THE SEARCH FOR CONJUGATIVE TRANSPOSON IN RUMEN BACTERIUM *Prevotella bryantii* B14

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Received March 15, 2009; accepted November 27, 2009.

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Only few plasmids and bacteriophages have been described to date in ruminal prevotella strains, therefore it appears plausible that the genetic exchange in these organisms must exploit other routes. Large conjugative transposons make possible the gene exchange process in bacteria from the genus *Bacteroides*, the phylogenetic relatives of ruminal prevotellas. The access to fully or partially finished genome sequences of *Bacteroides* and *Prevotella* representatives made possible the search for conserved regions within putative conjugative transposons. Multiple sequence alignment of known and putative conjugative transposon gene sequences of *Bacteroides thetaiotaomicron*, *Prevotella intermedia*, *Bacteroides fragilis* and *Tanerella* sp. was used to locate partially conserved regions within most preserved conjugative transposition genes, traG, and to construct appropriate degenerated oligonucleotide primers. These were used to amplify genome fragments from ruminal prevotella strains. Sequence analysis of the subcloned PCR products revealed the presence of a hypothetical gene in the genome of *Prevotella bryantii* B14, similar to the ORF BF2880 from *B. fragilis* YCH46, which is a part of a large conjugative transposon. Inverse PCRs were designed and performed to confirm the initial findings. A partial map of *P. bryantii* B14 putative conjugative transposon region was constructed, indicating an intergeneric horizontal gene transfer.

**Key words:** microbiology / molecular genetics / conjugative transposon / *Prevotella bryantii* / *Bacteroides fragilis*

Iskanje konjugativnega transpozona v vampni bakteriji *Prevotella bryantii* B14


**Ključne besede:** mikrobiologija / molekularna genetika / konjugativni transpozon / *Prevotella bryantii* / *Bacteroides fragilis*
1 INTRODUCTION

Prevotella bryantii is a Gram-negative, strictly anaerobic member of the evolutionary distinct phylum Bacteroidetes, also known as the CFB group (Paster et al., 1994; Shah and Collins, 1990). It is an important environmental commensal bacterium inhabiting the rumen (Avguštin, 1992; Hobson, 1997; Ramšák et al., 2000). In this complex ecosystem some important functions have been assigned to ruminal prevotellas, e.g. degradation of proteins and certain plant cell polysaccharides (Daniel et al., 1995; Hobson, 1997; Teather et al., 1997). Due to its rather distinct capability to survive at lower pH values than other rumen bacteria, this organism was proposed as a suitable model organism for genetic manipulation studies in ruminal ecosystem (Russel and Dombrowski, 1980). The substantial evolutionary distance to well studied bacteria like E.coli is probably the main reason for poor understanding of prevotella genetics (Avguštin et al., 1994, Accetto and Avguštin, 2007). The lack of suitable genetic tools hindered the progress and brought it almost to standstill in the mid 1990’s. The recent progress in the genetic research of their relatives i.e. the prevotellas and the bacteroides from the human colon revived the interest in the genetics of ruminal prevotellas in last couple of years (Shoemaker et al., 1992; Nikolich et al., 1994; Vercoe and White, 1997; Flint and Scott, 2000; Accetto et al., 2005, Accetto and Avguštin, 2007).

If an organism is to be a suitable genetic model, its horizontal gene transfer capacity should be known as well potential barriers for it. In addition to strong non-specific deoxyribonuclease activity of Prevotella bryantii B 4 (Flint and Thomson, 1990), only few plasmids and bacteriophages have been described from ruminal prevotella strains (Flint and Stewart, 1987; Ogata et al., 1996; Accetto and Avguštin, 1997; Ambrožič et al., 2001), therefore it appears plausible that the genetic exchange in these organisms must exploit other routes.

