°C. Acid is produced without gas formation from arabinose, ribose, sorbitol, galactose, dextrin, dextran, mannose, glucose, maltose, sucrose, fructose, mannitol and lactose. No acid formation from: sorbose, raffinose, xylose, starch was detected. Acid production from rhamnose is variable. Strains are tolerant up to 8.0% NaCl and present multiple resistance to antibiotics.

All these results as well as the API 50 CHL data, made us presume that our strain belongs to the *Lactobacillus plantarum* (**Table 3**) and, therefore, will be referred as *L.plantarum* CMGB-1 in the present paper.

Table 3. Characteristics of analyzed (CMGB - 1) and reference (ATCC 8014) *Lactobacillus* strains.

CHARACTERISTICS	STRAINS	
	ATCC 8014	**CMGB -1**
MORFOPHYSIOLOGICAL CHARACTERISTICS		
Pigments	White cream	White cream
Cell shape	Single rods, in pairs and short chains	Single rods, in pairs and short chains
Gram stain	+	+
Growth on solid MRS	Circular to slight irregular and smooth	Circular to slight irregular and smooth
Growth on liquid MRS	Uniform turbidity with sediment	Uniform turbidity with sediment
Optimal pH	5.0- 7.0	4.0- 8.0
Temperature (^{0}C)	30 – 37	28 – 42
Tolerance to NaCl (% NaCl)	4 – 8	0.5 - 8
BIOCHEMICAL CHARACTERISTICS		
Acid formation from:		
L – Arabinose	+	+
Ribose	+	+
D- Xylose	–	–
Galactose	+	+
D- Glucose	+	+
D-Fructose	+	+
D- Mannose	+	+
Rhamnose	–	–/ +
Mannitol	+	+
Sorbitol	+	+
Sorbose	–	–
Raffinose	+	–
Maltose	+	+
Lactose	+	+
Sucrose	+	+
Dextrin	ND	+
Dextran	ND	+
Fermentative type	Homofermentative	Homofermentative
Enzyme activity		
Catalase	–	–
Amylase	–	–
Nitratreductase	ND	–
Lysine decarboxylase	ND	+
Arginine decarboxylase	ND	–
Ornithine decarboxylase	+	+
Lipase	ND	–
Bile resistance	ND	+
H_2S production	ND	+
ANTIBIOTIC RESISIANCE	ND	Resistant to β – lactamic antibiotics, cefalosporines, quinolones, tetracycline; sensitive to aminoglycosidic and macrolidic antibiotics.
TAXONOMICAL IDENTIFICATION		
Family	*Lactobacillaceae*	*Lactobacillaceae*
Genus	*Lactobacillus*	*Lactobacillus*
Species	*Lactobacillus plantarum*	*Lactobacillus plantarum*

Note: ND + not determined, += positive results, -= negative results, -/ += variable results.

Determination of Guanine + Cytosine Content of the Genomic DNA (mol% GC)

Spectrophotometric (**Figure 1**) and electrophoretic analysis (**Figure 2**) revealed that chromosomal DNA had adequate concentration (5.34 μg/μL), purity (A_{260}/A_{280} = 1.88; A_{260}/A_{230} = 2.33) and high level of molecular integrity, therefore was suitable for further manipulations.

