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Characteristics of Two Types of *in vitro* Constructed Plasmid Vectors for Bacterium *Erwinia citreus**

Osobitosti dva tipa *in vitro* konstruiranih plazmidnih vektora za bakteriju *Erwinia citreus*

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Summary

Two types of plasmids were used for construction of cloning vectors for bacterium **Erwinia citreus**: *cryptic plasmid denoted pPZG500 (3.8 kb) isolated from* **E. citreus** *ATCC 31623 and plasmid pUC19. Cryptic plasmid pPZG500 was inserted in plasmid pSELECT TM-1 which confers tetracycline resistance gene. Three new recombinant plasmids were obtained and denoted pPZG501A, pPZG501B and pPZG501AA according to the orientation of pPZG500 in pSELECT TM-1. Their orientation was proved by restriction analysis. All of the recombinant plasmids after transformation expressed tetracycline resistance in* **E. citreus.** *For further studies pPZG501AA (13.3 kb) was used which surely confers an intact replicon of native plasmid pPZG500. As an additional antibiotic resistance marker, fragment of 906 bp from plasmid pE194 harboring erythromycin resistance gene was inserted into multicloning site of plasmid pUC19. New recombinant plasmid pPZG650 (3.5 kb) after transformation expressed erythromycin resistance in* **E. citreus.** *Structure of recombinant plasmid was proved by Southern hybridization and restriction analysis. It was shown that both cloned markers (Tc^R and Er^R) are suitable for recombinant selection. Studies of plasmid stability of two constructed vectors have shown that pPZG501AA segregates stable during a great number of generations, whereas plasmid pPZG650 is gradually disappearing from the population.*

Sažetak

Upotrijebljena su dva tipa plazmida u konstrukciji vektora za kloniranje u bakteriji **Erwinia citreus**: *kriptički plazmid označen pPZG500 (3,8 kb) iz* **E. citreus** *ATCC 31623 i plazmid pUC19. Plazmid pPZG500 ugrađen je u plazmid pSELECT TM-1 koji ima gen za rezistenciju na tetraciklin. Dobivena su tri nova rekombinantna plazmida, te su označena: pPZG501A, pPZG501B i pPZG501AA, ovisno o orijentaciji pPZG500 u pSELECT-u. Njihova orijentacija dokazana je restrikcijskom analizom. Svi rekombinantni plazmidi pokazuju rezistenciju na tetraciklin u bakteriji* **E. citreus.** *Za daljnje analize korišten je plazmid pPZG501AA (13,3 kb) koji ima neoštećeni replikon nativnog plazmida pPZG500. Novi gen biljeg za rezistenciju na eritromicin, u fragmentu od 906 bp iz plazmida pE194, ugrađen je u višestruko mjesto za kloniranje plazmida pUC19. Rekombinantni plazmid pPZG650 (3,5 kb) nakon transformacije u stanice* **E. citreus** *eksprimirao je rezistenciju na eritromicin. Hibridizacijom i restrikcijskom analizom potvrđena je struktura konstruiranog vektora. Pokazalo se da su klonirani geni biljezi (Tc^R i Er^R) pogodni za selekciju rekombinantnih sojeva. Segregacijska nestabilnost oba tipa konstruiranih plazmidnih vektora pokazala je da se pPZG501AA stabilno održava u stanicama tijekom velikog broja generacija, dočim se pPZG650 postupno gubi iz populacije stanica.*

Introduction

Bacteria of genus *Erwinia* attract interest of scientific community for two reasons. The first is that they are known as major plant pathogens causing soft-rot and fireblith diseases in many economically important plants. Their pathogenicity is attributed to extracellular enzymes secreted by members of this genus cleaving components of plant cell wall (for review see: *(1)*).

The second is that some representatives of the genus are used for creation of novel metabolic routes by gene cloning, for conversion of glucose to 2-keto-L-gulonic

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acid, the last intermediate in synthesis of L-ascorbic acid. Recombinant strains of *Erwinia* constructed by gene manipulation allow biotechnological production of vitamin C (for review see: *(2)*).