Conjugative transposons are distinct DNA segments that are normally integrated into bacterial chromosome and transfer by conjugation from donor to recipient bacterium. These genetic elements are integrated in the host genome except during transfer, therefore no method for their identification exists, analogous to plasmid isolation (Salyers et al., 1995). All studies of conjugative transposition in ruminal Prevotella strains were linked to conjugative transposition elements in Bacteroides spp. In vitro experiments showed that bidirectional transfer of native conjugative transposition element Tc' Em' 12256 from B. fragilis clinical isolate can occur between closely related human commensal species Bacteroides uniformis and B. thetaiotaomicron, and ruminal P. bryantii strains (Shoemaker et al., 1992). Additionally, mating experiments confirmed in vitro mobilization of Tc' Em' 7853 conjugative transposition element in P. bryantii B 4 strain (Nikolich et al., 1994). Nevertheless, to date the presence and identity of a conjugative transposon in any ruminal Prevotella species remains unproven.

2 MATERIAL AND METHODS

2.1 BACTERIAL STRAINS AND DNA ISOLATION

Two strictly anaerobic ruminal strains from genus Prevotella were used in this study: P. bryantii B 4 (Russel, 1983) and P. bryantii TC 1-1 (Van Gylswyk, 1990). Strains were grown under strict anaerobic conditions in M2 medium (Hobson, 1969) by modification of the Hungate technique for cultivation of anaerobic bacteria, as described by Bryant (1972). Eight ml of M2 medium was inoculated and incubated under anaerobic conditions for 14–24 hrs at 37 °C, until optical density at 654 nm reached 0.9–1.4. Total genomic DNA was isolated by modification of the CTAB/NaCl isolation protocol, as described in “Current Protocols in Molecular Biology” (Ausubel et al., 1987).

2.2 MULTIPLE SEQUENCE ALIGNMENT AND DEGENERATE PRIMER CONSTRUCTION

Multiple sequence alignment program tool Clustal X (Thompson et al., 1997) was used to align known and putative conjugative transposon transfer gene sequences of B. thetaiotaomicron, P. intermedia, B. fragilis and T. periodontalis sp. Largest consensus regions within traG gene were located and, considering their appropriate length and reciprocal location, used to construct a pair of degenerate oligonucleotide primers CTf1310 and CTr2270 (see results).

2.3 DEGENERATE PCR, DNA CLONING, SEQUENCING AND SEQUENCE ANALYSIS

Degenerate PCR was used to amplify the putative homologs of the traG gene in P. bryantii B 4 and P. bryantii TC 1-1 strains. 25 μl of reaction mixture contained 2.5 μl of the 10x Taq Buffer (Fermentas), 2 mM MgCl2, 0.2 mM deoxynucleoside triphosphate mixture (dATP, dGTP, dTTP, dCTP), 1.4 μM of each degenerate primer, 0.75 U Taq DNA polymerase (Fermentas) and 20 ng of genomic DNA, extracted from cultured ruminal prevotella strains. A series of PCR reactions was performed to determine the final amplification conditions: an ini-
tial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min. PCR was prolonged by final extension at 72 °C for 7 min. PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized under UV light using GelDoc 1000 trans-illuminator (BioRad). Degenerate PCR products containing specific DNA bands were excised from the gel, purified with QIAQUICK Gel Extraction Kit (Qiagen) and subcloned with TOPO TA Cloning Kit (Invitrogen). DNA sequences of the pBAD/Thio-TOPO vector inserts were determined by Microsynth GmbH (Switzerland). Sequence data were analyzed using BLAST analysis and compared to sequences from the NCBI and TIGR CMR databanks.

2.4 INVERSE PCR AND PRIMER WALKING

Retrieved sequences were used as a template for construction of specific primer pair, oriented outwards of the retrieved sequence, and used for inverse PCR (Ochman et al., 1988). 5 μg of genomic P. bryantii B14 DNA was partially digested using restriction endonucleases EcoRI (Gibco), HindIII (Promega), NotI or PstI (Fermentas), followed by purification, agarose electrophoresis size selection and quantification of digested DNA. A series of self-ligation reactions was set, using 0.1, 0.25, 0.5, 0.75, 1.0; 5 and 10 ng/μl DNA with 5 U of T4 DNA ligase (Fermentas) per reaction. Self-ligation reaction was carried out at 22 °C for 1 h, again followed by purification, agarose electrophoresis size selection and DNA quantification.

