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Characterisation of plasmids purified from *Acetobacter pasteurianus* 2374

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Abstract

Four cryptic plasmids pAP1, pAP2, pAP3, and pAP4 with their replication regions AP were isolated from Gram-negative bacteria *Acetobacter pasteurianus* 2374 and characterised by sequence analyses. All plasmids were carrying the kanamycin resistance gene. Three of four plasmids pAP2, pAP3, and pAP4 encode an enzyme that confers ampicillin resistance to host cells. Moreover, the tetracycline resistance gene was identified only in pAP2 plasmid. All plasmids are capable to coexist with each other in *Acetobacter* cells. On the other hand, the coexistence of more than one plasmid is excluded in *Escherichia coli*. The nucleotide sequence of replication regions showed significant homology. The nucleotide and protein sequence analyses of resistance genes of all plasmids were compared with transposons *Tn*3, *Tn*10, and *Tn*903 which revealed significant differences in the primary structure, however no functional changes of gene were obtained.

Acetobacter spp. are Gram-negative bacteria many of which are used in differential biotechnological processes such as vinegar, cellulose, sorbose production, etc. [1]. Most of acetic acid bacteria possess a wide range of extra chromosomal plasmid DNAs [2–4]. More than 90% of acetic acid plasmids have been isolated from the species capable of vinegar fermentation, isolated from bacterial strains capable for glucose oxidation and cellulose synthesis [5]. The plasmids have been used for the construction of cloning vectors in the same manner as natural plasmids isolated e.g., from *Escherichia coli* and other commonly used strains in molecular biology techniques [6–8] and pMVC21 [9]. From group of plasmids are the best characterised plasmid pAH4 [10], plasmid pAP12875 isolated from *Acetobacter pasteurianus* [11], and plasmid pJK2-1 from *Acetobacter europaeus* JK2 [12]. Plasmid pAC1 (18.5–19 kb) has been identified in *A. pasteurianus* 3612 [13–15], which was used for construction of cloning vectors successfully transformed and expressed not only in *E. coli*, *Acetobacter*, but also were able to replicate in some Gram-negative and Gram-positive bacteria [16,17].

This paper presents the results obtained by the characterisation of genes that encoded antibiotic resistance in separate plasmids pAP1, pAP2, pAP3, and pAP4 purified from *Acetobacter pasteurianus* 2374.

Materials and methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Acetobacter pasteurianus* 2374 were grown in YPG liquid medium [13] at 28 °C and *E. coli* DH1 and *E. coli* JM109 strains were routinely grown in Luria-Bertani (LB) broth medium at 37 °C supplemented with antibiotics: tetracycline 12.5 μg/ml, kanamycin 30–50 μg/ml, and ampicillin 100 μg/ml.

Plasmid DNA and plasmid purification. The plasmids used in this work are listed in Table 1. Plasmid DNA from *E. coli* cells was isolated by the standard Birnboim and Doly [18] method and from *A. pasteurianus* cells by modified Grones and Turňa [15] method.

Cloning procedure. Construction of all recombinant molecules was performed by using the procedure described in laboratory manual [19]. Restriction endonuclease, T4 DNA ligase, and DNA modified enzymes were obtained from Amersham. *Escherichia coli* cells were transformed according to the methods of Mandel and Higa [20]. Plasmid DNA was separated on 0.7–1% agarose gel in TAE buffer medium [19]. DNA bands were visualised by transillumination with an UV light source and photographed.