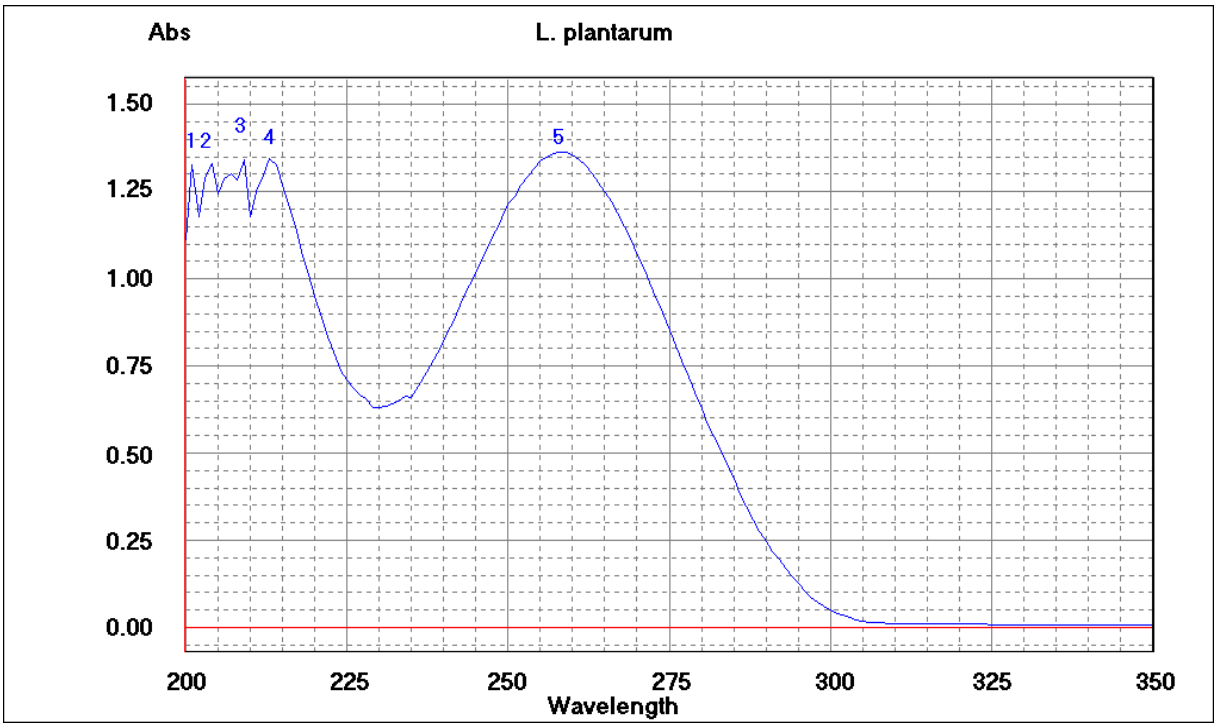

Figure 1. Absorbtion spectrum between λ = 200 – 350 nm of L.plantarum CMGB-1 genomic DNA.

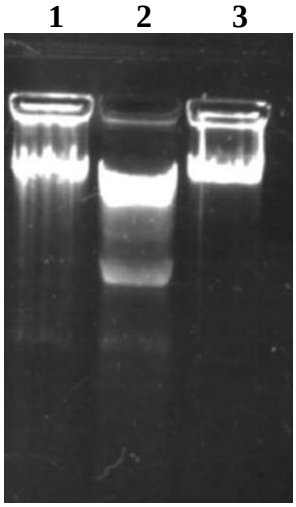

Figure 2. Agarose gel electrophoresis of chromosomal DNA Lanes: 1 L.plantarum - CMGB-1; 2-L. acidophilus CMGB –3; 3- L. plantarum ATCC 8014.

Estimation of molar percentage of guanine + cytosine in chromosomal DNA was performed using the thermal denaturation technique. *Lactobacillus plantarum* ATCC 8014 was used as test strain.

Our results on the thermal denaturation of *L.plantarum* CMGB-1 chromosomal DNA (**Figure 3**) showed that the hyperchromic shift profile presents a relatively constant increase between 20 ^{o}C – 100^{o}C.

