Construction of shuttle vectors from pAP1 plasmid for cloning into *Escherichia coli* and *Acetobacter* strains

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Summary

Plasmid pAP1 (3 080 bp), previously isolated from *Acetobacter pasteurianus* 2374 and sequenced, was used for construction of several deletion derivatives. Two cloning and expression vectors were prepared from these derivates. Vector pMK10 contains *lacZ'* gene from pUC19 plasmid and a replication region from pAP1 plasmid. The second vector pMK20 contains P_L and P_R promotors and cI^{857} repressor from bacteriophage lambda. The stability of both shuttle vectors was high in *E. coli* and *Acetobacter pasteurianus* cells at cultivation in non-selective media.

Keywords

Acetobacter pasteurianus; plasmid; replicon; gene of resistance; plasmid stability

Acetic acid bacteria are Gram-negative, ellipsoidal to rod-shaped cells that have an obligatory aerobic metabolism with oxygen as the terminal electron acceptor. They can use such substrates as glucose, ethanol, lactate or glycerol as energy sources. However, most of these compounds are not completely oxidized to CO_2 and water, and several metabolites are accumulated in the growth medium, especially acetic acid. *Acetobacteriaceae* are commonly found in nature. Because of their high resistance to acidity and the variety of substrates that they can use, they belong to main food spoilage microorganisms. On the other hand, their presence in food is mostly related to intentional food modification and human activities for food preservation [1].

Acetic acid is one of the main products of *Acetobacter* metabolism and it is found in many foods as the result of the presence and activity of these bacteria. Acetic acid is the major volatile acid in wine. High population of acetic acid bacteria grown in grape must produce and accumulate gluconic and acetic acids.

Acetic acid bacteria are producers of not only organic acids, but also of restriction endonuclease [2, 3], insertion sequence [4] and wild palette of plasmid DNAs.

Plasmids isolated from *Acetobacter* genus include pAH4 [5], pAP12875 [6], pAP1, pAP2, pAP3 and pAP4 isolated and characterized from *Acetobacter pasteurianus* 2374 [7], pJK2-1 from *Acetobacter europaeus* JK2 [8] and plasmid pAG20 from *Acetobacter aceti* 3620 [9]. Based on cryptic plasmid pAC1 from *Acetobacter pasteurianus,* several plasmid vectors have been constructed [10, 11]. These vectors were used for cloning and were successfully transformed and expressed not only in *E. coli* and *Acetobacter*, but also were able to replicate in some other Gram-negative and Gram-positive bacteria [12, 13].

Plasmid pAP1 from *Acetobacter pasteurianus* was previously isolated and characterized in our laboratory [7]. Here we describe the deletion derivates prepared from this plasmid and the construction of new cloning and expression shuttle vectors for cloning into *E. coli* and *Acetobacter* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and transformation procedures

Bacterial strains and plasmids used in this study are listed in Table 1. YPG [16] and LB media were used for routine cultivation of both *Acetobacter pasteurianus* and *Escherichia coli*. When necessary, kanamycin (Kn) and ampicillin (Ap) were added to the medium at a concentration of

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Tab. 1. Bacterial strains and plasmid DNA.

Strains or plasmids	Relevant characterisation	References
Bacterial strains		
A. pasteurianus 2374		CCM
A. pasteurianus 3614		CCM
E. coli DH1	F⁻ *recA1 endA1 gyrA96* (Nal^r) *thi-1 rsdR17 supE44 relA1*	[14]
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)	[15]
E. coli MC1061λ	F⁻ *araD139* Δ(ara-leu)*7696 galE15 galK16* Δ(lac)*X74 rpsL* (Str^r) *hsdR2* (r_K–m_K+) *mcrA mcrB1*	[15]
Plasmids		
pUC19	Ap^r, *ColE1* replicon	[16]
pKC30	Ap^r, *ColE1* replicon, P_L and P_R promoter from lambda	[17]
pAP1	Kn^r, *AP1* replicon	[7]
pMK1	Kn^r, *AP1* replicon	This study
pMK2	Kn^r, *AP1* replicon	This study
pMK3	Kn^r, *AP1* replicon	This study
pMK4	Kn^r, *AP1* replicon	This study
pMK5	Kn^r, *AP1* replicon	This study
pMK11	Kn^r, *AP1* replicon, *lacZ'*	This study
pMK20	Kn^r, *AP1* replicon, P_L and P_R promoter from lambda	This study

