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RŪTA KANANAVIČIŪTĖ

DEVELOPMENT OF A GENETIC TRANSFORMATION SYSTEM FOR BACTERIA OF THE GENUS *GEOBACILLUS*

Summary of doctoral dissertation

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Scientific consultant:

prof. dr. Donaldas Čitavičius (Vilnius University, Biomedical sciences, Biology – 01B).

The defense council:

Chairman:

dr. Saulius Serva (Vilnius University, Biomedical sciences, Biology – 01B),

Members:

dr. Saulius Kulakauskas (Micalis institute, French National Institute for Agricultural Research, Biomedical sciences, Biology – 01B),

dr. Vida Časaitė (Vilnius University, Biomedical sciences, Biology – 01B),

prof. habil. dr. Aniolas Sruoga (Vytautas Magnus University, Biomedical sciences, Biology – 01B),

dr. Vytautas Boleslovas Melvydas (Nature Research Centre, Biomedical sciences, Biology – 01B).

The thesis defense will take place at 15:00 on January 15th, 2016 at the open meeting of the Council of Biology in the prof. J. Dagio auditorium at the Faculty of Natural Sciences, Vilnius University.

Address: M.K. Čiurlionio g. 21/27, LT-03101 Vilnius, Lithuania.

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VILNIAUS UNIVERSITETAS

RŪTA KANANAVIČIŪTĖ

GEOBACILLUS GENTIES BAKTERIJŲ GENETINĖS TRANSFORMACIJOS SISTEMOS KŪRIMAS

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Biomedicinos mokslai, Biologija (01B)

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Mokslinis konsultantas – prof. dr. Donaldas Čitavičius (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B).

Disertacija ginama Vilniaus universiteto Biologijos mokslo krypties taryboje:

Pirmininkas:

dr. Saulius Serva (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B).

Nariai:

dr. Saulius Kulakauskas (Micalis institutas, Prancūzijos nacionalinis žemės ūkio tyrimų institutas, biomedicinos mokslai, biologija – 01B),

dr. Vida Časaitė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B),

prof. habil. dr. Aniolas Sruoga (Vytauto Didžiojo universitetas, biomedicinos mokslai, biologija – 01B),

dr. Vytautas Boleslovas Melvydas (Gamtos tyrimų centras, biomedicinos mokslai, biologija – 01B).

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INTRODUCTION

Bacteria of the genus *Geobacillus* are Gram positive, endospore-forming thermophiles. Since their discovery, geobacilli have attracted the attention of many scientists as a model organism for investigation of the physiology and metabolism of thermophilic organisms. Moreover, these bacteria were shown to have tremendous possibilities to be used in various biotechnological areas.

First of all, geobacilli are source of many thermostable enzymes (proteases, cellulases, DNA polymerases, restriction endonucleases and many others), which could be useful for various industrial and research purposes (McMullan et al., 2004, Mead at al., 1991, Vellore et al., 2004). In some biotechnological processes, not only purified enzymes, but also the whole *Geobacillus* cells could be used, which synthesize all biocatalysts required for bioconversion and production of a desirable product.

In spite of huge interest in geobacilli, there are only several examples of their successful employment. The main reason that retards the research and application of these thermophiles is the lack of reliable and effective genetic transformation systems. Genetic manipulation tools and techniques are prerequisite to manipulate their genome, introduce or knockout a particular gene and subsequently to acquire new knowledge about their physiology or enhance the output of their bioprocesses in industry.

Genetic transformation system of a bacterial strain consist of (1) vector, which serves as a carrier of a particular gene or other DNA fragment and (2) DNA transfer method, which enables the entry of the vector to the cell. Currently the choice of vectors suitable for geobacilli is limited and genetic transfer protocols are developed only for several *Geobacillus* strains.

Therefore, this work was designed to widen genetic transformation possibilities for these relevant thermophilic bacteria of the genus *Geobacillus*.

The aim of the work:

- Development of the genetic system for transformation of *Geobacillus* spp.

The following main tasks of the work:

- Search and characterization of new low-molecular-size plasmids of *Geobacillus* spp.

- Employment of newly characterized plasmids in the construction of *Geobacillus* spp. shuttle vector.
- Elaboration of the DNA transfer method.

The defensive statements:

- Newly isolated *Geobacillus* spp. strains can serve as a source of novel plasmids, that differ from all other described bacterial plasmids and which can provide new insights into plasmid biology area.
- Characterization of novel plasmids expands the assortment of molecular tools suitable for *Geobacillus* spp., as they can be employed for construction of vectors.
- Vectors replicating by RCR mode can be stably maintained in the host cells
- Electroporation is a feasible method for the transformation of *Geobacillus stearothermophilus* NUB3621R strain.

Scientific novelty:

Two novel *Geobacillus* spp. plasmids, pGTG5 and pGTD7, are described in this work. Both are small (1.5 kb and 3.3 kb, respectively) plasmids, attributed to the group of rolling-circle-replicating (RCR) plasmids that are rarely found in geobacilli. Plasmid pGTG5 is the smallest plasmid isolated from bacteria of this genus. Bioinformatic analysis revealed that the plasmid pGTG5 should belong to a new family of RCR plasmids, whose mechanism of replication may differ from other known plasmids. The results obtained by studying this plasmid contribute to the investigation of plasmid biology.

The second plasmid, pGTD7, is a member of well-characterized pC194/pUB110 plasmid family. The replicon of this plasmid was shown to be functional not only in its native host, but also in *G. stearothermophilus* NUB3621R strain. Moreover, high copy number and high segregational stability are characteristic to the plasmid bearing pGTD7 replicon. All these features show that the plasmid pGTD7 is an excellent source for the construction of new *Geobacillus* spp. vectors. Thus it expands the assortment of molecular tools available for geabacilli.

As a host for plasmids constructed in this study one of the most studied *Geobacillus* strains, *G. stearothermophilus* NUB3621R was used. In the works of other researchers, transformation of this strain was achieved only by using protoplast transformation

technique, which is laborious and time-consuming. Therefore, in this work a protocol was elaborated of a much more convenient method, electro-transformation, which results in approximately 5×10^3 transformants per μg of plasmid DNA.

The possibility to use a simple and convenient technique for the transformation of this relative *Geobacillus* strain facilitates its further investigation and accelerates its employment in various biotechnological fields.

MATERIALS AND METHODS

Bacterial strains. Strains 610 and 1121 of Gram-positive endospore-forming bacteria bearing low molecular weight plasmids were obtained from the collection of the Department of Microbiology and Biotechnology (Vilnius University, Lithuania). These strains were identified as described in the section "Sequencing and bioinformatics analysis". *E. coli* DH5α (F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK−, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) was used for molecular cloning. *G. stearothermophilus* strain NUB3621R (Hsr^- Hsm^- Rif^R), obtained from Bacillus Genetic Stock Center was used for genetic transformation with plasmids containing thermostable replicons. *G. stearothermophilus* cells transformed with plasmids pUCG18 and pUCK7 were named NUB3621R_pUCG18 and NUB3621R_pUCK7, respectively.

Cultivation of strains. *E. coli* DH5α was cultured in LB broth (Sambrook and Russell, 2001) at 37 °C. Thermophilic bacterial strains were routinely grown in Nutrient broth (NB, 1% peptone from casein, 0.5% meat extract, 0.5% NaCl) at 55 °C. Solid medium was prepared by adding 1.5% agar to the broth. Transformed *E. coli* strains were cultivated in the media supplemented with ampicillin or kanamycin to a final concentration of 100 μg/ml or 30 μg/ml, respectively. Media for transformed *Geobacillus* strains was supplemented with kanamycin to a final concentration of 12.5 μg/ml.

