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INVESTIGATION OF ARTHROBACTER SPP. PLASMIDS

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BIOCHEMIJOS INSTITUTAS

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ARTHROBACTER SPP. PLAZMIDŽIŲ TYRIMAS

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INTRODUCTION

Arthrobacter spp. are Gram-positive GC-rich bacteria (Jones & Keddie, 2006) that belong to the family *Micrococcaceae* of *Actinomycetales* (Stackebrandt & Schumann, 2006; Zhi et al., 2009). Different species are either psychrophilic or mesophilic (Jones & Keddie, 2006). The most common habitat of these bacteria is soil. Although *Arthrobacter* sp. bacteria are the most numerous soil microorganisms (Jones & Keddie, 2006), new species have been also isolated from alpine, sea and glacier ice (Margesin et al., 2004), wastewater sediment and sludge (Bieszkiewicz et al., 1995; Kampfer et al., 1996; Kim et al., 2008; Li et al., 2008; Molokwane et al., 2008; Wu et al., 2010), animal (Osorio et al., 1999) and human clinical speciments (blood, urine), and wounds (Funke et al., 1996; Wauters et al., 2000; Huang et al., 2005; Mages et al., 2008). The extreme resistance to drying and to starvation explains the predominance of this bacterial genus. The nutritional versatility of the commonly occurring species that are capable to degrade natural or man-made aromatic and heterocyclic compounds undoubtedly also plays a part in their predominance (Jones & Keddie, 2006).

Recently, the interest in mesophilic and psychrophilic *Arthrobacter* sp. bacteria has increased because of new enzymes that could be used in the branches of industry and biotechnology. It has been determined that catabolic genes generally are plasmid located (Pohlenz et al., 1992; Hayatsu et al., 1999; Chauhan et al., 2000; Turnbull et al., 2001; Eaton, 2001; Mongodin et al., 2006; Igloi & Brandsch, 2003; Parschat et al., 2007; Monnet et al., 2010). To date, degradation genes for phthalic acid (benzene-1,2-dicarboxylic acid) (Eaton, 2001), nicotine (Igloi and Brandsch, 2003), herbicides atrazine (Sajjaphan et al., 2004; Mongodin et al., 2006), phenylcarbamate (Pohlenz et al., 1992), and diuron (Turnbull et al., 2001), were investigated. Unfortunately, degradation genes-encoded recombinant enzymes cannot be easily produced, since most of the proteins, over-expressed in *Escherichia coli* are inactive. Reliable hybrid shuttle or expression vectors would solve this problem.

The replicons of small arthrobacterial plasmids would be very helpful for the construction of hybrid vectors. However, at present only three such plasmids, pA3 (Grosse, 1993), p54 (Miteva et al., 2008), pRE117-2 (Monnet et al., 2010), are known.

Plasmid p54 was used for the construction of the vector pSVJ1 and, to date, it is the first and only hybrid vector containing an *Arthrobacter* spp. replicon (Miteva et al., 2008). Several hybrid vectors for this bacterial genus were created earlier, but, in all cases, replicons from other *Actimocytales* bacteria were used.

The aim of this study was:

to investigate *Arthrobacter* spp. catabolic and small plasmids, and to construct the hybrid vector(s), containing *Arthrobacter* sp. replicon. Towards this goal, the following tasks had been formulated:

- To determine the phenotype of large molecular weight plasmids from *Arthrobacter* sp. 68b;
- To investigate phthalic acid degradation pathway;
- To investigate 2-methylpyridine and pyridine degradation pathway;
- To determine the location of 2-hydroxypyridine degradation genes in *Arthrobacter rhombi* PRH1 and VP3 genomes.
- To investigate small plasmids from *A. rhombi* PRH1 and VP3.
- To construct hybrid vector(s) using *Arthrobacter* sp. replicon.

Scientific novelty:

Arthrobacter sp. 68b bacteria are able to degrade pyridine, 2-methylpyridine, nicotine, and phthalic acid. It was determined that some enzymes of pyridine and 2-methylpyridine degradation pathways are shared. Degradation genes are located in one of two catabolic plasmids. In both cases, the putative monooxygenase is induced.

Phthalic acid degradation genes of 68b strain form an operon and are located in the same plasmid as pyridine and 2-methylpyridine degradation genes. These bacteria degrade phthalic acid by 3,4-dioxygenase pathway, that is common to Gram-positive bacteria. *Arthrobacter* sp. 68b cells, pre-grown with phthalic acid, are able to convert quinolinic acid by adding a hydroxyl group into the heterocyclic ring. Generally, it is believed that only phthalate 4,5-dioxygenase and 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase harbouring microorganisms are capable of such transformation.

Both *Arthrobacter rhombi* PRH1 and VP3 strains, capable to degrade 2-hydroxypyridine, harbour one large likely catabolic plasmid and one small (< 10 kb) plasmid. During this work it was determined that in the case of PRH1 strain 2-hydroxypyridine degradation genes are plasmid-located. Nucleotide sequences of both small plasmids were determined, open reading frames were designated, and encoded proteins were identified. The minimal replicon of the plasmid pPRH from the strain PRH1 is *repAB*. Based on the homology of replication proteins, the composition of minimal replicon, and the similarity of the *ori* region, it was concluded that the plasmid pPRH belongs to theta replication C class pAL5000 subfamily of ColE2 family.

Hybrid vectors pRMU824, pRMU824Km and pRMU824Tc were constructed using pPRH minimal replicon. Hybrid plasmids successfully replicate in *Arthrobacter* spp., *Rhodococcus* spp. and *Escherichia coli* DH5α strains.

Defensive statements:

- *Arthrobacter* sp. 68b bacteria harbour at least two large molecular mass catabolic plasmids, containing genes, involved in degradation of piridine, 2-methylpyridine, phthalic acid, and nicotine.
- *Arthrobacter* sp. 68b bacteria degrade phthalic acid via 3,4-dioxygenase pathway. Phthalate pregrown cells are capable to hydrolyse quinolinic acid.
- Pyridine and 2-methylpyridine induce the synthesis of the hypothetical monooxygenase in *Arthrobacter* sp. 68b bacteria.
- 2-Hydroxypyridine degradation genes are plasmid-located in *Arthrobacter rhombi* PRH1.
- The minimal replicon of plasmid pPRH is the *repAB*. The plasmid belongs to theta replication type subfamily pAL5000 of ColE2 family.
- Hybrid vectors pRMU824, pRMU824Km, pRMU824Tc for *Arthrobacter* spp. and *Rhodococcus* spp. bacteria were constructed using the minimal replicon of the plasmid pPRH.

Contents of the doctoral thesis

The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature review, Materials and Methods, Results and Discussion, Conclusions, List of References (177 positions), Tables (6), and Figures (44). Total 149 pages.

MATERIALS AND METHODS

Reagents

2-hydroxypyridine, 2-methylpyridine, 3-methylpyridine, 4-methylpyridine were purchased from Aldrich, Germany. Kanamycin, Tris were from AppliChem, Germany. Glicerol was from Barta a Cihlar, Czech Republic. Acetic acid was purchased from Chempur, Poland. 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), isopropyl-β-D-galactopyrano-side (IPTG), restriction endonucleases, T4 ligase, CIAP, FastAP, T4 polinucleotid-kinase, Proteinase K, Gene Ruler DNA ladder Mix, MassRuler High Range DNA Ladder, Maxima Hot Start Green PCR Master Mix, DNA extraction kit, TrueStart Taq DNA Polymerase, TrueStart Taq bufferis, $MgCl_2$ were from Fermentas – Thermo Fisher Scientific, Lithuania. *β*-merkaptoethanol was purchased from Ferak, Germany. Amonium acetate, ampicillin, quinolic acid, potassium acetate, tetracycline were purchased from Fluka, Germany. K_2HPO_4 was from Girochem, Slovenia. AccuPrime Pfx DNA polymerase was from Invitrogen, USA. Glucose was purchased from Lach-Ner, Czech Republic. KCl, $MgSO_4$, NaCl were from Lachema, Czech Republic. Agar, dimethylformamide (DMF), NaOH, nicotine, *p*-nitroaniline were from Merck, Germany. BHI (brain heart infusion), NA (nutrient agar), NB (nutrient broth), yeast extract were purchased from Oxoid, UK. $CaCl_2$ was from Penta, Czech Republic. $CaCl_2x2H_2O$, chloroform, $FeSO_4x7H_2O$, phthalic acid, succinate, K_3PO_4, KH_2PO_4, lysocime, $MgCl_2$, $MgSO4x7H_2O$, $MnSO_4x4H_2O$, Na_2HPO_4, $NaNO_2$, NH_4Cl, $(NH_4)_2SO_4$, pyridine were from Reachim, Russia. Ethylenediaminetetraacetic acid (EDTA), phenol-chloroform were from Roth, Germany. Ethanol was purchased from Vilniaus Degtinė, Lithuania. Agarose, ethidium bromide, chloramphenicol were purchased from Serva, Germany. Sodium dodecilsulfate (SDS) was from Sigma, USA. ZYMO Plasmid PREP kit was from ZYMO Research Corporation, USA.
Primers were from Metabion, Germany; Integrated DNA technology, Germany.

Bacterial strains and plasmids

Escherichia coli DH5α (ϕ80dlacZΔM15 Δ(lacZY-argF) U169 deoR recA1 endA1 hsdR17($r_K^-m_K^+$) sup E44 thi-1 gyrA96 relA1) (Pharmacia). *Arthrobacter rhombi*: PRH1, VP3, KA3, KA4; *A. oxydans* PY21 (capable to degrade 2-hydroxypyridine) (Semėnaitė et al., 2003). *Arthrobacter* sp. 68b (capable to degrade 2-methylpyridine, nicotine, phthalic acid, pyridine), *Arthrobacter* sp. 68b mut4, mut5 (*Arthrobacter* sp. 68b 2-methylpyridine and pyridine catabolism mutants), *Arthrobacter* sp. 83; 85 (capable to degrade 2-methylpyridine and nicotine), *Rhodococcus* sp. TMP1 (capable to degrade 2,3,5,6-tetramethylpyrazine) are laboratory strains. *Arthrobacter globiformis* (NRRL B-2979) (ARS culture collection). *Rhodococcus erythropolis SQ1* (Quan & Dabbs, 1993).
pTZ57R (Mead et al., 1986), pACYC184 (Chang & Cohen, 1978; Rose, 1988), p34S-Tc (Dennis & Zylstra, 1998), pART2, pART2gfp (Sandu et al., 2005). pVP3, pVP3Sal24 (Meškienė, personal communication). pPRH was isolated during this work. Plasmids pPRHHind4, pAPrepAB4, pAPrepA2, pRMU8, pRMU824, pRMU824Km, pRMU824Tc, pVPAD2 and pVPAM11 were constructed during this work.

Media and growth conditions

BHI (brain heart infusion) 37 g/L; **NA** (nutrient agar) 28 g/L; **NB** (nutrient broth) 13 g/L; **EFA** 10 g/L, K_2HPO_4 4 g/L, KH_2PO_4 1 g/L $(NH_4)_2SO_4$, yeast extract 0.5 g/L, $MgSO_4 \times 7H_2O$ 0.4 g/L, $CaCl_2 \times 2H_2O$ 2g/L, 1 g/L $MnSO_4 \times 4H_2O$, 0.5 g/L $FeSO_4 \times 7H_2O$, all components dissolved in 0.1N HCl.

All media were autoclaved for 30 min at 121 °C and 1 atm.

E. coli were cultivated at 37 °C, and *Actinomycetales* – at 30 °C on agar plates. In liquid medium all bacteria were cultivated at 30 °C with aeration.

DNA isolation

Plasmid DNA from *E. coli*, *Arthrobacter,* and *Rhodococcus* spp. was isolated by alkaline lysis method (Sambrook et al., 1989). For isolation of DNA from *Actinomycetales* bacteria, lysozyme (10 mg/mL) was added (Denis–Larosa et al., 1997). Total DNA from *Arthrobacter* sp. strains was isolated by method proposed by Woo et al. (1992) using TNE buffer and Triton X-100.

DNA digestion, dephosphorylation and phosphorylation

DNA was digested with appropriate DNA restriction endonucleases, dephosphorylated using CIAP (calf intestine alkaline phosphatase) or FastAP (thermosensitive alkaline phosphatase) or phosphorylated using T4 polynucleotide kinase according to the recommendations of manufacturer.