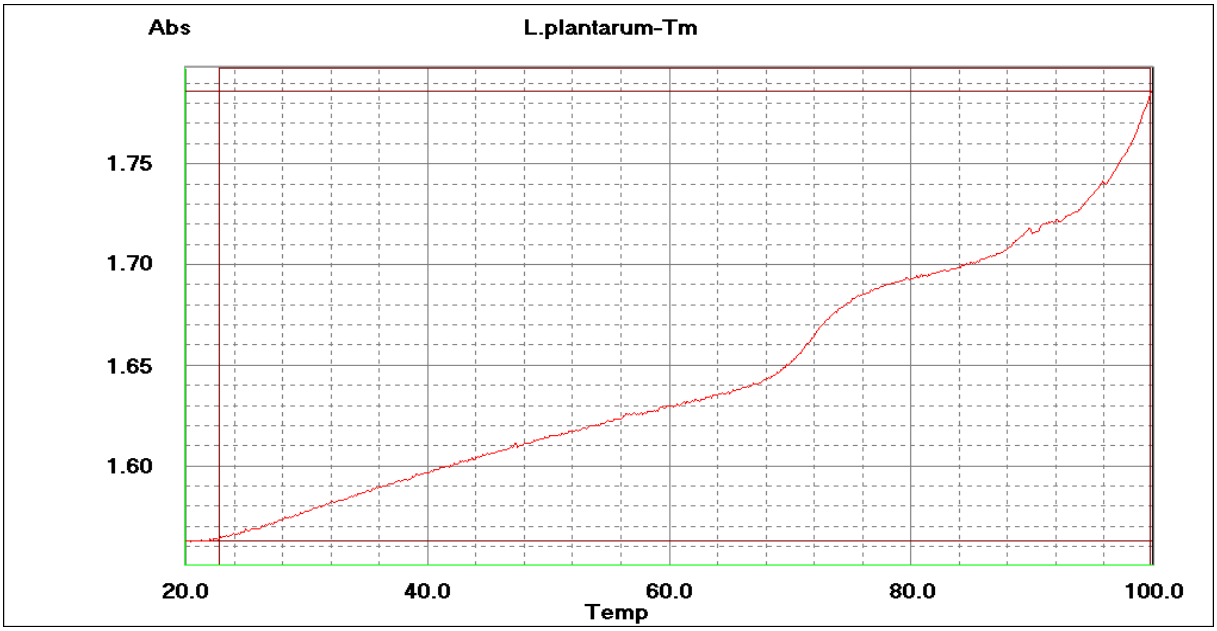

Figure 3. Hyperchromic shift in the thermal denaturation of L.plantarum CMGB-1 genomic DNA.

L. plantarum ATCC 8014	Tm = 70.86 ^{o}C mol %	GC = 40.98
L. plantarum CMGB-1	Tm = 72.30 ^{o}C mol %	GC = 43.98

As several papers (15, 16) give mol% GC ranging between 41 and 46, we predict that our results on the ATCC strain confirm the accuracy of the technique we used, as well as the validity of the mol% GC for the CMGB-1 strain. On the other hand, the two natural plasmids present in the CMGB-1 strain are low copy plasmids, so that they have no significant influence on the mol% G+C in the total bacterial DNA.

Our data regarding the G+C content in chromosomal DNA places the tested LAB strain (CMGB-1) in the *L.plantarum* species, thus confirming our microbiological and biochemical results. We also underline that the mol% GC is nowadays considered to be a very important taxonomic parameter and is listed in most international manuals on systematic and determinative bacteriology.

Our results also allow us to consider that the adapted protocol we used for the isolation and purification of bacterial genomic DNA is very accurate, providing DNA samples having high level of molecular integrity and minimal contamination degree with proteins, sugars and low weight RNAs.

Electrotransformation Results

In order to develop cloning vector systems for biotechnological *Lactobacillus* strains, we have first isolated and purified pC194 and pHV41 as plasmid vectors from two *B. subtilis* strains.

The two purified vector samples were spectrophotometrically analyzed (**Table 4**) and proved to have minimum protein contamination. DNA concentration was 5.5 μg μL^{-1} for pC194 and, respectively, 3.4 μg μL^{-1} for pHV41. Both these values are considered to be high enough for electroporation experiments (min.10μg plasmid DNA /sample).

Table 4. Spectrophotometric analysis of pHV41 and, respectively, pC194 samples.

