CLONING OF *pac* GENE AND ITS FLANKING REGIONS USING MINI-MU BACTERIOPHAGE

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Summary. – An *in vitro* method for cloning and mapping *Escherichia coli* genes by means of mini-Mu phage and its application to the penicillin G acylase (*pac*) gene of *E. coli* PAC2 strain with its flanking regions is described. The gene was marked by insertion of a fragment bearing kanamycin resistance (Km^r). Most of Km^r clones obtained from mini-Mu transductants contained the whole *pac* gene with its flanking regions. Localization of *pac* gene to 98.5 min of *E. coli* PAC2 chromosome was confirmed by an *in vivo* P1 phage transduction.

Key words: mini-Mu phage; in vivo cloning; penicillin acylase; E. coli ATCC 11105; E. coli PAC2

Introduction

Transposable bacterioghage Mu and its mini derivatives present useful tools for genetic analysis of bacteria that upon lysogenization generate mutations in the host with the highest transposition frequency and the most random insertion specificity at the same time, comparing to other known transposons. Mini-Mu derivatives are suitable for *in vivo* cloning, because they transpose hundreds of times and they replicate when derepressed for their lytic functions. Many mini-Mu derivatives containing different antibiotic markers, replicons and/or reporter genes for the gene fusion have been constructed (Groisman *et al.*, 1984). We have constructed mini-Mu derivatives (plasmids) that can be regulated not only in transposition but also in replication (Osusky *et al.*, 1987). These plasmids contain an origin of replication from the filamentous phage fd and their replication depends on fd gene 2, which can be carried on charon (lambda) phage ch616 (Geider *et al.*, 1985).

Penicillin G acylase is an industrially important enzyme utilized in penicillin bioconversion into its semisynthetic derivatives. The *pac* is present in *E. coli* W ATCC 11105, localized on the non-homologic island (Oh *et al.*, 1987), but it is not present in the laboratory strain K12 of *E. coli*. This gene is also unique in it structure, regulation and post-translation maturation, which is atypical for prokaryotes like *E. coli*. Therefore we decided to clone *pac* gene with its flanking regions for more detailed studies.

In this paper we describe the use of the mini-Mu derivative pJT2 for *in vivo* cloning of *pac* gene with its flanking regions originating from *E. coli* PAC2 strain, because it would be obviously difficult to clone such a large molecule into multicopy vectors. The average cloning capacity of the mini-Mu pJT2 vector is about 20 kb (Stuchlik *et al.*, 1992). In the first step we prepared *E. coli* PAC2 strain with the *pac* gene marked by kanamycin resistance (Km^r) gene, using the *in vivo* method of gene replacement with the pMAK 705 vector (Hamilton *et al.*, 1989). As we couldn't use this strain for straight *in vivo* cloning with mini-Mu, because it contained the Mu phage resistance, the second step was conjugation of the whole region into the *E. coli* RR1 strain. After preparing a recombinant plasmid bearing the *pac* gene with its flanking regions by mini-Mu phage transduction, we subcloned the 5′ flanking region of *pac* gene and subjected it to nucleotide sequence analysis.

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Abbreviations: 6-APA = 6-aminopenicilin acid; Ap = ampicilin; Cm = chloramphenicol; lacZ = β-galactosidase Z gene; kbp = kilobase pair; Km^r = kanamycin resistance gene; MCS = multiple cloning site; pac = penicillin G acylase; rep(ts) = thermosensitive replicon; Sm^r = streptomycine resistance gene; Tc^r = tetracycline resistance gene

