Acta virol. **37**: 369–376, 1993

CLONING OF TOXIC GENES WITH MINI-MU DERIVATIVE OF BACTERIOPHAGE MU

S. STUCHLÍK, V. JANITOROVÁ, J. TURŇA*

Department of Molecular Biology, Comenius University, Faculty of Natural Sciences, Mlynská dolina B-2, 842 15 Bratislava, Slovak Republic

Received November 23, 1992; revised December 14, 1992

Summary. – We describe an *in vivo* cloning method using mini-Mu phage for genes, which cannot be cloned on multicopy vectors, mainly for their toxicity. We have successfully cloned succinate dehydrogenase (*sdh)* gene *E. coli* which was inactivated with defined insertion of fragment Km^r by this method. The most of obtained Km^r clones of mini-Mu transductants have contained the sequence of whole *sdh* gene. The intact gene of *sdh* can be reconstructed by site-directed mutagenesis.

Key words: mini-Mu phage; in vivo cloning; succinate dehydrogenase; Enterobacteriaceae

Introduction

Cloning of DNA sequences has become an important step in many biological studies. It usually involves the joining to vector of DNA sequences capable of replication when introduced into an appropriate host. An alternative to *in vitro* DNA cloning systems is the use of transposons in cloning of genes (Berg *et al.*, 1989).

Bacteriophage Mu was discovered as a temperate phage which upon lysogenization generated mutations in the host with a high frequency. Mu can grow in *E. coli* as well as in many species of *Enterobacteriaceae* family. The host range of Mu was extended further by preparation a recombinant of Mu with the related tail genes of phage P1 (MuhP1) (Csonka *et al.*, 1981). Only products of two phage genes, A and B, and short sequences of the both ends of phage linear genome are needed for its transposition. These features make bacteriophage Mu and its deletion derivatives (mini-Mu) the transposable element of choice to carry out *in vivo* genetic engineering.

Mu is especially suitable for *in vivo* cloning due to its very intensive transposition – more than 100-times per cell within its lytic cycle. Groisman *et al.* (1984) have used the Mu vectors for *in vivo* cloning for the first time. They have constructed mini-Mu derivatives with different antibiotic markers and replicons. These derivatives carry also a segment of *lac* operon which enables to construct

* to whom correspondence should be addressed

fusions at either transcription or translation levels (Groisman and Casadaban, 1986). Osuský *et al.* (1987) have constructed mini-Mu derivatives which can be regulated not only in transposition but also in their replication. These plasmids contain origin of replication of filamentous phage fd and their replication is dependent on expression of fd gene 2, which can be carried on charon λch616 (Geider *et al.*, 1985). Groisman *et al.* (1991) have constructed mini-Mu for direct expression and determination of transcriptional orientation of *in vivo* cloned genes from T7 promoter which was placed near the Mu right end.

In this paper we describe the use of mini-Mu pJT2 for *in vivo* cloning of sdh gene of *E. coli*, which is very difficult to clone on multicopy vectors, mainly for its toxicity. Moreover, fumarate reductase (*frd*) gene can replace the function of *sdh* gene. This fact was confirmed also in *sdh* auxotrophic mutant of *E. coli*, when *frd* was amplified on plasmid vector (Guest, 1981). Therefore we used for *sdh* cloning an insertion mutant *sdh*C::Km.

Materials and Methods

Bacterial strains, phages and plasmids are described in Table 1.

Table 1. E. coli strains, phages and plasmids

Strains, phages and plasmids		Genotype	Source or references
Strains *E. coli*	SM1	*Δfnr, Δ(argF-lac), U169, araD137, rpsL150, deoC1, relA1, flbB5301, rbsR, ptsF25*	Melville and Gunsalus (1990)
	DW33	*zjd::*Tn*10, Δ(frdABCD)102, Δ(argF-lac), U169, araD139, rpsL150, deoC1, relA1, flbB5301, rbsR, ptsF25*	Westenberg *et al.* (1990)
	XL1 Blue	*endA1, hsdR17* (m_K^-, r_K^+), *supE44, thi-1, λ⁻, recA1, gyrA96, relA1, lac⁻* [*F',trad36,proAB,lacI^qZΔM15,Tn10(tet^R)*]	Stratagene
	TG1	K12, *Δ(lac-pro), supE, thi, hsdD5,* [*F',traD36,proAB,lacI^qZΔM15*]	Sambrook *et al.* (1989)
	JT4	TG1, *Δfnr, Mucts62*	this work
	JT6	TG1, *Δfnr, sdh::Km, Mucts62*	this work
	JT110	TG1, *Δfnr, Δ(frdABCD)*102, *sdh::Km, Mucts62*	this work
Bacterio-phages	Mucts62		Howe (1973)
	P1cam	P1::Tn*9clr-100*	Silhavy *et al.* (1984)
Plasmids	pEG5166	*Cm^r*, pMB1, *oriT, lac*	Groisman and Casadaban (1986)
	pJT2	pEG5166 with deletion of *Bam*HI-*Sal*I *lac* fragment	this work
	pJT Km	*Cm^r, Km^r*	this work
	pTZ19R	*Ap^r*, pMB1, *f1 ori*	Pharmacia
	pTZKm	*Ap^r, Km^r*	this work

