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ISOLATION AND USE OF *Prevotella ruminicola* TC18 PLASMID pTC18 IN *Escherichia coli-P. ruminicola* SHUTTLE VECTOR CONSTRUCTION

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Isolation and use of Prevotella ruminicola TC18 plasmid pTC18 in Escherichia coli-P. ruminicola shuttle vector construction

A cryptic plasmid of approximately 3 kilobases named pTC18 was discovered in a ruminal *Prevotella ruminicola* TC18 strain and cloned into *Escherichia coli*. Based on pTC18, several shuttle vectors, containing *Prevotella*/*Bacteroides tetQ* selection marker and *E. coli* vector pUC19 inserted at two different positions in pTC18 were constructed. The shuttle vectors, protected with *Hae*III methylase against the *P. ruminicola* 23 restriction were electroporated into *P. ruminicola*. Despite numerous attempts a tetracycline resistant recombinant strain 23 was not obtained. The possible causes for electroporation failure are discussed.

Key words: microbiology / anaerobic bacteria / Prevotella ruminicola / shuttle vector / rumen

Osamitev plazmida pTC18 seva Prevotella ruminicola TC18 in njegova uporaba v razvoju prenosljivih vektorjev Escherichia coli-P. ruminicola

V vampnem sevu *Prevotella ruminicola* TC18 smo odkrili 3 kilobazne pare dolgo plazmidno DNA, jo poimenovali pTC18 in klonirali v *Escherichia coli*. Na njeni osnovi smo razvili več različic prenosljivega plazmida, ki je poleg pTC18 vseboval še selekcijski marker *tetQ* iz sevov rodu *Bacteroides* in plazmidni vektor *E. coli* pUC19. Prenosljive vektorje smo s *Hae*III metilazo zaščitili proti restrikciji v *P. ruminicola* 23 in jih nato poskusili vnesti v *P. ruminicola* 23 z elektrotransformacijo. Kljub mnogim poskusom nismo uspeli pridobiti proti tetraciklinu odpornih sevov *P. ruminicola* 23.

Ključne besede: mikrobiologija / anaerobne bakterije / Prevotella ruminicola / prenosljivi vektor / vamp

1 INTRODUCTION

Prevotella ruminicola is thought to be the most numerous among the strictly anaerobic gram negative rumen bacteria from the genus *Prevotella* which apparently play important roles in the rumen ecosystem (Tajima *et al*., 2001; Miyazaki *et al*., 2003). The genome of the *P. ruminicola* type strain 23 is currently being sequenced at former TIGR, now J. Craig Venter Institute (http://www.jcvi.org/rumenomics/). However, even the most basic genetic tools such as gene introduction system, which would enable verification of ideas that may originate from the genome data analysis, are undeveloped for this bacterial species. It was shown previously (Purdy *et al*., 2002) in *Clostridium difficile* that plasmids, native to species to be genetically manipulated are needed and restriction barriers must be characterized and circumvented in order to develop a successful gene transfer system. To construct shuttle vectors for *P. ruminicola*, native *P. ruminicola* plasmids are therefore needed. Plasmids, however are surprisingly scarce in this bacterial genus (Peterka *et al*., 2003). One of the few reported *P. ruminicola* plasmids was found in *P. ruminicola* strain TC18 but was not characterized nor exploited as a shuttle vector (Avguštin, 1992). Recently, the type II restriction-modification system of *P. ruminicola* 23 was described as well as a procedure using *Hae*III methylase to protect DNA against it was developed (Accetto *et al*., 2005).

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2 MATERIAL AND METHODS

2.1 STRAIN, PLASMID, MEDIUM AND GROWTH

P. ruminicola TC18 (van Gylswyk, 1990) was grown anaerobically in M2 medium (Hobson, 1969) according to the Bryant's modification of the Hungate technique (Bryant, 1972). Source of *tetQ* alele was *E. coli-Bacteroides* shuttle plasmid pRH3 (Daniel *et al.*, 1995).

The plasmid DNA was extracted using standard alkaline lysis. Cleavage with restriction endonucleases, ligation and transformation of *Escherichia coli* were all done using standard molecular biology techniques (Sambrook, 2001). The DNA was protected against the *P. ruminicola* 23 restriction using *Hae*III methylase (NEB, USA) according to manufacturers instructions in reactions which contained S-adenosyl methionine as the methyl donor. The protected plasmid DNA was electroporated into *P. ruminicola* TC18 as described previously (Accetto *et al.*, 2005). Briefly: growth of *P. ruminicola* TC18 culture was stopped during exponential growth at OD_{600} = 0.5 by chilling on ice. The cells were then washed three times in anaerobic ice-cold 10% glycerol, electroporated at 12.5 kV/cm, resuspended in fresh M2 medium and left at 37 °C for an hour. Subsequently, the 0.1 ml portions of cells were transferred on tetracycline containing M2 agar plates in an anaerobic chamber.