Since gene cloning in certain genus of bacteria, is dependent on a good cloning vector system and the host cell, it was interesting to find and construct plasmid vector(s) for gene cloning in genus *Erwinia*. Vectors used so far for gene cloning in studying *Erwinia* pathogenicity were constructs, based mainly on artificial plasmids pUC8/9 *(3)*, pUC18/19 *(4,5)*, pBluescript or pBR322 *(6)*. A similar example of application in biotechnology was *Corynebacterium* 2,5-diketo-L-gluconate reductase gene, cloned in genus *Erwinia* for single-step conversion of D-glucose to 2-keto-L-gulonic acid. In these processes for cloning vector construction derivatives of pBR322 *(7)* or pUC8 *(8)* were used.

Since gene cloning and stability of constructed plasmids depends on the characteristics of vectors (markers, copy number, structural and segregational instability etc.) and host, the aim of this study was to construct versatile useful cloning vector for genus *Erwinia*, based on *Erwinia* cryptic plasmid and introducing new selective markers.

Experimental

Bacterial strains and plasmids

Bacterial strains and plasmids used in experiments are listed in Table 1.

Table 1. Bacterial strains and plasmids
Tablica 1. Bakterijski sojevi i plazmidi

Strains and plasmids Sojevi i plazmidi	Characteristics Svojstva	Reference or source Izvor
Strains		
Escherichia coli JM109		Stratagen, USA
Erwinia herbicola		ATCC 21998
Erwinia citreus		ATCC 31623
Erwinia citreus C-4	plasmid free	PLIVA Collection
Erwinia citreus	plasmid pCBR13	(8), DSM 3404
Erwinia punctata		ATCC 3162
Bacillus subtilis BD170	plasmid pE194	PLIVA Collection
Plasmids		
pUC19	Ap^R	Stratagen, USA
pSELECT TM-1	Tc^R	Promega, USA
pE194	Er^R	*(9)*
pCBR13	Ap^R	*(8)*
pPZG500	Cryptic plasmid from *E. citreus* ATCC 31623	(Bilić et al. in preparation)

Media and growth conditions

For all experiments *E. coli* and *B. subtilis* were cultivated overnight in LB liquid or solid medium at 37 °C. Bacteria from genus *Erwinia* were cultivated overnight in MA liquid (mannitol 2.5 %; yeast extract 0.5 %; peptone 0.3 %) or solid (MA + agar 2.0 %) medium at 28 °C. Antibiotic sensitivity of used bacterial strains was tested by streaking culture onto solid LB medium containing different concentrations of antibiotics. *B. subtilis* BD170 free of plasmid is sensitive to less than 1 μg/mL of erythromycin *(10)*. Bacteria from genus *Erwinia* are sensitive to 12.0 μg/mL of tetracycline.

Chemicals

Antibiotics (from PLIVA, d. d.) for transformants selection were added to the solid medium as follows: ampicillin 100.0 μg/mL; tetracycline 12.0 μg/mL; azithromycin 50.0 μg/mL; erythromycin 50.0 μg/mL. Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim, Germany.

DNA manipulation

Plasmid isolation was performed according to Ausubel et al. *(11)* and gel electrophoresis according to Sambrook et al. *(12)*. Gel electrophoresis was run on 25 cm × 14 cm × 0.5 mm agarose gel at 5 V per cm. Transformation was performed according to Hanahan *(13)*. Southern hybridization was performed by DIG-Kit from Boehringer Mannheim, Germany, according to the manufacturer's instructions.

Plasmid stability

Plasmid stability was investigated in *Erwinia citreus*. For determination of generation time, bacteria were cultivated in MA liquid medium in 500 mL Erlenmeyer flasks with 80 mL of medium shaken at 250 rpm at 28 °C. Samples were taken from growing culture at regular time intervals and optical density (OD_{660}) was measured. Generation time was determined in exponential phase of growth.

For studying plasmid stability, cultures were grown in liquid medium containing ampicillin, tetracycline or azithromycin, respectively. After 18 hours of cultivation 800 μL of culture was transferred to 80 mL new fresh liquid medium in the absence of antibiotic selection. Samples were taken every 12 hours and plated in dilution on solid medium with and without selection pressure. At the same time a new fresh medium was inoculated for further generation. Plasmid stability was determined as the percentage of colonies appearing on the medium with antibiotic (Ap^R, Tc^R or Az^R) in comparison with the medium without antibiotic.

Results

Recently discovered cryptic plasmid pPZG500 of 3.8 kb (Bilić et al., experiments currently in progress) from *Erwinia citreus* ATCC 31623 does not exert useful characteristics for recombinant selection.