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, kanamycin (Km) 25 mg/l. Minimal medium was prepared according Maniatis *et al.* (1982), and as a source of carbon sodium succinate (40 mmol/l) was used.

DNA biochemistry. Restriction enzymes *Bam*HI, *Eco*RV, T4 DNA ligase and λ DNA was from the Realization Center of Comenius University, Bratislava. Other enzymes were from Boehringer Mannheim, FRG. Plasmid DNA was isolated as described by Birnboim and Doly (1979). The other protocols were used according Maniatis *et al.* (1982).

Preparation and using of Mu lysates. The lysates of mini-Mu/Mucts62 double lysogens were prepared by a modified method of Groisman and Casadaban (1986) with thermal induction of the donor strain. Overnight culture grown at 28 °C in liquid LB medium with Cm was diluted 1:100 in 10 ml LB medium without Cm, but with $MgSO_4$ (10 mmol/l) in 250 ml Erlenmeyer flask and incubated at 28 °C with good aeration until A_{600} reached approximately 0.5. Then $CaCl_2$ was added to the culture to final concentration 5 mmol/l and the culture was shifted to 42 °C for 20 min and then to 37 °C until visible lysis was observed. Chloroform (1 % of the volume) was added to the lysate and vortexed for 10 sec. Cell debris were removed by centrifugation and the supernatant was used for determination of the phage titer (PFU/ml).

In vivo cloning with mini-Mu replicons. To infect the recipient cells 100 μl of lysate was mixed with 200 μl of cells from overnight culture and incubated at 20 °C for 45 min without shaking. 1 ml LB medium was then added to the tube and incubation was carried out for 1 hr at 28 °C with shaking to allow for expression of the drug resistance marker in the mini-Mu. Cells were plated in appropriate medium containing the corresponding antibiotic and incubated overnight at 28 °C.

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

Results

Construction of JT4 and JT110 strains

The JT4 and JT110 strains were constructed by P1 transduction of a Δ*fnr* mutation from SM1 into TG1 and from DW33 into JT6 respectively and by subsequent lysogenization with Mucts62.

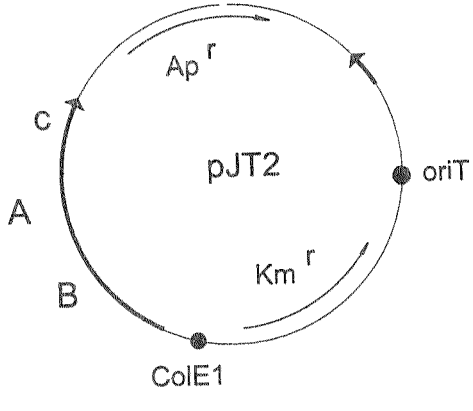

Fig. 1
Physical map of mini-Mu pJT2

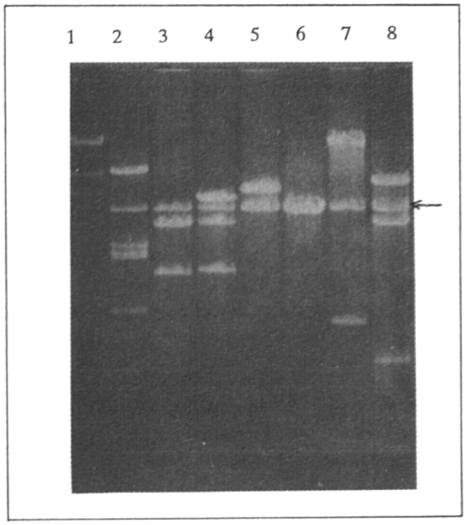

Fig. 2
Restriction analysis of plasmids from *in vivo* cloning of *sdh* gene
Molecular weight marker λ DNA cut with *Hind*III (lane 1); seven different plasmids cut with *Hind*III and *Xho*I (lanes 2–8). Arrow indicates 5.1 kb *Hind*III fragment of frd gene.