Plasmid isolation and molecular cloning. Plasmids used in this study are listed in table 1. Plasmid DNA from *E. coli* cells was isolated using GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Plasmids from *Geobacillus* strains were extracted by the alkaline lysis method as described by Sambrook and Russell (2001) with some modifications. Briefly, harvested cells were treated with lysozyme before the lysis step. All enzymes (restriction endonucleases, Taq polymerase,

T4 ligase) used in this study were purchased from Thermo Fisher Scientific. Endonuclease digestions, PCR, DNA ligation and other enzymatic reactions were performed according to the supplier's instructions. Primers used in this work are listed in table 2.

Table 1. Plasmids used in this study

Plasmid	Features	Reference
pGTD7	Cryptic plasmid from *Geobacillus* sp. 610	This study
pGTG5	Cryptic plasmid from *Geobacillus* sp. 1121	This study
pTZ57R/T	TA cloning vector, *lac*Z, Amp^R	Thermo Fisher Scientific
pUC19	Cloning vector *lac*Z, Amp^R	Thermo Fisher Scientific
pUCG18	*E. coli – Geobacillus* spp. shuttle vector, pBST1 replicon, Kan^R (*TK101*)	Taylor et al. 2008
pUCK	pUC19 with inserted gene *TK101*	This study
pUCK7	pUCK with inserted replicon of pGTD7	This study

Table 2. Primers used in this study.

Primer pair	Sequence	Reference
27F 1495R	3`-GAGAGTTTGATCCTGGCTCAG-5` 3`-CTACGGCTACCTTGTTACGA-5`	Studholme et al., 1999
5r1 5f1	3`-AACTTAAACGCGACCCTCACATCA-5` 3`-CGGAAGGCGCTCGAAACAGA-5`	Kananavičiūtė et al., 2014
7f1 7r1	3`-CGGGCGATCGATACGGTTCAT-5` 3`-AGCCTTACGCCAGCGACAATCC-5`	Kananavičiūtė et al., 2014
adk_F adk_R	3`-AAGCCAGGCGTTTGCGATAA-5` 3`-AGCGGCTGCGTTTGTTTGAC-5`	This study
M13/pUC M13/pUC rev.	3`-GTAAAACGACGGCCAGT -5` 3`-CAGGAAACAGCTATGAC-5`	Thermo Fisher Scientific
TK_F TK_R	3`-GGGGATGATGTTAAGGCTATTGG-5` 3`-GCTGAACTCTGCTTCCTCTGTTG-5`	This study
TK101_F TK101_R	3`-TGAGAGTGCACCATATGTCAAAATG-5` 3`-GTTCATATGATTTGGAGGAAGGTTTACAC-5`	This study
7pK_F 7pK_R	3`-TGGCCCGTTTGTTGAACTCTTTGATCGATA CGGTTCATGGGT-5` 3`-GCAGCCTGAATGGCGAATGGCGTCGCAACC AAAGTTGCT-5`	This study

Sequencing and bioinformatics analysis. Strains bearing plasmids were identified by the analysis of 16S rRNA gene sequences. The 16S rRNA gene was PCR-amplified from the genomic DNA using the universal primer pair 27F/1495R. The amplified fragments were purified using GeneJET™ PCR Purification Kit (Thermo Fisher Scientific) and sequenced at the Institute of Biotechnology, Vilnius University (Lithuania). A similarity search of the 16S rRNA gene sequences against National Centre for Biotechnology Information (NCBI) database entries was performed using the Basic Local Alignment Search Tool (BLAST) program provided by the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST).

Detection of ssDNA. DNA-DNA hybridization was performed to detect the ssDNA form of investigated plasmids. Total DNA samples of strains 610 and 1121 were treated or not with S1 nuclease. After DNA separation in TAE agarose gel, DNA was transferred to SensiBlot Plus Nylon Membrane (Thermo Fisher Scientific) by upward capillary transfer with or without previous alkaline treatment. DNA transfer and hybridization conditions and buffers were used as described in protocols supplied by Thermo Fisher Scientific. The biotinylated probe was prepared from pGTG5 plasmid fragment amplified with primers 5f1 and 5r1, and from pGTD7 plasmid fragment obtained with primers 7f1 and 7r1 using Biotin DecaLabel DNA Labeling Kit (Thermo Fisher Scientific). Detection of hybridized DNA was conducted using Biotin Chromogenic Detection Kit (Thermo Fisher Scientific).

Electrotransformation of *E. coli*. Electrocompetent *E. coli* DH5α cells were prepared and transformed as described by Sambrook and Russell (2001).

Electrotransformation of *G. stearothermophilus* NUB3621R. To prepare electrocompetent *G. stearothermophilus* NUB3621R cells, the strain was grown in 5 different media (listed in table 3) to an appropriate value of OD_{590} (0.75, 1.0, 1.15, 1.3, 1.4). Then the cells were harvested washed four times with ice-cold electro-transformation medium and suspended in the same medium to reach an OD_{590} of 100. Four different ETM were used: G (10 % glycerol), SMG (10 % glycerol, 0.5 M sorbitol, 0.5 M mannitol), SMGM (SMG medium supplemented with 1 mM $MgCl_2$) and TG (10 % glycerol, 0.5 M trehalose). The suspension was divided into 50 µl aliquots and used for transformation immediately or stored at -70 °C until further use.

Prepared electro-competent cells were mixed with 100 ng of plasmid DNA. Then, the cells were loaded into an ice-cold 1-mm gap electroporation cuvette and subjected to a single pulse generated by Eppendorf Multiporator (exponentially-decaying pulse, 5 ms time constant, 600 Ω resistance, 10 µF capacitor). The values of applied voltage were: 1.6, 1.8, 2.0, 2.2 or 2.4 kV. After electric pulse, the cells were immediately transferred into 1.5 ml of a medium identical to their growth medium and incubated at 52 °C for 2 h with shaking (180 rpm) to allow expression of the antibiotic resistance gene. Finally, transformants were selected by spreading the cell suspension on ANB agar medium with kanamycin (12.5 µg/ml). Plates were incubated overnight at 55 °C.

Table 3. Growth media used for the preparation of electrocompetent NUB3621R cells.

Medium	Composition
TSB (Merck)	1.7 % peptone from casein, 0.3 % peptone from soya, 0.25 % glucose, 5% NaCl, 0.25 % K_2HPO_4
LB	1% peptone, 0.5% NaCl, 0.5% yeast extract
mLB (Zeigler, 2001)	LB, supplemented with: 1.05 mM nitrilotriacetic acid, 0.59 mM $MgSO_4$, 0.91 mM $CaCl_2$, 0.04 mM $FeSO_4$
NB	1 % peptone; 0.5 % NaCl; 0,5 % meat extract
ANB	NB supplemented with: 2.30 mM $CaCl_2$, 1.10 mM $MgCl_2$, 0.07 mM KCl, 0.91 µM $ZnSO_4$, 0.47 µM $MnSO_4$

Cell viability determination. Evaluation of cell viability was performed by counting colony-forming units (CFU). The prepared electrocompetent cells were treated with electric pulse of appropriate strength and then the cells were spread on agar medium and incubated at 55 ºC. The CFU was counted after 12 h incubation. The CFU after electroporation were compared to the non-treated samples (control). The viability was determined as the percentage of CFU in respect to the control.