For the purpose of vector construction we decided to introduce tetracycline resistance gene as a marker to plasmid pPZG500. Plasmid DNA from pPZG500 was *Hin*dIII cut open and ligated with T4 DNA ligase to pSELECT TM-1 opened with *Hin*dIII in multicloning site. After transformation of *E. coli* JM109, transformants resistant to tetracycline were selected, plasmid isolated and analyzed by gel electrophoresis (Fig. 1). The results have shown that during ligation three types of constructions occurred (Fig. 1, lanes 1, 2 and 3).

To analyze the obtained constructs, plasmid DNA was isolated and cut with different restriction enzymes. Asymmetric *Eco*RI and *Eco*RV restriction sites of pPZG500 allow determination of its orientation in pSELECT TM-1. The

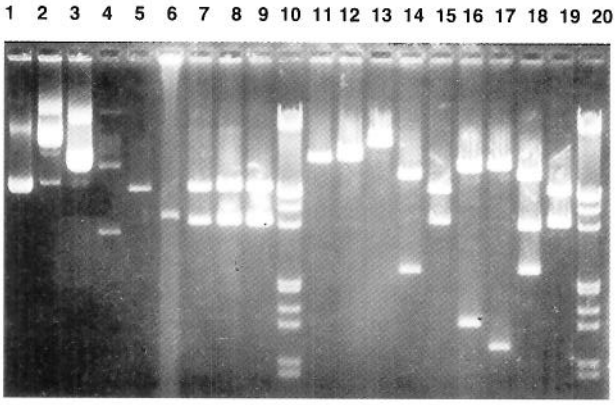

Fig. 1. Electrophoretic analysis of constructed plasmids pPZG501A, pPZG501B and pPZG501AA. Lanes: 1) pPZG501A, 2) pPZG501B, 3) pPZG500AA, 4) pSELECT, 5) pSELECT + *Hin*dIII, 6) pPZG500 + *Hin*dIII, 7) pPZG501A + *Hin*dIII, 8) pPZG501B + *Hin*dIII, 9) pPZG501AA + *Hin*dIII, 10) λ + *Hin*dIII + *Eco*RI, 11) pPZG501A + *Pst*I, 12) pPZG501B + *Pst*I, 13) pPZG501AA + *Pst*I, 14) pPZG501A + *Eco*RI, 15) pPZG501A + *Eco*RV, 16) pPZG501B + *Eco*RI, 17) pPZG501B + *Eco*RV, 18) pPZG501AA + *Eco*RI, 19) pPZG501AA + *Eco*RV, 20) λ DNA + *Hin*dIII + *Eco*RI.
Slika 1. Elektroforetska analiza konstruiranih plazmida pPZG501A, pPZG501B i pPZG501AA. Linije: 1) pPZG501A, 2) pPZG501B, 3) pPZG500AA, 4) pSELECT, 5) pSELECT + *Hin*dIII, 6) pPZG500 + *Hin*dIII, 7) pPZG501A + *Hin*dIII, 8) pPZG501B + *Hin*dIII, 9) pPZG501AA + *Hin*dIII, 10) λ + *Hin*dIII + *Eco*RI, 11) pPZG501A + *Pst*I, 12) pPZG501B + *Pst*I, 13) pPZG501AA + *Pst*I, 14) pPZG501A + *Eco*RI, 15) pPZG501A + *Eco*RV, 16) pPZG501B + *Eco*RI, 17) pPZG501B + *Eco*RV, 18) pPZG501AA + *Eco*RI, 19) pPZG501AA + *Eco*RV, 20) λ DNA + *Hin*dIII + *Eco*RI.