Construction of mini-Mu pJT2

The *Bam*HI-*Sal*I fragment of *lac* operon was removed from mini-Mu pEG5166 plasmid. 5'-protruding ends were filled up using Klenow fragment of DNA polymerase I and then ligated. Physical map of mini-Mu pJT2 is shown in Fig. 1. The plasmid pJT2 is able to transduce larger DNA fragments than pEG5166 itself.

In vivo cloning of sdh gene

During the *in vivo* cloning *E. coli sdh* gene a complementation of mutant *sdh*::Km in a recipient strain was used. The cells grew on minimal medium with sodium succinate as a sole source of carbon. The lysate of JT4 (pJT2) strain was prepared and after transduction 14 colonies grew on the minimal medium with

Table 2. In vivo cloning of sdh and sdh::Km

donor	mini-Mu	PFU/ml	recipient	Cm^r/PFU	sdh^+/Cm^r	sdh^+/Cm^r
JT4	pJT2	2.3×10^9	JT6	4×10^{-2}	1.2×10^{-4}	–
JT110	pJT2	1.3×10^9	JT4	6.4×10^{-2}	–	9.4×10^{-5}

Cm^r/PFU – ratio between number of colonies Cm^r and titer of lysate (frequency of transduction of Cm resistance); sdh^+/Cm^r – ratio between number of colonies sdh^+ and number of colonies Cm^r; $sdhKm^+/Cm^r$ – ratio between number of colonies Km^+ and number of colonies Cm^r

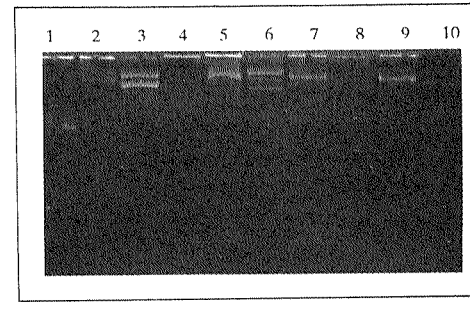

Fig. 3
Gel electrophoresis of plasmids from in vivo cloning of *sdh*::Km
pJT2 plasmid (lane 1); plasmids from Km resistant clones (lanes 2–10).

succinate and Cm. The obtained results are shown in Table 2. However, we found out after restriction analysis, that all the positive clones contain *Hind*III fragment of approximately 5.1 kb, which is characteristic for the *frd* gene (Fig. 2). These results fit those of Guest (1981) indicating that the *frd* gene is able replace the *sdh* function in multicopy plasmids.

In vivo cloning of sdh::Km

We used *E. coli* JT110 (pTJ2) for the *in vivo* cloning of the *sdh*::Km fragment. Using thermal induction a lytic cycle and transposition of Mu phage and mini-Mu derivative pJT2 were induced. The lysate was prepared and its titer was estimated. This lysate was used for the transfer of Cm resistance gene and *sdh*::Km fragment into JT4 recipient strain (Table 2). From 5 positive clones, which grew on LB medium with Cm and Km, preparations of plasmid DNA were analyzed (Fig. 3). After cleavage with *Bam*HI in three of them there were fragments, which were as large as *sdh* gene with insertion of Km cassette from Tn*903*. From a chosen clone, which was designated pJTSDHKm4, the mentioned fragment was removed using *Bam*HI and recloned into pTZ19R plasmid. Selection was carried out on the medium containing Ap and Km. The recombinant plasmid was designated pTZSDHKm3 (Fig. 4).

Discussion

Mini derivatives of Mu bacteriophage were successfully used for *in vivo* cloning of several genes (Sirko *et al.*, 1987; Sirko and Hulanicka, 1988; Groisman and Casadaban, 1987; Wang *et al.*, 1987; Gramajo *et al.* 1988; Srivastava *et al.*, 1989; Strauch *et al.*, 1989; Roncero and Casadaban, 1992). *In vivo* cloning is convenient provided there is a possibility of selection of recombinants using suitable selective medium. This method can considerably shorten the time, especially in cloning of analogous genes, when it is not necessary to isolate DNA