Plasmid	A_{260}/A_{280}	DNA concentration (μg μl^{-1})
pC194	2.120	5.5
pHV41	2.298	3.4

Results of the electroporation experiment are presented in **Figure 4** and **Figure 5** and demonstrate that the most important parameter – transformation frequency – varies with vector type and field intensity. Transformed colonies were first selected after incubation on media supplemented with appropriate antibiotics in lower concentrations (10 μg mL^{-1} chloramphenicol and 6.6 μg mL^{-1} kanamycin, respectively). Transformants were further grown on MRS supplemented with 30 μg mL^{-1} chloramphenicol and 20 μg mL^{-1} kanamycin, respectively. At these antibiotic concentrations no spontaneous antibiotic-resistant mutants have emerged on control plates. The *L. plantarum* GMGB - 1 strain presented a higher transformation frequency with pC194 than with pHV41. For pC194 the maximum frequency was at 7.0 kV cm^{-1} and for pHV41 at 8.0 kV cm^{-1} (**Figure 4** and **Figure 5**).

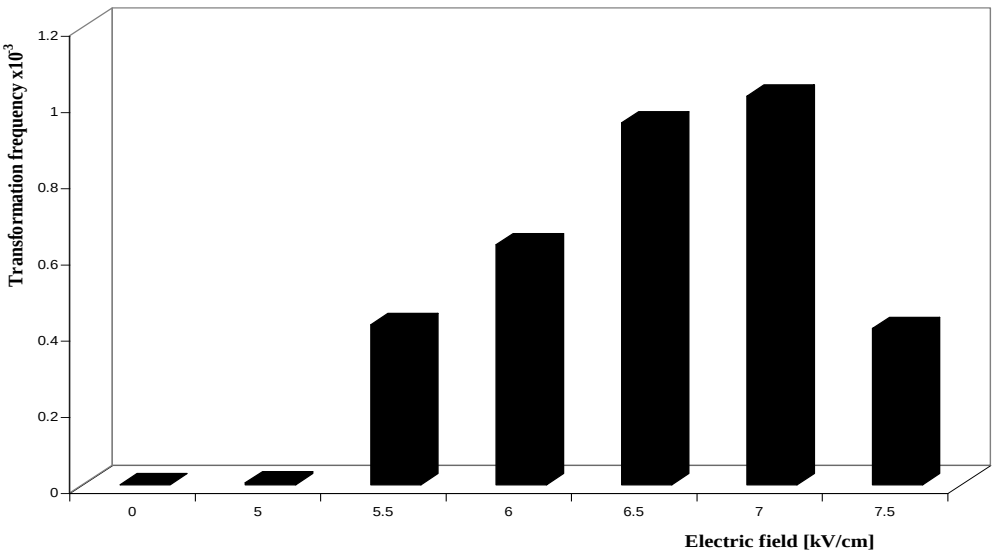

Figure 4. Electrotransformation frequency vs electric field for L. plantarum CMGB-1 using pC194 vector (Conditions: Cell suspension 200μl $4.7x10^8$cell/ml; DNA pC194- 13.8μg; Electric field 0-8KV).

