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MOLECULAR CLONING OF THE rRNA GENES OF *Prevotella bryantii* $B_1$4

Matjaž PETERKA [a)] and Gorazd AVGUŠTIN [b)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, M.Sc.
[b)] Same address, Assoc.Prof., Ph.D., M.Sc.

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ABSTRACT

Parts of the rRNA operons of anaerobic rumen bacteria *Prevotella bryantii* $B_1$4 were cloned. 350 colonies carrying pBluescriptII with *Hind*III DNA fragments were screened with 16S rDNA specific probe and six positive clones were obtained. Inserts with 16S rRNA genes were identified by hybridisation with digoxigenin labelled *Prevotella-Bacteroides* 16S rRNA specific probe. All six inserts were approximately 2 kb long and contained large parts of the 16S rRNA gene, 16S-23S rRNA internal spacer region and approximately 100 bp of 23S rRNA gene. The origin of 16S rRNA genes were confirmed by PCR with *P. bryantii* 16S rRNA specific primer.

Key words: microbiology / bacteria / *Prevotella bryantii* / molecular genetics / ribosomal RNA operons / cloning / rumen

MOLEKULSKO KLONIRANJE GENOV ZA rRNK BAKTERIJE *Prevotella bryantii* $B_1$4

IZVLEČEK

Klonirali smo dele operonov rRNK anaerobne vampne bakterije *Prevotella bryantii* $B_1$4. S sondo, specifično za gen za 16S rRNK, smo pregledali 350 bakterijskih kolonij z vektorjem pBluescripII. Našli smo šest pozitivnih klonov, katerih vektorji so vsebovali gen za 16S rRNK. Fragmente, ki so vsebovali gene za 16S rRNK, smo identificirali s hibridizacijo z digoksigeninom označeno sondo, ki je bila specifična za gen za 16S rRNK rodov *Prevotella* in *Bacteroides*. Fragmenti so bili dolgi 2 kb in so vsebovali gen za 16S rRNK, 16S-23S rRNK interno medgensko regijo in približno 100 bp gena za 23S rRNK. Poreklo genov za 16S rRNK smo potrdili z verižno reakcijo s polimerazo (PCR) z začetnim oligonukleotidom specifičnim za gen za 16S rRNK vrste *P. bryantii*.

Ključne besede: mikrobiologija / bakterije / *Prevotella bryantii* / molekularna genetika / operoni ribosomskih RNK / kloniranje / vamp

INTRODUCTION

Prevotella bryantii is a strictly anaerobic, Gram-negative rumen bacterial species with DNA G+C content between 39 and 43 mol% (Avguštin *et al.*, 1997). It is a noncellulolytic bacteria that actively degrades xylan, starch, CMC, proteins and peptides (Wallace and Brammall, 1985; Marinšek-Logar, 1999). Little is known about genetics of *P. bryantii*. Until now only few genes of this bacterial species were cloned and analysed (Matsushita *et al.*, 1990, 1991; Gasparič *et al.*, 1995; Wulffstrobel and Wilson, 1995). For phylogenetic purposes, sequence coding for 16S rRNA gene was determined (Flint unpublished, GeneBank Accession. No. AJ006457). Recent

work showed that *P. bryantii* possess 6 rRNA operons with 16S-23S rRNA internal spacer region of 510 bp (Peterka and Avguštin, 1998).

In bacterial rRNA operons genes are usually linked in 16S rRNA-23S rRNA-5S rRNA order with internal spacer regions (ISR) between them (Krawiec and Riley, 1990). A common assumption that copies of rRNA genes within the same organism are identical has been questioned recently since several reports described considerable differences in nucleotide sequences between copies of rRNA genes in single organism (Mylvaganam *et al.*, 1992; Wang *et al.*, 1997; Dennis *et al.*, 1998). Such findings are important from phylogenetic as well as functional aspect. To analyse heterogeneties, individual copies of rRNA genes must be cloned and sequenced. The aim of our work was to develop procedure for cloning and subsequent identification of 16S rRNA genes from rRNA operons of *P. bryantii* and related anaerobic bacterial species from rumen.