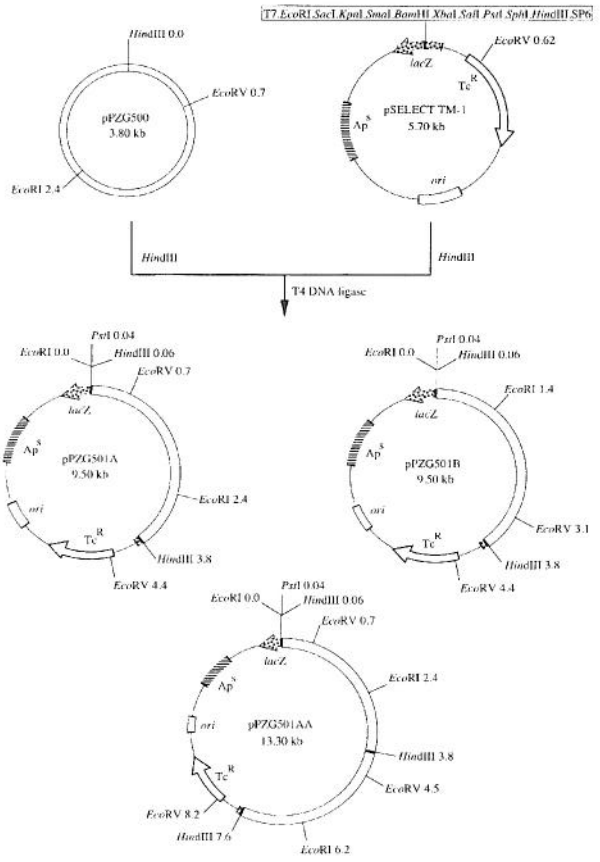

Fig. 2. Construction of plasmid vectors from cryptic plasmid pPZG500 and pSELECT TM-1 conferring tetracycline resistance. MCS = multicloning site.
Slika 2. Konstrukcija plazmidnih vektora od pPZG500 i pSELECT TM-1 s rezistencijom na tetraciklin. MCS = višestruko mjesto za kloniranje.

results of digestion with *Hin*dIII, *Pst*I, *Eco*RI and *Eco*RV (Fig. 1, lanes 5 to 20) confirmed that during ligation processes cryptic plasmid pPZG500 was inserted in two orientations in pSELECT TM-1 (pPZG501A and pPZG501B, respectively) and also two molecules of pPZG500 were tandemly ligated in the same orientation in pSELECT TM-1 (pPZG501AA)(Fig. 2). All three types of isolated recombinant plasmids were separately used for transformation of *E. citreus* C-4 and have shown to replicate in host. Tetracycline resistance (Tc^R) from pSELECT TM-1 in recombinant plasmids allow clear-cut selection of *Erwinia* transformants on MA medium.

Table 2. Antibacterial activity of erythromycin (Er) and azithromycin (Az) to the strains used in this work
Tablica 2. Antibakterijska aktivnost eritromicina (Er) i azitromicina (Az) na sojeve korištene u ovom radu

Bacteria	Antibiotic	Concentration/(μg/mL)							
		0	1	5	10	50	100	200	500
E. coli JM109	Az	+++	+++	+++	+++	+++	+++	+++	–
	Er	+++	+++	+++	+++	–	–	–	–
B. subtilis/pE194	Az	+++	+++	+++	+++	+++	+++	+++	+++
	Er	+++	+++	+++	+++	+++	+++	+++	+++
Erwinia citreus	Az	+++	+++	+++	+	–	–	–	–
ATCC 31623	Er	+++	+++	+++	+	–	–	–	–
Erwinia herbicola	Az	+++	+++	+++	+	–	–	–	–
ATCC 21998	Er	+++	+++	+++	+	–	–	–	–
Erwinia punctata	Az	+++	++	++	++	–	–	–	–
ATCC 31626	Er	+++	++	++	–	–	–	–	–

+++ good growth dobar rast + poor growth slabi rast
++ medium growth srednji rast – no growth bez rasta

For further analysis recombinant plasmid pPZG501AA was used, because of the possibility that in this construct an intact replicon of pPZG500 could be functional in *Erwinia citreus*. In the case of other two constructs (pPZG501A and pPZG501B) a replicon of pPZG500 could be inactivated by insertional inactivation due to insertion of pSELECT TM-1 in *Hin*dIII restriction site of pPZG500.

The second approach for better cloning vector construction and recombinant selection in genus *Erwinia* was to introduce a new marker useful for selection of *Erwinia* transformants. Antibiotic erythromycin was not frequently used as a selection marker in gram-negative bacteria; accordingly it was first decided to check antibiotic activity of new erythromycin derivative azithromycin *(14)* on three different strains of *Erwinia* (Table 2).

As shown in Table 2, all three species of genus *Erwinia* are susceptible to azithromycin at concentration of 50 μg/mL. *B. subtilis* harboring plasmid pE194 determining erythromycin resistance (Er^R) gene is growing on more than 500 μg/mL of each of two antibiotic in the medium. *E. coli* JM109 was more sensitive to azithromycin than to erythromycin allowing even usage of this marker in that host.