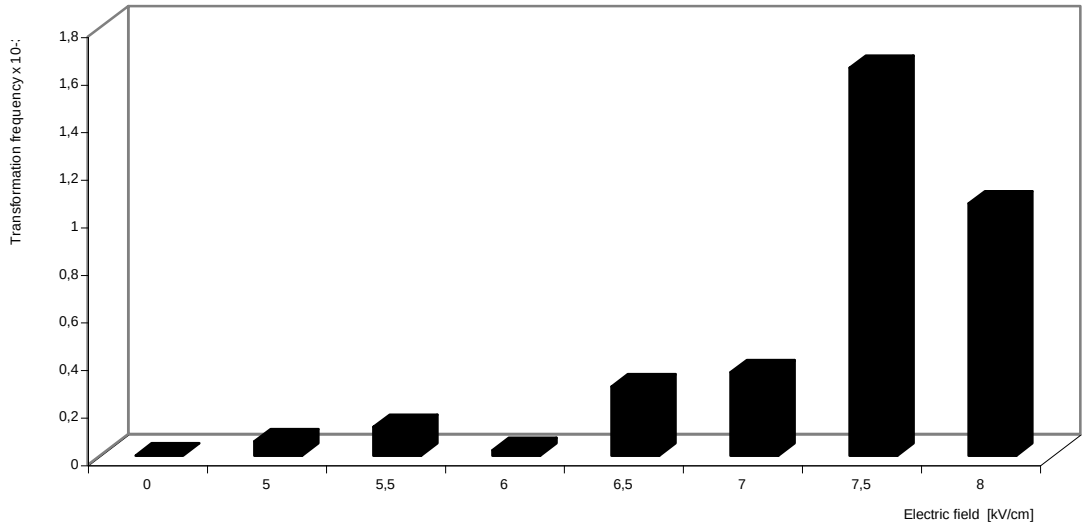

Figure 5. Electrotransformation frequency vs electric field for L. plantarum CMGB-1 using pHV 41 vector (Conditions: Cell suspension 200µl 4.7x10^8cell/ml; DNA pHV41- 8.0µg; Electric field 0-8KV).

It is important to emphasize that in both experiments the maximum transformation frequencies have been obtained at rather low cell viability values (15-20%), related to 7-7.5kV cm^{-1} (data not shown). Compared to our data, Aymerich et al. [5] reported best electrotransformation results with a *Lactobacillus curvatus* strain at 50% cell survival.

Compared to our results, highest transformation frequency at 7 $kVcm^{-1}$ for pC194 and at 7.5 $kVcm^{-1}$ for pHV41 respectively, other papers communicated the same voltage values, e.g. 7 $kVcm^{-1}$ for *Lactobacillus curvatus* and *L.sake* [5].

The most significant transformation frequencies for the pC194 and pHV41 plasmids were noticed at 7 kV cm^{-1} for the former, at 7.5 $kVcm^{-1}$ for the latter respectively. Similar results, e.g. voltage values of 7kV cm^{-1} corresponding to the highest transformation frequency in *Lactobacillus curvatus* and *L. sake* were reported in other papers.

The plasmid profile of the recipient *L.plantarum* CMGB-1 strain consists of two natural plasmids of approx. 4.7 and, respectively, 8.0 Kbp (**Figure 6** and **Figure 7**). Molecular analysis of pC194-electrotransformants reveals the presence of the transformant pC194, along with the two natural plasmids (**Figure 6**).

The pHV41-transformants gel electrophoresis (**Figure 7**) revealed only two bands: the natural 4.7 Kbp plasmid and a band with intermediate size between pHV41 (approx. 9.0 Kbp) and the 8 Kbp-natural plasmid. We can presume that after entering the recipient cells, a recombination process might have taken place between the two plasmid species generating a new one that confers the antibiotic resistance to the receptor strain.

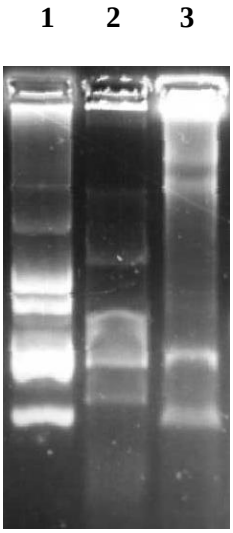

Figure 6. Identification of pC194 plasmid in electrotransformed L.plantarum CMGB –1. The DNA was separated on a 0.7% agarose gel, stained with EtBr. From left to right E. coli K12 V517 (lane 1); transformed cells (lane 2) ; B. subtilis pC194 (lane 3).

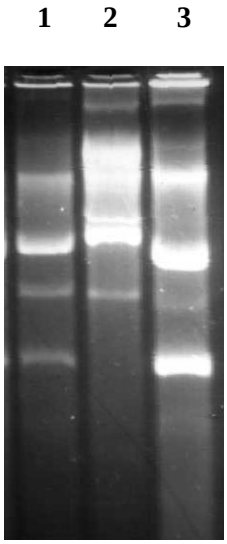

Figure 7. Identification of pHV41 plasmid in electrotransformed L. plantarum CMGB – 1. The DNA was separated on a 0.7% agarose gel stained with EtBr. From left to right: transformed cells (lane 1); B. subtilis pHV41 (lane 2); L. plantarum CMGB – 1 – receptor (lane 3).

