Studies on *Prevotella* nuclease using a system for the controlled expression of cloned genes in *P. bryantii* TC1-1

Tomaž Accetto and Gorazd Avguštin

University of Ljubljana, Biotechnical Faculty, Zootechnical Department, Chair for Microbiology and Microbial Biotechnology, 1230 Domžale, Slovenia

Available tools for genetic analysis in the anaerobic rumen bacterium *Prevotella bryantii* are limited to only two known systems for gene delivery, and no genes, with the exception of plasmid maintenance and selection genes, have been successfully expressed from plasmids in any species of the genus *Prevotella* until now. It is shown here that nucB, a newly cloned nuclease gene from *P. bryantii*, can be controllably expressed from shuttle vector pRH3 in *P. bryantii* strain TC1-1, depending on the tetracycline concentration in the growth medium. nucB expression is also growth-medium dependent and this regulation presumably takes place at the translational level. His-tagged NucB was purified from *P. bryantii* TC1-1 culture supernatant and was shown to degrade DNA as well as RNA; it is most likely a minor 36 kDa *P. bryantii* non-specific nuclease.

INTRODUCTION

Members of the bacterial genus *Prevotella* have been found in the rumen as well as in the oral cavity and large intestine of man and animals (Edwards *et al.*, 2004; Paster *et al.*, 2001; Duncan *et al.*, 2003; Eckburg *et al.*, 2005; Leser *et al.*, 2002). In the rumen they are symbionts contributing to the degradation of plant cell-wall polysaccharides (Miyazaki *et al.*, 2003) and protein metabolism (Walker *et al.*, 2003), whereas oral prevotellas are implicated in dental plaque (Paster *et al.*, 2002), periodontitis (Paster *et al.*, 2001), advanced caries (Martin *et al.*, 2002), oro-pharyngeal abscesses (Brook, 2004) and noma, a grotesque childhood oro-facial gangrene, now confined mostly to sub-Saharan Africa (Enwonwu *et al.*, 2006). The genetic tools that would make possible more thorough studies of prevotellas are still undeveloped despite their ecological and medical importance. This appears to be primarily due to the large phylogenetic distance between prevotellas and other well-characterized micro-organisms, and as a consequence of the rather specific genetic elements, exemplified by promoters, containing distinct −7/−33 consensus sequences that are recognized by unusual primary σ factor (Vinggaard, 2005). Nevertheless, in two studies (Shoemaker *et al.*, 1991; Accetto *et al.*, 2005) the plasmid replicons and tetQ selectable marker derived from *Prevotella* and related colonic *Bacteroides* strains were used successfully for shuttle-vector introduction into the xylan-degrading ruminal species *Prevotella bryantii*, which forms a distinct phylogenetic lineage apparently somewhat closer to oral prevotellas than the other ruminal *Prevotella* species (Avguštin *et al.*, 2001). Based on the conjugal transfer protocol developed in the first study, a *P. bryantii* B14 carboxymethylcellulase gene, fused to a cellulose-binding domain of *Thermomonospora fusca* cellulase, and driven by a *Prevotella ruminicola* 23 xylanase promoter, was introduced into *P. bryantii* B14. The gene was expressed in *Bacteroides uniformis* 1108, but not in *P. bryantii* B14, possibly due to *P. bryantii* B14 not recognizing the *P. ruminicola* 23 xylanase promoter (Gardner *et al.*, 1996). In the second study, a shuttle vector pRH3, constructed on the basis of *Prevotella* sp. 223/M2/7 plasmid pRR12 (Daniel *et al.*, 1995), and protected against restriction, was successfully transferred to *P. bryantii* TC1-1 by electroporation (Accetto *et al.*, 2005). These studies highlighted, among other factors, the importance of enzymic barriers preventing the successful establishment of foreign DNA in the microbial cell. Besides restriction enzymes, non-specific nucleases may be involved too, as in the case of *Vibrio cholerae* and *Vibrio vulnificus*, where representatives of these mostly extracellular enzymes having no sequence and sugar specificity (Rangaranjan & Shankar, 2001; Jarvill-Taylor *et al.*, 1999) have been shown to inhibit transformation (Focareta & Manning, 1991; Wu *et al.*, 2001). Besides inhibiting transformation, they have been ascribed various roles in nutrition (Benedik & Strych, 1998), in the processing of DNA during natural transformation, e.g. in *Bacillus subtilis* and *Streptococcus pneumoniae* (Provvedi *et al.*, 2001; Dubnau 1999), and in virulence (Bendjennat *et al.*, 1999). Most *P. bryantii* strains have high nucleolytic activity (Avguštin *et al.*, 1997) and strain B14 was shown to possess a 45 kDa non-specific nuclease (Accetto & Avguštin, 2001). The electrotransformation frequency of *P. bryantii
TC1-1 with pRH3 (Accetto et al., 2005) could thus be risen considerably if the non-specific nuclease genes in the recipient strain were inactivated. We report here on the cloning of a P. bryantii B4 non-specific nuclease gene named nucB and an attempt to express it in its native background.