Accordingly, for new vector construction, *B. subtilis*/pE194 was cultivated in liquid medium, plasmid pE194 isolated and cut with *Sac*I-*Nsi*I restriction enzymes. Small fragment of 906 bp conferring erythromycin resistance gene was isolated and ligated to pUC19 opened by *Sac*I-*Pst*I in the multicloning site (Fig. 3).

Fragments were ligated by T4 DNA ligase and recombinant plasmids were selected after transformation

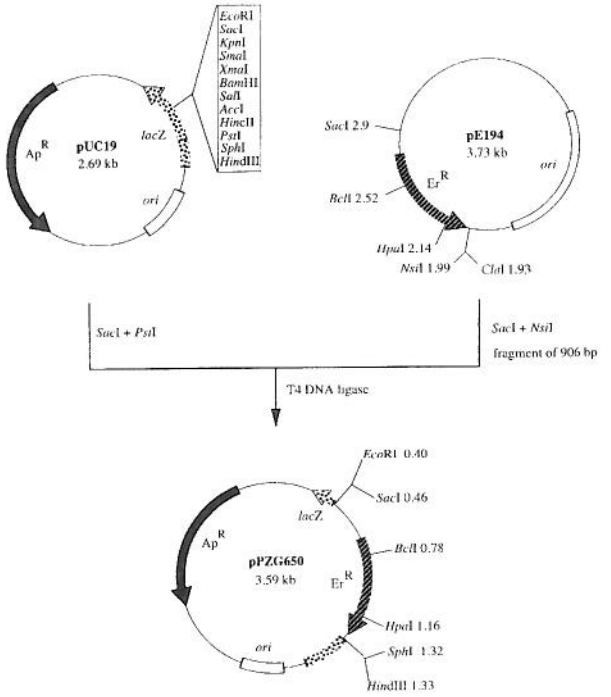

Fig. 3. Construction of plasmid pPZG650 conferring erythromycin and ampicillin resistence gene
Slika 3. Konstrukcija plazmida pPZG650 s genima za rezistenciju na eritromicin i ampicilin