We emphasize that the recombination-generated molecular hybrid identified in pHV41-transformants might contain three genetic regions: ***(i)*** cryptic DNA fragments from the 8 Kbp-natural plasmid; ***(ii)*** genes coding for Cm^R and Km^R from pHV41 vector; and ***(iii)*** ***rep-inc*** sequences *("replicon-type"*) either from pHV41 or from the 8 Kbp-natural plasmid.

Taking into account that *E.coli* is one of the original hosts of pHV41 and also the high stability of this rather big hybrid plasmid (~ 8-9 Kbp) even after 100 generations, the second configuration seems to be more probable than the first one.

Vector Stability in Electrotransformants

Agarose gel electrophoresis (**Figure 6** and **Figure 7**) confirmed the presence of the transformant vectors even after 100 cell generations and even in a non-selective media. This fact demonstrates the high stability of the two vector species into the recipient strain *L.plantarum* CMGB-1.

Plasmid DNA analysis for one electrotransformant obtained with pHV41 vector (noted as *Lactobacillus plantarum* A1) was performed by restriction analysis (**Figure 8**). We found *Eco*RI to be the best enzyme, i.e. it gave reproducible digestion patterns and complete DNA digestion into suitable number of fragments. Comparative analysis of *Eco* RI digested vectors and plasmid DNA from transformants confirmed the successful transfer of pHV41 into the receptor strain *Lactobacillus plantarum* CMGB – 1 (**Figure 8**).

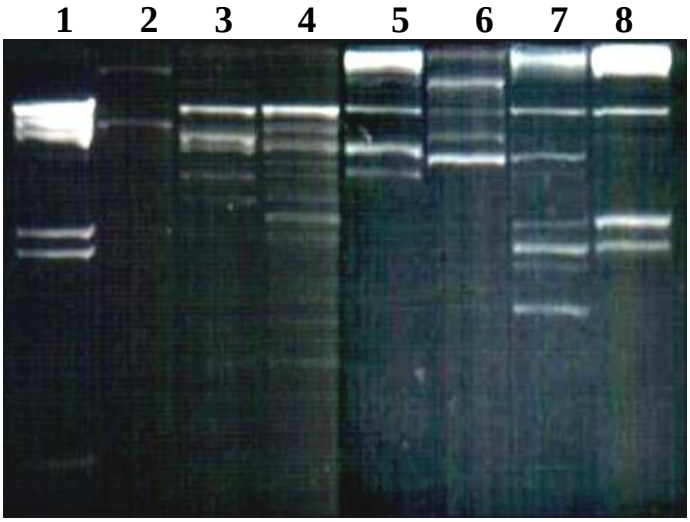

Figure 8. Agarose gel electrophoresis of restriction digests of a pHV41 electrotransformant. From left to right: λ–DNA/Hind III (lane 1); pHV41/ Eco RI (lane 2); plasmids from transformant/EcoRI (lane 3); plasmids from L.plantarum GML-1/EcoR I (lane 4); undigested plasmids from the transformant (lane 5); undigested pHV41 (lane 6); plasmids from E.coli K-12 V517 (lane 7); undigested plasmids from receptor L.plantarum CMGB –1 (lane 8).

Although the molecular mechanism for the plasmid DNA electrotransfer through bacterial cell membrane remains still unclear, we confirm and extend earlier observation that electroporation is an efficient technique to promote gene transfer in biotechnological important microorganisms especially in lactic acid bacteria [24, 25].

References

1. A. M. P. GOMES, F. X. MALCATA, *J. Appl. Microbiol.*, **85**, 893 – 848 (1998).
2. L. DE VUYST, E. J. VANDAMME, *Antimicrobial potential of lactic acid bacteria*, in *Bacteriocins of Lactic Acid Bacteria*, Ed. De Vuyst, L. and Vandamme, E.J., London: Chapman and Hall, 91–142 (1994).

