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THE SEARCH FOR CONJUGATIVE TRANSPOSON IN RUMEN BACTERIUM *Prevotella bryantii* $B_1 4$

Gregor GORENC [1], Tomaž ACCETTO [1], Gorazd AVGUŠTIN [2]

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The search for conjugative transposon in rumen bacterium Prevotella bryantii $B_1 4$

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* $B_1 4$, 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* $B_1 4$ 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 $B_1 4$

Ker sevi rodu *Prevotella* iz vampa le izjemoma posedujejo plazmide in je opisanih le nekaj bakteriofagov, je zelo verjetno, da izmenjava genov pri teh organizmih vključuje druge poti. Veliki konjugativni transpozoni omogočajo prenos genov pri rodu *Bacteroides*, filogenetskih sorodnikih vampnih prevotel. Dostop do delno ali v celoti sekvenciranih genomov predstavnikov *Bacteroides* in *Prevotella* je omogočil iskanje ohranjenih regij znotraj domnevnih konjugativnih transpozonov. S poravnavo več sekvenc znanih ali domnevnih genov konjugativnih transpozonov iz vrst *Bacteroides thetaiotaomicron*, *Prevotella intermedia*, *Bacteroides fragilis* in rodu *Tanerella* smo določili delno ohranjene regije v najbolj ohranjenem genu konjugativne transpozicije, *traG*, in jih uporabili za izdelavo primernih začetnih oligonukleotidov za pomnoževanje dela gena pri vampnih sevih iz rodu *Prevotella*. Analiza sekvenc subkloniranih pomnožkov je pri *P. bryantii* $B_1 4$ razkrila prisotnost hipotetičnega gena, podobnega odprtemu čitalnemu okvirju BF2880 seva *B. fragilis* YCH46, ki je del velikega konjugativnega transpozona. Z inverzno verižno reakcijo s polimerazo smo potrdili prvotne ugotovitve. Izdelali smo delno mapo regije domnevnega konjugativnega transpozona pri *P. bryantii* $B_1 4$, ki nakazuje, da je prišlo do medrodovnega horizontalnega prenosa genov.

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

1 Univ. of Ljubljana, Biotechnical Fac., Dept. of Animal Science, Groblje 3, SI-1230 Domžale, Slovenia, Ph.D.
2 Same address, Prof., Ph.D., e-mail: gorazd.avgustin@bfro.uni-lj.si

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; Ramšak *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 nonspecific deoxyribonuclease activity of *Prevotella bryantii* $B_1$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^r Em^r 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^r Em^r 7853 conjugative transposition element in *P. bryantii* $B_1$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_1$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 *Tanerella 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_1$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 $MgCl_2$, 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* $B_1$4 DNA was partially digested using restriction endonucleases *EcoR*I (Gibco), *Hind*III (Promega), *Not*I or *Pst*I (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-ACA-ART-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.

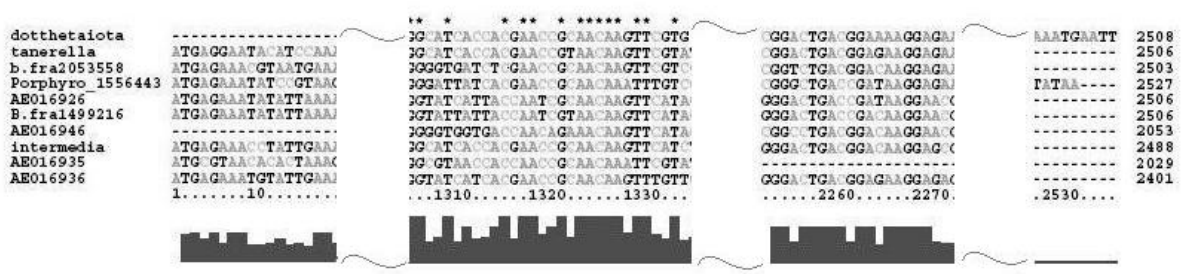

Figure 1: *Multiple sequence alignment of known and putative conjugative transposition transfer genes from sequenced members of the Bacteroidetes phylum. Partial display of the aligned 2.5 kbp region with the two largest partially conserved regions, used for construction of degenerated oligonucleotide 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 sekvenca genoma. 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 *traI* gene from *P. intermedia* strain 17 cojugative 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 *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 *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-ligation reactions under most suitable conditions.