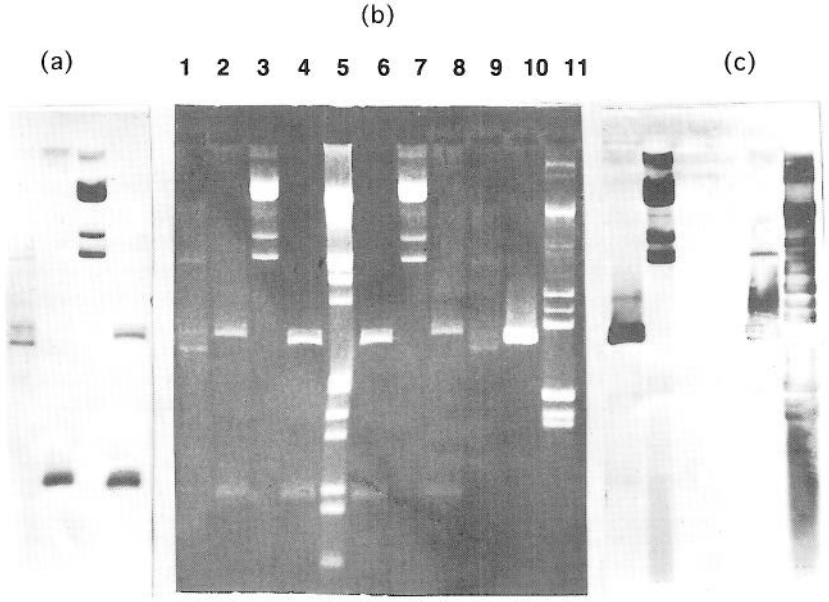

Fig. 4. Electrophoretic and Southern analysis of constructed plasmid pPZG650. (a) Southern blot analysis with pE194 (*Sac*I-*Cla*I fragment of 957 bp from pE194). (b) Ethidium bromide-stained gel of pUC19, pE194 and pPZG650 digested with restriction enzymes. Lanes: 1) pE194, 2) pE194 + *Sac*I + *Cla*I, 3) pPZG650, 4) pPZG650 + *Sac*I + *Hin*dIII, 5) λ DNA + *Hin*dIII + *Eco*RI, 6) pPZG650 + *Sac*I + *Hin*dIII, 7) pPZG650, 8) pE194 + *Sac*I + *Cla*I, 9) pE194, 10) pUC19 + *Eco*RI, 11) pUC19. (c) Southern blot analysis with pUC19 (linearized with *Eco*RI).
Slika 4. Elektroforetska i Southern analiza konstruiranog plazmida pPZG650. a) Southern analiza sa pE194 (957 bp *Sac*I-*Cla*I DNA fragment iz plazmida pE194). b) Cijepanja plazmida pUC19, pE194 i pPZG650 sa restrikcijskim enzimima u gelu agaroze obojenom etidij-bromidom. Linije: 1) pE194, 2) pE194 + *Sac*I +*Cla*I, 3) pPZG650, 4) pPZG650 + *Sac*I + *Hin*dIII, 5) λ DNA + *Hin*dIII + *Eco*RI, 6) pPZG650 + *Sac*I + *Hin*dIII, 7) pPZG650, 8) pE194 + *Sac*I + *Cla*I, 9) pE194, 10) pUC19 + *Eco*RI, 11) pUC19. c) Southern analiza sa pUC19 (lineariziran sa *Eco*RI).

of *E. coli* JM109 on LB medium with ampicillin. Ampicillin resistant transformants were checked for resistance to azithromycin and an isolated recombinant clone, named pPZG650, was analyzed by restriction analysis and Southern hybridization for the presence of *B. subtilis* DNA fragment harboring Er^R gene (Fig. 4).

As shown by Southern blotting, very strong hybridization signal was observed with a small fragment of pPZG650 cut with *Sac*I-*Hin*dIII when *Sac*I-*Cla*I fragment of pE194 was used as a probe (Fig. 4, (a)). Absence of an hybridization signal with the large fragment of pPZG650 confirmed that the construct was composed of two different plasmid DNA. On the other hand, when hybridized with pUC19 as a probe very specific hybridization was observed with the large fragment (Fig. 4, (b)).

To check the efficiency of newly constructed plasmids as possible vectors, bacterial strain *E. citreus* C-4 was transformed separately with pPZG501AA and pPZG650 and transformants were selected according to plasmid harboring marker gene.

Plasmid stability was investigated by cultivating transformants in liquid medium in comparison with *Erwinia citreus* harboring plasmid pCBR13 *(8)* which confers Ap^R gene (Fig. 5).

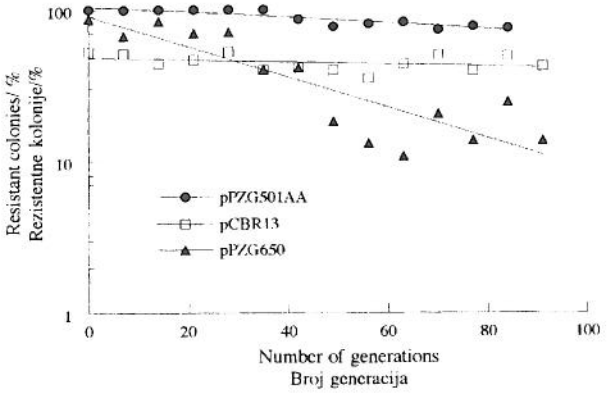

Fig. 5. Comparison of plasmid stability of pCBR13 (Ap^R), pPZG501AA (Tc^R) and pPZG650 (Er^R) in *E. citreus* C-4
Slika 5. Usporedba stabilnosti plazmida pCBR13 (Ap^R), pPZG501AA (Tc^R) i pPZG650 (Er^R) u *E. citreus* C-4

The results have shown that pCBR13 and pPZG501AA plasmids have higher stability during prolonged cultivation in comparison with pPZG650. After the same period of time (after 90 generations) 85 % of pPZG501AA population was tetracycline resistant. In the case of pCBR13, the plasmid is present in population in the range of cca 50 %, whereas construct pPZG650 harboring erythromycin resistant gene gradually loses the plasmid. At the same generation time only about 15 % of population still contains the recombinant plasmid.