DNA electrophoresis in agarose gels

Horizontal DNA electrophoresis was performed using 0.8–1 % agarose gels in TAE buffer as described by Sambrook et al. (1989). Gels were stained with ethidium bromide and analyzed by UV using Herolab Transilluminator UVT-28ME (Germany).

Pulse field electrophoresis (PFGE) was used as following: DNA samples were mixed with 1.2 % low-melting agarose and loaded into a 1.2 % agarose gel. PFGE was performed using the Gene Navigator system from Pharmacia Biotech in 0,5 % TBE buffer for 24 h at 8° C. Pulse time: 10 s – 4 h; 15 s – 4 h; 20 s – 6 h, 25 s – 10 h. The gel was stained for 30 min with ethidium bromide (0.5 μg/ml) and visualized on a UV box.

DNA extraction from the agarose gel

DNA extraction Kit (Fermentas) was used. Procedure was performed according to the protocol of manufacturer.

DNA ligation

The mixture of insert DNA, linearized vector, T4 DNA ligase buffer, and T4 DNA ligase was prepared and incubated overnight at 10 °C or 1–2 hours at room temperature (22 °C). Ligase was inactivated by heating the mixture for 10 min at 65 °C and then cooled.

Preparation of electrocompetent cells and electroporation

E. coli competent cells were prepared by the method described by Sharma and Schimke (1996). *Arthrobacter* sp. and *Rhodococcus* sp. competent cells were prepared following the method proposed by Gartemann and Eichenlaub (2001). Briefly, DNA was mixed with 100 μL of ice-cold competent cells. Later, transferred to the electrocuvette (100 μL

capacity) and subjected to 1.8-2.0 kV electric pulse with duration of 4,6□5,6 ms. Pulsed cells were immediately diluted with 1 mL NB medium. *E. coli* cells were incubated for 30□45 min at 37 °C, whereas *Arthrobacter* sp. and *Rhodococcus* sp. cells-overnight at 30 °C. After the recovery, cells were spread on plates containing appropriate antibiotics and/or substrates.

Nucleotide sequence determination and analysis

Plasmid DNA was purified using ZYMO Plasmid PREP. The concentration of DNA was determined by electrophoresis in agarose gel using Mass Ruler^{TM} DNA Ladder (High Range). The nucleotide sequences were determined at the Sequencing Centre in the Vilnius University Institute of Biotechnology, Vilnius, Lithuania or at Macrogen (South Korea). The chromatograms of sequencing were analyzed using Chromas 2.24 program (http://www.technelysium.com.au/chromas.html) and VectorNTI AdvanceTM 9.0 program (Gorelenkov ir kt., 2001). The alignments were performed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The phylogenetic analysis was performed using MEGA 5.0 (Tamura ir kt., 2011). The evolutionary history was inferred using the Nieghbor-joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from the 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches, corresponding to partitions reproduced in less than 50 % bootstrap replicates, are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using Poisson correction method (Zuckerkandl & Pauling, 1965) and are represented in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

The phylogenetic analysis of replication proteins was performed using Mr. Bayes-3.1.2 program (Huelsenbeck, Ronquist, 2001).

Genome sequencing

Arthrobacter sp. 68b genome was sequenced using 454 platform and ILLUMINA technology by Macrogen (South Korea) and GATC (Germany).

Protein *de novo* sequencing

Proteins were extracted from polyacrylamide gel after separation. Samples were prepared for the mass spectrometry analysis according to the specifications of Proteomics centre in Vilnius University Institute of Biochemistry. Sequence of gene, encoding the protein of interest, was determined using obtained protein sequences and the BioEdit 7.1.3 program (Hall, 1999).

Plasmid curing

Cultivation at higher than optimal temperature. Bacteria were cultivated in 1 mL NB medium at 35 °C, 37 °C, 40 °C, 42 °C and 45 °C temperature overnight (18 hours) with aeration. Next day, 20 µL of cell extract were spread on NA or EFAA media, both containing 0.2 % of 2-hydroxypyridine.

Cultivation with novobiocin. Bacteria were cultivated in 5 mL of NB with 5, 10 and 15 µg/mL of novobiocin (or without it as the control) overnight (18 hours) at 30 °C

temperature with aeration. Then, the optical density (A600) was measured to evaluate the effect of novobiocin on the growth of bacteria. 20 µL of cells were spread on NA medium containing 0.2 % of 2-hydroxypyridine. Subsequently, viable bacterial clones were spread on EFAA containing 0.2 % of 2-hydroxypyridine.

PCR reactions

During this work (except for the amplification of fragments used for the construction of shuttle vectors), 2X Maxima Hot Start Green PCR Master Mix was used according to the recommendations of manufacturer. PCR was performed with T-personal thermocycler (Biometra) and *Mastercycler ep* gradient S (Eppendorf) devices.

qPCR for the determination of the copy number of plasmid pRMU824Km in *Arthrobacter* sp. 68b.

Total DNA was isolated from the overnight cultures of *Arthrobacter* sp. 68b (negative control) and *Arthrobacter* sp. 68b, harbouring plasmid pRMU824Km, by the method described by Woo et al. (1992). DNA samples (50 µg/mL) were diluted 100- and 1000-fold before the analysis. Quantitative real-time PCR amplification was carried out using a Rotor-Gene Q 6plex instrument (Qiagen). qPCR was conducted in 0.1-mL tubes containing 15 µL of reaction mixture: 200 nM of each primer, 200 µM dNTP, 3 mM MgCl2, 1.5 µM Syto9 (Invitrogen-Molecular Probes), 0.04 U/µL, TrueStartTM Taq DNA Polymerase, TrueStartTM Taq buffer and 1 µL of the DNA tested. The qPCR was initiated by 4 min of incubation at 95 °C, followed by 35 cycles of 95 °C for 20 s, 56 °C for 60 s and 72 °C for 60 s. Fluorescence data were recorded after the annealing steps. All experiments were carried out in triplicate.

A genome target, encoding the glycine oxidase (primers GlyOX68F and GlyOX68R), was used as a single-copy reference. The repAB genes (primers DP2 and RP2) were used as a plasmid target. The amplification efficiency for both targets was 1.12 and 1.06, respectively. The template-free negative control was used to estimate nonspecific binding. The copy number was calculated from the threshold cycle (C^T). The C^T values were calculated automatically according to the amplification plot. The difference between the mean C^T value of the single-copy reference and the mean C^T value of the vector target was calculated.

Determination of the minimal replicon

Plasmids pAPrepAB4 and pAPrepA2 were created by replacing pCG100 origin of replication (2.1 kb *Bgl*II–*Sal*I) in the pART2 plasmid with the appropriate DNA fragments from pPRH plasmid containing *ori* sequence with *repAB* operon (1.9 kb *Bam*HI-*Sal*I) for pAPrepAB4 plasmid, and *ori* sequence with *repA* gene (1.6 kb *Bam*HI–*Xho*I) for pAPrepA2 plasmid.

PCR for the construction of *E. coli-A. rhombi* shuttle vector

All PCRs were performed using *T Personal* thermocycler (Biometra) and AccuPrime Pfx DNA polymerase (Invitrogen). The reaction mixtures (total volume 25 µl) contained 0.5 µl of template DNA (50–100 ng), 2.5 µl 10X AccuPrime *Pfx* reaction mix, 0.5 µl of each primer (final concentration 2 µM) and 0.5 µl of AccuPrime *Pfx* DNA polymerase (1.25 units). The amplification conditions were as follows: 1 cycle of 95°C for 5 min, 30

cycles of 95°C for 30 s, 30 cycles of 52–62°C for 30 s, 30 cycles of 72°C for 1 min per kb, and 1 cycle of 72°C for 5 min.

Plasmid stability test

The method described by Picardeau et al. (2000) was used to determine the segregational stability of the vector pRMU824Km in *Arthrobacter* sp. 68b and *Rhodococcus erythropolis* SQ1 strains.

Plasmid compatibility test

The compatibility of plasmids pPRH and pART2gfp was investigated in *Arthrobacter oxydans* PY21. Bacteria, harbouring pRMU824Tc plasmid were transformed with pART2gfp. Subsequently, cells were spread on NA medium with tetracycline and kanamycin. Plasmid DNA was extracted from several clones and transformed into *E. coli*. DNA extracted from *E. coli* cells was analyzed by digestion with restriction endonucleases and horizontal gel electrophoresis.

Determination of capability to degrade various substrates

Arthrobacter sp. 68b strain was spread on agarized EFA medium with 0.1 % pyridine, 0.2 % 2-hydroxypyridine, 0.1 % 3-methylpyridine, 0.1 % 4-methylpyridine, 0.1 % nicotine, 0.1 % phthalic acid, 0.3 % succinic acid, 0.5 % glycerol, and without the substrate as the control. Plates were incubated for 72 hours at 30 °C.

Evaluation of optimal concentration of phthalic acid

For measuring growth rate, 1 mL of *Arthrobacter* sp. 68b bacteria night culture, grown in NB medium, was transferred into 20 mL of mineral EFA medium, containing 0.1–1 % of phthalic acid. Medium without the carbon source was used as a control. Optical density was measured by spectrophotometer at 600 nm wavelength every 24 hours.

Detection of phthalate 4,5-dioxygenase activity

To detect phthalate 4,5-dioxygenase activity, a diazotized *p*-nitroaniline was used as described by Nomura et al. (1989). The reagent was prepared by mixing 50 volumes of 0.3% (w/v) solution of *p*-nitroaniline in 0.8 N HCl and 3 volumes of 5% $NaNO_2$ solution immediately before use. *Arthrobacter* sp. 68b bacteria were cultivated at 30° C with aeration for 48 hours in EFA medium containing 0.5% phthalic acid. Cells were harvested by centrifugation (20000 *g* for 1 min). Later, they were washed with 20 mM Tris-HCl buffer (pH 8.0), and resuspended in the same buffer containing 2.5 mM of quinolinic acid. The cell suspension was incubated at 30° C, and samples were taken after 1 and 2 hours. Cells were eliminated by centrifugation, and 2 µL of diazotized *p*-nitroaniline reagent was added to 1 ml of supernatant. The absorption spectrum was read over the range from 300 to 700 nm.

Resting cells reaction

Arthrobacter sp. 68b cells were grown with aeration for 48 hours at 30 °C in 20 mL of EFA medium with 0.1 % 2-methylpyridine or pyridine and with 0.5 % phthalic acid. Cells were harvested by centrifugation at 20000*g* for 1 min and washed thrice with the buffer (detailed further).

Cells from 2.25 mL growth culture were used for **pyridine and 2-methylpyridine bioconversion**. They were washed with 25 mM potassium phosphate buffer (pH 7.2) and resuspended in 1 mL of the same buffer, and used as the resting cells. Then the substrate was added. The initial concentration of 2-methylpyridine and pyridine was 0.05 and 0.25 μM, respectively.

Cells from 1.5 mL growth culture were used for **the bioconversion of phthalic and quinolinic acids.** Cells were washed with 20 mM potassium phosphate buffer (pH 7.5), resuspended in 1 mL of the same buffer, and used as the resting cells. Then the substrate was added. The initial concentrations of phthalic and quinolinic acids were 1 mM and 0.4 mM, respectively.

After the preparation of reaction mixture, cells were resuspended and immediately harvested by centrifugation. Then the supernatant was transferred to cuvette and the primary spectrum was read over the range of 200 to 350 nm. Later, the supernatant was transferred to the same reaction tube; cells were resuspended and incubated in Thermomixer compact (Eppendorf) orbital shaker (350 rpm) at 30° C temperature. Spectra were read in chosen time intervals (detailed further in Results and discussion).

Preparation of cell-free extracts

The cells grown in liquid media were harvested by centrifugation and washed twice with 25 mM potassium phosphate buffer (pH 7.2), then resuspended in the same buffer, and disrupted by sonification (2 min at 22 kHz, in ice-water bath, 40 % of amplitude). The cell debris was removed by centrifugation (3220 *g* for 5 min).

Protein analysis by SDS-PAGE

Analysis was performed in the gel containing 10 % acrylamide for the separating gel, and 4.5 % acrylamide for the stacking gel. Gel was stained with PageBlue^{TM} Protein Staining Solution, Unstained Protein Molecular Weight Marker (14.4□116) was used as the standard. The procedure was performed following the manufacturer's recommendations.