3 RESULTS AND DISCUSSION

Plasmid DNA was isolated from *P. ruminicola* TC18 (Fig. 1A). Restriction enzymes *Hind*III, *Bam*HI, *Kpn*I in *Xho*I all convert plasmid DNA into a linear, approximately 3100 base pairs long DNA. The plasmid was named pTC18 and its restriction map is presented in Fig. 1B.

*Hind*III cleaved pTC18 was ligated into multiple cloning site of pUC19 and transformed into *E. coli* TOP10 (Invitrogen, USA). The resulting construct was cleaved using *Sst*I and ligated to *tetQ* allele. The latter was obtained by cleavage of pRH3 with *Sst*I and subsequent isolation of 2.6 kilobase pair fragment from the agarose gel. The ligation products were transformed into *E. coli* TOP10 and restriction analysis of plasmid DNA was performed on several recombinant strains to obtain strains harbouring both possible *tetQ* orientations (Fig. 2)

Since it is possible that *Hind*III site lies within the pTC18 replication region and thus cloning into this site would most likely inactivate replication in *Prevotella* hosts, we have also constructed shuttle vectors using the pTC18 *Kpn*I site. The procedures were essentially the same as above yielding constructs presented in figure 3.

All four shuttle vector constructs were subsequently protected against the *P. ruminicola* 23 restriction enzyme *Pru2*I using *Hae*III methylase and electroporated into *P. ruminicola* 23 cells. Despite numerous attempts we were unable to obtain a tetracycline resistant *P. ruminicola* 23 strain harbouring the shuttle vector. The electroporation parameters i.e. DNA concentration, electrocompetent cells density and electroporation time constant were essentially the same as in the previously described successful electroporation of plasmid pRH3 into *P. bryantii* TC1-1 strain (Accetto *et al.*, 2005). Several explanations for the failure of electroporation are possible: (i) both, *Hind*III and *Kpn*I site are placed within the region essential for pTC18 replication (ii) *P. ruminicola* 23 harbours

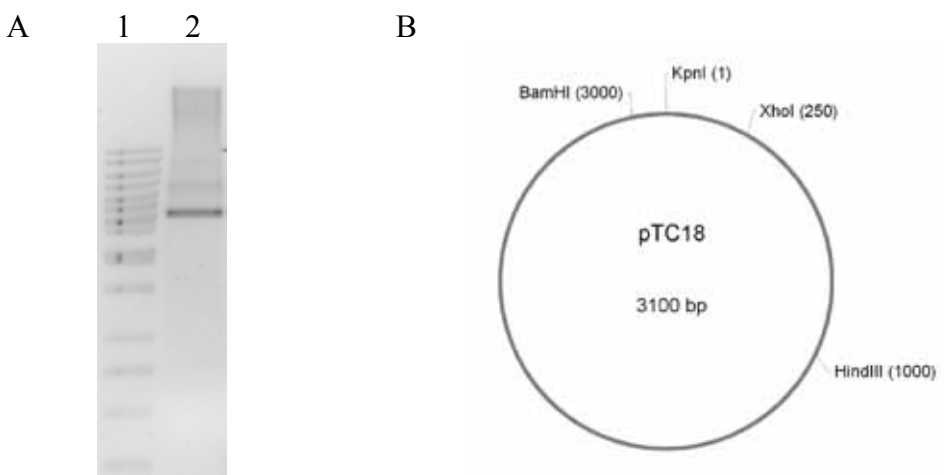

***Figure 1: A**: Plasmid DNA isolated from P. ruminicola TC18, agarose DNA electrophoresis. 1: marker generuler 1kb dna ladder (Fermentas); 2: plasmid DNA isolated from P. ruminicola TC18. **B**: Restriction map of pTC18.*
***Slika 1: A**: Plazmidna DNA iz P. ruminicola TC18, agarozna DNA elektroforeza. 1: velikostni standard generuler 1kb dna lestvica (Fermentas); 2: plazmidna DNA, osamljena iz P. ruminicola TC18. **B**: Restrikcijska mapa pTC18.*