3. T. F. O'SULLIVAN, G. F. FITZGERALD, *J. Appl. Microbiol.*, **86**, 275 –283 (1999).
4. N. .M. CALVIN, P. C. HANAWALT, *J. Microbiol*., **170**, 2796-2801, (1988).
5. M. T. AYMERICH, M. HUGAS, M. GARRIGA, R. F. VOGEL, J. M. MONFORT, *J. Appl. Bacteriol.*, **75**, 320-325 (1993).
6. Q. W. MING, C. .M. RUSH, J. M. NORMAN, L. M. HAFNER, J. ROLAND, R. J.. EPPING, P. TIMMS, *Elsevier J. Microbiol. Meth*., **21**, 97-109 (1995).
7. W. AUKRUST, M. B. BRURBERG I. F. NES, *Transformation of Lactobacillus by electroporation,* in *Methods in Molecular Biology 47, Electroporation Protocols for Microorganisms.*, Ed. By Jac A Nickoloff. Humana Press Totowa, New Jersey, pp. 201-208, 1995.
8. F. BERTHIER, M. ZAGOREC, M. CHAMPOMIER-VERGES, S. D. EHRLICH, F.. MOREL-EVILLE, *Microbiol*., **142**, 1273-1279 (1996).
9. N. ITOH, T. KOUZAI, Y. KOIDE, *Biosci. Biotech.*, **58**, 1306-1308 (1994).
10. S. SIXOU, N. EYNARD, , J. M. ESCONBAS, E. WERNER , J. TEISSIE, *Biochim. Biophys. Acta*., **1088**, 135-138 (1991).
11. M. B. M. JOS, VAN DER VOSSEN, J. KOK, D. A. VENEMA, *Appl. Env. Microbiol.*, **50**, 540-542 (1985).
12. K. SCHERWITZ HARMON, L. L. MCKAY, *Appl. Env. Microbiol.*, **53**, 1171-1174 (1987).
13. J. K. THOMPSON, K. J. MCCONVILLE, C. MCREYNOLDS, M. .A. COLLINS, *Appl. Env. Microbiol.*, **65**, 1910-1914 (1999).
14. J. C. DE MAN, M. ROGOSA, M. E. SHARPE, *J. Appl. Bacteriol*, **23**, 130-135, (1960).
15. H. DE ROISSART, F. M. LUQUET, *Bacteries Lactiques, Aspect fundamentaux et technologiques tome 1*, Ed. Lorica, pp. 380-410, 1994.

16. F. AUSUBEL, *Short protocols in molecular biology*, Third Edition, Ed. J. Wilei & Sons, Inc., 1995.
17. T. MANIATIS, E. F. FRITSCH, J. SAMBROOK, *Molecular Cloning. A Laboraory Manual*, Cold Spring Harbor Laboratory, USA, 1982.
18. S. D. EHRLICH, *Proc. Natl. Acad.. Sci. USA*, **74**, 1680-1682 (1977).
19. H. C. BIRBOIM, J. DOLY, *Nucleic Acids Research*, **7,** 1513-1523 (1979).
20. D. ISH-HOROWICZ, F. J. BURKE, *Nucleic Acids Res*., **9**, 2989-2999 (1981).
21. A. ARGNANI, R. J. LEER, N. VAN LUIJK, P. H. POUWELS, *Microbiol*., **142**, 109-114 (1996).
22. A. P. GLEAVE, A. MOUNTAIN, C. M. THOMAS, *J. Gen. Microbiol.*, **136**, 905-912 (1990).
23. J. M. WELLS, P. W. WILSON, R. W. F. LE PAGE, *J. Appl. Bacteriol.*, **74**, 629-636 (1993).
24. D. VUJAKLIJA, J. DAVIES, *J Antibiotics*, **48**, 635 – 637 (1995).
25. D. J. O'SULLIVAN, T. R. KLAENHAMMER, *Appl. Env. Microbiol.*, **59**, 2730–2733 (1993).