RESULTS AND DISCUSSION

Investigation of plasmids and catabolic pathways in *Arthrobacter* sp. 68b bacteria

The strain 68b capable to degrade 2-methylpyridine as a sole carbon and energy source was previously isolated from the soil. 16S rRNA gene analysis revealed that it belongs to *Arthrobacter* genus (Gasparavičiūtė, 2008). The genome sequence analysis allowed identification of the entire 16S rRNA gene. The phylogenetic analysis (Fig. 1) revealed that *Arthrobacter* sp. 68b bacteria are most closely related to *Arthrobacter* sp. AD27 and T3AB1 bacteria isolated from soil and capable to degrade atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine). Since a distinct branch was clearly formed, these microorganisms may belong to a new species.

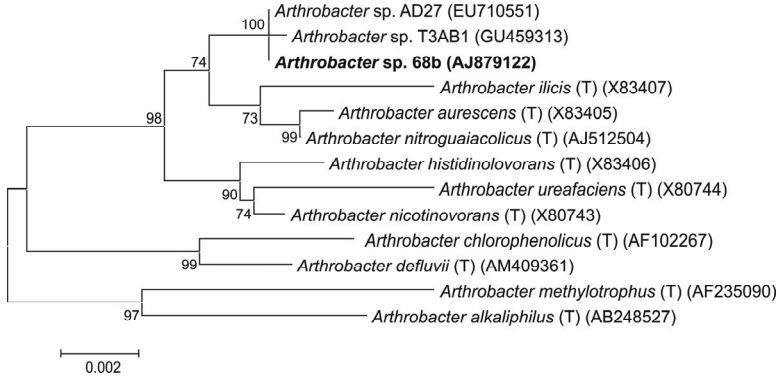

Fig. 1. The phylogenetic tree of the bacterial strain *Arthrobacter* sp. 68b capable to degrade 2-methylpyridine and its nearest relatives based on 16S rRNA gene sequence analysis, using the neighbour-joining method. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. Scale bar represents 2 expected substitutions per 1000 nucleotides. The culture collection accession number or the GenBank accession numbers are indicated for each strain. The phylogenetic method used here is described in detail in the Materials and methods.

It is known that *Arthrobacter* spp. bacteria are metabolically versatile. Thus, the capability of *Arthrobacter* sp. 68b strain to degrade other heterocyclic compounds (pyridine, 2-hydroxypyridine, 3-methylpyridine, 4-methylpyridine, and nicotine), phthalic and succinic acids, and glycerol as a sole carbon source was investigated. It was determined that this strain is able to utilise pyridine, nicotine, phthalate, succinate, and

glycerol. *Arthrobacter* sp. 68b mutants (mut4, mut5) unable to degrade 2-methylpyridine were previously obtained (Gasparavičiūtė, 2008). Further investigations revealed that mutant mut4 is not able to use any of the compounds that wild-type 68b bacteria can utilise as carbon source. Other mutant (mut5) was able to degrade nicotine, succinic acid, and glycerol.

It is known that pyridine and its derivatives degrading bacteria in general harbour large catabolic plasmids, containing degradation genes (Agapova et al., 1992; Mohan et al., 2003; Vedler, 2009). Commonly, the inability to degrade certain compounds is related with the plasmid loss (Kolenbrander & Weinberger, 1977; Weinberger & Kolenbrander, 1979; Overhage et al., 2005). Thus, the results of the substrate degradation of *Arthrobacter* sp. 68b and its mutants raised the hypothesis that these bacteria could harbour several catabolic plasmids.

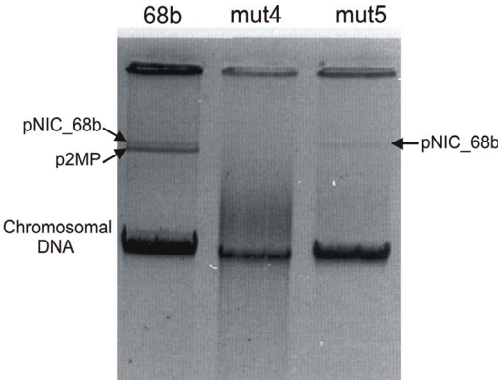

Fig. 2. Analysis of the total DNA from *Arthrobacter* sp. 68b, and its mutants (mut4 and mut5) by the agarose gel electrophoresis.

Total DNA isolated from *Arthrobacter* sp. 68b and its mutants (mut4 and mut5) was analyzed using horizontal and pulsed-field gel electrophoresis. Such analysis revealed that *Arthrobacter* sp. 68b bacteria harbour at least two large molecular weight plasmids, mut5 contains only one (the larger) plasmid, and mut4 has lost both plasmids (Fig. 2). The comparison of data, obtained during substrate specificity determination experiments, with DNA electrophoresis data revealed that the smaller plasmid, named p2MP, contains genes involved in pyridine, 2-methylpyridine, and phthalate degradation, whereas the larger plasmid, named pNIC_68b, harbours nicotine degradation genes.

The genome of *Arthrobacter* sp. 68b was sequenced using 454 platform and ILLUMINA technology. Sequencing data analysis allowed the identification of the

plasmid pNIC_68b-coded DNA fragment, containing nicotine degradation genes, and ~113 kb circular plasmid, encoding putative phthalic acid as well as pyridine and 2-methylpyridine degradation proteins. Comparative sequence analysis revealed that plasmid-borne nicotine degradation genes and their products identified show high percent of nucleotide sequence and amino acid similarity with homologous genes and proteins from *Arthrobacter nicotinovorans* 165 kb plasmid pAO1 (Igloi and Brandsch, 2003). Based on the results mentioned, it could be presumed that the size of pNIC_68b is similar to that of pAO1. *Arthrobacter* sp. 68b plasmid pNIC was not studied during this work, since the plasmid of the same structure had been already published.

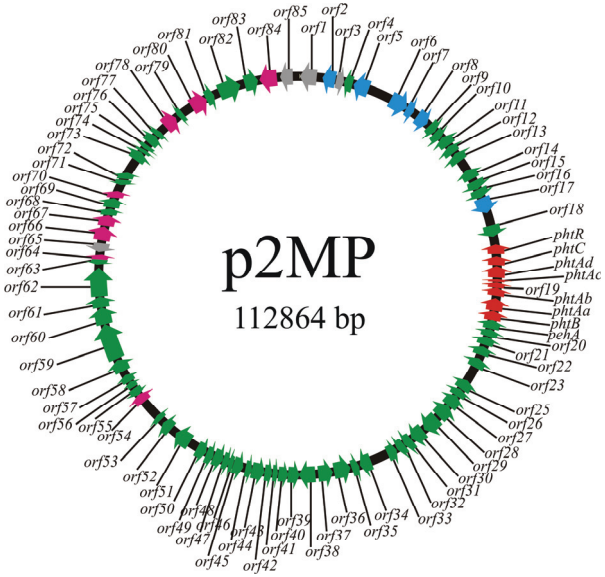

Fig. 3. *Arthrobacter* sp. 68b catabolic plasmid p2MP. Genes encoding putative proteins are indicated as follows: blue – 2-methylpyridine and pyridine degradation, red – phthalic acid degradation, pink – DNA replication, recombination, conjugation and partitioning; grey – tranposases and integrases; green – other functions.

The plasmid p2MP is 112864 bp in length with GC content of 61 mol%, which is similar to that of the chromosomal DNA of *Arthrobacter* spp. In total, 93 *orfs* were detected (Fig. 3) and their corresponding proteins were predicted (Table 1).

Table 1. *orf* analysis of *Arthrobacter* sp. 68b plasmid p2MP.

orf	Encoded protein length, a.a.	Homology	Accesion no. of data bases	Identical aa/ total aa (%)	E value	Reference
orf1	490	transposase IS4 family protein *Nakamurella multipartita* DSM 44233	YP_003199534	248/528 (47%)	4e-143	Tice et al., 2010
orf2	384	luciferase-like monooxygenase *Rhodococcus pyridinivorans* AK37	ZP_09310356	147/360 (41%)	2e-82	Kriszt et al., 2012
orf3	167	integrase catalitical region *Saccharopolyspora spinosa* NRRL 18395	ZP_08877666	49/81 (60%)	2e-19	Pan et al., 2011
orf4	165	riboflavin kinase/FAD syntetase *Amycolatopsis mediterranei* U32	YP_003764712	66/138 (48%)	2e-32	Zhao et al., 2010
orf5	465	succinate semialdehyde dehydrogenase *Streptomyces* sp. S4	ZP_09178778	330/456 (72%)	0	Seipke et al., unpublished
orf6	558	amidohydrolase *Nocardiopsis dassonvillei subsp. dassonvillei* DSM 43111	YP_003678962	289/544 (53%)	0	Sun et al., 2010
orf7	178	flavin reductase, FMN-binding protein *Mycobacterium* sp. MCS	YP_638703	86/154 (56%)	9e-52	Copeland et al., unpublished
orf8	384	luciferase-like monooxygenase *Rhodococcus pyridinivorans* AK37	ZP_09310356	147/360 (41%)	2e-82	Kriszt et al., 2012
orf9	88	unnamed protein product *Arthrobacter* sp. FB24	YP_833278	39/71 (55%)	1e-17	Copeland et al., unpublished
orf10	76	hypothetical protein Asphe3_34180 *Arthrobacter phenanthrenivorans* Sphe3	YP_004242657	22/55 (40%)	9e-06	Lucas et al., unpublished
orf11	230	3,4-dihydroxy-2-butanon-4-phosphate syntase *Arthrobacter globiformis* NBRC 12137	ZP_09280002	155/200 (78%)	2e-104	Miyazawa et al., unpublished
orf12	85	hypothetical protein ARGLB_036_00300 *Arthrobacter globiformis* NBRC 12137	ZP_09279305	22/44 (50%)	0,029	Miyazawa et al., unpublished
orf13	139	hypothetical protein SchaN1_07861 *Streptomyces chartreusis* NRRL 12338	ZP_09951866	51/126 (40%)	1e-22	Doroghazi et al., 2011

orf14	396	hypothetical protein SACE_3483 *Saccharopolyspora erythraea* NRRL 2338	YP_001105682	128/361 (35%)	8e-51	Oliynyk et al., 2007
orf15	115	glyoxylase/bleomycin resistant protein/dioxygenase *Pseudonocardia dioxanivorans* CB1190	YP_004335127	73/112 (65%)	6e-37	Sales et al., 2011
orf16	100	D-fructose-6-phosphate amidotransferase *alpha proteobacterium* BAL199	ZP_02188273	37/94 (39%)	5e-14	Hagstrom et al., unpublished
orf17	494	succinate semialdehyde dehydrogenase *Arthrobacter phenanthrenivorans* Sphe3	YP_004240547	439/486 (90%)	0	Lucas et al., unpublished
orf18	337	AraC family transcriptional regulator *Rhodococcus jostii* RHA1	YP_700292	166/315 (53%)	2e-91	McLeod et al., 2006
phtR	284	PhtR family transcriptional regulator *Arthrobacter phenanthrenivorans* Sphe3	YP_004243277	221/269 (82%)	6e-157	Lucas et al., unpublished
phtC	249	3,4-dihydroxyphthalate decarboxylase *Arthrobacter phenanthrenivorans* Sphe3	YP_004243276	201/247 (81%)	6e-140	Lucas et al., unpublished
phtAd	411	phthalate 3,4-dioxygenase, ferredoxin reductase subunit *Arthrobacter phenanthrenivorans* Sphe3	YP_004243275	333/409 (81%)	0	Lucas et al., unpublished
phtAc	65	ferredoxin *Arthrobacter phenanthrenivorans* Sphe3	YP_004243274	55/64 (86%)	1e-32	Lucas et al., unpublished
orf19	111	hypothetical protein Arth_4365 *Arthrobacter* sp. FB24	YP_829383	92/109 (84%)	1e-59	Copeland et al., unpublished
phtAb	202	phthalate 3,4-dioxygenase subunit beta *Arthrobacter phenanthrenivorans* Sphe3	YP_004243272	169/195 (87%)	5e-124	Lucas et al., unpublished
phtAa	474	phthalate 3,4-dioxygenase, alpha subunit *Arthrobacter phenanthrenivorans* Sphe3	YP_004243447	430/473 (91%)	0	Lucas et al., unpublished
phtB	289	3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase *Arthrobacter keyseri*	AF331043_13	221/288 (77%)	2e-163	Eaton, 2001
pehA	222	putative phthalate ester hydrolase *Arthrobacter keyseri*	AF331043_12	190/214 (89%)	1e-140	Eaton, 2001
orf20	267	nitrate/sulfonate/bicarbonate ABC transporter permease *Arthrobacter phenanthrenivorans* Sphe3	YP_004243451	236/265 (89%)	1e-144	Lucas et al., unpublished
orf21	267	nitrate/sulfonate/bicarbonate ABC transporter permease *Arthrobacter phenanthrenivorans* Sphe3	YP_004243451	236/265 (89%)	1e-144	Lucas et al., unpublished