Table 1. *E. coli* strains, phages and plasmids

Genotype	Source or reference		
E. coli strains	BB4	*sup*F58, *sup*E44, *hsd*R514, *gal*K2, *gal*T22, *trp*R55, *met*B1, *ton*A, D*lac*U169 F´[*pro*AB, *lacI*^{q} *lac*ZDM15, Tn*10*(Tc^R)]	Bullock *et al.*, 1987
	DH5αF´	F´, *end*A1, *hsd*R17, (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A(Nal^r), *rel*A1, D(*lac*IZYA-*arg*F)U169, *deo*R, (F80*dlac* D(*lacZ*)M15)	Woodcock *et al.*, 1989
	MC4100	F-,*ara*D139 D(*argF-lac*)U169, *rps*L150, (Str^R), *rel*A1, *flb*B5301, *deo*C1, *pts*F25, *rbs*R	Silhavy *et al.*, 1984
	PAC2	*pac*$^+$	Biotika a.s., Slovenská Ľupča
	PACKm13	PAC2 *pac*$^+$::Km^r	This work
	RR1	F-, D(*gpt-pro*A)62, *leu*B6, *sup*E44, *ara*14, *gal*K2, lacY1, D(*mcr*C-*mrr*), rpsL20, (Str^R), *xyl*-5, *mlt*-5, *rec*A13, *rec*A$^+$, Km^R, *pac*.	Maniatis *et al.*, 1982
	RR1Km2.13	RR1 *pga*::Km^r	This work
	TG1	K12, D(*lac-pro*), *sup*E, *thi* , *hsd*D5, F´[*tra*D, *pro*AB, *lacI*^{q} *lac*ZDM15]	Sambrook *et al.*, 1989
Phages	Mu*cts*62		Howe, 1973
	P1 cam	P1::Tn9*clr-100*	Silhavy *et al.*, 1984
Plasmids	pBluescript	ColE1 ori, Ap^r	Stratagene
	pJT2	pEG5166 Dlac (*Bam*HI-*Sal*I)	Stuchlik *et al.*, 1993
	pRJT16	pJT2, Cm^r , pac::Km^r	This work
	pRJT45	pJT2, Cm^r , pac::Km^r	This work
	pRJT50	pJT2, Cm^r , pac::Km^r	This work
	pRJT82	pJT2, Cm^r , pac::Km^r	This work
	pRR1	PUC21, pac, Ap^r	This work
	p3B	ColE1 ori, Ap^r	This work
	p8B	ColE1 ori, Ap^r	This work

Materials and Methods

Bacterial strains, phages and plasmids are described in Table 1. pPR1 plasmid was obtained from Dr. R. Kormuťáková and Dr. A. Laczová, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava.

Media. E. coli was grown either in liquid or solid LB medium (Maniatis *et al.*, 1982). The concentrations of antibiotics were: ampicillin (Ap) 50 mg/l, chloramphenicol (Cm) 30 mg/l, and kanamycin (Km) 30 mg/l.

DNA biochemistry. Restriction enzymes *Bgl*II, *Eco*RV, *Hind*III, *Hpa*I and T4 DNA ligase from the Realization Center of Comenius University, Bratislava. Plasmid DNA was isolated as described by Birnboim and Doly (1979). The other protocols were according to Maniatis *et al.* (1982).

Conjugation was performed according to Low (1991).

Mu phage lysates. The lysates of mini-Mu/Mu*cts*62 double lysogenes were prepared by the method of Groisman and Casadaban (1986) with thermal induction of the donor strain modified by Stuchlik *et al.* (1993).

In vivo cloning with mini-Mu replicons. To infect the recipient cells the lysate was mixed with an overnight culture in a ratio of 1:2 and incubated at 20°C for 45 mins without shaking. An aliquot (1 ml) of the culture was then incubated in a tube at 28°C for 1 hr with shaking. Cells were then plated on an appropriate solid medium in Petri dishes containing the corresponding antibiotic and incubated at 28°C overnight.

Bacterial genetic techniques. Transformation of *E. coli* was done according to Cohen *et al.* (1973). P1 phage transduction and preparation of P1 lysates were carried out according to Silhavy *et al.* (1984).

Results

Marking the pac gene with Km^r

For the *in vivo* cloning of *pac* gene it was necessary to mark it with a selective marker. A DNA fragment bearing Km^r and originating from the Tn903 transposon. The marker was inserted into the unique *Bgl*II site of pPR1 plasmid, located in the central part of *pac* gene (Fig.1). The resulting recombinant plasmid was designated pTKm7.

Construction of the E. coli PACKm13 strain

The *Hpa*I fragment cut out from pTKm7 plasmid was recloned into the pMAK705 vector, resulting in pRR20 plasmid. The Km^r marker was introduced into the *E. coli* PAC2 chromosome by the gene replacement method based on pMAK705 vector (Hamilton *et al.*, 1989). Selecting the clones on the basis of Km^r and Cm sensitivity we obtained a stabile clone designated *E. coli* PACKm13 with *pac* gene marked with Km^r.