A series of inverse PCRs with an annealing temperature span of 50–60 °C was set. 50 μl of reaction mixtures contained 5.0 μl of the 10x Long PCR Buffer+Mg (Fermentas), 0.5 mM deoxynucleoside triphosphate mixture (dATP, dGTP, dTTP, dCTP), 0.5 μM of each inverse primer, 2.5 U Long PCR Enzyme Mix (Fermentas) and 1.0 μl of purified self-ligation reactions containing 0.1 to 10 ng/μl DNA per reaction. Amplification conditions consisted of an initial denaturation step of 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 15 s, annealing for 30 s and extension at 68 °C for 10 min. 27 cycles followed, consisted of denaturation at 94 °C for 15 s, annealing for 30 s and extension at 68 °C for 10 min, with extra 5 s added each cycle. PCR was prolonged by final extension at 68 °C for 10 min. PCR products were examined by electrophoresis, purified and sequenced (Microsynth GmbH, Switzerland). The acquired sequences were analyzed and used to construct primers for next part of the sequence in primer walking procedure. Sequences were linked and compared to known genome sequences of related species.

3 RESULTS AND DISCUSSION

Multiple sequence alignment of known and putative conjugative transposition traG genes from sequenced members of the Bacteroidetes phylum showed that no conserved oligonucleotides larger than 5 nucleotides exist within the aligned 2.5 kbp region (complete alignment not shown). Thus two largest partially conserved regions were identified, spanning approx. 960 bp long region (Fig. 1). The mismatch positions were used as 2–4-base degeneracies for the primer construction. 19 bp forward primer CTf1310 (5’-CSA-AYM-GHA-ARA-TYR-T-3’) with 192-fold degeneracy and 17 bp reverse primer CTr2270 (5’-TCC-TTN-TCS-GTC-AGN-CC-3’) with 32-fold degeneracy were constructed and subsequently used in degenerate PCR.

Figure 1: Multiple sequence alignment of known and putative conjugative transposition transfer genes from sequenced members of the Bacteroidetes phylum. Partial display of the aligned 2.5 kbp region with the two largest partially conserved regions, used for construction of degenerated oligonucleotide primers CTf1310 and CTr2270, is shown.

Slika 1: Poravnava sekvenc znanih in domnevnih genov prenosa konjugativne transpozicije članov debla Bacteroidetes, pri katerih je že znana celotna sekvensa genome. Prikazan je delni prikaz poravnave 2,5 kbp regije z dvema najobsežnejšima delno ohranjenima regijama, ki smo ju uporabili za izdelavo degeneriranih začetnih oligonukleotidov CTf1310 in CTr2270.
In degenerate PCR the competitive inhibition due to high primer degeneracy may occur. Primers anneal to the correct template but are not extended by the polymerase due to unstable 3’-ends, which results in high inefficiency of the first few PCR cycles. This can be overcome by increasing PCR cycles, which in return may increase nonspecific background and decrease the yield of specific PCR product. Only \( P. \) bryantii B14 reaction showed the presence of expected fragment of approximately 1 kbp (Fig. 2), which was isolated and subcloned.

The analysis of the sequenced region revealed the presence of a complete open reading frame sharing 24\% identity with \( \text{traI} \) gene from \( P. \) intermedia strain 17 conjugative transposon and 58\% identity with the ORF BF2880 from the putative conjugative transposon of the \( B. \) fragilis strain YCH46, at the amino acid level. The 24\% identity with the \( \text{traI} \) gene from \( P. \) intermedia 17 is on the border as far as assigning of the function or recognition of homology is concerned. However, the rather high degree of similarity with the ORF BF2880 from \( B. \) fragilis provides strong evidence for intergeneric horizontal gene transfer, especially if we bare in mind that the average DNA:DNA homology of total chromosomal DNA from \( Bacteroides \) species and ruminal \( \text{Prevotella} \) species is less than 5\% (Johnson in Harich, 1986).