orf22	276	nitrate/sulfonate/bicarbonate ABC transporter ATPase *Arthrobacter phenanthrenivorans* Sphe3	YP_004243266	247/275 (90%)	1e-178	Lucas et al., unpublished
orf23	335	nitrate/sulfonate/bicarbonate ABC transporter system periplasmic protein *Arthrobacter phenanthrenivorans* Sphe3	YP_004243265	284/334 (85%)	0	Lucas et al., unpublished
orf24	326	oxidoreductase *Rhodococcus jostii* RHA1	YP_706605	171/328 (52%)	6e-111	McLeod et al., 2006
orf25	176	hypothetical protein RHA1_ro06673 *Rhodococcus jostii* RHA1	YP_706604	71/152 (47%)	1e-32	McLeod et al., 2006
orf26	390	hypothetical protein AK37_11196 *Rhodococcus pyridinivorans* AK37	ZP_09309319	215/369 (58%)	3e-123	Kriszt et al., 2012
orf27	428	Rieske (2Fe-2S) protein *Rhodococcus imtechensis* RKJ300	ZP_10002289	356/427 (83%)	0	Kumar et al., unpublished
orf28	514	IclR family transcriptional regulator *Rhodococcus pyridinivorans* AK37	ZP_09309323	124/239 (52%)	4e-72	Kriszt et al., 2012
orf29	434	transport protein *Rhodococcus imtechensis* RKJ300	ZP_10002293	250/391 (64%)	1e-173	Kumar et al., unpublished
orf30	140	hypothetical protein AAur_1893 *Arthrobacter aurescens* TC1	YP_947642	55/68 (81%)	3e-28	Mongodin et al., 2006
orf31	98	hypothetical protein Achl_0077 *Arthrobacter chlorophenolicus* A6	YP_002486169	29/58 (50%)	1e-07	Lucas et al., unpublished
orf32	294	family 2 glycosyl transferase *Arthrobacter chlorophenolicus* A6	YP_002487344	135/254 (53%)	2e-64	Lucas et al., unpublished
orf33	332	cell division ATP-binding protein FtsE *Arthrobacter* sp. FB24	ABK04044	135/221 (61%)	3e-86	Copeland et al., unpublished
orf34	152	hypothetical protein pSI-1_30 *Arthrobacter* sp. AK-1	YP_001741970	126/142 (89%)	8e-88	Jerke et al., 2008
orf35	590	putative conjugative transfer gene complex protein *Arthrobacter* sp. AK-1	YP_001741969	572/589 (97%)	0	Jerke et al., 2008
orf36	452	hypothetical protein pSI-1_28 *Arthrobacter* sp. AK-1	YP_001741968	437/451 (97%)	0	Jerke et al., 2008
orf37	503	hypothetical protein pSI-1_27 *Arthrobacter* sp. AK-1	YP_001741967	492/502 (98%)	0	Jerke et al., 2008
orf38	418	hypothetical protein pSI-1_26 *Arthrobacter* sp. AK-1	YP_001741966	316/426 (74%)	1e-142	Jerke et al., 2008
orf39	292	hypothetical protein pSI-1_25 *Arthrobacter* sp. AK-1	YP_001741965	127/252 (50%)	9e-61	Jerke et al., 2008

orf40	90	hypothetical protein pSI-1_24 *Arthrobacter* sp. AK-1	YP_001741964	89/89 (100%)	1e-51	Jerke et al., 2008
orf41	197	hypothetical protein pSI-1_23 *Arthrobacter* sp. AK-1	YP_001741963	188/196 (96%)	3e-132	Jerke et al., 2008
orf42	478	hypothetical protein pSI-1_22 *Arthrobacter* sp. AK-1	YP_001741962	443/477 (93%)	0	Jerke et al., 2008
orf43	133	hypothetical protein pSI-1_21 Arthrobacter sp. AK-1	YP_001741961	119/132 (90%)	1e-79	Jerke et al., 2008
orf44	359	CHAP domain-containing protein *Arthrobacter* sp. AK-1	YP_001741960	311/359 (87%)	0	Jerke et al., 2008
orf45	116	hypothetical protein Arth_4511 *Arthrobacter* sp. FB24	YP_829261	74/111 (67%)	1e-38	Copeland et al., unpublished
orf46	144	hypothetical protein Arth_4512 *Arthrobacter* sp. FB24	YP_829262	117/142 (82%)	5e-78	Copeland et al., unpublished
orf47	340	hypothetical protein Arth_4513 *Arthrobacter* sp. FB24	YP_829263	174/324 (54%)	6e-108	Copeland et al., unpublished
orf48	189	hypothetical protein Arth_4514 *Arthrobacter* sp. FB24	YP_829264	108/181 (60%)	1e-69	Copeland et al., unpublished
orf49	284	hypothetical protein Arth_4515 *Arthrobacter* sp. FB24	YP_829265	186/282 (66%)	8e-130	Copeland et al., unpublished
orf50	612	hypothetical protein Achl_4545 *Arthrobacter chlorophenolicus* A6	YP_002478472	435/618 (70%)	0	Lucas et al., unpublished
orf51	257	cobyrinic acid a,c-diamide synthase *Arthrobacter* sp. FB24	YP_829398	232/256 (91%)	5e-170	Copeland et al., unpublished
orf52	108	hypothetical protein Arth_4175 *Arthrobacter* sp. FB24	YP_829397	66/107 (62%)	1e-25	Copeland et al., unpublished
orf53	337	hypothetical protein AAur_pTC10275 *Arthrobacter aurescens* TC1	YP_950144	216/330 (65%)	2e-82	Mongodin et al., 2006
orf54	200	putative site-specific recombinase *Arthrobacter* sp. AK-1	YP_001741953	188/199 (94%)	2e-130	Jerke et al., 2008
orf55	86	conserved hypothetical protein *Nocardioides* sp. JS614	YP_921291	39/82 (48%)	7e-11	Coleman et al., 2011
orf56	174	hypothetical protein ARGLB_008_00050 *Arthrobacter globiformis* NBRC 12137	ZP_09278157	97/174 (56%)	4e-50	Miyazawa et al., unpublished
orf57	380	DNA-cytosine methyltransferase *Cellvibrio gilvus* ATCC 13127	YP_004602009	190/352 (54%)	3e-131	Lucas et al., unpublished

orf58	1097	hypothetical protein OPAG_00219 *Rhodococcus opacus* PD630	EHI41030	350/1017 (34%)	5e-169	Holder et al., 2011
orf59	520	hypothetical protein RER_40510 *Rhodococcus erythropolis* PR4	YP_002767498	187/498 (38%)	4e-94	Sekine et al., 2006
orf60	333	hypothetical protein A20C1_10409 marine actinobacterium PHSC20C1	ZP_01129291	125/314 (40%)	1e-66	Murray et al., unpublished
orf61	1030	hypothetical protein GOAMR_50_01130 *Gordonia amarae* NBRC 15530	ZP_09216759	470/1063 (44%)	0	Takarada et al., unpublished
orf62	105	hypothetical protein Achl_4499 *Arthrobacter chlorophenolicus* A6	YP_002478426	75/104 (72%)	2e-47	Lucas et al., unpublished
orf63	103	hypothetical protein Arth_0995 *Arthrobacter* sp. FB24	YP_830492	87/102 (85%)	1e-65	Copeland et al., unpublished
orf64	180	ATP-dependent DNA ligase *Arthrobacter phenanthrenivorans* Sphe3	YP_004243397	101/133 (76%)	1e-65	Lucas et al., unpublished
orf65	508	transposase *Micrococcus luteus* NCTC 2665	YP_002957277	352/480 (73%)	0	Lucas et al., unpublished
orf66	242	DNA replication protein *Arthrobacter phenanthrenivorans* Sphe3	YP_004239991	208/241 (86%)	3e-150	Lucas et al., unpublished
orf67	189	RNA polymerase sigma factor, sigma-70 family *Arthrobacter phenanthrenivorans* Sphe3	YP_004243335	98/175 (56%)	4e-60	Lucas et al., unpublished
orf68	287	peptidase M56 family protein *Arthrobacter globiformis* NBRC 12137	ZP_09279059	178/278 (64%)	9e-112	Miyazawa et al., unpublished
orf69	142	conserved hypothetical protein *Streptomyces lividans* TK24	ZP_06531183	63/132 (48%)	1e-37	Fischbach et al., unpublished
orf70	84	DNA ligase *Arthrobacter phenanthrenivorans* Sphe3	YP_004243362	62/83 (75%)	7e-34	Lucas et al., unpublished
orf71	110	hypothetical protein pSI-1_88 *Arthrobacter* sp. AK-1	YP_001742026	68/96 (71%)	4e-36	Jerke et al., 2008
orf72	143	hypothetical protein Asphe3_41650 *Arthrobacter phenanthrenivorans* Sphe3	YP_004243364	117/142 (82%)	1e-83	Lucas et al., unpublished
orf73	329	hypothetical protein Arth_4331 *Arthrobacter* sp. FB24	YP_829349	160/335 (48%)	7e-93	Copeland et al., unpublished
orf74	50	hypothetical protein Asphe3_41720 *Arthrobacter phenanthrenivorans* Sphe3	YP_004243371	23/46 (50%)	2e-05	Lucas et al., unpublished

orf75	71	hypothetical protein pSI-1_75 *Arthrobacter* sp. AK-1	YP_001742015	41/68 (60%)	5e-18	Jerke et al., 2008
orf76	357	putative smf family protein *Arthrobacter* sp. AK-1	YP_001742013	311/383 (81%)	0	Jerke et al., 2008
orf77	104	hypothetical protein *Arthrobacter nicotinovorans*	CAD48021	55/102 (54%)	7e-28	Grether-Beck et al., 1994
orf78	470	parB-like partition protein *Arthrobacter chlorophenolicus* A6	YP_002478569	374/450 (83%)	0	Lucas et al., unpublished
orf79	103	hypothetical protein pSI-1_71 *Arthrobacter* sp. AK-1	YP_001742011	91/102 (89%)	2e-57	Jerke et al., 2008
orf80	601	putative relaxase *Arthrobacter* sp. AK-1	YP_001742009	437/457 (96%)	0	Jerke et al., 2008
orf81	253	hypothetical protein pSI-1_68 *Arthrobacter* sp. AK-1	YP_001742008	217/252 (86%)	1e-134	Jerke et al., 2008
orf82	819	hypothetical protein Achl_0171 *Arthrobacter chlorophenolicus* A6	YP_002486262	266/785 (34%)	1e-114	Lucas et al., unpublished
orf83	411	hypothetical protein AoriK_08859 *Actinomyces oris* K20	ZP_08126590	269/407 (66%)	0	Yamane and Nambu, unpublished
orf84	518	putative site-specific recombinase *Arthrobacter* sp. AK-1	YP_001741999	247/521 (47%)	1e-157	Jerke et al., 2008
orf85	371	integrase family protein Mycobacterium rhodesiae JS60	EHB53325	208/363 (57%)	7e-145	Lucas et al., unpublished

It was determined that the majority of predicted proteins are homologous to those from *Arthrobacter* spp. and *Rhodococcus* spp. The fragments of 11,3 kb (*orf*34–*orf*44) and 2,5 kb (*orf*45–*orf*49) are similar to DNA fragments of *Arthrobacter* sp. AK-1 pSI-1 and *Arthrobacter* sp. FB24 pFB24-136 plasmids, respectively. The plasmid p2MP contains genes for transposases, integrases, and nitrate/sulfonate/bicarbonate ABC transporter as well as those essential for such plasmid functions as replication, recombination, partitioning and conjugation.

The genes for degradation of phthalic acid, pyridine and 2-methylpyridine were also identified in the plasmid p2MP. The metabolic pathways and the degradation proteins of the compounds mentioned are being analysed further.