Correctness of this recombinant clone was proved phenotypically, e.g. by the inability to grow on the minimal broth with phenyl acetic acid as the unique C source and also by the loss of *pac* activity.

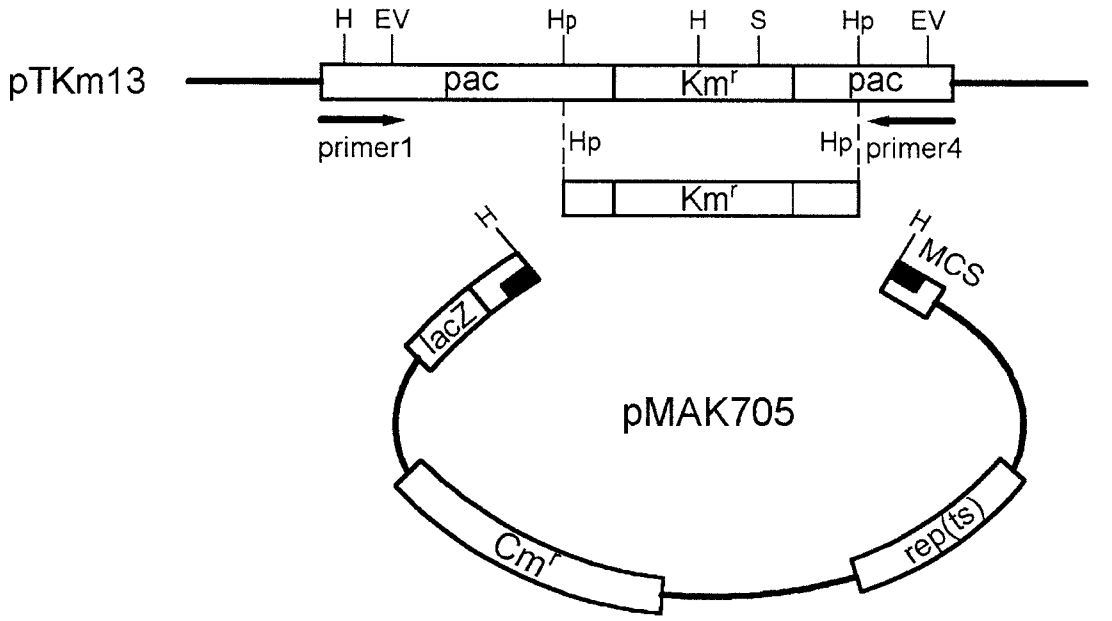

Fig. 1

Physical mapping of pTKm13 and introducing Km^r with *pac* gene *Hpa*I fragments into pMAK705 plasmid

H = *Hind*III, Hp = *Hpa*I, EV = *Eco*RV, S = *Sma*I, lacZ = β-galactosidase gene, rep(ts) = thermosensitive replicon, MCS = multiple cloning site. Arrows indicate the PCR primers origin.

Transfer of the pac::Km^r region into the RR1 E. coli strain by conjugation

Since the original *E. coli* PAC2 strain as well as the recombinant PACKm13 strain are resistant to P1 and Mu bacteriophages, we transferred the *pac::Km* region from the PACKm13 strain into the RR1 strain by conjugation. The *E. coli* BB4 strain was the donor of F plasmid marked by Tc^r. This plasmid was introduced into the PACKm13 strain by conjugation with the RR1 strain, which lasted 30 mins. The conjugates obtained were selected on Sm^r and Km^r. As the selected strains RRKm1.30 and RRKm2.13, both with Sm^r and Km^r were Tc sensitive, we could conclude that they carried a stable insertion of the *pac::Km* region in their chromosome. The constructs were proved also by PCR amplification using the *pac* gene primers (Fig. 1).