All bacterial strains and plasmids were from Collection in Department of Molecular Biology, Comenius University, Bratislava, Slovakia.
CCM - Czech Collection of Microorganisms, Brno, Czech republic.

30 μg.ml^{-1} and 100 μg.ml^{-1}, respectively. Transformation of *A. pasteurianus* was performed as previously described [18], with plasmid DNA concentration up to 0.5 mg.ml^{-1} in the transformation mixtures. Transformation of *E. coli* was carried out as described previously [19].

Isolation of plasmid DNA

Plasmid DNA from *E. coli* cells was isolated according to Birnboim and Doly [20] and from *A. pasteurianus* cells using the method of Grones and Turňa [16].

Molecular genetic techniques

Restriction enzymes digests, isolation of DNA fragments from agarose gels and molecular cloning were performed using standard techniques [21]. *E. coli* cells were transformed according to the methods of Mandel and Higa [22]. Plasmid DNA was separated in 0.7 to 1% agarose gel in TAE buffer [21]. DNA bands were visualised under UV light and photographed.

Segregational stability of the plasmid

Plasmid segregational stability was determined as the fraction of culture that maintained the tested plasmid after growth for 250 generations without selection on LB or YPG medium at 37 °C or 28 °C. Culture was analysed for plasmid maintenance at every 50 generations of growth by testing 100 randomly selected colonies for the plasmid-borne antibiotic resistance phenotype. Correlation between the phenotype and plasmid content was confirmed by analysing the plasmid profile of resistant colonies and examined by agarose gel electrophoresis. Segregational stability of each plasmid was defined as (the number of kanamycin resistant colonies)/(total number of colonies) × 100%.

RESULTS AND DISCUSSION

Construction of cloning vectors

The small plasmid pAP1 (3 080 bp) was isolated and purified from *Acetobacter pasteurianus* 2374 [7]. Isolated plasmid was retransformed into *E. coli* DH1 and transformants were selected on LB medium supplemented with kanamycin. Analysis of the primary nucleotide sequence determined one site for BamHI, ClaI, two sites for AvaI, and four sites for HaeII restriction endonuclease. After partial digestion of plasmid pAP1 by HaeII, three different deletion derivatives were prepared.

The first deletion plasmid pMK1 (2 671 bp) was constructed by partial cleavage of plasmid pAP1 with HaeII in position 1386–1795 bp. New deletion derivate pMK1 is about 409 bp smaller than pMK1 (Fig. 1). The second derivative, plasmid pMK2 (2 153 bp), about 927 bp smaller than pMK1, was constructed after deletion of fragment

cleaved by AvaI in position 1074 bp and 2011 bp in plasmid pAP1. The third derivative plasmid pMK3 (2 063 bp) was prepared from pMK2 after deletion of 86 bp large fragment between EcoRI (in position 1 bp) and HaeII (in position 86 bp) restriction sites. The last derivative plasmid pMK4 (2 994 bp) was prepared after deletion of EcoRI (position 1) and HaeII (position 86 bp) fragment. The fourth recognition site for HaeII (2 222 bp) is situated in the replication region of pAP1 plasmid.

Plasmid pAP1 was used for construction of two different cloning vectors. The first vector is an analogue of pUC19 vector with *lacZ*' gene [16] and the second contains P_L and P_R promoters and cI^{857} repressor from bacteriophage lambda [17].