MATERIALS AND METHODS

Bacterial strains, vector and media

The bacterial strain *P. bryantii* B_14 has been described previously (Avguštin *et al.*, 1994; Avguštin *et al.*, 1997). Strain stored in semi-solid (containing 0.7% agar) M2 medium (Hobson, 1969) was cultured according to Bryant modification of Hungate technique for cultivation of anaerobic micro-organisms (Bryant, 1972). *E.coli* DH5α (Gibco BRL) was used as the host strain for clones made in phagemid pBluescript®II KS (Stratagene). *E.coli* cells carrying phagemid were grown on LB medium containing 100 μg ampicilin per ml.

Chromosomal and phagemid DNA extraction

Total DNA was extracted from *P. bryantii* B_14 by modified method from "Current Protocols in Molecular Biology" (Ausubel *et al.*, 1987). Bacterial culture was grown overnight in liquid M2 medium. The cells were harvested and resuspended in TE buffer (pH 8.0). A 0.5 ml of 10% SDS (pH 7.2) and 50 μl of proteinase K (20 mg/ml) were added and suspension was incubated for 20 minutes at 37° C. After that 1.8 ml of 5 M NaCl and 1.5 ml of CTAB/ NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) were added, mixed and incubated for 20 minutes at 65° C. The mixture was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). Following the centrifugation at 6000 g and room temperature for 10 minutes, the aqueous phase was transferred to a new vessel. DNA was recovered by spooling after precipitation with 0.6 volume of isopropanol, and than redissolved in 4 ml of TE buffer. Chromosomal DNA was further purified by CsCl – ethidium bromide density gradient centrifugation. The ethidium bromide was removed by sequential extraction with water-saturated *n*-butanol, samples were dialysed against TE buffer at 4° C, and the DNA was concentrated by ethanol precipitation. Phagemid DNA was extracted by alkali lysis method from *E.coli* DH5α cells grown overnight on LB medium containing100 μg ampicilin per ml (Sambrook *et al.*, 1989).

DNA Library construction

DNA of *P. bryantii* B_14 and pBluescriptII was completely digested with *Hind*III. Digested pBluescriptII DNA was defosforilated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Ligation mixture contained 100 ng of digested pBluescriptII DNA, eqimolar amount of digested B_14 DNA, 1 x ligase buffer and 2U of T4 DNA ligase (Promega). Ligation was performed overnight at 15° C. Transformation was carried out according to standard protocol

(Karcher, 1995). Frozen competent DH5α cells were thawed on ice and ligation mixture was added. Cells and DNA were incubated on ice for 30 minutes, followed by heat shock at 42° C for 90 second. 0.4 ml of LB medium was added and mixture was incubated at 37° C for 60 minutes. 100 µl of transformed cells were plated on LB plates containing ampicilin, X-gal and IPTG.

Cloning of 16S rDNA

16S rDNA of *P. bryantii* was amplified with universally conserved 16S bacterial primer FD1 (Weisburg *et al.*, 1991) and universaly conserved reverse 16S rDNA primer (aagcttgcggccgcACGGGCGGTGTGTRC - 3’, R=A/G) (Olsen *et al.*, 1986). Both primers were modified to contain the linker sequence including restriction sites for *Not*I and *Hind*III, as described previously (Peterka *et al.*, 1997). PCR products were digested with *Not*I and *Hind*III and cloned in pBluescriptII that was also digested with *Not*I and *Hind*III. Clones carrying 16S rDNA were used as positive control for colony hybridisation.