Determination of plasmid copy number per chromosome. The relative copy number of plasmids pUCG18 and pUCK7 was determined by quantitative PCR (qPCR). Total DNA of *G. stearothermophilus* NUB3621R_pUCG18 and NUB3621R_pUCK7 strains were ten-fold serially diluted and the PCR reactions were performed in triplicate using Thermo Fisher Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Fragments of KNT-ase gene residing in both plasmids and a single copy chromosome gene adk encoding adenylate kinase (GeneBank # AY729037) were amplified using primer pairs TK_R/TK_F and adk_R/adk_F (stated in the table 2). For amplification and detection, BioRad CFX96 Real-Time Detection System was used. After amplification, melting curve analysis and gel electrophoresis were performed to confirm the specificity of the used primers. Relative copy numbers were calculated according to Skulj et al. (2008).

Determination of segregational stability of plasmids. *G. stearothermophilus* NUB3621R_pUCG18 and NUB3621R_pUCK7 strains were grown in ANB medium without kanamycin. After every 12-th generation, samples of grown culture were spread on (1) ANB agar and (2) ANB agar supplemented with kanamycin. After overnight growth, CFU was counted and percentage of kanamycin resistant cells was determined. To prove that kanamycin resistant colonies were formed by plasmid bearing cells, plasmid isolation and restriction analysis was performed.

3. RESULTS AND DISCUSSION

3.1. Screening for plasmid-bearing strains and their identification.

Sixty-eight unidentified thermophilic Gram-positive endospore-forming strains, isolated from soil (Lithuania), were screened for low-molecular-weight plasmids. After plasmid extraction and restriction analysis, it was shown that only two isolates have one plasmid each (fig. 1). Plasmid pGTD7 (3.3 kb) was found in strain 610 and another plasmid pGTG5 (1.5 kb) was isolated from strain 1121. Interestingly, the plasmid pGTD7 was shown to have a form of DNA highly resistant to various restriction nucleases used in this study.

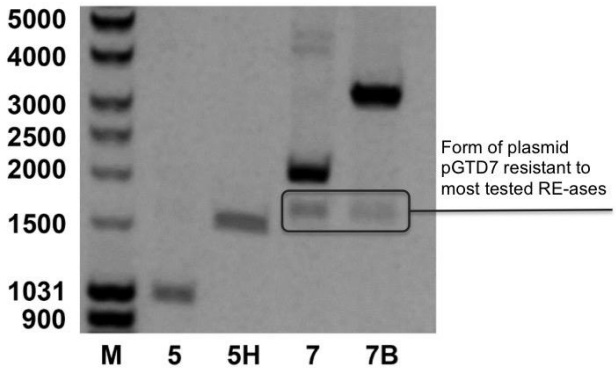

Figure 1. Plasmid DNA from strains 1121 and 610. Plasmid DNA from strain 1121 was not treated (5) or treated with HincII (5H). Plasmid DNA from strain 610 was not treated (7) or treated with BamHI (7B). Both RE-ases have single restriction sites on plasmids that they cleave. Marker lane is indicated by abbreviation M and molecular weights (bp) of marker bands are specified on the left.

Subsequently, strains 610 and 1121 were identified by 16S rDNA analysis. Using primer pair 27F/1495R, partial sequences of analyzed gene were obtained. A sequence similarity search by BLASTN indicated that strain 610 is 100% identical to *G. thermodenitrificans* SSCT85 (accession No. AB210952), while strain 1121 showed 100% identity to some *G. thermoglucosidasius* strains (accession Nos. FJ491390, AY608990, AY608984) and *G. stearothermophilus* (accession No. AY608989). Both strains were assigned to the genus *Geobacillus* and designated as *Geobacillus* sp. 610 and *Geobacillus* sp. 1121, respectively. Their 16S rDNA sequences have been deposited in GeneBank under accession Nos. KC013279 (strain 610) and KC243779 (strain 1121).

3.2. Sequencing of plasmids and their bioinformatic analysis

Further, in this work, the sequences of both plasmids DNA were determined. Plasmid pGTD7, isolated from *Geobacillus* sp. 610, is 3279 bp in length and has the GC content of 45.90 %. Another plasmid, pGTG5, extracted from *Geobacillus* sp. 1121, consists of 1540 bp with an overall GC content of 46.04 %.

The GC content of both plasmids falls into the GC content range characteristic to genomes of the genus *Geobacillus*, which varies from 42 to 55 %. However, GC content of both plasmids is lower than that of *G. thermodenitrificans* and *G. thermoglucosidasius* (species which, according to 16S rDNA analysis, are most similar to native strains of these plasmids). These results conform the findings of Nishida et al. (2012), who determined that plasmids and other genetic mobile elements possess a GC content that is usually about 10% lower than the GC content of host chromosome.

3.2.1 Plasmid pGTD7

Plasmid pGTD7 encodes two open reading frames (ORFs), which are oriented in the same direction (Fig. 2). The product of the first ORF (position 372–1397 bp) is a 341 aa-length protein, named Rec7. Its sequence is most similar to some predicted recombinases encoded in the genome of *Sporosarcina newyorkensis* and in plasmid pAO1 of *Aeribacillus pallidus*. Moreover, conservative domain (DNA_BRE_C) characteristic to site-specific tyrosine recombinases was identified. Recombinases are associated with segregational stability of plasmids, because they resolve plasmid multimers, which arise by homologous recombination (Grainge and Sherratt, 2007). Thus the protein Rep7 may function as a recombinase and may be required for the stability of plasmid pGTD7.

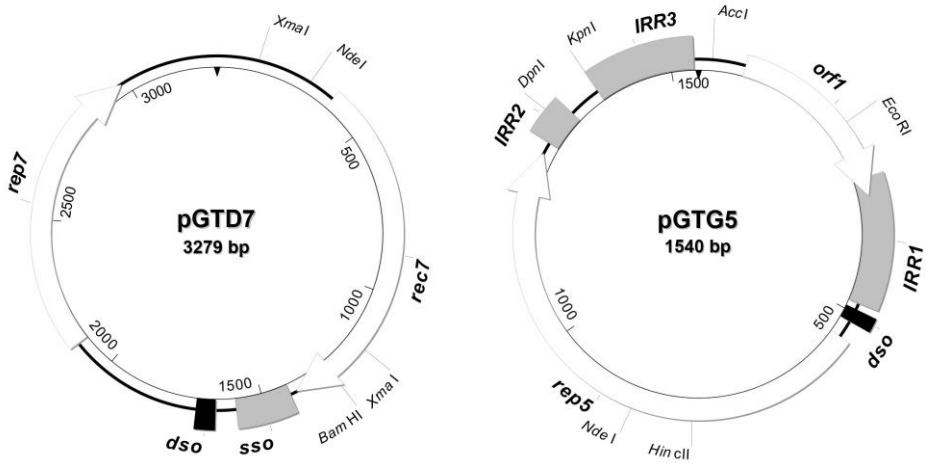

Figure 2. Genetic organization of plasmids pGTD7 and pGTG5. The putative genes are indicated with white arrows, the *dso* and *sso* are depicted with black and grey boxes. Some restriction enzymes sites are also marked.