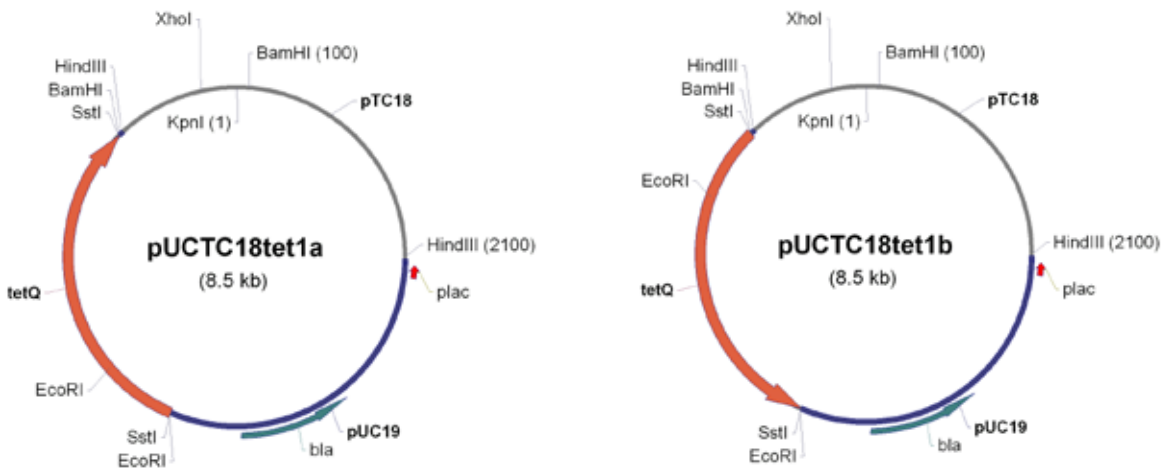

Figure 2: *Restriction maps of shuttle vectors based on pTC18 cleaved with HindIII and with different orientations of tetQ gene.*
Slika 2: *Restrikcijska mapa prenosljivih vektorjev osnovanih na pTC18 cepljenim s HindIII z različnima usmeritvama tetQ.*

another, non type II restriction system (iii) *P. ruminicola* 23 contains a cryptic plasmid that cannot be isolated by ordinary means or its relicts, but in both cases they belong to the same incompatibility group as pTC18 does and (iv) *tetQ* gene is lethal to or does not function in *P. ruminicola* 23.

4 CONCLUSIONS

The novel *Prevotella* plasmid pTC18 based shuttle vectors were unable to transform *P. ruminicola* 23. Several strategies to overcome this may be envisaged: transformation of other *P. ruminicola* strains preceded by protection of transforming DNA using cell free extract of strains to be transformed (Accetto *et al.*, 2005); the *tetQ* antibiotic resistance gene can be exchanged with *cfXA2* cephalosporinase resistance gene, known to reside in several oral *Prevotella* isolates (Giraud-Morin *et al.*, 2003) and finally, the other two unique restriction sites *BamH*I and *Xho*I can be exploited as cloning sites for antibiotic resistance gene and *E. coli* replicon in order to evade the pTC18 replication region supposedly inactivated by cloning into *Hind*III and *Kpn*I sites.

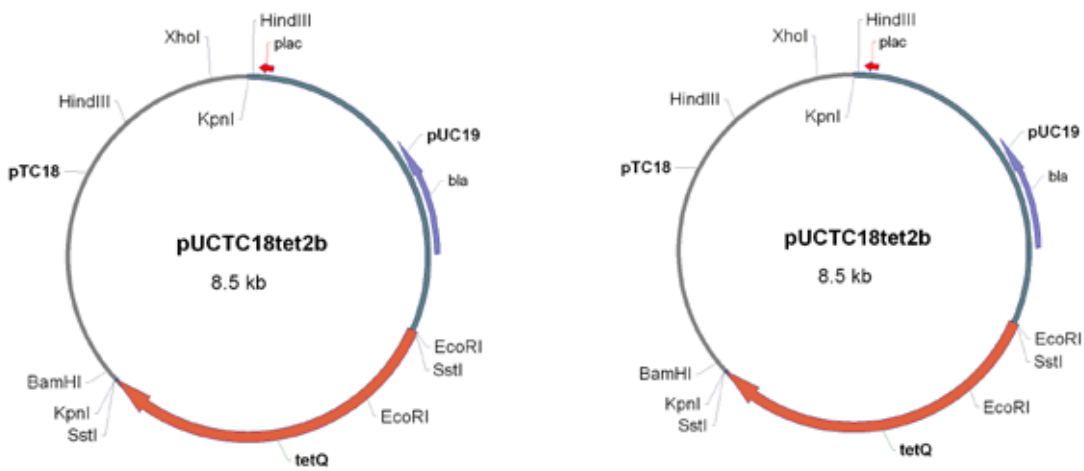

Figure 3: *Restriction map of shuttle vectors based on pTC18 cleaved with KpnI and with different orientation of tetQ gene.*
Slika 3: *Shema prenosljivih vektorjev osnovanih na pTC18 cepljenim s KpnI z različnima usmeritvama tetQ.*

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