Determination of the complete sequence of the cloned \( P. \) bryantii B14 genome fragment made possible the construction of specific primer set, which was subsequently used for inverse PCR in order to extend the known sequence in both directions. Figure 3 shows a series of inverse PCR amplifications from self-igation reactions of \( P. \) bryantii B4 genomic DNA flanking the targeted region. Reactions were performed at 54°C annealing temperature, using 0.1 to 10 ng/μl DNA per reaction. \( M \) – DNA size marker O’GeneRuler 1 kb DNA Ladder, 2 μl; 10 μl reaction samples: 1 – 0.1 ng/μl DNA; 2 – 0.25 ng/μl DNA; 3 – 0.5 ng/μl DNA; 4 – 0.75 ng/μl DNA; 5 – 1.0 ng/μl DNA; 6 – 5 ng/μl DNA; 7 – 10 ng/μl DNA.

Figure 3: Agarose gel electrophoresis of inverse PCR amplifications from self-igation reactions of \( P. \) bryantii B4 genomic DNA flanking the targeted region. Reactions were performed at 54 °C annealing temperature, using 0.1 to 10 ng/μl DNA per reaction. \( M \) – DNA size marker O’GeneRuler 1 kb DNA Ladder, 2 μl; 10 μl reaction samples: 1 – 0.1 ng/μl DNA; 2 – 0.25 ng/μl DNA; 3 – 0.5 ng/μl DNA; 4 – 0.75 ng/μl DNA; 5 – 1.0 ng/μl DNA; 6 – 5 ng/μl DNA; 7 – 10 ng/μl DNA.
Figure 4: Partial genetic map of P. bryantii B4 genomic region homologous to the putative conjugative transposon region of the B. fragilis YCH46. Arrows denote ORFs; gene homologs of Bacteroides fragilis YCH46 are indicated.

Slika 4: Delna genetska mapa genomske regije P. bryantii B14 homologue domnevnemu konjugativnemu transpozonom seva B. fragilis YCH46. Puščice označujejo odprte boljši okvirje ORFs; prikazani so homologi genov pri Bacteroides fragilis YCH46.

level (TIGR; http://tigrblast.tigr.org/cmrblast/) showed the highest similarity with BF2879 – BF2884 ORFs (with 69%, 61%, 45%, 71% and 44% identities, respectively) in B. fragilis YCH46, which are placed within the larg, 120 kbp putative conjugative transposon CTn3Bf (Kuwahara et al., 2004). Comparison of the mapped P. bryantii B4 region to the available genomic sequences of the related Bacteroidetes (TIGR CMR; http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) shows a similar gene organization. These findings confirm that these genetic elements were likely transferred to or from P. bryantii B4. Nevertheless, additional work is needed to reveal the nature of conjugative transposition in ruminal Prevotella species, to determine the presence of an active conjugative transposition element and its complete sequence. We are currently optimizing the genomic DNA primer walking procedure and preparing a cosmid genomic library in order to reveal the sequence of this interesting region in P. bryantii B4.

4 CONCLUSIONS

Multiple sequence alignment of known and putative conjugative transposition traG genes from sequenced members of the Bacteroidetes phylum showed that no conserved oligonucleotides larger than 5 nucleotides exist within the aligned region, therefore appropriate degenerate oligonucleotide primers had to be constructed to amplify homologous gene fragments from ruminal prevotella strains. Sequence analysis of degenerate PCR products revealed the presence of an ORF homologous with ORF BF2880 from the putative conjugative transposon of B. fragilis YCH46, showing 58% identity at the amino acid level. Inverse PCRs making possible the amplification of the flanking regions in P. bryantii B4 were set and their products sequenced. A partial map of P. bryantii B4 genomic DNA homologous to the putative conjugative transposon region of B. fragilis YCH46 was constructed, showing the same gene organization and high gene similarity. The observed indices suggest that the conjugative transposition elements of P. bryantii B4 were introduced through an intergeneric horizontal gene transfer.

5 REFERENCES


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