Plasmid pMK4 (2 994 bp) was used for construction of the first cloning vector. This plasmid was cleaved with AvaI and 5' ends were blunted by DNA polymerase Klenow fragment. Plasmid was ligated with HaeII fragment (444 bp) that encodes *lacZ'* gene from pUC19 plasmid, and blunted with Klenow fragment. Positive blue recombinants in *E. coli* JM109 were selected on LB medium with kanamycin, X–gal and IPTG. New cloning and expression vector pMK10 (2 500 kb) with kanamycin resistance gene, origin from plasmid pAP1 from *Acetobacter* and multi-cloning sites from plasmid pUC19 (Fig. 2) was selected. The vector was successfully used for sequence analysis of plasmids pAP2 [7], pAG20 [9] and pAC7 (unpublished) from *Acetobacter* and cloning *rep* protein of plasmid pAG20.

The second expression vector was prepared from plasmid pMK5 (2 585 bp; Fig. 3). Plasmid pMK5 was linearized by PstI and partially cleaved with AvaI. Linearized plasmid was ligated with a 1.35 kb EcoRI and PstI cleavage fragment from plasmid pCE30 [17]. The fragment contains P_L and P_R promoters and cI^{857} represor from bacteriophage lambda. 5' ends of both fragments were

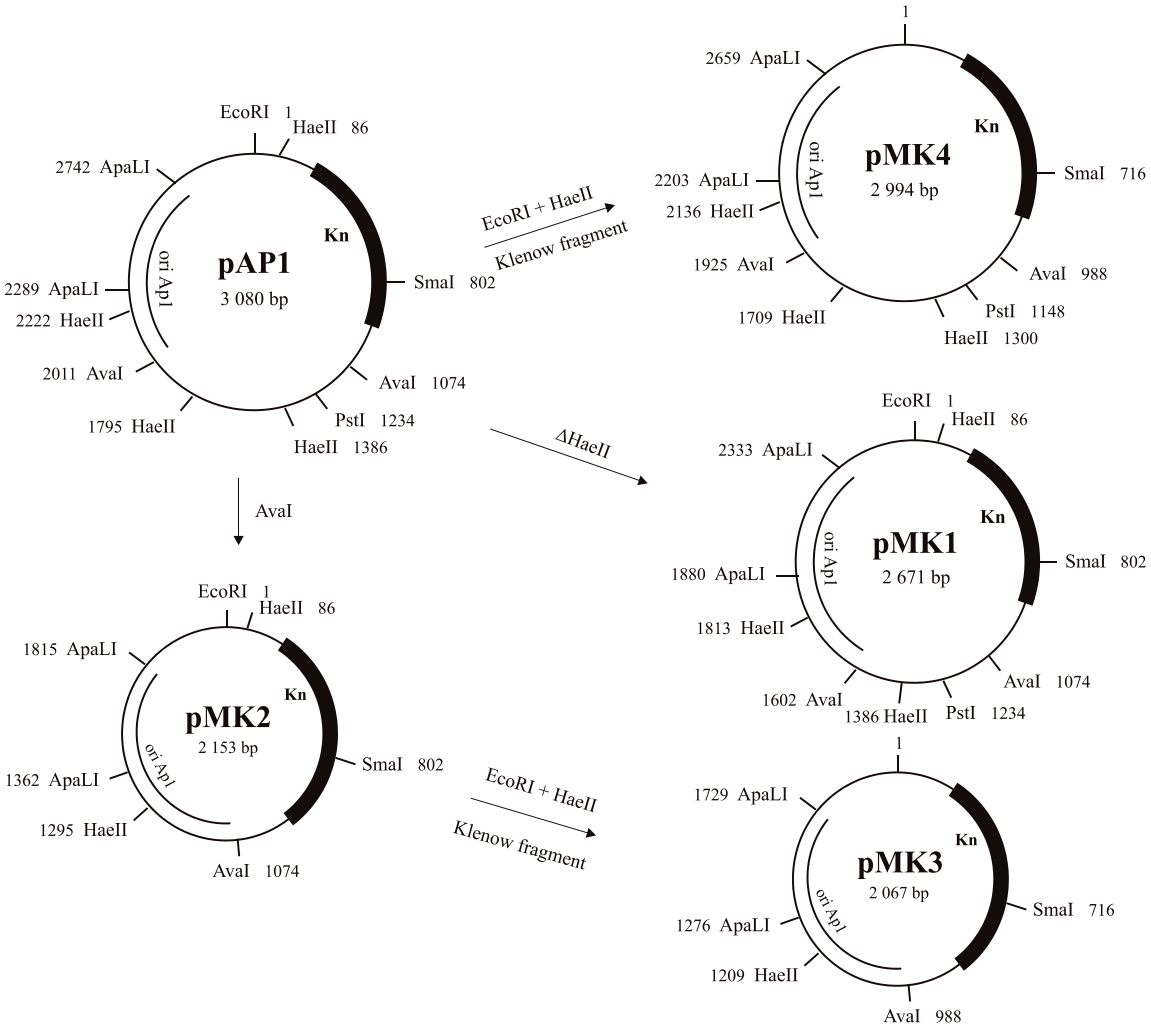

Fig. 1. Construction of deletion derivatives of plasmid pAP1 purified from *Acetobacter pasteurianus* 2374.

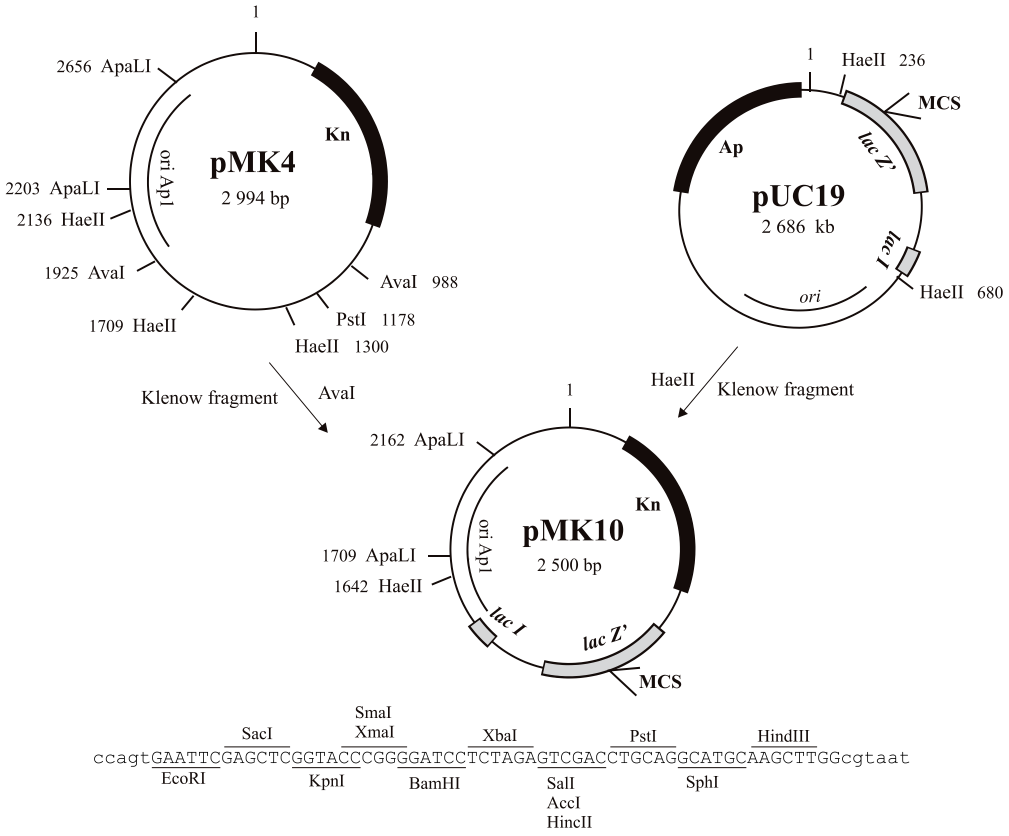

Fig. 2. Construction of pMK10 cloning vector with *lacZ'* gene and replication region from pAP1 plasmid isolated from *Acetobacter pasteurianus* 2374.