Colony hybridisation

White colonies were streaked on fresh LB plates containing ampicilin and were grown overnight. Colonies were transferred to circular nylon membranes using modified method described by Karcher (1995). Membranes were sequentially soaked into lysis solution 1 (25% sucrose, 50 mM Tris, 10 mM EDTA, 1.5 mg lysozyme/ml), lysis solution 2 (0.5 N NaOH, 0.1% SDS), denaturation solution (0.5 N NaOH, 1.5 M NaCl) and neutralization solution (0.5 M Tris pH 7.5, 1.5 M NaCl). DNA was UV cross linked at 254 nm for 3 minutes. After that membranes were incubated in 2 x SSC with 100 µg/ml proteinase K for 90 minutes at 37° C and 2 x SSC/ 0.5% SDS for 120 minutes at 65° C. Colonies carrying 16S rRNA genes were detected with BacPre probe, which is specific for bacteria from genus *Bacteroides* and *Prevotella* (Avguštin *et al.*, 1994). Hybridisation with digoxigenin labelled probe was performed according to manufacturer instructions at 50° C (DIG DNA Labelling and Detection Kit, Boehringer Mannheim). Fragments containing 16S rRNA genes were identified by Southern blot of *Hind*II digested phagemid DNA and subsequent hybridisation with BacPre probe at 50° C.

PCR

Parts of the *P. bryantii* $B_1$4 16S rRNA genes were amplified with conserved bacterial primer EUB 338 (GCTGCCTCCCGTAGGAGT -3' (Amann *et al.*, 1995) and primer PBB$_1$4 (CGCTTCCT GTGCACTCAAGT-3') specific for *P. bryantii* (Avguštin *et al.*, 1994), as described previously (Tepšič, 1999) with annealing temperature 72° C.

RESULTS AND DISCUSSION

P. bryantii $B_1$4 genomic DNA *Hind*III fragments were cloned in pBluescriptII vector with intention to isolate 16S rRNA genes and develop procedure for cloning and subsequent identification of 16S rRNA genes from bacteria belonging to the genus *Prevotella*. 350 colonies carrying pBluescriptII with inserted *Hind*III fragments were screened with BacPre probe and six positive clones, designated 128, 142, 219, 47, 427 and 428, were obtained (Figure 1). Vectors DNA from positive clones was extracted by alkali lysis method and digested with *Hind*III (Figure 2). Inserts carrying 16S rRNA genes were identified by Southern blot and hybridisation with BacPre probe (Figure 3).

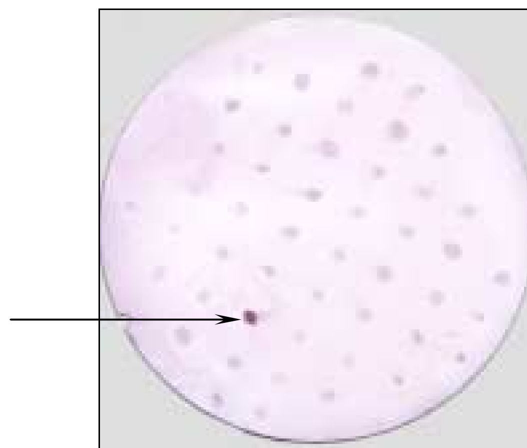

Figure 1. Colony hybridisation; clones carrying pBluescriptII with inserted 16S rRNA genes were detected with BacPre probe.
Slika 1. "Colony" hibridizacija; odkrivanje klonov z pBluescriptII vektorjem, ki ima vključen gen za 16S rRNK, s sondo BacPre.

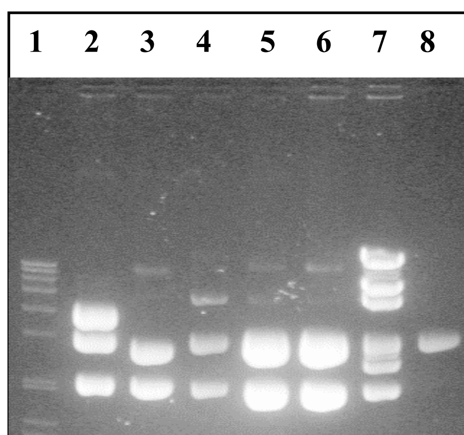

Figure 2. *Hind*III digestion of plasmid DNA.
Lane 1=standard VII (Boehringer Mannheim); 2=clone 128; 3=clone142; 4=clone 219; 5=clone 47; 6=clone427; 7=clone 428; 8=pBluescript digested with *Xho*I
Slika 2. Razkroj plazmidne DNK pozitivnih klonov.
Linija 1=velikostni standard VII (Boehringer Mannheim); 2=klon 128; 3=klon 142; 4=klon 219; 5=klon 47; 6=klon 427; 7=klon 428; 8=pBluescriptII razrezan z *Xho*I