Another identified ORF (position 2111–2980) encodes a protein of 289 aa that was named Rep7. Rep7 showed the highest similarity to replication proteins of plasmids isolated from bacteria belonging to *Bacillaceae* family (pL3 from *Halobacillus halophilus*, pBC1 from *Bacillus coagulans* and pAO1 from *Aeribacillus pallidus*). Multiple amino acid sequence alignment of Rep7 and replication proteins of other related plasmids revealed three conservative motifs characteristic to the Rep proteins from plasmids and phages replicating via the RC mechanism (Koonin and Ilyina, 1993). The sequences of these motives coincide with the consensus sequences typical to proteins of Rep_1 family (table 4).

Besides Rep protein, replication of RCR plasmids requires two distinct replication origins (*cis* acting elements): *dso* (double strand origin) and *sso* (single strand origin). The Rep protein binds a specific region in the *dso*, called *bind*. Then it nicks *dso* at another highly conserved region adjacent to the *bind* site, called *nick*, and initiates the leading strand synthesis (Guglielmetti et al., 2007). The consensus sequence of *dso nick* region was found also on the plasmid pGTD7 in the region upstream of the predicted *rep7* at position 1662–1651. According to similarities between amino acid sequences of Rep proteins and nucleotide sequences of *dso*, RCR plasmids are divided into families. The sequences of these replicon elements of plasmid pGTD7 allow assigning it to the pC194/pUB110 plasmid family (Koonin and Ilyina, 1993).

Table 4. Alignment of amino acids sequences of conservative Rep motifs and nucleotide sequences of *dso nick* regions. Amino acids and nucleotides, matching those found in plasmids pGTD7 or pGTG5 are shown in yellow. Slashes in *dso* sequence indicate the predicted nick site.

Plasmid	Conservative sequences of Rep proteins			*Dso nick* region	Organism
	Motif I	Motif II	Motif III		
	pC194/pUB110 family				
pGTD7	FLTLTVRN	YHPHFHVLLCV	ILEVSKYPVKDTDV	TTCTTTCTTATCTTG/AT	*Geobacillus* sp. 610
pAO1	FLTLTVRN	YHPHFHVLLAV	ILEVSKYPVKDTDV	TTTTTTCTTATCTTG/AT	*A. pallidus*
pFL5	FLTLTVRN	YHPHFHVLVPV	VLEISKYPVKDTDI	TCTTTTCTTATCTTG/AT	*B. thuriengiensis*
pUB110	FLTLTVKN	YNQHMHVLVCV	--ETAKYPVKDTDF	TTCTTTCTTATCTTG/AT	*Staphylococcus aureus*
pC194	FLTLTTPN	YNPHFHVLIAV	LYEMAKYSGKDSDY	TTCTTTCTTATCTTG/AT	*S. aureus*
Consensus	FLTLTxxN	yxxHUHvLUxV	ExxKYxxKxxDU		
	Plasmids and phages similar to ΦX174				
pGTG5	FFTLTFRE	GAVHYHVVVFN	GAYVTKYMTKNMPY	TATTACTTTACTTG/ATTAT	*Geobacillus* sp. 1121
pHT926	FMTLTFAE	GAVHYHCVFFN	GAYVTKYMQKNLPF	ATCCTGTTAACTTG/ATTAT	*Br. borstelensis*
pBRLA07	FITLTYEE	GAVHYHMM-SN	GAYISKYMMKNLPY	CCTTATTAGACTTG/ATTTA	*Br. laterosporus*
chp2	FLTLTYED	QRPHYHLLIYN	AGYVARYSLKNYDF	TAACAGTCTACTTG/ATCTG	Chlamydia phage
ΦX174	FDTLTLAD	GRLHFHAVHFM	GFYVAKYVNKMRTL	GAAAAATTATCTTG/ATAAA	Enterobacteria phage
Consensus	futLt	gxuHUHuxuu	gxYuakYuxk		

Another origin of the RC-type plasmids, *sso*, is required for the replication initiation of lagging strand synthesis. *Sso* sequences are mainly identified by the ability to form imperfect stem-loop structures and are divided into several types according to the form of these secondary structures and/or their nucleotide sequences. Plasmids of the same family may have different types of *ssos* (del Solar ir kt., 1998; Khan, 2000; Guglielmetti ir kt., 2007; Ruiz-Masó ir kt., 2015). The region that may function as *sso* was determined on plasmid pGTD7 upstream of the *dso* at the nucleotides 1417–1583. This DNA fragment contains palindromic sequences, which could generate extensive secondary structure. Moreover, two conservative motives, RS_B and $RS_{B(C)}$, typical to *sso*A type origins (Krammer et al., 1998) were identified at the 5' and 3' ends of the region.

3.2.2. Plasmid pGTG5

Sequence analysis of plasmid pGTG5 revealed two putatively protein-coding ORFs that are transcribed in the same direction (fig. 2). First identified gene, named *orf1*, encodes a protein of 87 aa. Its amino acid sequence has low homology to some hypothetical proteins of plasmids from bacteria of the family *Bacillaceae* (Accesion Nos.: AFQ20077; NP_044340). A short amino acid stretch (6–48 aa) showed similarity to Ribbon–helix–helix (RHH) motif of these predicted proteins. RHH motif has a DNA

binding capability and is characteristic of transcription factors (Schreiter and Drennan, 2007) including bacterial plasmid-encoded repressors CopG that are involved in plasmid copy regulation. Thus, it can be presumed that protein Orf1 could have a regulatory function.

The second ORF (position 539–1273) encodes a protein of 244 aa that was named Rep5. It shares a highest similarity with the putative replication protein of *Brevibacillus borstelensis* plasmid pHT926 and some other sequenced but not characterized proteins. Rep5 contains conservative motifs found in all RC-replicating plasmids and phages (table 4). However, the sequences of these motifs didn't match any consensus sequence of known plasmid protein family. Instead, the region from 78 to 176 aa (includes all three motifs) showed similarity (E-value: 3.61e-03) to a conserved domain of PHA00330 superfamily proteins, whose members are putative replication initiation proteins of *Microviridae* phages. The main hallmark of initiation proteins of RCR phages is that the active site-constituting motif III harbors two conservative tyrosine residues (like Rep5), which participate in the initiation and termination of plasmid replication (Koonin & Ilyina, 1993; Van Mansfeld et al., 1986). Meanwhile, most Rep proteins of bacterial RCR plasmids (including previously described plasmid pGTD7) contain only one such residue and the second tyrosine is replaced by another amino acid (for example, in plasmids of pC194 family, this second aa is glutamine) (Marsin & Forterre, 1999; Zhou et al., 2008).

A highly conservative sequence 5'-CTTGAT-3', present in *dso-nick* regions of all RCR plasmids of pC194 and phage ΦX174 families, was found 38 nucleotides upstream of the rep5 start codon. Moreover, the similarity of this region with the corresponding sequence of plasmid pHT926 (Ebisu et al., 1995) endorse the assumption that it functions as an origin of leading strand synthesis. The obtained results suggest that plasmid pGTG5 is RCR plasmid but together with its closest relatives (pHT926, pBRLA07) it should be assigned to a new family of bacterial RC plasmids.

No known *sso* types were detected on this plasmid, but three regions rich in inverted repeats, IRR1 (position 306 – 408; upstream of the *dso*), IRR2 (position 1289 – 1343; downstream of *rep5*) and IRR3 (position 1390 – 1533) were identified. IRR2 showed the potential to form a large imperfect stem-loop structure, similar to that of *ssoA* type, but lacking any sequence similarity with it. It should be determined experimentally which of the mentioned three regions act as an origin of lagging strand synthesis.