Discussion

Many cryptic plasmids were discovered and isolated from different *Erwinia* strains *(3,15,16)*. In spite of that fact, none of the isolated plasmids were used for construction of plasmid vector for gene cloning in this important group of microorganisms. The main reason for this neglect could be that even pathogenicity, as one of the most recognizable features of this genus, was not unequivocally attributed to isolated plasmids and also there were no other suitable selection markers for recombinant isolation. Such obstacles present serious disadvantage for using members of genus *Erwinia* in processes for biotehnological production by, for instance, cloned microorganisms. This is specially important when time consuming biosynthetic processes are performed with cloned host, particularly cultivated without selection pressure, mainly antibiotics.

Accordingly, selection pressure or marker inserted into the cloning vector, as well as segregational instability, are the most serious problem for application of this genus in biotehnological processes. For cloning purposes these shortcomings could be solved by insertion of a particular fragment of DNA (fragment of interest) into host genome or by cloning the fragment of interest in a suitable plasmid vector.

Ampicillin frequently used as a selection marker for many bacteria seems not to be a suitable choice for recombinant selection in genus *Erwinia,* since Anderson et al. *(9)* stated that at least *E. herbicola* was naturally resistant to this antibiotic. For this reason it was decided to introduce a tetracycline and erythromycin resistance gene as a marker in plasmid vectors. Constructed plasmid pPZG501AA with tetracycline resistant gene is more suitable for transformants selection giving clear response after transformation of competent *Erwinia* cells. As evident from plasmid segregational instability study (Fig. 5) maintenance of constructed plasmid in *E. citreus* cytoplasm is stable, since after almost 90 generations about 85–90 per cent population inherited this plasmid. It is possible that this feature could be attributed to the part (57 %) of plasmid DNA coming from cryptic plasmid pPZG500 of *E. citreus* bearing its own origin of plasmid replication recognizable by the host cell.

Macrolide type of antibiotics are not frequently used as markers in gene cloning for gram negative bacteria. Azithromycin, a new macrolide antibiotic which belongs to a recently described class of antibiotics known as azalides *(14)*, turns out to be also a good marker for selection of recombinants in genus *Erwinia*. All three tested species of the genus are susceptible to 50 μg/mL of azithromycin concentration, which could be used for transformant selection resulting in better recombinant colony resolution on the plate. Hence, erythromycin resistance gene from *B. subtilis* plasmid pE194 (*Sac*I-*Nsi*I fragment) was used for vector construction. Cells of *E. citreus* were efficiently transformed with a new plasmid pPZG650 and selection of transformants performed on medium containing azithromycin. The newly constructed plasmid has two selection markers (Ap^R and Er^R) and unique restriction sites in Er^R gene (*Hpa*I and *Bcl*I) useful for cloning by insertional inactivation. Plasmid pPZG650 is less stable in *Erwinia citreus* as shown by plasmid loss (Fig. 5) followed during almost 90 generations than for instance pCBR13. This phenomenon is not easily explainable since both plasmids (pCBR13 and pPZG650) confer the same origin of replication from pUC which obviously operate in *E. citreus*.

Studying segregational instability of *E. citreus* harboring plasmid pCBR13, it was shown that at the beginning of cultivation only 50 % of population was ampicillin resistant and that range remained during 90 generations in nonselective medium. Possible explanation for this is that population of *E. citreus* harboring pCBR13 plasmid is not homogenous according to the plasmid copy number. The expression of β-lactamase by the plasmid, which is constitutive, breaks down ampicillin. So plasmid bearing cells can grow up to a certain ampicillin concentration depending on the number of the plasmids. In a similar system producing β-lactamase has been shown that the production of this enzyme is proportional to the plasmid copy number up to 60 *(17,18)*.

In this study, only one ampicillin concentration (100 μg/mL) was used for quantification of plasmid segregational instability during generations and probably only high copy number plasmid containing cells were selected, eliminating in this way cells with lower copy number of pCBR13. This explanation is supported by results obtained by Lamotte et al. *(18)* studying segregational instability of *E. coli* harboring GAPDH gene on plasmid pBR322 in tetracycline gene region and selection on different concentration of ampicillin.

In conclusion, two new resistant markers (Az^R and Tc^R) turn out to be suitable for recombinant selection and gene cloning in genus *Erwinia*, although plasmid vector having erythromycin resistant gene is not stable in the course of long cultivation under described conditions.

Acknowledgments

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