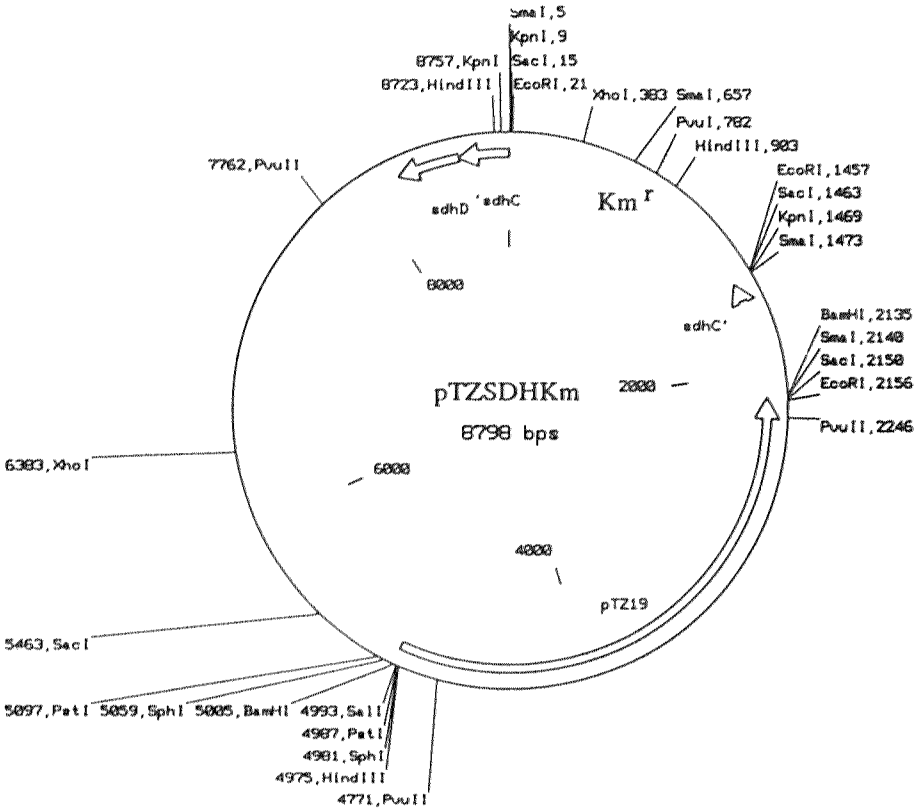

Fig. 4
Physical map of plasmid pTZSDHKm4

and to construct genomic library. Preparation of Mu phage lysates on a competent donor is incomparably simpler than the *in vitro* library construction.

Our primary aim was to clone the *sdh::* gene from several strains of *Enterobacteriaceae*. After Mu transduction we have received several clones which grew on the minimal medium with sodium succinate. The analysis of clones has shown that only the *frd* gene was in all recombinant plasmids. It is known that *frd* gene in high copy number can compensate for the *sdh* function (Guest, 1981). *E. coli* was chosen as a donor of *sdh* gene for the analysis of this problem because the sequences of both genes are known and it considerably simplifies the restriction analysis of transductants. In this case we analyzed 14 transductants growing on the minimal medium with succinate as a sole source of carbon. Only the *frd* sequences was found in all recombinants. The ratio *frd:sdh*

14:0 indicates some principal reason, why the clones with *sdh* have not been cloned. *E. coli sdh* gene has been cloned using λ transduction bacteriophage (Spencer and Guest, 1982). Because of that we supposed that *sdh* gene present on multicopy plasmid can be toxic for the cell or there could be other toxic determinants flanking this gene. Therefore in the next work we used nonfunctional *sdh* gene split with Km cassette. In this case we selected clones with Km resistance.

We supposed that *sdh* sequence would be present in the most of Km resistant clones which containing fragments 14 kb in average. Using this method we were able to clone the whole *sdh* gene sequence, that was confirmed by restriction analysis as well. The fragment with *sdh*::Km was recloned into the pTZ19R plasmid, in which it is possible to reconstruct the functional *sdh* gene by directed mutagenesis.

Our results show that difficulties with *sdh* gene cloning are probably connected with higher level of gene product using multicopy plasmids and not directly with the *in vivo* cloning. Similar results were obtained in cloning the determinant for tellurite resistance (Burian *et al.*, personal communication). We assume that the use of low copy number replicon in mini-Mu represents one way how to solve the problem of direct *in vivo* cloning of toxic genes. The method used in our work can be also applied in the *in vivo* cloning of other toxic genes.

Acknowledgement. The authors wish to express thanks to Dr. R. P. Gunsalus for gift of *E. coli* strains SM1 and DW33.

References

Berg, C. M., Berg, D. M., and Groisman, E. A. (1989): In D. E. Berg and M. M. Howe (Eds): *Mobile DNA*, p. 879. American Society for Microbiology, Washington, D. C.

Birnboim, H. C., and Doly, J. (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513–1523.