3.3. Confirmation of replication mechanism of plasmids pGTD7 and pGTG5

Bioinformatic analysis of the plasmids pGTD7 and pGTG5 suggest that they both duplicate by the RC mechanism. Another evidence of RC replication is the detection of ssDNA that forms as an intermediate product and then the synthesized new strand (leading strand) displaces the old DNA strand (te Riele et al., 1986). To detect ssDNA form of the plasmids, Southern blot was performed. The samples of total DNA from *Geobacillus* sp. 610 and *Geobacillus* sp. 1121, with or without previous denaturation, were transferred to nylon membrane and hybridized with biotin-labeled fragments of plasmid pGTD7 or pGTG5, respectively. Total DNA samples were pretreated or not with S1 nuclease, an enzyme that degrades only ssDNA. The ssDNA was detected as a faint band, which migrated in agarose–gel faster than the dsDNA, which disappeared after the treatment with S1 nuclease and which was seen even after hybridization without previous alkaline DNA denaturation. Such bands were observed in the samples of both plasmids, confirming that the plasmids pGTD7 and pGTG5 replicate by the RC mode.

3.4. Construction of vectors bearing replicons of plasmids pGTG5 and pGTD7

After the replicons of both plasmids were identified *in silico* and the replication mechanism was confirmed by ssDNA detection, the next step of my study was to demonstrate the functionality of these replicons *in vivo* and to investigate their suitability to be used in the construction of new vectors. For this reason, vector pUCK was constructed. It consists of universal *E. coli* cloning vector pUC19 with an inserted gene of thermostable kanamycin nucleotidyltransferase (KNT-ase; a thermostable variant of KNT-ase from plasmid pUB110 of *Staphylococcus aureus*). Interestingly, it was shown that the kanamycin resistance gene was not suitable for the selection of *E. coli* transfromants. Similar results were also obtained by Narumi et al. (1993).

The constructed vector pUCK retains the intact multicloning site (MCS) of pUC19. Thus, it is suitable for (1) convenient insertion of DNA fragments encompassing replicons of the investigated plasmids and for (2) effective blue-white screening for recombinant plasmids in *E. coli* cells. Moreover, the thermostable KNT-ase gene allows a further selection of recombinant plasmids with functional thermostable replicon in geobacilli.

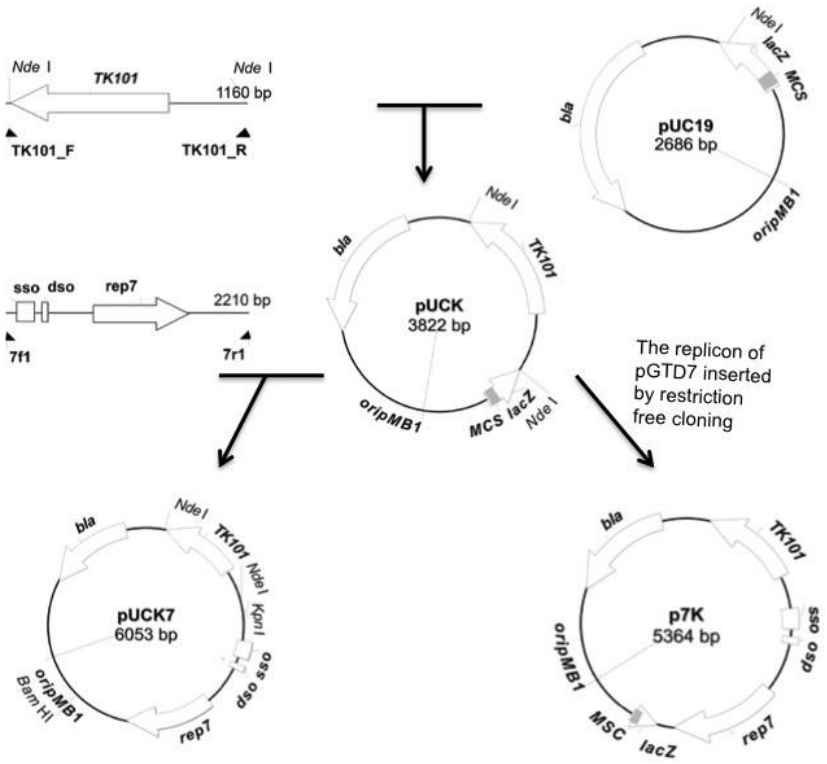

Fig. 3. Construction of vector pUCK and plasmids pUCK7 and p7K containing the replicon of plasmid pGTD7.

Subsequently, several different plasmids were constructed by inserting different fragments of pGTD7 (fig. 3) or pGTG5 (fig. 4) to pUCK. The obtained constructs were transferred to *G. stearothermophilus* NUB3621R cells under the conditions that were determined to give best electrotransformation efficiency of the strain (determination of these conditions is described in chapter 3.5). Transformation with plasmids bearing pGTG5 replicon didn't succeed. This could be explained by several possible causes: (1) plasmid pGTG5 may have a narrow host range and its replicon isn’t functional in the NUB3621R strain, (2) developing both constructs, DNA sequences essential for replication or regulation of pGTG5 replicon may be interrupted, or (3) insufficient transformation efficiency has impeded transfer of the plasmids to the cells.

However, transformants of NUB3621R were obtained with plasmid pUCK7. Hence, it was proven that the inserted fragment comprising replicon of pGTD7 ensures the plasmid replication not only in its native host but also in another strain of the same genus. Further, the segregational stability and plasmid copy number (PCN) of pUCK7 were assessed.

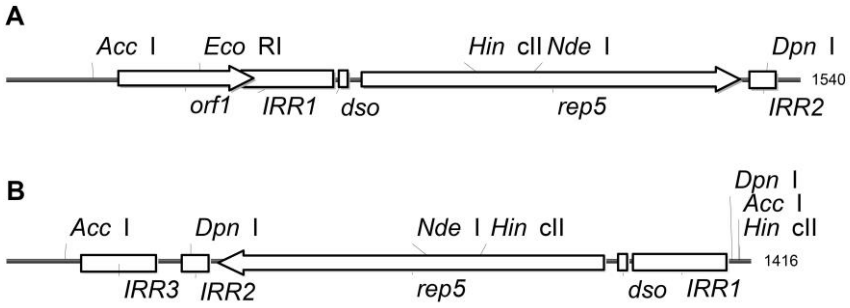

Fig. 4. Fragments of pGTG5 inserted into vector pUCK. (A) The fragment contained in plasmid pUK5K. (B) The fragment contained in plasmid pUK5EH.

3.4.1 Segregational stability and PCN of plasmids pUCK7 and pUCG18

PCN and segregational stability are important characteristics that determine suitability of a plasmid to be used in a construction of a genetic vector. Therefore in the next step, these two features of plasmid pUCK7 in *G. stearothermophilus* NUB3621R cells were assessed. For comparison, a known *Geobacillus–E. coli* shuttle vector pUCG18, which contains theta-type replicon derived from *G. stearothermophilus* 1102 plasmid pBST1 (Liao et al., 1986; Taylor et al., 2008), was also analyzed.