In vivo cloning the pac::Km^r gene region

E. coli RRKm2.13 strain was used as a donor strain for the *in vivo* cloning, using the mini-Mu derivative pJT2. In the first step it was lysogenised by Mu*cts*62 phage, followed by mini-Mu pJT2 transformation in the second step. After selection on Cm^r ce born on pJT2 plasmid the phage lysate was prepared using the Mu*cts* lysogen. The lysate was used for infection of lysogenic *E. coli* strains TG1 and MC4100 and selected on Cm^r and Km^r. Some of these clones were proved to harbor the *pac* gene by PCR amplification. From these, the clones pRJT16, pRJT45 and pRJT50 were chosen for further physical analysis (Table 2). The most suitable mini-Mu recombinant plasmid containing the longest *pac* gene flanking regions was pRJT45.

Table 2. Recombinant clones obtained by mini-Mu transduction

Mini-Mu recombinant	Insert length[a] (kbp)	PCR[b]	*Hind*III fragment 3420 bp[c]	*Hind*III fragment 2900 bp[c]
pRJT 16	12	+	+	+
pRJT 45	14	+	+	+
pRJT 50	18	+	–	+
pRJT 82	18	–	ND	ND

[a]Approximate sizes of DNA fragments inserted into pJT2 vector.
[b]Results of PCR amplification of *pac* gene with primers 1 and 4.
[c]Control *Hind*III restriction analysis.
ND = not done.

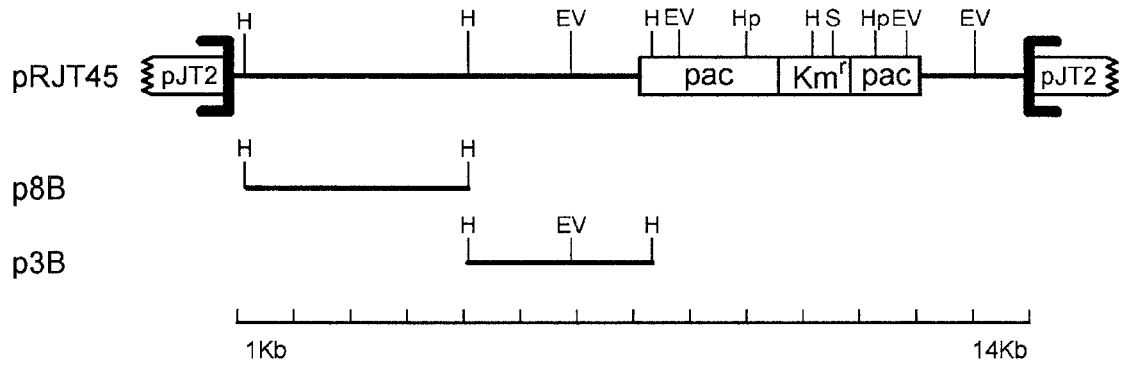

Fig. 2

Physical mapping of pRJT45 showing the position of subclones p3B and p8B with the molecular mass scale at the bottom

pJT2 = original mini-Mu derivative. For other abbreviations see Fig. 1.

Subcloning and sequencing the pac gene 5´flanking region

To establish the nucleotide sequence of the *pac* gene 5´flanking region of it was necessary to prepare subclones of the mini-Mu plasmid pRJT45. For the subcloning we used the restriction endonuclease *Hind*III (Fig. 2). The defined 3420 bp and 2900 bp *Hind*III DNA fragments were cloned into a standard pBluescript plasmid, transfected into DH5α *E. coli* cells and selected on LB with ampicillin. From the transformants two deletants, designated p8B and p3B, were isolated. Their subclones were checked by physical mapping and subjected to nucleotide sequence analysis.

The nucleotide sequences obtained were compared with *E. coli* K12 deposited at the EMBL database using the BLAST software (http://www.ncbinlm.nih.gov/blast/Blast.cgi?). The comparison revealed a 90% homology of the 5´-end a 93% homology of the 3´-end of the p8B subclone with the 98.5 min of the *E. coli* K12. The p3B subclone revealed a 93% homology of its 3´-end with the same region of *E. coli* K12 but a 97% homology of its 5´-end with the regulatory region of the *pac* gene of *E. coli* ATCC 11105.

Localization of potential non-homologic island into 98.5 min of *E. coli* K12 genome was proved also by an *in vivo* method of P1 transduction. We followed the frequency of the *pac* gene transfer together with the threonine operon transfer, which is localized close to this region. The results of the P1 phage transduction confirmed the results mentioned above.