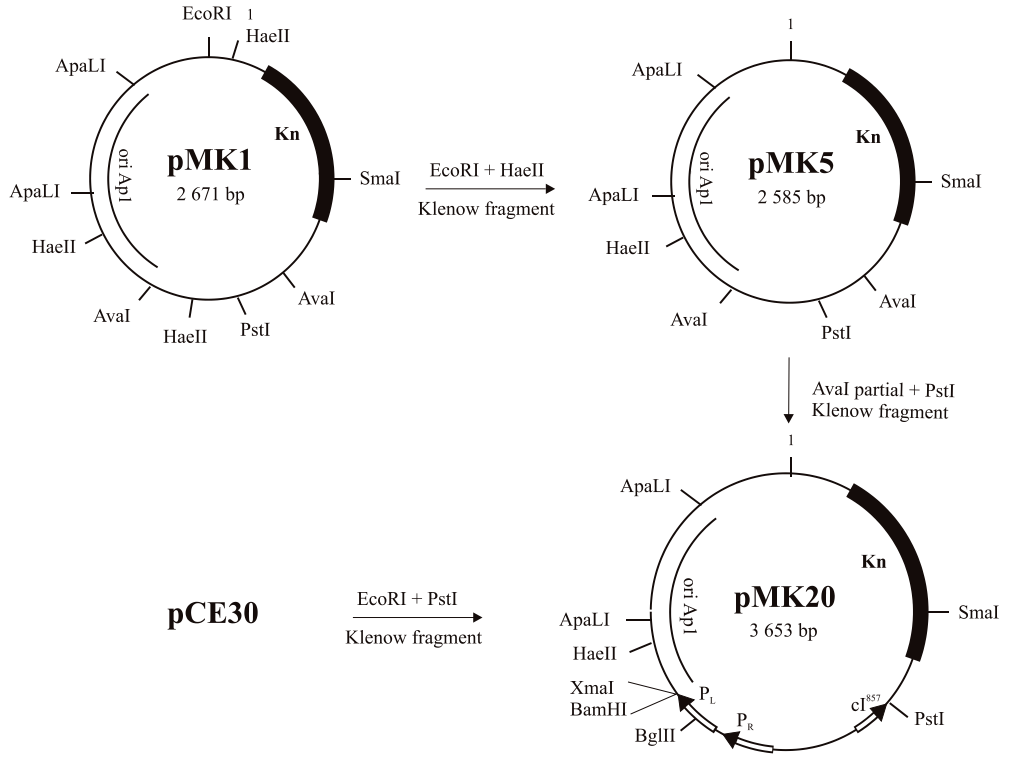

Fig. 3. Construction of pMK20 expression vector with strong promoters P_L and P_R from lambda bacteriophage and origin from pAP1 plasmid from *Acetobacter pasteurianus* 2374.

blunted by Klenow fragment. Positive transformants in *E. coli* MC1061λ were selected on LB medium with kanamycin. New expression vector pMK20 (3 653 bp; Fig. 3) was successfully used for cloning and expression of membrane protein from *Salmonella* (unpublished).

The segregational stability of cloning vectors

The segregational stabilities of pMK10 and pMK20 plasmids in strains *A. pasteurianus* 3614, *E. coli* MC1061λ and *E. coli* DH1 were determined after 250 generations. All plasmids were stably maintained in the populations of *E. coli* MC1061λ, *E. coli* DHI and *A. pasteurianus* 3614 grown in the absence of kanamycin after the entire tested period. The stability ranged from 96% to 98%. DNA electrophoresis confirmed that the kanamycin-resistant cells contained analysed plasmids' DNA. Such high segregation stability can be expected in the case of plasmids' replication analogical to the high copy number plasmids.

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