PCN of pUCK7 and pUCG18 was estimated in NUB3621R cells grown in media with kanamycin. Total DNA was extracted and relative copy number of these plasmids was determined by qPCR using primers for fragments of (1) KNT-ase gene residing in both plasmids and (2) a single copy chromosome gene *adk* encoding adenylate kinase. Analysis of the results revealed that the relative copy numbers of plasmids pUCK7 and pUCG18 were 447.5 and 558.0, respectively. Such high amount of pUCG18 copies was unexpected, because theta-type plasmids are usually maintained at low copy number (1–10 copies) per cell. Presumably, vector pUCG18 has incomplete or disrupted sequences required for plasmid copy control of plasmid pBST1.

The obtained pUCK7 copy number shows that pGTD7 replicon also ensures high PCN in *G. stearothermophilus* NUB3621R cells. This feature is characteristic to many RCR plasmids. High PCN is a desirable feature for some vectors, especially for the expression vectors when an expression system lacks strong promoters (promoters suitable for protein expression in geobacilli are still under investigation). It is also worth to mention that the PCN of both analyzed plasmids could be influenced by the host strain, growth

media composition (concentration of antibiotic) and also growth phase of the cells (Zhong et al., 2011).

To test plasmid stability, cells of NUB3621R strain bearing pUCK7 or pUCG18 were grown in a medium without kanamycin (without selective pressure). After every 12 generations, the numbers of all cells (CFU formed on ANB agar medium) and only those cells that retained the plasmids (CFU formed on ANB agar medium with kanamycin) were estimated. The obtained results (fig. 5) show that the vector pUCG18 is readily eliminated from NUB3621R strain cells and after 12 generations only 2.5% of the cells still bear the plasmid. Such results agree with data of Suzuki & Yoshida (2012), who determined low segregational stability of the vector pUCG18 in another *Geobacillus* species, *G. kaustophilus* HTA426.

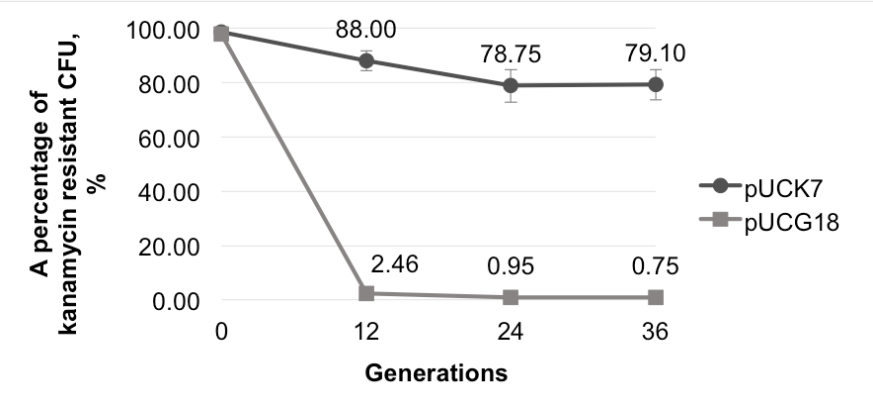

Figure 5. Segregational stability of plasmids pUCK7 and pUCG18.

Contrary to pUCG18, plasmid pUCK7 showed much higher stability. Even after 24 generations, only 20 % of NUB3621R cells lost this plasmid. Observing the dynamics of plasmid pUCK7 maintenance, the percentage of cells bearing the plasmid changed only insignificantly. These experimental results verified that the replicon of pGTD7 provides high segregational stability to it bearing plasmid. Stability of the plasmids that do not encode an active segregation system is based on high copy number. On the other hand, high PCN dooms hosts to bear an unavoidable metabolic burden. Therefore, the cells tend to lose plasmids if they didn't provide any advantage over plasmid-free cells. However, there are usually some percentages of cells in the population that maintains the plasmid even without selective pressure. This phenomenon is explained by compensatory

adaptation – some plasmid carrying cells undergo mutations, which lower plasmid maintenance costs. In this way, the plasmids may persist in a population even without active segregation system and without selective pressure (San Millan et al., 2014).

In summary, these results, together with other determined characteristics of pGTD7, demonstrate that plasmid pGTD7 could be useful for the development of new replicative vectors for genetic manipulations of *Geobacillus* spp.

3.5. Electrotransformation of *Geobacillus stearothermophilus* NUB3621R

G. stearothermophilus NUB3621R was used as a host for all plasmids constructed in this study. The chosen strain of *Geobacillus* is claimed to be deficient in active restriction-modification system (Blanchard et al., 2014; Wu & Welker, 1989). Thus it should be more easily transformable. Wu and Welker (1989) have elaborated a protoplast transformation protocol that enables genetic engineering of NUB3621R. However, this protocol does not work, when Kan^R based selection system is used and, in general, protoplast transformation is cumbersome and a time-consuming procedure. Therefore I used electrotransformation, a much more convenient method. Because this technique hasn't been previously used for the transformation of NU3621R, the conditions for genetic transfer by electroporation were determined in this study. For this goal, we used a well-known *Geobacillus – E. coli* shuttle vector, pUCG18 (Taylor et al., 2008) (because, if a newly constructed plasmid would be used and negative results were obtained, it would be difficult to say whether the plasmid doesn't replicate in the host, or the applied electrotransformation conditions are not suitable for transfer of the plasmid to the host).

The results of electrotransformation of different bacterial strains depend highly on various factors, such as preparation of electrocompetent cells, parameters of electroporation and the conditions of transformed cell recovery (Assad-García et al., 2008; Cao et al., 2011; Lu et al., 2012; Shen et al., 2013; Lu et al., 2014; Landete et al., 2014; Meddeb-Mouelhi et al., 2012; Rhee et al., 2007; Rodríguez et al., 2007; Turgeon et al., 2006; Xue et al., 1999; Yang et al., 2010; Zhang et al., 2011; Zhang et al., 2015). The influence of all these factors on the efficiency of transformation is species or even strain dependent. Thus, to determine the best conditions for transformation of *G. stearothermophilus*, the influence of different electrotransformation factors was tested.

3.5.1. Preparation of electrocompetent cells

First, the influence of a growth medium on the transformability of NUB3621R cells was investigated. Various components of a growth medium modify the physiology and metabolism of bacteria (Egli & Zin, 2003). At the same time they could also change transformability of the cells (Zhang et al., 2011; Nováková et al., 2014). Searching for the medium that would be suitable for the preparation of electrocompetent NUB3621R cells, they were grown in several different media: (1) LB, (2) mLB (LB supplemented with Mg^{2+}, Ca^{2+} ir Fe^{2+} salts), (3) NB (medium composition similar to LB, but yeast extract is replaced by meat extract), (4) ANB (NB supplemented with Mg^{2+}, Ca^{2+}, K^{+}, Zn^{2+}, Mn^{2+} salts) and (5) TSB (rich medium containing glucose and KH_2PO_4).

Observing the bacterial growth in different media, it is clear that some salts, like $MgCl_2$ and $CaCl_2$, positively affect the growth rate of NUB3621R. Supplementation of LB and NB media with the components listed above in the brackets significantly enhances the growth of strain NUB3621R. High growth rate is also achieved when cultivating the cells in TSB medium rich in various nutrients including glucose. However, the cell culture grown in this medium undergoes lysis immediately after its OD_{590} reaches the maximum value. This phenomenon could be explained by the effect of glucose present in the medium. Pavlostathis et al. (2006) working with another *Geobacillus* species, *G. thermoleovorans*, has shown that higher amount of glucose causes accelerated growth followed by intensive cell death and lysis.