Degradation of phthalic acid

Gram-negative and Gram-positive bacteria degrade phthalic acid to protocatechuate via 2 different pathways, 4,5-dioxygenase and 3,4-dioxygenase, respectively. It could be supposed that Gram-positive *Arthrobacter* sp. 68b strain should degrade phthalic acid via 3,4-dioxygenase pathway. Thus, the metabolic pathway and respective degradation genes were investigated. The optimal phthalic acid concentration for growth of the *Arthrobacter* sp. 68b was determined as described in the Materials and methods. After 48 hours, the highest amount of cells was observed with 1% phthalic acid concentration (data not shown). After 72 hours, the decrease of the optical density was observed in all cultures.

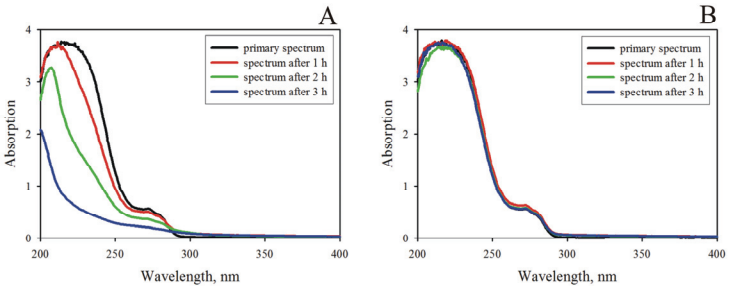

Fig. 4. Bioconversion of phthalic acid using *Arthrobacter* sp. 68b resting cells, pre-grown with phthalic acid (A) and succinate (B).

Bioconversion of phthalic acid was analyzed using *Arthrobacter* sp. 68b resting cells, pre-grown with phthalate and succinate. Initial substrate concentration was 1 mM. The spectra of supernatant from the reaction mixture were recorded after 1, 2, 3, and 4 hours. It was found that *Arthrobacter* sp. 68b cells could use phthalic acid only if they were pre-grown in the presence of this substrate (Fig. 4 A). The cells cultivated in the presence of succinic acid were not able to utilise phthalic acid (Fig. 4 B). Hence, it is obvious that phthalate utilization is an inducible process in the *Arthrobacter* sp. 68b cells.

Degradation of quinolinic acid (pyridine-2,3-dicarboxylic acid)

Nomura et al. (1989) described the method to determine the activity of 4,5-dioxygenase and 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase. They also showed that *Pseudomonas putida*, capable to degrade phthalic acid via 4,5-dioxygenase pathway, were also capable to convert quinolinic acid to a hydroxylated product (Nomura et al., 1989). Using this method it was determined that in the case of *Arthrobacter* sp. 68b bacteria, only 3,4-dioxygenase pathway exists. The ability of phthalate-induced *Arthrobacter* sp. 68b cells to consume quinolinic acid was also tested. It was found that the cells converted quinolinic acid if they were pre-grown in the presence of phthalic acid. Changes in the UV-VIS spectrum during the biotransformation are illustrated in Fig. 5 A. The cells cultivated in the presence of succinic acid were not able to use quinolinic acid (Fig. 5 B).

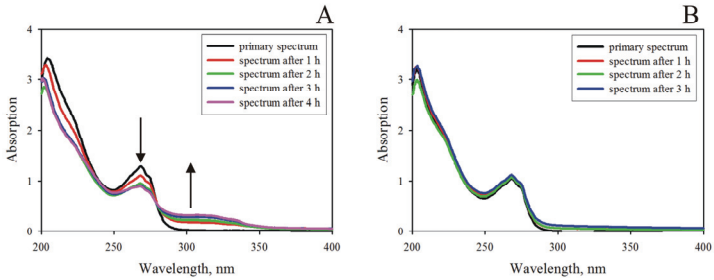

Fig. 5. Bioconversion of quinolinic acid using *Arthrobacter* sp. 68b resting cells pre-grown with phthalic acid (A) and succinate (B). The arrows show changes in absorption during the bioconversion.

Hence, an induction of phthalate catabolic genes was necessary for the utilization of quinolinic acid by *Arthrobacter* sp. 68b cells. Moreover, the increase of absorbance in 300–350 nm spectral region during bioconversion procedure was observed. This indicates the introduction of a hydroxyl group into the heterocyclic ring (Taylor & King, 1987). Bioconversion of quinolinic acid by *Arthrobacter* sp. 68b cells opens the possibility for biocatalytic synthesis of new hydroxylated *N*-heterocyclic compounds.

Analysis of the phthalate degradation operon

It is known that phthalic acid operon (*pht*) generally consists of six genes, encoding phthalate degradation to protocatechuate (Eaton, 2001). The analysis of the *Arthrobacter* sp. 68b *pht* operon showed that it consists of 7 instead of 6 *orfs* (Fig. 6), as an additional *orf19* was detected. Genes encoding the conversion of phthalate to protocatechuate and the regulatory gene are arranged in the *phtBAaAbAcAdCR* order and transcribed in the same direction (Fig. 6).

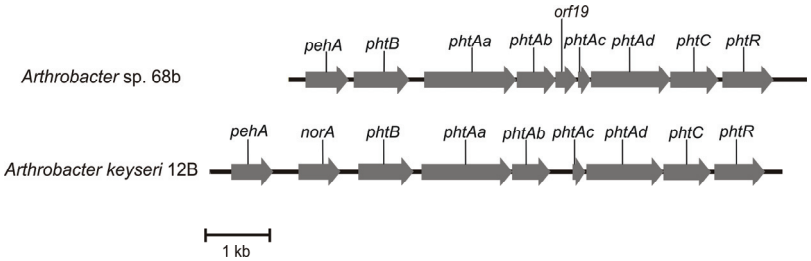

Fig. 6. Phthalate degradation operons and the organization of genes in *Arthrobacter* sp. 68b and *Arthrobacter keyseri* 12B (Eaton, 2001).

The comparison of the organization of genes involved in phthalate metabolism in *Arthrobacter* sp. 68b and *Arthrobacter keyseri* 12B revealed that the genes of both operons are arranged in the same order and transcribed in the same direction. The gene *pehA*, encoding a putative phthalate ester hydrolase, is located upstream of the *pht* operon in both cases. This gene is transcribed in the same direction as genes of phthalate degradation operon. As the organization of both operons was similar, genes of the phthalate operon from *Arthrobacter* sp. 68b were named in the manner of those from *A. keyseri* 12B (Eaton, 2001).

A homology search revealed that all gene products are most similar to phthalate degradation proteins from other *Arthrobacter* spp. strains. In all cases, the phylogenetic analysis of proteins revealed that they are mostly related to *Arthrobacter* spp. proteins and form a separate group in the phylogenetic tree (data not shown).

Based on the results of homology search and the reaction with diazotized *p*-nitroaniline, it can be concluded that *Arthrobacter* sp. 68b bacteria degrade phthalic acid via 3,4-dioxygenase pathway. Cells, pre-grown with phthalic acid, are capable to use quinolinic acid. It was determined that catabolic proteins analyzed here differ from their related counterparts in *Arthrobacter* spp.

Degradation of pyridine and 2-methylpyridine

According to literature, the bacteria capable to degrade pyridine generally are also able to use one or more of its derivatives (Shukla, 1974; Shukla & Kaul, 1986; Agapova et al., 1992; Khasaeva et al., 2011). *Arthrobacter* sp. 68b bacteria can degrade 2-methylpyridine as well as pyridine.

The investigation of pyridine and 2-methylpyridine bioconversion was carried out using *Arthrobacter* sp. 68b resting cells pre-grown in EFA medium with pyridine, 2-methylpyridine, succinate, or without the substrate. Pyridine and 2-methylpyridine were used at an initial concentration of 0.25 μM and 0.05 μM respectively.

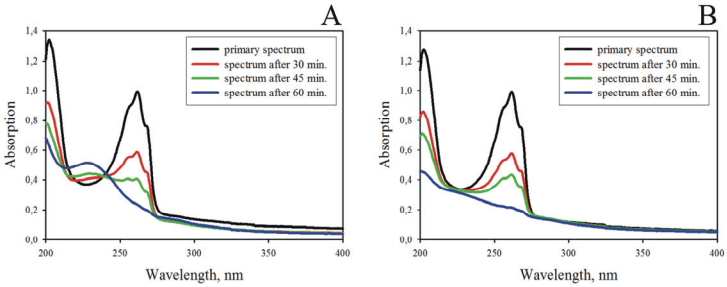

Fig. 7. Bioconversion of 2-methylpyridine using *Arthrobacter* sp. 68b resting cells, pre-grown with 2-methylpyridine (A) and pyridine (B).

It was determined that cells, cultivated with succinic acid or without any substrate could utilize neither 2-methylpyridine nor pyridine (data not shown). Cells, pre-grown with 2-methylpyridine or pyridine, were able to degrade both compounds (Fig. 7 and Fig. 8) without the addition of cofactors, co-substrates or metal ions. Thus,

the results of bioconversion indicate that the degradation of pyridine and 2-methylpyridine are inducible processes, and that some of the enzymes in the degradation pathways are shared.

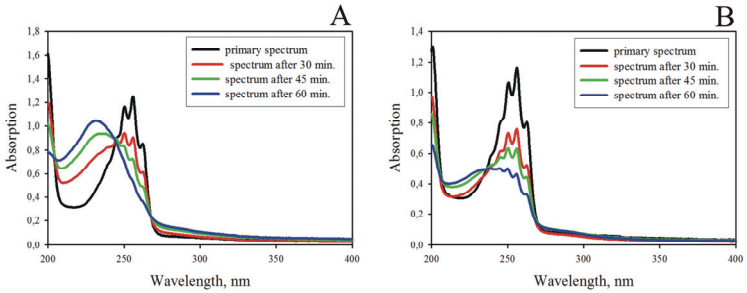

Fig. 8. Bioconversion of pyridine using *Arthrobacter* sp. 68b resting cells, pre-grown with 2-methylpyridine (A) and pyridine (B).

Investigation of proteins, induced by pyridine and 2-methylpyridine

In order to confirm that 2-methylpyridine and pyridine degradation pathways share the same enzymes, proteins induced by these compounds were analysed. *Arthrobacter* sp. 68b bacteria were grown in EFA medium, containing 0.2 % of 2-methylpyridine, pyridine, 0.5 % of glucose and succinic acid (control). The composition of cell proteins was investigated in the polyacrylamide gel under denaturing conditions. It was determined that 2-methylpyridine and pyridine induce protein of similar molecular weight (Fig. 9).

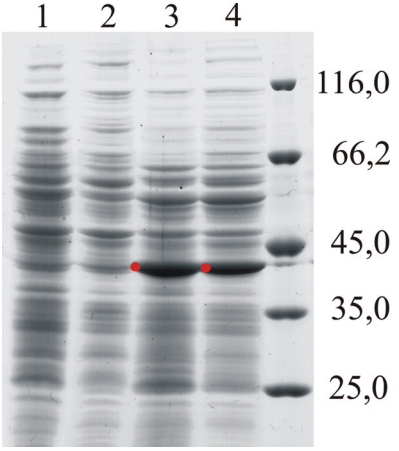

Fig. 9. *Arthrobacter* sp. 68b proteins induced by 2-methylpyridine and pyridine. *Arthrobacter* sp. bacterium cultivated in EFA medium with: 1 – succinic acid, 2 – glucose, 3 – 2-methyl-pyridine, 4 – pyridine. The size of protein markers are indicated in kDa.

The 40 kDa protein induced by 2-methylpyridine and pyridine was prepared for *de novo* sequencing. Four potential peptides were identified and their putative amino acid sequences were determined (Fig. 10). Since the molecular weight of leucine (L) and isoleucine (I) is the same, and the molecular weight of lysine (K) (146,19 Da) and glutamine (Q) (146,15 Da) is very similar, the mass spectrometer used in this work could not separate them, hence, L and I or K and Q were interpreted equally.

The predicted sequences of peptides were used to identify the target gene (encoding the protein of interest) in partially sequenced genome of *Arthrobacter* sp. 68b. All peptides were found to be present in the predicted amino acid sequence of a single protein (Fig. 10). Based on this sequence, the gene was identified using BioEdit 7.1.3 program. The analysed protein was found to be most homologous to luciferase-like monooxygenase from *Rhodococcus pyridinivorans* AK37 (Kriszt et al., 2012).