Sequencing procedure. Plasmid DNA cloned into *E. coli* was isolated from cells by using the Wizard Plus Midipreps DNA Purification

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Table 1
Bacterial strains and plasmids DNA

Bacterial strains and plasmids DNA		References
A. pasteurianus 2374		CCM
E. coli DH1	F$^-$ *recA1 endA1 gyrA96* (Nal^r) *thi-1 rsdR17 supE44 relA1*	[20]
E. coli JM109	F′ tra D36 lacI^{q}Δ (lacZ)M15 proAB/recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_K-m_K+) sup E44 relA1 Δ(lac-proAB)	[21]
E. coli RR1	F$^-$ *recA*$^+$ *hsd*S20 (r_B-m_B-) *leu supE44 ara14 galK2 lacY1*, *proA2 rpsL20* (Str^r) *xyl5 mtl1 recA13 mctB*	[19]
pBlue SK+	Ap^r, *ColE1* replicon	Stratagene
pAP1	Kn^r, *AP1* replicon	This study
pAP2	Ap^r, Kn^r, Tc^r, *AP2* replicon	This study
pAP3	Ap^r, Kn^r, *AP3* replicon	This study
pAP4	Ap^r, Kn^r, *AP4* replicon	This study

All bacterial strains and plasmids were from Collection in Department of Molecular Biology in Bratislava.

System (Promega). Nucleotide sequences of both strains were determined by the subcloning of deletion plasmid's derivatives into pUC19 or pBlue SK+. Cloned products were sequenced by using M13 universal and reverse primers. Sequences were analysed in sequence machine ABI PRISM 310 and compared with the information deposited in the GenBank/EMBL Database.

Results and discussion

Series of four different plasmids pAP1 (3.1 kb), pAP2 (4.5 kb), pAP3 (8 kb), and pAP4 (7 kb) in slowly grown bacteria *A. pasteurianus* 2374 was identified. The mixture of plasmids purified from *Acetobacter* was transformed into *E. coli* DH1 cells with an aim to separate single plasmid. Transformants were selected on LB medium with kanamycin. By analyses of transformants, cells with separate plasmid were identified, and were used for molecular analyses. Plasmids in *Acetobacter* cells are capable of coexistence. On the other hand, the coexistence of more than one plasmid is excluded in *E. coli*. Restriction analyses of plasmid DNAs confirmed that *Pst*I nuclease recognise one place in pAP1, three places in pAP2, pAP3, and pAP4.

By analyses of all plasmids, gene of kanamycin resistance was determined. Plasmids pAP2 and pAP3 encode genes of ampicillin resistance and plasmid pAP2 gene of tetracycline resistance, too. Restriction analyses of pAP2, pAP3, and pAP4 plasmids by *Pst*I nuclease suggested three patterns with different lengths. Small and middle fragments were of the same size in all plasmids and the largest fragments of all plasmids were of various sizes. Identification of a replicon region of studied plasmids acknowledge of this present just on the largest fragment of these plasmids. The complete nucleotide sequence was determined for plasmids pAP1, pAP2, and partial nucleotide sequence for plasmids pAP3 and pAP4.

In particular, the state from the series of four plasmids has plasmid pAP3. Molecular analyses of pAP3 confirmed to existence of dimeric form of plasmid molecules. Restriction pattern indicates parallel arrangement of two identical molecules of plasmids as shown in Fig. 1. High stability of dimeric form was approved after transformation of *E. coli* RR1 (*recA*$^+$) and *E. coli* DH1 (*recA*$^-$) with plasmid pAP3. Analyses of the transformants show that almost from a hundred percent of transformants in both testing cells were in dimeric form.

Primary nucleotide structure of all plasmids determined of the existence of three genes of resistance to ampicillin, four genes for kanamycin, and one gene for tetracycline. Comparison of nucleotide sequences with *Tn*3 and *Tn*903 transposons and gene tetracycline re-

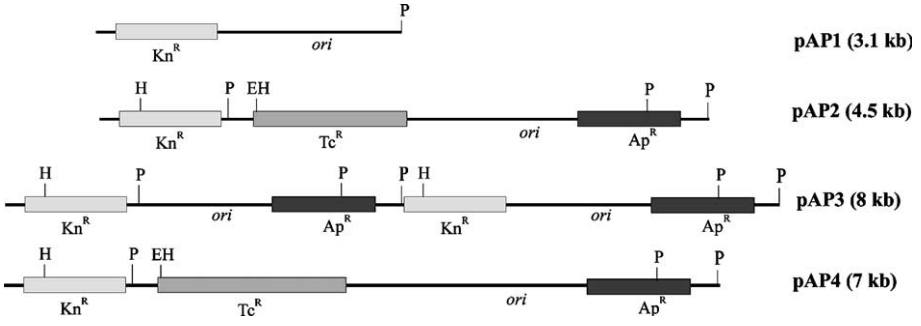

Fig. 1. Physical maps of plasmids pAP1, pAP2, pAP3, and pAP4 purified from *Acetobacter pasteurianus* 2374.