Depending on bacterial growth in different media, the cells were harvested at an OD_{590} of 1 or 0.75. Then, the cells were suspended in SMG medium, mixed with DNA of pUCG18 (extracted from *E. coli* DH5α) and subjected to a 22 kV/cm pulse. Transformants were obtained only from cells grown in ANB medium. It should be noted that the growth curve of strain NUB3621R in ANB was very similar to that of cells grown in mLB medium. Apparently, similar growth of the cells in different media not always ensures similar physiological state at an appropriate growth time. This implies that the components of growth medium have significant impact on the electrocompetency of NUB3621R cells.

The obtained transformation efficiency was 120 transformants/μg of plasmid DNA. Interestingly, it was observed that plasmid pUCG18 isolated from NUB3621R tarnsformants transform this strain >2 times more effectively than the plasmid extracted

from *E. coli* DH5α. Thus, although the strain NUB3621R is considered as lacking functional restriction modification system (Wu & Welker, 1989), somehow it is able to distinguish its own DNA from the foreign one and this leads to the lower transformation efficiency using plasmid DNA extracted from other bacterial strains. In the further work, ANB medium was used for the preparation of electrocompetent cells and plasmid pUCG18 isolated from strain NUB3621R was used for transformation.

The next task was the determination of the OD_{590} value of the growing NUB3621R culture, a point where the harvested cells were most electrocompetent. Electrocompetent cells of various other bacteria are prepared from the exponentially growing cells. However, harvesting cells at different points of the exponential phase results in different transformation efficiency and the optimal harvesting point is species and strain dependent (Lu et al., 2012; Shen et al., 2013).

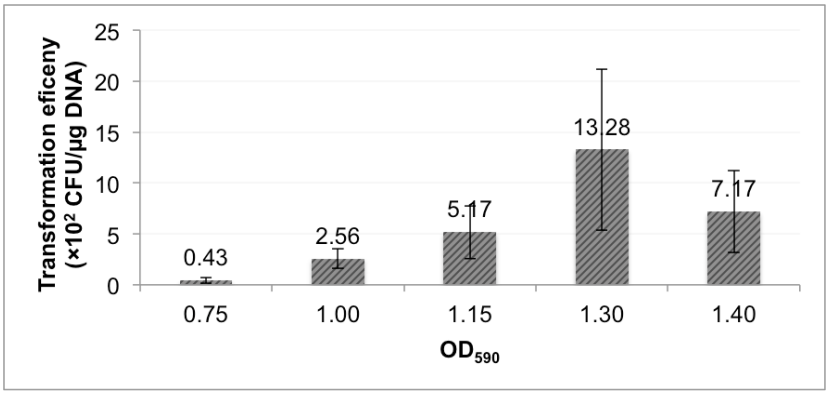

Figure 6. The transformation efficiency of NUB3621R cells grown until different growth phases in ANB medium.

G. stearothermophilus NUB3621R strain was grown to several different values of OD_{590} (0.75, 1.0, 1.15, 1.3 and 1.4). Harvested cells were suspended in SMG medium mixed with plasmid DNA and shocked by a 22 kV/cm pulse. Results (fig. 6) indicate that transformants can be obtained from the culture grown to OD_{590} from 0.75 to 1.4. However, the highest transformation efficiency is reached by harvesting cells at OD_{590} of 1.3.

Furthermore, the influence of the electrotransformation medium (ETM) on the transformation efficiency was evaluated. Various components of the ETM may alter the resistance of the sample and affect cell viability and/or mobility of DNA. Thus, in this step, NUB3621R strain cells collected at OD_{590} of 1.15 were suspended in four different ETM and treated by a 22 kV/cm pulse. Results are presented in table 5.

Table 5. The effect of different ETM on transformation efficiency

ETM	Composition	Efficiency
G	10 % glycerol	0.0
SMG	10 % glycerol, 0.5 M sorbitol, 0.5 M mannitol	$(5.2 \pm 2.5) \times 10^2$
SMGM	10 % glycerol, 0.5 M sorbitol, 0.5 M mannitol, 1 mM $MgCl_2$	$(4.7 \pm 4.3) \times 10^3$
TG	10 % glycerol, 0.5 M trehalose	$(2.8 \pm 2.6) \times 10^2$

The medium composed only of 10 % glycerol (G medium) is used in the conventional electrotransformation protocols of *E. coli* and some other bacteria. However, this medium was not suitable for the transformation of *G. stearothermophilus* NUB3621R. Supplementation of the ETM with compatible solutes, such as sorbitol, mannitol, or trehalose enhances cell viability after electrical pulsing procedures and subsequently it may increase transformation efficacy (Cao et al., 2011; Shen et al., 2013; Xue et al., 1999). Positive effect of these substances was also shown in this study; SMG and TG media yielded 10^2 transformants/μg DNA. Additional supplementation of SMG medium with 1 mM $MgCl_2$ raised efficiency by almost nine fold to 4.7×10^3. Thus SMGM medium was proven to be most suitable for the transformation of strain NUB3621R.

3.5.2. Electroporation parameters

The applied strength of the electric field is one of the most important electroporation parameters influencing transformation efficiency. Higher electric field strength correlates with higher membrane permeability and subsequently higher transformation efficiency, but at the same time it negatively affects cell viability (Trevors et al., 1992). Thus, optimal electric field intensity for electrotransformation is when the cells are maximally permeabilized but cell viability is still sufficient to obtain high number of transformants.

To determine this value, we used electrocompetent cells prepared from the culture grown to an OD_{590} of 1.3. Harvested cells were suspended in SMG, mixed with plasmid DNA and exposed to electric field strengths ranging from 18 to 24 kV/cm. After electric shock, the transformation efficacy and the cell viability were evaluated and depicted in figure 7. About 90% of the cells remain viable after an 18 kV/cm pulse. The further increase of the voltage results in a remarkable viability drop. In contrary, transformation efficiency positively correlates with higher electric field strength and reaches a peak at 20-

22 kV/cm with transformation efficiencies of 1.3×10^3 of transformants/µg DNA. The pulse of 24 kV/cm is already too detrimental to the cells and results in decreased transformation efficiency.

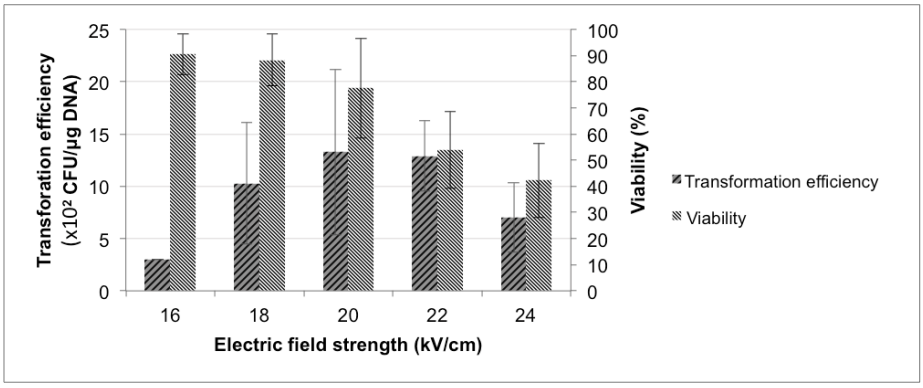

Figure 7. The effect of applied electric field on cell viability and transformation efficiency.