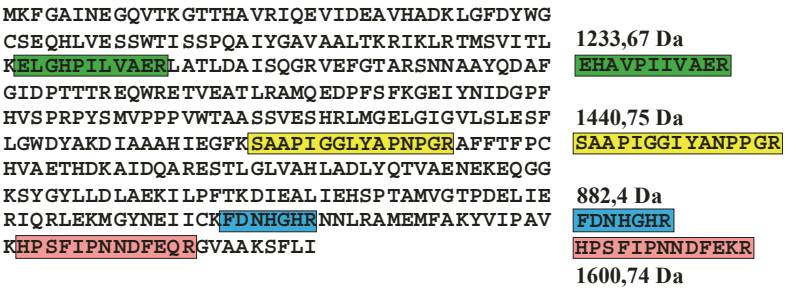

Fig. 10. The amino acid sequences of 4 *de novo* determined potential peptides and their location in the predicted sequence of protein encoded by *orf*2/*orf*8.

Luciferase-like monooxygenases from various *Actinomycetales* species as well as those from Gram-negative bacteria are found among the homologues of the predicted protein. The phylogenetic analysis revealed that *Arthrobacter* sp. 68b monooxygenase is most closely related to *R. pyridinovorans* AK37, *Pseudomonas putida* S16 and *Burkholderia ambifaria* AMMD luciferase family proteins, all of which form a distinct branch on the phylogenetic tree (Fig. 11).

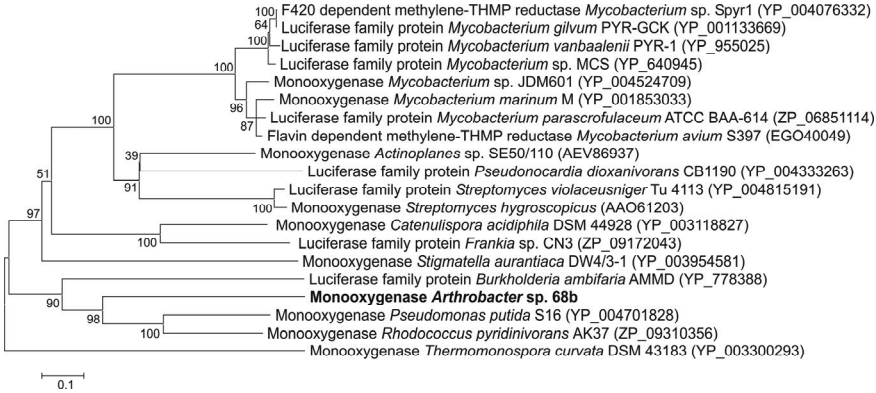

Fig. 11. Neighbour-joining tree based on the amino acid alignment of *orf2*/*orf8* encoding protein from *Arthrobacter* sp. 68b and its nearest relatives. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Scale bar represents expected amino acid substitutions per position. The GenBank accession number is indicated for each protein. Details of phylogenetic method are given in Materials and methods. THMP – tetrahydromethanopterin.

Phthalic acid degradation proteins described above are most closely related to *Arthrobacter* spp. proteins. Meanwhile, the determined monooxygenase is homologous to proteins from several species, including Gram-negative bacteria. This indicates that the origin of analysed proteins could be different, despite the fact that the degradation genes of phthalic acid, 2-methylpyridine, and pyridine are located on the same plasmid. It could be stated that the gene for monooxygenase, related to that from Gram-negative bacteria, had most likely been obtained via the horizontal gene transfer.

Putative pyridine and 2-methylpyridine degradation genes

The gene for monooxygenase was identified in the 22 kb fragment of *Arthrobacter* sp. 68b plasmid p2MP. It contains 17 *orfs* (Fig. 12). The putative functions of predicted proteins are listed in Table 2. *orf2* and *orf8* encode proteins that show the highest degree of sequence homology with the luciferase-like monooxygenase from the *Rhodococcus pyridinivorans* AK37 (Kriszt ir kt., 2012). The nucleotide sequences of both *orfs* are identical, suggesting the gene duplication. Two *orfs* (*orf5* and *orf17*) encoding putative succinate semialdehyde dehidrogenase were also identified. A homology search results indicated that protein, encoded by *orf5* (72 % identity, and 84 % similarity), is less homologous to the succinate semialdehyde dehydrogenase than that

(89 % identity, 94 % similarity), encoded by *orf*17. Previously, it was revealed that the protein encoded by *orf17* is induced by 2-methylpyridine, and its activity was proved by the reaction of resting cells with succinate semialdehyde (Bachmatova & Marcinkevičienė, personal communication). Based on all mentioned results, it could be predicted that succinate semialdehyde is produced by *Arthrobacter* sp. 68b cells during the degradation of 2-methylpyridine and pyridine.

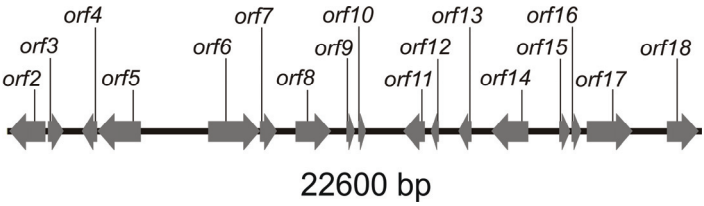

Fig. 12. *Arthrobacter* sp. 68b DNA fragment containing putative 2-methylpyridine and pyridine degradation genes.

Hypothetical pathway of pyridine and 2-methylpyridine degradation

Based on the obtained results, a multi-step degradation pathway of 2-methylpyridine and pyridine in *Arthrobacter* sp. 68b bacteria could be proposed (Fig. 13). The monooxygenase (*orf2*/*orf8*) catalyzes the first step leading to the cleavage of the pyridine ring between C2 and C3 atoms, and in the case of 2-methylpyridine, *N*-[(1*Z*)-4-oksobut-1-en-1-yl]acetamide is formed. A flavin reductase (*orf7*) probably also participates in this reaction. The intermediate may isomerize to *N*-[(1*Z*)-4-oxobutiliden]acetamide or may be cleaved to (3*Z*)-4-aminobut-3-enal and acetic acid by hydrolase. (3*Z*)-4-aminobut-3-enal isomerizes into 4-iminobutanal that is hydrolyzed spontaneously or by amidohydrolase (*orf6*) to succinic acid dialdehyde. The dialdehyde may be formed from *N*-[(1*Z*)-4-oxobutilideno]acetamide by the reaction of amidohydrolase (*orf6*) when acetamide is detached. The succinate dialdehyde dehydrogenase (*orf5*) and succinate semialdehyde dehydrogenase (*orf17*) catalyze the oxidation of succinate dialdehyde to succinic acid, that is further converted in the tricarboxylic acid cycle. The degradation of pyridine may proceed by analogous pathway, except that at first *N*-[(1*Z*)-4-oksobut-1-en-1-yl]formamide is formed. It either isomerizes to *N*-[(1*Z*)-4-oxobutiliden]formamide or may be cleaved to formic acid and

(3*Z*)-4-aminobut-3-enal. As mentioned above this compound isomerizes to 4-iminobutanal that is hydrolyzed to succinic acid dialdehyde, which is also formed from *N*-[(1*Z*)-4-oxobutiliden]formamide when formamide is detached. Enzymes catalyzing described reactions are the same as in the case of 2-methylpyridine degradation.

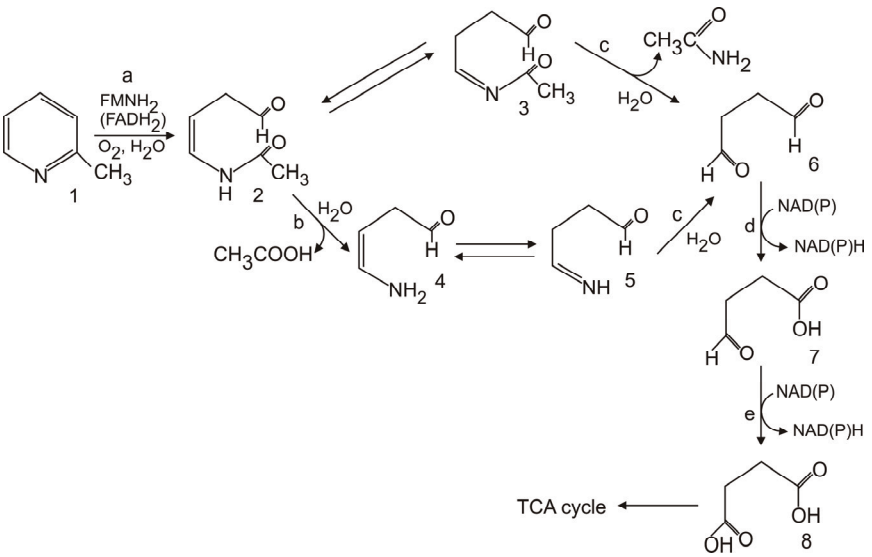

Fig. 13. The hypothetical degradation pathway of 2-methylpyridine and pyridine in *Arthrobacter* sp. 68b bacteria. 1 – 2-metilpiridine, 2 – *N*-[(1*Z*)-4-oxobut-1-en-1-yl]acetamide, 3 – *N*-[(1*Z*)-4-oxobutilideno]acetamide, 4 – (3*Z*)-4-aminobut-3-enal, 5 – 4-iminobutanal, 6 – succinate dialdehyde, 7 – succinate semialdehyde, 8 – succinic acid; a – monooxygenase and flavin reductase, b – hydrolase, c – amidohydrolase, d – succinate dialdehyde dehydrogenase, e – succinate semialdehyde dehydrogenase; TCA cycle – tricarboxylic acid cycle.

The putative bacterial degradation pathways of pyridine most often starts with reduction or hydroxylation reactions. Later, the pyridine ring is cleaved between C2 and C3 atoms by dioxygenases or hydrolases. Although many different pyridine degradation pathways are proposed, in most cases, succinate semialdehyde and succinic acid are produced (Watson & Cain, 1975; Sims et al., 1986; Korosteleva et al., 1981; Khasaeva et al., 2011b). In *Arthrobacter* sp. 68b the pyridine ring is neither hydrated nor hydroxylated. As mentioned above, the degradation starts by oxydation, and the pyridine ring is cleaved between C2 and C3 atoms. Later, succinate semialdehyde and succinic acid are produced as in pathways described above.

Plasmids of the strains, capable to degrade 2-hydroxypyridine

Based on the series of experiments conducted previously at the department of Molecular biology and biotechnology, it was determined that bacteria from *Arthrobacter rhombi* strains PRH1 and VP3, capable to degrade 2-hydroxypyridine, harbour one smaller than 10 kb plasmid (Semenaite, 2003; Meskiene, personal communication) (Fig. 14 A). Later, the additional large plasmid was also detected in both strains (Fig. 14 B).

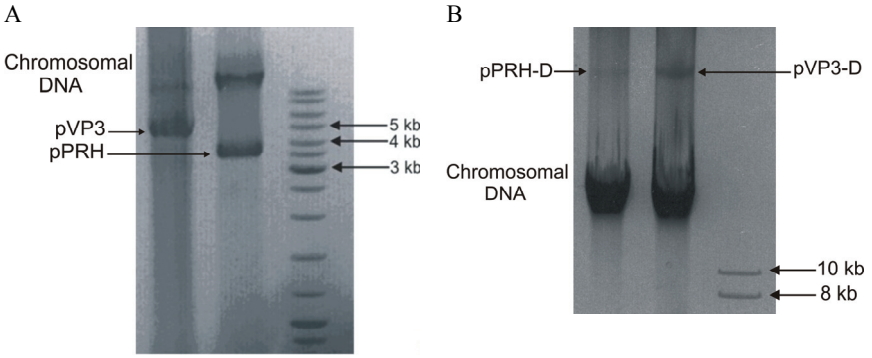

Fig. 14. *Arthrobacter rhombi* PRH1 and VP3 plasmids: small (A), large (B).

Large plasmids of PRH1 and VP3 strains.

It is known that 2-hydroxypyridine degrading bacteria, cultivated on solid medium, produce the brilliant metallic green pigment (Kolenbrander & Weinberger, 1977). It was noticed that some *Arthrobacter rhombi* PRH1 bacteria colonies, cultivated on EFAA medium, containing 2-hydroxypyridine, had lost the ability to extract the pigment. Such spontaneous mutants were hypothesised to be unable to degrade 2-hydroxypyridine. This phenomenon could be related to the loss of plasmid.