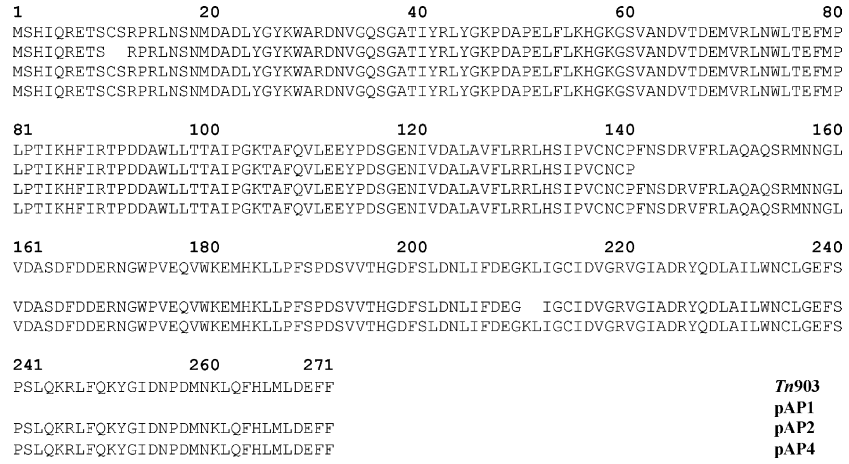

Fig. 2. Alignment of amino acids sequence of kanamycin genes with plasmids pAP1, pAP2, and pAP4 purified from *Acetobacter pasteurianus* 2374 compared with transposon *Tn*903.

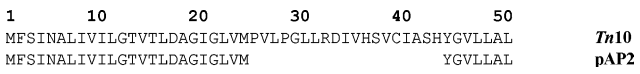

Fig. 3. Compare segment of amino acids sequence of tetracycline gene in plasmid pAP2 purified from *Acetobacter pasteurianus* 2374 with gene in transposon *Tn*10.

sistance in plasmid pBR322 suggests of specific difference in monitored genes. Gene of ampicillin resistance of all plasmids is identical with the gene in transposon *Tn*3. Different situation is in the comparison of the genes of kanamycin resistance. A basic gene is a derivative of the gene of transposon *Tn*903 with same differences. Kanamycin gene of plasmid pAP1 has only 420 nk, it is 51.6% of full kanamycin gene and in position 28–34 nk is presented in a gap of six nucleotides (Fig. 2). Nucleotide sequence of the gene of kanamycin resistance in plasmid pAP2 is identical to 98.03% as the gene in *Tn*903 transposon. In position 627–632 nk is similar deletion of 6 nucleotides as in plasmid pAP1, but these deletions do not approach the function of kanamycin genes. Nucleotide sequence of gene of kanamycin resistance in plasmid pAP4 is similar to *Tn*903 transposon identical to 97.8% (Fig. 2).

Plasmid pAP2 involves besides genes of ampicillin and kanamycin also gene of tetracycline resistance. Primary nucleotide structure of tetracycline gene was compared with similar gene in transposon *Tn*10 (Fig. 3). Result showed that tetracycline gene in plasmid pAP2 is shorter 57 nk, between 72 and 129 nk as gene in *Tn*10. Reduction of this gene in studied plasmid has no effect on the function of gene to tetracycline resistance and cells with plasmid were grown on media with 20 $\mu g\,ml^{-1}$ of tetracycline.

Results showed that four plasmids with identical replicons from cells of *A. pasteurianus* 2374 are degraded products of the biggest plasmid. This degradation has probably risen by the activity of insertion sequence or transposon, which caused deletion mutation by the loss of the gene of kanamycin resistance.

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