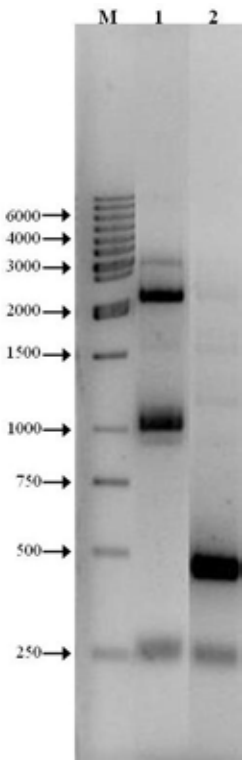

Figure 2: *Agarose gel electrophoresis of degenerate PCR multiplication of putative traG homologs in P. bryantii $B_1$4 and P. bryantii TC1-1. M – DNA size marker O'GeneRuler 1 kb DNA Ladder, 2 µl; 1 – Prevotella bryantii $B_1$4; 2 – Prevotella bryantii TC 1-1.*
Slika 2: *Agarozna gelska elektroforeza pomnoževanja domnevnega homologa gena traG z degenerirano verižno reakcijo s polimerazo pri P. bryantii $B_1$4 in P. bryantii TC1-1. M – velikostni standard O'GeneRuler 1 kb DNA Ladder, 2 µl; 1 – Prevotella bryantii $B_1$4; 2 – Prevotella bryantii TC 1-1.*

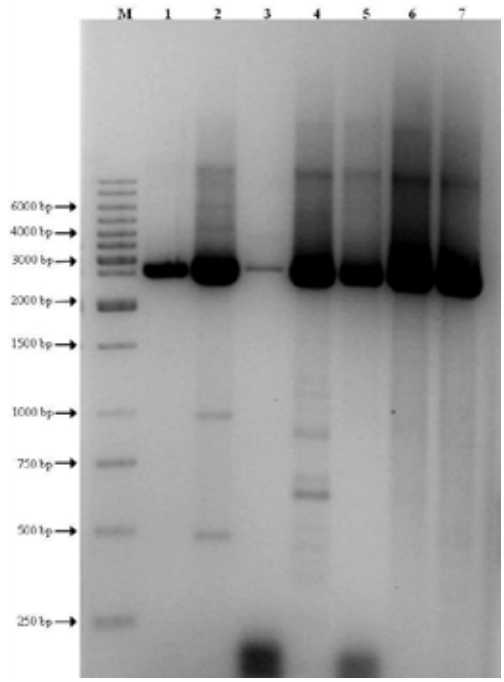

Figure 3: *Agarose gel electrophoresis of inverse PCR amplifications from self-ligation reactions of P. bryantii $B_1$4 genomic DNA flanking the targeted region. Reactions were performed at 54 °C annealing temperature, using 0,1 to 10 ng/µl DNA per reaction. M – DNA size marker O'GeneRuler 1 kb DNA Ladder, 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.*
Slika 3: *Agarozna gelska elektroforeza produktov inverzne verižne reakcije genomske DNK-P. bryantii $B_1$4. Reakcije so bile izvedene pri 54 °C z 0,1 do 10 ng/µl DNA na reakcijo. M – velikostni standard O'GeneRuler 1 kb DNA Ladder, 2 µl; vzorci po 10 µl na jamico: 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.*

All inverse PCR reactions showed the presence of 2.5 kbp products. The products of 0.1 ng/µl DNA reaction without additional multiple or smeared bands were used for subsequent sequencing, primer construction and gene walking procedure. The retrieved sequence data was analyzed and used to construct a partial map of *P. bryantii* $B_1$4 genomic region homologous to the putative conjugative transposon region of the *B. fragilis* YCH46 . Its structure and organization is shown in Figure 4.

Five ORFs with 162, 231, 108, 102 and 164 amino-acid residues were identified, with the first two being completely sequenced. BLAST analysis at amino-acid

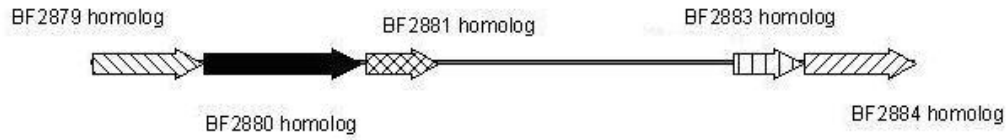

Figure 4: *Partial genetic map of P. bryantii $B_1$4 genomic region homologous to the putative conjugative transposon region of the B. fragilis YCH46. Arrows denote ORFs; gene homologs of Bacteroides fragilis YCH46 are indicated.*
Slika 4: *Delna genetska mapa genomske regije P. bryantii B14 homologne domnevnemu konjugativnemu transpozonu seva B. fragilis YCH46 . Puščice označujejo odprte bralne okvirje ORFs; prikazani so homologi genov pri Bacteroides fragilis YCH46.*

level (TIGR; http://tigrblast.tigr.org/cmr-blast/) 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* $B_1$4 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* $B_1$4. 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* $B_1$4.

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* $B_1$4 were set and their products sequenced. A partial map of *P. bryantii* $B_1$4 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* $B_1$4 were introduced through an intergeneric horizontal gene transfer.

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