In order to prove this hypothesis, total DNA was extracted from the bacteria of *A. rhombi* PRH1 strain and its spontaneous mutant (PRH1mut1). The agarose gel electrophoresis revealed that PRH1mut1 bacteria had lost the plasmid (data not shown), which led us to the conclusion that 2-hydroxypyridine degradation genes were plasmid located. The results were confirmed by the PCR using *hpyD* F/R, *hpyE* F/R primers that were created to amplify the corresponding 2-hydroxypyridine degradation genes of *Arthrobacter* sp. PY22 strain (Gasparavičiūtė, 2008). The PCR products were obtained

only when PRH1 DNA was used (Fig. 15). Thus, 2-hydroxypyridine degradation genes of PRH1 strain are located in a large plasmid, which could be named catabolic.

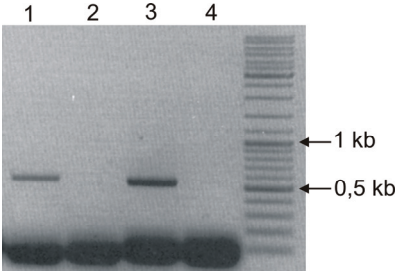

Fig. 15. The results of DNA from *Arthrobacter rhombi* PRH1 and its mutant PRH1mut1 PCR analysis. 2-hydroxypyridine primer pairs were used as follows: 1, 2 - hpyD primer pair; 3, 4 - hpyE primer pair. 1, 3 PRH1 DNA; 2, 4 PRH1mut1 DNA.

The obtained results raised the hypothesis that 2-hydroxypyridine degradation genes in *A. rhombi* VP3 bacteria could also be plasmid located. In this case, the spontaneous loss of green pigment extraction was not observed. Therefore, *A. rhombi* VP3 cells were cured from plasmid in order to obtain mutants, incapable to produce the pigment. Two different strategies were adopted for this purpose: (i) growth of the strains at higher than optimal temperature (Ghosh et al., 2000), (ii) incubation of the strains with novobiocin (Hooper et al., 1984). In both cases, a green pigment non-producing strains were not detected. Thus, it was proposed that in the case of *A. rhombi* VP3 bacteria, 2-hydroxypyridine degradation genes are chromosome-located.

Plasmid pVP3

Small cryptic plasmid, extracted from *A. rhombi* VP3 strain, and named pVP3, was cloned into the plasmid pTZ18R by using the restriction endonuclease *Sal*I cloning site. Hybrid plasmid pVP3Sal24 was used to determine the nucleotide sequence of the cloned cryptic plasmid.

pVP3 is 6135 bp in length, and its GC content is 64,6 mol%. Sequence analysis revealed 9 open reading frames (*orfs*) longer than 200 bp (Fig. 16). Only 4 (Orf2–Orf5) out of 7 (Orf1–Orf7) encoded proteins have reliable homologues in databases (Table 2). The sequence analysis revealed that Orf1 shares conserved domains with serine recombinase and PinR proteins. Both mentioned proteins belong to resolvase/invertase family. The members of this family catalyze site-specific recombination of DNA. Orf6 and Orf7 encode putative mobilization proteins. Orf6 shares the conserved domain with the MobC protein; meanwhile Orf7 contains a relaxase domain. In general, the *mob*

regions contain mobile plasmids. Relaxase, protein that initiates DNA mobilization, is the important element of relaxosome formed by Mob proteins (Francia et al., 2004).

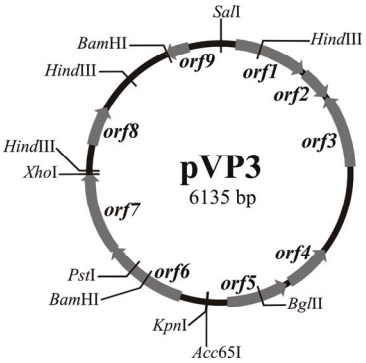

Fig. 16. Plasmid pVP3 from *Arthrobacter rhombi* VP3 strain. The sites of several restriction endonucleases and putative open reading frames (*orf*) are indicated.

Table 2. Orf analysis of *Arthrobacter rhombi* VP3 plasmid.

orf	Encoded protein length aa	Homology	Accesion no. of data bases	E value	Reference
orf1	186	resolvase protein domain *Mycobacterium tusciae* JS617	ZP_09685077	2e-71	Lucas et al., unpublished
orf2	80	hypothetical protein AARI_30370 *Arthrobacter arilaitensis* Re117	YP_003918210	3e-07	Monnet et al., 2010
orf3	187	hypothetical protein AARI_pII00110 *Arthrobacter arilaitensis* Re117	YP_003915171	5e-27	Monnet et al., 2010
orf4	120	hypothetical protein Mlut_09640 *Micrococcus luteus* NCTC 2665	YP_002957037	4e-13	Lucas et al., unpublished
orf5	156	hypothetical protein AARI_10730 *Arthrobacter arilaitensis* Re117	YP_003916254	0,0002	Monnet et al., 2010
orf6	210	mobilisation protein *Thiomonas intermedia* K12	YP_003643626	9e-08	Lucas et al., unpublished
orf7	246	mobilisation protein *Arthrobacter arilaitensis* Re117	YP_003915166	1e-73	Monnet et al., 2010
orf8	100	no homology			
orf9	62	no homology			

Since pVP3 plasmid does not encode replication proteins, it is rather difficult to determine its replication type. It is known that the replicons of theta replication B class do not encode Rep proteins. Such plasmids do not harbour a typical *ori* region; their replication is initiated by the RNA primer and depends on the host replication proteins (Bruand et al., 1993; Meijer et al., 1995). This type of replication is specific to ColE1 and related plasmids from *Enterobacteriaceae* (Del Solar et al., 1998). Recently, it was announced that the replication of *Vibrio nigripulchritudo* plasmid pB1067, which does not encode any replication proteins, depends on RNA as well (Le Roux et al., 2011). Hence, it could be assumed that the replication of the pVP3 plasmid is also RNA-dependent. However, in order to prove this statement, further investigations are needed.

Plasmid pPRH

The small plasmid pPRH1 was isolated from *A. rhombi* PRH1 bacteria. Using restriction endonuclease *Hind*III, it was cloned into pTZ57R, and the plasmid pPRHHind4 was obtained. pPRH (GenBank accession no. HQ624979) plasmid is 5000 bp in length with the GC content of 66 mol%. It contains six putative *orfs* (Table 3) and a putative promoter (859–899 nt) (Fig. 17). No homologues were found for Orf1. A search against the GenBank protein database revealed that Orf4 and Orf5 share the amino acid sequence homology with a few hypothetical proteins. Orf6 contains domains, conserved in serine recombinase and PinR proteins, as it was also observed in the case of Orf1 from the plasmid pVP3.

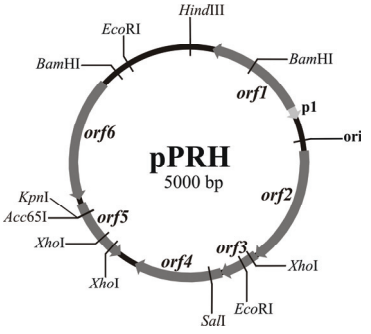

Fig. 17. Plasmid pPRH from the *Arthrobacter rhombi* PRH1 strain. The sites of several restriction endonucleases and putative open reading frames (*orf*) are indicated.

A structural homology search revealed that Orf2 and Orf3 encode putative replication proteins RepA and RepB, respectively. Two conserved (replicase and primase) domains were found in Orf2. C-terminal domain of Orf3 was found to be very similar to the region 4 of sigma-70 like sigma factor that binds to the –35 element of promoter (Campbell et al., 2002). Both Orf2 and Orf3 share significant amino acid sequence similarity with homologous proteins from *Rhodococcus* sp., *Brevibacterium* sp., *Mycobacterium* sp., and *Corynebacterium* sp. plasmids, belonging to theta replication pAL5000 subfamily of ColE2 family.

Table 3. Orf analysis of *Arthrobacter rhombi* PRH1 plasmid.

orf	**Encoded protein length aa**	**Homology**	**Accesion no. of data bases**	**E value**	**Reference**
orf1	234	no homology			
orf2	280	replication protein RepA *Rhodococcus rhodochrous*	YP_001019181	9e-59	Matsui et al., 2007
orf3	89	DNA-binding protein *Rhodococcus ruber*	BAE06129	2e-11	Matsui et al., 2007
orf4	204	hypothetical protein AARI_pII00110 *Arthrobacter arilaitensis* Re117	YP_003915171	3e-33	Monnet et al., 2010
orf5	152	hypothetical protein TERTU_4246 *Teredinibacter turnerae* T7901	YP_003075508	2e-21	Yang et al., 2009
orf	275	putative resolvase *Arthrobacter arilaitensis* Re117	YP_003915172	2e-78	Monnet et al., 2010

These findings raised the hypothesis that pPRH plasmid might also belong to pAL5000 subfamily. In order to prove the hypothesis, it was decided to determine the minimal replicon of the plasmid pPRH. For that purpose, pAPrepAB4 plasmid, containing *repAB* genes, and pAPrepA2 plasmid, harbouring the *repA* gene, were constructed. pAPrepAB4 was successfully transformed to *Arthrobacter oxydans* PY21, *Arthrobacter rhombi* VP3, *Arthrobacter* sp. 68b, and *Rhodococcus* sp. SQ1. By using a second derivative, pAPrepA2, no transformants were obtained in all *Arthrobacter* and *Rhodococcus* spp. strains tested. Therefore, it was determined that the minimal replication operon of pPRH consists of *repAB* genes. This is in accordance with the

previous findings that both *repA* and *repB* are required for the replication of plasmids belonging to theta replication pAL500 subfamily: pAL5000 (Stolt & Stoker, 1996), pFAJ2600 (De Mot et al., 1997), pBLA8 (Leret et al., 1998) and pCASE1 (Tsuchida et al., 2009).