Cohen, S. N., Chang, A. C. Y., and Hsu, L. (1973): Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. natn. Acad. Sci. U. S. A.* **69**, 2110–2114.

Csonka, L. N., Howe, M. M., Ingraham, J. L., Pierson III, L. S., and Turnbough Jr., C. L. (1981): Infection of *Salmonella typhimurium* with coliphage Mu d1 (Ap^r *lac*): construction of *pyr::lac* gene fusions. *J. Bacteriol.* **165**, 299–305.

Geider, K., Hohmeyer, C., Haas, R., and Meyer, T. F. (1985): A plasmid cloning system utilizing replication and packaging functions of filamentous bacteriophage fd. *Gene* **33**, 341–349.

Gramajo, H. C., Viale, A. M., and de Mendoza, D. (1988): Expression of cloned genes by *in vivo* insertion of *tac* promoter using a mini-Mu bacteriophage. *Gene* **65**, 305–314.

Groisman, E. A., Castilho, B. A., and Casadaban, M. J. (1984): *In vivo* DNA cloning and adjacent gene fusing with a mini-Mu-*lac* bacteriophage containing a plasmid replication. *Proc. natn. Acad. Sci. U. S. A.* **81**, 1480–1483.

Groisman, E. A., and Casadaban, M. J. (1986): Mini-Mu bacteriophage with plasmid replicons for *in vivo* cloning and *lac* gene fusing. *J. Bacteriol.* **168**, 357–364.

Groisman, E. A., and Casadaban, M. J. (1987): Cloning of genes from members of the family *Enterobacteriaceae* with mini-Mu bacteriophage containing plasmid replicons. *J. Bacteriol.* **169**, 687–693.

Groisman, E. A., Pagratis, N., and Casadaban, M. J. (1991): Genome mapping and protein coding region identification using bacteriophage Mu. *Gene* **99**, 1–7.

Guest, J. R. (1981): Partial replacement of succinate dehydrogenase function by phage-and plasmid-specified fumurate reductase in *Escherichia coli*. *J. gen. Microbiol.* **122**, 171–179.

Howe, M. M. (1973): Prophage deletion mapping of bacteriophage Mu-1. *Virology* **54**, 93–101.

Maniatis, T., Fritsch, E. E., and Sambrook, J. (1982): *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Melville, S. B., and Gunsalus, R. P. (1990): Mutations in *fnr* that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli*. *J. biol. Chem.* **265**, 18733–18736.

Osuský, M., Turňa, J., Hybenová, J., Brezovská, Z., and Kozák, J. (1987): Construction of novel cloning vectors based on bacteriophage Mu. *Biológia* (Bratislava) **42**, 1141–1148.

Roncero, C., and Casadaban, M. J. (1992): Genetic analysis of the genes involved in synthesis of the lipopolysaccharide core in *Escherichia coli K-12* – 3 operon in the Rfa locus. *J. Bacteriol.* **174**, 3250–3260.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989): *Molecular cloning: a laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Silhavy, T. J., Berman, M. L., and Enquist, L. W. (1984): *Experiments with gene fusions.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Sirko, A. E., Zatyka, M., and Fulanicka, M. D. (1987): Identification of the *Escherichia coli cysM* gene encoding *O*-Acetylserine sulphydrolase B by cloning with mini-Mu-*lac* containing a plasmid replicon. *J. gen. Microbiol.* **133**, 2719–2725.

Sirko, E, A., and Hulanicka, M. D. (1988): Cloning of *cysE* gene of *Escherichia coli* with a mini-Mu-*lac* containing a plasmid replicon. *Acta biochim. Polonica* **35**, 51–55.

Spencer, M. E., and Guest, J. R. (1982): Molecular cloning of four tricarboxylic acid cycle genes of *Escherichia coli*. *J. Bacteriol.* **151**, 542–552.

Srivastava, R., Sinha, V. B., and Srivastava, B. S. (1989): Chromosomal transfer and *in vivo* cloning of genes in *Vibrio cholerae using RP4::mini-Mu*. *Gene* **75**, 253–259.

Strauch, K. L., Johnson, K., and Beckwith, J. (1992): Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. *J. Bacteriol.* **171**, 2689–2696.

Wang, B., Liu, L., Groisman, E. A., Casadaban, M. J., and Berg, C. M. (1987): High efficiency generalized transduction by miniMu plasmid phage. *Genetics* **116**, 201–206.

Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A. C., and Cecchini, G. (1990): Electron transfer from menaquinol to fumarate. Fumarate reductase anchor polypeptide mutants of *Escherichia coli*. *J. biol. Chem.* **265**, 19560–19567.