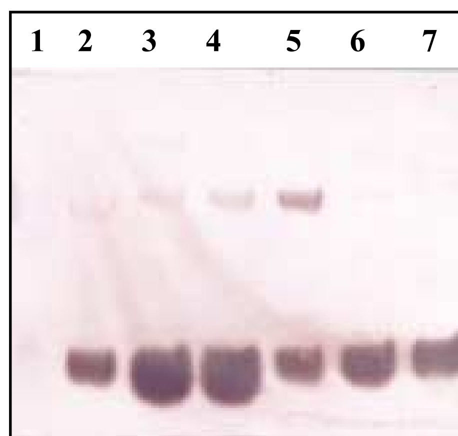

Figure 3. Hybridisation of *Hind*III digested plasmids DNA with BacPre probe.
Lane 1= pBluescript digested with *Xho*I; 2=clone428; 3=clone 427; 4=clone 47; 5=clone 219; 6=clone 142; 7=clone 128
Slika 3. Hibridizacija s *Hind*III razrezane plazmidne DNK in sonde PreBac.
Linija 1= pBluescriptII razrezan z *Xho*I; 2=klon 428; 3=klon 427; 4=klon 47; 5=klon 219; 6=klon 142; 7=klon 128;

In all six positive clones hybridising fragments with 16S rRNA genes were approximately 2 kb long. Sequence of 16S rRNA gene of *P. bryantii* B_14 (Accession. No. AJ006457) was obtained from the GeneBank database and analysed with WebCutter (BCM Search Launcher; http://dot.imgen.bcm.tmc.edu:9331/seq-util/seq-util.html). Computerised restriction analysis showed that *Hind*III cuts the 16S rRNA gene of *P. bryantii* B_14 at the position 88. Our previous work demonstrated that 16S-23S rRNA internal spacer region of *P. bryantii* contain approximately 510 nucleotides (Peterka and Avguštin, 1998). According to this findings, the

inserts of clones 128, 142, 219, 47, 427 and 428 contained most of the 16S rRNA gene, 16S-23S rRNA internal spacer region and approximately 100 bp of 23S rRNA gene (Figure 4). In all 16S rDNA positive clones inserts were approximately of the same size. Two explanations seem possible. First, all clones represent the same rRNA operon or second, rRNA operons of *P. bryantii* $B_1$4 are identical or contain microheterogeneities that can be identified only by sequencing. In order to confirm that cloned genes are actually from the *P. bryantii* $B_1$4 and to exclude possible contamination, part of the 16S rRNA gene was amplified with pair of primers specific for *P. bryantii* (Figure 5). Characteristic 327 bp fragments were observed in all six clones.

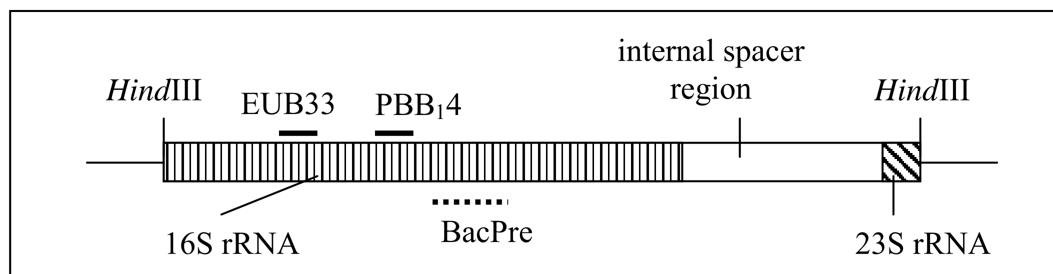

Figure 4. Schematic representation of cloned *Hind*III fragments with positions indicating the aligment sites of probe BacPre and primers EUB338 and $PB_1$4.

Slika 4. Shematski prikaz ostanka z označenim mestom naleganja sonde BacPre in začetnih oligonukleotidov EUB338 in $PB_1$4.