Summarizing all results obtained by determining best conditions for the transformation of NUB3621R strain, derivatives of plasmids pGTD7 and pGTG5 constructed in this work, were transferred to the cells of NUB3621R by the following protocol. The cells were grown in ANB medium to an OD_{590} of 1.15 – 1.3, then suspended in ETM supplemented with $MgCl_2$. After that the cell suspension was mixed with plasmid derivatives (constructed and propagated in *E. coli*) and subjected to a 22 kV/cm pulse. As mentioned in section 3.4, transformants were obtained only with plasmid pUCK7 (contains replicon of plasmid pGTD7). Transformation efficiency was 4.6×10^2 of tarnsformants per µg of DNA.

CONCLUSIONS

1. The plasmid pGTD7 isolated from *Geobacillus* sp. 610 and described in this work is a suitable source for the development of new *Geobacillus* spp. replicative high-copy-number vectors.
2. RC-replicating plasmid pUCK7 containing replicon of pGTD7 possesses higher segregational stability than the vector pUCG18 replicating by θ-mechanism.
3. *Geobacillus* sp. 1121 plasmid pGTG5 should be assigned to a new RC-plasmid family which members encode replication initiation proteins, according to amino acid sequences, similar to those of bacteriophages.
4. Transformation of *G. stearothermophilus* NUB3621R by electroporation results in 10^3 of transformants/μg of plasmid DNA.

List of publications by the author of the dissertation:

1. Kananavičiūtė, R., Butaitė, E., Čitavičius, D., 2014. Characterization of two novel plasmids from *Geobacillus* sp. 610 and 1121 strains. Plasmid 71: 23–31.
2. Kananavičiūtė, R., Čitavičius, D., 2015. Genetic engineering of *Geobacillus* spp. J Microbiol Methods. 111:31-39.
3. Kananavičiūtė, R., Kanišauskaitė, I., Novickij, V., Čitavičius, D., 2015. *Geobacillus stearothermophilus* NUB3621R genetic transformation by electroporation. Biologija. 61:101–108.

List of the conferences where the results of the thesis were presented:

1. Kananavičiūtė, R., Čitavičius, D. Optimization of electroporation of thermophilic bacteria *Geobacillus stearothermophilus* NUB3621R. FEMS 2013: 5th Congress of European Microbiologists, Leipzig, Germany, July 21-25, 2013 [abstracts]. Art. no. 83.
2. Kananavičiūtė, R., Kanišauskaitė, I., Kalėdienė, L., Lastauskienė, E., Čitavičius, D. Improvement of genetic transformation system for thermophilic bacteria *Geobacillus stearothermophilus* NUB3621R. Journal of biotechnology. 2014, vol. 185, suppl.: European Biotechnology Congress 2014. ISSN 0168-1656 p. S101.

SANTRAUKA

Geobacillus genties bakterijų genetinės transformacijos sistemos kūrimas

Geobacillus genties bakterijos yra vertingi termofiliniai mikroorganizmai, kurie yra tyrinėjami ne tik mokslinio pažinimo tikslais, bet ir siekiant juos išnaudoti įvairių bioprocesų pagerinimui. Tiek šių termofilų tyrimai, tiek ir efektyvus jų praktinis pritaikymas yra neįmanomi be patikimų metodų ir įrankių, kurie leistų manipuliuoti genomu ir keisti jų genų raišką. Deja, šiuo metu geobacilų genetinių priemonių nėra daug, o jos pačios vis dar yra tobulintinos. Būtent dėl to šiame darbe siekta praplėsti *Geobacillus* spp. bakterijų genetinės inžinerijos galimybes.

Tam kad būtų praplėstas negausus geobaciloms tinkamų vektorių pasirinkimas, pirmiausia buvo tiriamos naujos plazmidės, išskirtos iš dviejų laukinių kamienų priklausančių *Geobacillus* genčiai. Viena plazmidžių, pGTD7, remiantis jos bioinformatine analize ir viengrandės plazmidinės DNR formos nustatymu, yra priskirtina riedančio rato (RR) mechanizmu besireplikuojančioms plazmidėms ir priklauso pUB110 plazmidžių šeimai. Parodyta, kad šios plazmidės replikonas yra funkcionalus ne tik savo natyviame, bet ir kitame *Geobacillus* genties kamiene. Be to nustatyta, kad pGTD7 replikonas suteikia jį turinčiai plazmidei aukštą segregacinį stabilumą ir didelį kopijų skaičių ląstelėje. Taigi, ši plazmidė yra tinkama daugiakopijinių replikatyvinių vektorių kūrimui.

Antroji darbe tirta plazmidė, pGTG5, taip pat replikuojasi RR mechanizmu. Tačiau skirtingai nei plazmidės pGTD7 atveju, jos koduojamo replikacijos baltymo sekoje buvo nustatyti motyvai, kurie yra panašesni ne į kurios nors žinomos bakterijų plazmidžių šeimos, o į RR bakteriofagų atitinkamus konservatyvius motyvus. Dėl to daroma prielaida, kad ši plazmidė, kartu su kitomis į ją panašiomis plazmidėmis, yra priskirtina naujai neaprašytai plazmidžių šeimai.

Šeimininku šiame darbe sukurtiems plazmidžių dariniams buvo pasirinktas vienas daugiausiai tirtų *Geobacillus* genties kamienų, *G. stearothermophilus* NUB3621R. Darbo metu buvo sukurta metodika, leidžianti transformuoti *G. stearothermophilus* NUB3621R kamieną elektroporacijos būdu. Tai yra daug patogesnis metodas nei iki šio darbo

NUB3621R kamienui naudota protoplastų transformacija. Galimybė, patogiai transformuoti šį svarbų *Geobacillus* genties kamieną, palengvina jo tolimesnius tyrimus ir įgalina spartesnį jo pritaikymą įvairiems biotechnologiniams tikslams.

CURRICULUM VITAE

Name: Rūta Kananavičiūtė

Date and place of birth: 19.10.1983, Vilnius, Lithuania

Office address: Department of Microbiology and Biotechnology, Faculty of Natural Sciences, Vilnius University, M.K. Ciurlionio 21/27, LT-03101 Vilnius, Lithuania

E-mail: ruta.kananaviciute@gf.vu.lt

Education:

2008 Master degree in Microbiology, Vilnius University, Vilnius, Lithuania;

2006 Bachelor degree in Molecular biology, Vilnius University, Vilnius, Lithuania.

Employment:

2012–present Lecturer, Vilnius University, Vilnius, Lithuania.

2012–present Junior research associate, Vilnius University, Vilnius, Lithuania;

2007–2012 Technician, Vilnius University, Vilnius, Lithuania;

2006–2006 Senior technician, Vilnius University, Vilnius, Lithuania;

Scientific and academic activities:

2015–present Lecturer in Environmental microbiology.

2014–present Lecturer in Microbiology;

2013–2014 Laboratory exercises in Biotechnology;

2008–2013 Laboratory exercises in Microbiology;

Research interests:

Applied microbiology and biotechnology; genetic engineering of prokaryotes; synthetic biology, microorganism genetics.

Scientific publications:

- Kananavičiūtė, R., Butaitė, E., Čitavičius, D., 2014. Characterization of two novel plasmids from *Geobacillus* sp. 610 and 1121 strains. Plasmid 71: 23–31.
- Kananavičiūtė, R., Čitavičius, D., 2015. Genetic engineering of *Geobacillus* spp. J Microbiol Methods. 111:31–39.
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