Furthermore, on the complementary strand at 45 nt upstream the first *repA* nucleotide, the sequence similar (60 %) to the *ori* site of plasmids from ColE2 family, was determined. The comparative sequence analysis allowed to define a conservative core (TCAGAT) common to pAL500 related plasmids and ColE2 (Fig. 18).

```
pXZ10142   5′-CGTAACCAAGTCAGATGTTTCCGGG-3′    [65 bp]  ATG
pMB1       5′-GGGAACCAAGTCAGATATTTCAGCC-3′    [26 bp]  ATG
pAL5000    5′-GAGCTCCAAGTCAGATATTTCGCTG-3′    [45 bp]  GTG
pFAJ2600   5′-AGTCAGGTAGTCGAATTTTTGGAGC-3′    [89 bp]  ATG
pBLA8      5′-GAAAGCAATATCAGATGGTTCCGGC-3′    [68 bp]  ATG
pRBL1      5′-GAAAGCAATATCAGATGGTTCCTGC-3′    [68 bp]  ATG
pJD1       5′-ATAACCATAATCAGATAACAGCCCG-3′   [106 bp]  ATG
pPRH       5′-CACACCCTTATCAGATATTGGTGGT-3′    [45 bp]  ATG

ColE2      3′-ATAAGCCTTATCAGATAACAGCGCC-5′
                        **  **
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Fig. 18. Sequence alignment of the *ori* site of different pAL5000 subfamily ColE2-related plasmids. ColE2 *ori* consensus sequence (Yagyra *et al.*, 2006) is marked in bold. Conserved nucleotides are marked by the asterisk. Numbers in square brackets indicate the distance from the proposed *repA* start codon.

Based on the homology of replication proteins and the *ori* sequence as well as the constitution of minimal replicon, it was concluded that pPRH plasmid is the member of pAL5000 subfamily of ColE2 family (Stolt & Stoker, 1996), belonging to theta replication C class (Bruand et al., 1993).

Construction of the hybrid *Escherichia coli-Arthrobacter* vectors

The *E. coli-Arthrobacter* shuttle vector pRMU824, conferring resistance to chloramphenicol, was constructed according to the scheme presented in Fig. 19 A. The amplified fragments of pACYC184 (2120 bp) and pPRHHind4 (entire pPRH cloned into pTZ57R via *Hind*III site) (1223 bp) plasmids were ligated. After the electroporation, *E. coli* clones were selected for chloramphenicol resistance. Later the part of *lacZ* operon was cloned in order to obtain the vector with phenotypical selection system. To achieve that, the plasmid pRMU8 and the amplified fragment of pTZ57R (690 bp) were double

digested with *Bgl*II and *Xma*I. After ligation and electroporation cells were spread on NA plates containing chloramphenicol, IPTG and X-Gal. Blue colonies were investigated searching for the plasmid of proper structure. The selected plasmid was named pRMU824. In addition, either tetracycline or kanamycin resistance gene was inserted into the mentioned plasmid to expand the applicability of the vector. Plasmid pRMU824 and the amplified pART2 (884 bp) or p34S-Tc (1300 bp) fragments were hydrolysed with *Xma*I. After ligation and electroporation, kanamycin or tetracycline resistant clones were selected. Thus, additional two shuttle vectors, pRMU824Km and pRMU824Tc, were obtained. Constructed plasmids were re-sequenced, and the sequences were registered in GenBank with following accession numbers: pRMU824 (HQ624980), pRMU824Km (HQ624981), pRMU824Tc (HQ624982). The multiple cloning sites of vectors are given in figure 19 B. All shuttle vectors successfully replicated in *Arthrobacter* sp. 68b, 83, 85, *Arthrobacter oxydans* PY21, *Rhodococcus* sp. SQ1, and *E. coli*.

Upon investigation of the functionality of constructed vectors, 6 kb *hpy* fragment, containing 2-hydroxypyridine degradation genes, was cloned into the vector pRMU824Km, and plasmid pHYP1 was obtained. It was determined that proteins, encoded by inserted genes, degrade 2-hydroxypyridine to 2,3,6-trihydroxypyridine as a final product and as a substrate for the formation of blue (green) pigment. The pHYP1 plasmid was transformed to *Rhodococcus erythropolis* SQ1 strain, incapable to degrade 2-hydroxypyridine. Bacteria were spread on NA medium with 2-hydroxypyridine and kanamycin. All clones produced the pigment. Hence, it was suggested that the constructed vector is suitable for the screening of functional genes.

Approximately 9 copies of pRMU824Km vector per *Arthrobacter* sp. 68b cell were found by the qPCR. The segregational stability of pRMU824Km plasmid in *Arthrobacter* sp. 68b and *R. erythropolis* SQ1 strains was investigated. The analysis of plasmid loss during cultivation in rich medium without antibiotic pressure for 24 hours (equivalent of 10 generations) showed that 41 % of *Arthrobacter* sp. 68b and 74 % of *R. erythropolis* SQ1 cells retained the plasmid. No *Arthrobacter* sp. 68b cells, harbouring pRMU824Km plasmid after 48 hours cultivation under the same conditions were detected. Meanwhile, 69 % of *R. erythropolis* SQ1 cells did not lose the plasmid.

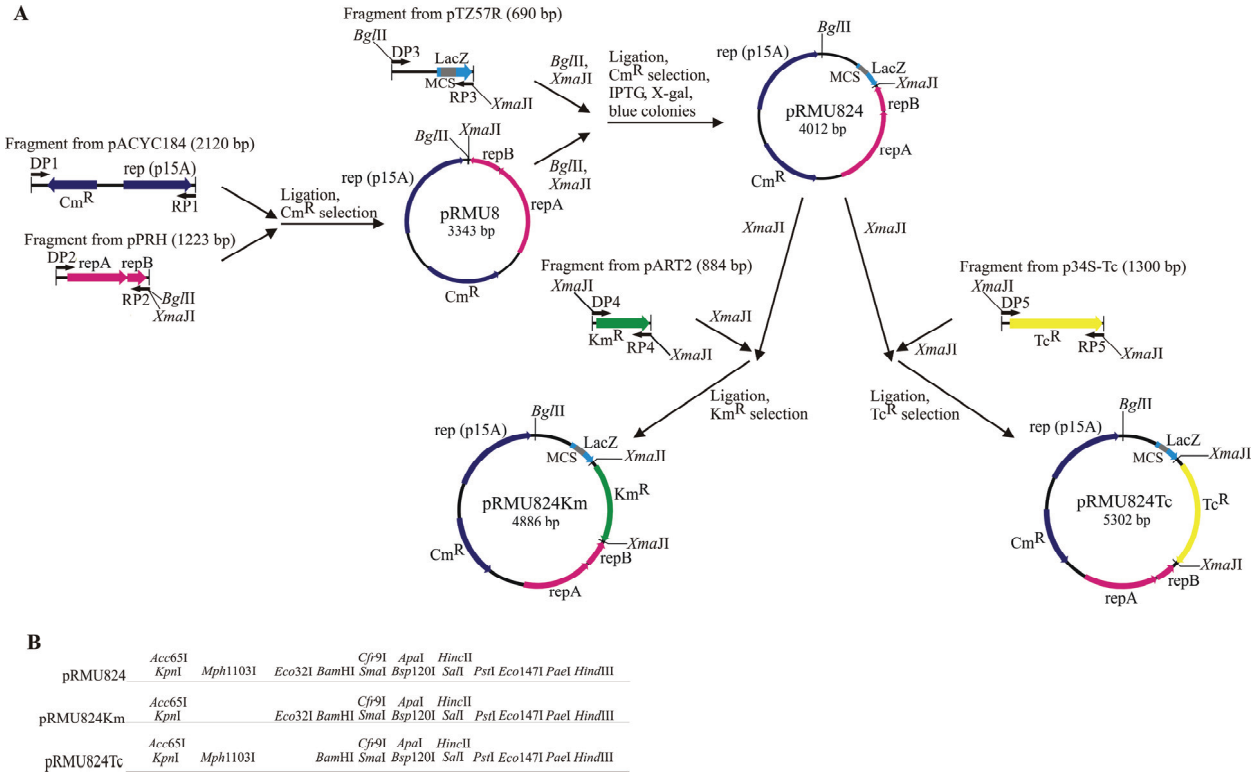

Fig. 19. Graphical representation of the construction of the shuttle vectors (A) and multiple cloning sites of pRMU824, pRMU824Km and pRMU824Tc (B). Solid arrows indicate the *orfs* and the direction of transcription. MCS – multiple cloning site, DP – direct primer, RP – reverse primer.

Since pRMU824Km was more stable in *R. erythropolis* SQ1, the segregational stability of the plasmid was proved to be a strain-dependent. The developed shuttle vectors were compatible with pART vectors (Sandu et al., 2005). Hence, these plasmids might be used as the original tools in genetic complementation studies as well as for a functional complementation based screening in both *Arthrobacter* and *Rhodococcus* species.

Summarizing the data obtained, it could be stated that *Arthrobacter* sp. 68b cells harbour at least two large molecular weight plasmids, meanwhile *A. rhombi* PRH1 and VP3 contain one large molecular weight plasmid and one smaller than 10 kb.

It was determined that in the case of *Arthrobacter* sp. 68b genes, encoding phthalic acid, 2-methylpyridine and pyridine degradation enzymes, are located on one 112864 bp p2MP plasmid, while those responsible for nicotine degradation are situated on the other plasmid pNIC. The degradation of phthalate, 2-methylpyridine and pyridine are inducible processes in these bacteria. Besides, it was proved that cells, pre-grown with phthalic acid, are capable to utilize quinolinic acid. 2-methylpyridine and pyridine induce the hypothetical monooxygenase that cleaves pyridine ring between C2 and C3 atoms. The hypothetical degradation pathway of mentioned compounds was proposed, suggesting the production of succinate semialdehyde and succinic acid as intermediate products.

It was determined that in the case of *A. rhombi* PRH1 strain, 2-hydroxypyridine degradation genes are plasmid-located. Meanwhile, analogous genes of *A. rhombi* VP3 strain are most probably located on chromosome. The small plasmids of both strains were sequenced, putative open reading frames were designated, and encoded proteins were identified. The replication type of pVP3 plasmid was not determined. However, the hypothesis that its replication could be initiated by the RNA primer was raised. After investigation of pPRH plasmid-encoded replication proteins, it was concluded that the plasmid in question belongs to theta replication pAL5000 subfamily of ColE2 family. Using the minimal replicon of pPRH, hybrid vectors pRMU824, pRMU824Km and pRMU824Tc suitable for functional gene screening were constructed for *Arthrobacter* spp. and *Rhodococcus* spp. bacteria.

CONCLUSIONS

- *Arthrobacter* sp. 68b bacteria harbours at least two large molecular weight catabolic plasmids. Degradation genes of 2-methylpyridine, pyridine and phthalic acid are located on one 112864 kb circular plasmid, while genes for nicotine degradation on the other plasmid.
- *Arthrobacter* sp. 68b bacteria degrade phthalic acid via 3,4-dioxygenase pathway.
- Pyridine and 2-methylpyridine induce the synthesis of hypothetical monooxygenase in *Arthrobacter* sp. 68b cells.
- 2-Hydroxypyridine degradation genes are plasmid-located in *Arthrobacter rhombi* PRH1.
- Sequenced *A. rhombi* pPRH and pVP3 plasmids are 5000 bp and 6135 bp in size, respectively. The minimal replicon of pPRH plasmid is *repAB*. It belongs to theta replication type class C subfamily pAL5000 of ColE2 family.
- Hybrid vectors pRMU824, pRMU824Km and pRMU824Tc were constructed using the minimal replicon of pPRH. They successfully replicate in *Arthrobacter* spp., *Rhodococcus* spp. and *Escherichia coli* DH5α cells.

LIST OF PUBLICATIONS

Articles

Stanislauskienė R, Rudenkov M, Karvelis L, Gasparavičiūtė R, Meškienė R, Časaitė V, Meškys R. Analysis of phthalate degradation operon from *Arthrobacter* sp. 68b. *Biologija*, 2011, 57: 45–54.

Stanislauskiene R, Gasparaviciute R, Vaitekunas J, Meskiene R, Rutkiene R, Casaite V, Meskys R. Construction of *Escherichia coli–Arthrobacter–Rhodococcus* shuttle vectors based on a cryptic plasmid from *Arthrobacter rhombi* and investigation of their application for functional screening. *FEMS Microbiol Lett*, 2012, 327: 78–86.

Conference poster

Stanislauskienė R, Meškienė R, Meškys R. New vectors for *Arthrobacter* and *Rhodococcus* spp. COST Action CM0701 "Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry" (CASCAT), Vilnius, Lithuania. On 08 – 11 September 2010.

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SANTRAUKA

Šio darbo metu buvo tiriamos *Arthrobacter* sp. 68b, *A. rhombi* PRH1 ir VP3 bakterijų didelės molekulinės masės katabolinės plazmidės, taip pat *A. rhombi* kamienų mažosios (<10 kb) plazmidės.

Nustatyta, kad *Arthrobacter* sp. 68b bakterijose genai, koduojantys ftalio rūgšties, 2-metilpiridino ir piridino skaidymo baltymus, yra susitelkę beveik 113 kb p2MP plazmidėje. Šiose bakterijose ftalato ir piridino bei jo darinio degradacija yra indukuojami procesai. Įrodyta, kad ląstelės, augintos su ftalio rūgštimi, gali panaudoti chinolino rūgštį. Nustatyta, kad ir 2-metilpiridinas, ir piridinas indukuoja hipotetinę monooksigenazę, kuri skelia piridino žiedą tarp antro ir trečio anglies atomų. Pasiūlytas minėtų substratų skaidymo kelias, kurio metu susidaro gintaro rūgšties pusiau aldehidas ir gintaro rūgštis.

Nustatyta, kad *A. rhombi* PRH1 bakterijose genai, koduojantys 2-hidroksipiridino skaidymo baltymus, yra didelės molekulinės masės plazmidėje. *A. rhombi* VP3 didelės plazmidės lemiamas fenotipas nenustatytas. Tiriant abiejų kamienų bakterijų mažąsias plazmides, buvo nustatytos jų nukleotidų sekos, aptikti atviro skaitymo rėmeliai ir įvardyti jų koduojami baltymai. Panaudojant *A. rhombi* PRH1 mažos plazmidės minimalų replikoną, buvo sukonstruoti hibridiniai vektoriai pRMU824, pRMU824Km, pRMU824Tc, skirti genų funkcinei atrankai, *Arthrobacter* spp. ir *Rhodococcus* spp. bakterijose.

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Scientific publications:

1. Stanislauskienė R, Rudenkov M, Karvelis L, Gasparavičiūtė R, Meškienė R, Časaitė V, Meškys R. Analysis of phthalate degradation operon from *Arthrobacter* sp. 68b. *Biologija*, 2011, 57: 45–54.
2. Stanislauskiene R, Gasparaviciute R, Vaitekunas J, Meskiene R, Rutkiene R, Casaite V, Meskys R. Construction of *Escherichia coli–Arthrobacter–Rhodococcus* shuttle vectors based on a cryptic plasmid from *Arthrobacter rhombi* and investigation of their application for functional screening. *FEMS Microbiol Lett*, 2012, 327: 78–86.