Discussion

Derivatives of Mu bacteriophage became useful molecular biology tools both for the *in vivo* (Groisman *et al.*, 1984; Groisman and Casadaban, 1986; Osusky *et al.*, 1994; Grones *et al.*, 1996) and *in vitro* (Lawes and Maloy, 1995; Haapa *et al.*, 1999) techniques. Mini-Mu phage cloning methods have many advantages, besides economical (skipping DNA isolation, restriction and ligation steps), the main advantage being the ability to clone relatively large regions of a chromosome and those bearing toxic or harmful genes that are difficult to clone using an *in vitro* system (Stuchlik *et al.*, 1993).

The first aim was to clone the *pac* gene with its flanking regions that we supposed to be too large for *in vitro* cloning. The *pac* gene encoding penicillin acylase is present in particular *E. coli* strains (e.g. ATCC 11105, Oh *et al.*, 1987) but is missing in the standard laboratory strain K12. Therefore we focused on the gene flanking region to find a closer localization in comparison with the laboratory strain K12 sequenced earlier (Blattner *et al.*, 1997). It is useful, for the *in vivo* mini-Mu phage cloning, to have a suitable selection marker. Natural marker of *pac* gene, penicillin acylase hydrolytic activity converting penicillin into 6-aminopenicillin acid (6-APA) is not suitable, because many *E. coli* strains carry β-lastamase and 6-APA activity as well. Therefore we decided to mark the *pac* gene with Km^r.

Km^r originating from the transposon Tn903 was inserted into *Bgl*II site in the central part of the *pac* gene. It was introduced into *E. coli* PAC2 chromosome by a gene replacement method, using the vector with a thermosensitive replicon, pMAK705 (Hamilton *et al.*, 1989). In the first step we cloned the *Hpa*I fragment of *pac* gene with Km^r into the vector pMAK705 and performed a gene replacement by homologous recombination with the production strain PAC2 of *E. coli*. Selecting on Km^r and Cm^s we obtained stable clones of chromosomal *pac* gene with Km^r. Disruption of *pac* gene was proved by phenotypical test – incapability to

grow on minimal media with phenylacetic acid as the unique C source and also by the enzymatic activity measurement (unpublished data). To exclude a false negativity and to prove the presence of disrupted *pac* gene we used the PCR amplification. The resulting *E. coli* strain, verified as given above, was designated PACKm13 and used in mini-Mu phage cloning. As the original as well as PACKm13 strains were P1 and Mu resistant, we decided to transfer the whole *pac*::Km fragment with its flanking regions into the mini-Mu phage-sensitive *E. coli* strain by conjugation. The recipient strain RR1 bearing chromosomal Sm^r was useful for further selection of Km^r Sm^r trans-conjugants. The presence of *pac*::Km region in the resulting trans-conjugant, designated RRKm2.13, was confirmed by PCR amplification of the gene fragment.

The obtained strain RRKm2.13 of *E. coli* was used for the *in vivo* mini-Mu phage cloning of *pac* gene with its flanking regions. By means of mini-Mu transduction it is possible to clone a chromosomal DNA fragment generally up to 20 kbp, but exceptionally up to 40kbp. For the localization of a non-homologous island bearing *pac* gene, it is sufficient to obtain a region of several hundreds of bp with homology to a known *E. coli* genomic sequence (Hinton, 1997). We obtained several mini-Mu recombinant plasmids. Based on fragment size, physical mapping and PCR analysis the clone named pRJT45 was chosen for our further studies to identify the bordering sequences of the non-homologous island bearing the *pac* gene.

The recombinant plasmid pRJT was subcloned with the aim to obtain the DNA sequence of the 5´-region of *pac* gene. The subclones p8B and p3B were sequenced and the sequences obtained, 500–600 bp from each side, were compared with those deposited at EMBL DNA database. The comparison revealed localization of the *pac* gene into 98.5 min of *E. coli* chromosome. This localization was confirmed by P1 transduction, in which the *pac* gene region was conjugated together with threonine operon that is localized in the same region.

Since we acquired characteristics of the 5´ flanking region of *pac* gene, it would be interesting to study also the 3´ flanking region to elucidate its origin and/or the horizontal transfer of this non-homologous island.

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