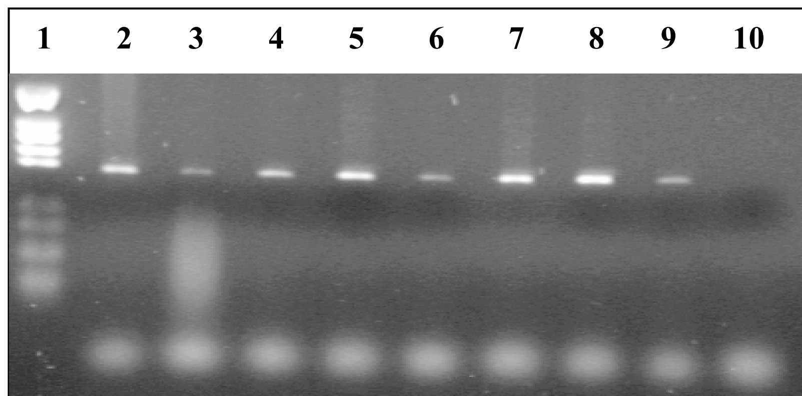

Figure 5. Amplification of the part of 16S rRNA gene with primers EUB338 and $PB_1$4.
Lane 1=standard pGEM (Promega); 2=$B_1$4 DNA; 3=clone 16S rRNA; 4=clone 128; 5=clone142; 6=clone 219; 7=clone 47; 8=clone427; 9=clone 428; 10=pBluescript

Slika 5. Namnoževanje dela gena za 16S rRNK z začetnimi oligonukleotidi EUB338 in $PB_1$4.
Linija 1=velikostni standard pGEM (Promega); 2=B14 DNK; 3=klon 16S rRNK; 4=klon 128; 5=klon 142; 6=klon 219; 7=klon 47; 8=klon 427; 9=klon 428; 10=pBluescriptII

Preliminary results were described showing that ribosomal RNA genes from anaerobic rumen bacteria can be successfully cloned into *E. coli* cells using the pBluescriptII vector. However, we failed to clone more than one operon although it was shown previously (Peterka and Avguštin, 1998) that at least 6 rRNA operons are present in this bacterial species and that there is substantial sequence variability between 16S rRNA gene copies in related bacteria *Prevotella*

ruminicola 23^T. We intend to further develop the cloning strategies that will allow the cloning of all ribosomal rRNA operons and the detailed analysis of their structure function.

POVZETEK

Prevotella bryantii je anaerobna vampna bakterijska vrsta, ki ima v vampu pomembno vlogo pri razkroju ksilana, škroba, beljakovin in peptidov. Vrsta, ki je bila pred kratkim na novo opisana, je na genetskem področju velika neznanka. Do sedaj so klonirali le nekaj genov, ki so vpleteni v razkroj polisaharidov in sekvencirali gen za 16S rRNK. Namen dela je bila priprava postopkov za kloniranje operonov ribosomskih RNK iz vrste *P. bryantii* in sorodnih bakterijskih vrst. Izolirali smo genomsko DNK seva *P. bryantii* $B_1 4$ in DNK vektorja pBluescriptII ter ju razrezali z restrikcijskim encimom *Hind*III. Kompetentne celice *E. coli* DH5α smo transformirali z vektorjem pBluecriptII spojenim z DNK fragmenti. S sondo specifično za gen za 16S rRNK smo pregledali 350 bakterijskih kolonij, ki so zrasle na ploščah z gojiščem LB, ki smo mu dodali 100 µg/ml ampicilina. Našli smo šest pozitivnih klonov, katerih vektorji so vsebovali gen za 16S rRNK. Fragmente, ki so vsebovali gene za 16S rRNK smo prepoznali s Southern prenosom in hibridizacijo z digoksigeninom označeno sondo, ki je bila značilna za rodova *Prevotella* in *Bacteroides*. Fragmenti so bili dolgi 2 kb in so vsebovali gen za 16S rRNK, 16S-23S rRNK interno medgensko regijo in 100 bp gena za 23S rRNK. Poreklo genov za 16S rRNK smo potrdili z verižno reakcijo s polimerazo (PCR) z začetnim oligonukleotidom, značilnim za vrsto *P. bryantii*.

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