**METHODS**

**Bacterial strains, plasmids, primers, media and growth conditions.** The P. bryantii strains B4 (the type strain) (Bryant et al., 1958) and TC1-1 (Van Gyswyk, 1990) were grown anaerobically at 37 °C in rumen fluid containing M2 medium (Hobson, 1969) or DSMZ medium M330, which was slightly modified: Na2S and glycerol were added. The rumen fluid containing M2 medium (Hobson, 1969) or DSMZ medium M330, which was slightly modified: Na2S and glycerol were added, and the glucose content was increased to 0.5 g l−1 to 2 g l−1. When needed, tetracycline was added to the medium at the final concentration of 2.5 μg ml−1 prior to inoculation. Escherichia coli TOP10 was obtained from Invitrogen and was grown on LB medium at 37 °C with the appropriate antibiotics. The shuttle vector pRH3 (Daniel et al., 1995) was kindly donated by Harry J. Flint (Rowett Research Institute, Aberdeen, Scotland). The cloning vector pUC19 was obtained from Promega. The oligonucleotide primers used in the study were synthesized at Microsynth at our request, and are shown in Table 1.

**Preparation of cell-free extracts.** Cells were broken with a bead-beater apparatus (BioSpec Products), the DNA was removed with streptomycin (Accetto et al., 2005), and the resulting extracts were used for determination of nuclease activity by means of the quick nuclease assay or DNA protection as described previously (Accetto et al., 2005).

**Preparation of cell-wash extracts.** The cells were washed once in TE, and then resuspended in one-tenth of the original volume in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% (v/v) Triton X-100, as described by Takahashi et al. (1996). After centrifuging, the cell-wash extracts were collected and, if necessary, concentrated using Microcon YM-10 centrifugal filter devices (Millipore).

**Quick nuclease assay.** Seventeen microlitres of cell extracts or culture supernatants were mixed with 2 μl of 0.25 μg μl−1 λ or plasmid DNA and 1 μl of 10× restriction enzyme buffer A from Roche, and incubated at 37 °C for 1 h. The proteins were then removed with phenol, and the samples were analysed by means of standard DNA gel electrophoresis.

**Sequencing.** The sequencing of plasmid and PCR DNA, as well as the direct sequencing of genomic DNA, was performed on request by Microsynth.

**Bioinformatic tools.** For computer-assisted DNA analysis we used BLAST (Altschul et al., 1997) at NCBI, PFAM search (Bateman et al., 2004) and SignalP signal peptide prediction (Bendtsen et al., 2004).

**PCR.** The following standard PCR reagent concentrations were used: 0.1 μM primers, 2 mM MgCl2, 0.2 mM dNTPs and 0.5 U Taq polymerase (Fermentas), in 20 μl reactions. When amplified DNA was used for expression or when performing inverse PCR, the high-fidelity PCR enzyme mix from Fermentas was used, and the reactions were set up according to the manufacturer’s instructions.

**Cloning of nucB.** Sequence analysis of the previously cloned chromosomal region of the P. bryantii TC1-1 (Accetto & Avgustin, 2001) showed that its hypothetical proteins share high amino acid sequence identity with Prevotella intermedia 17 proteins coded in loci PINA1518–1521, and that the gene order is conserved in this region. Preliminary genome sequence data of P. intermedia 17 were obtained from The Institute for Genomic Research web site (http://www.tigr.org). Survey of the flanking P. intermedia 17 genome regions revealed that a hypothetical non-specific nuclease lies only 4 kbp away. Subsequently 4.9 kb of P. bryantii B4 genomic sequence, extending from the above-mentioned cloned region, was retrieved with inverse PCR and sequenced. The gene order between P. bryantii B4, TC1-1 and P. intermedia 17 was found to be conserved. Thus the sequence of a P. bryantii B4 nucB, a homologue of the gene in locus PINA1511, was obtained.

**Inverse PCR.** Inverse PCR was performed in two rounds and essentially as described by Sambrook & Russell (2001). In the first round, the P. bryantii B4 genomic DNA was cleaved with PstI,

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**Table 1.** Primers used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>inv1</td>
<td>CTCTCGGTGCAACGTCATTTC</td>
</tr>
<tr>
<td>inv2</td>
<td>AGCATCTGTACAGTAG</td>
</tr>
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<td>inv3</td>
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</tr>
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<td>inv4</td>
<td>GGTGAATACCCGTACCATC</td>
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<tr>
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<tr>
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</tr>
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</table>
religated and amplified with the primers inv1 and inv2. For the second inverse PCR, the *P. bryantii* B4 genomic DNA was fragmented using *Hind*III. After ligation, the neighbouring region to the one obtained with the first inverse PCR was amplified using the primers inv3 and inv4. Both inverse PCR products were cloned using the pBAD-TOPO TA kit (Invitrogen) and sequenced.

**Expression of nucB in *P. bryantii* TC1-1.** The PCR-amplified *P. bryantii* B4 nucB gene variants were PstI-digested, ligated to the PstI linearized and shrimp alkaline phosphatase-treated vector pRH3, and transferred to *E. coli* TOP10. The orientation of nucB in the resulting clones was determined by restriction analysis. The constructs having the same orientation as tetQ gene were isolated, verified by sequencing using the primer kontrnuc, protected against the action of *Pbr*II restriction of *P. bryantii* TC1-1, and transferred to *P. bryantii* TC1-1 by electroporation (Accetto *et al.*, 2005). The culture supernatants of the resulting *P. bryantii* TC1-1-derived strains, which were grown with 2.5 μg tetracycline ml^-1^ unless stated otherwise, were assayed for nuclease activity either directly or were concentrated using Amicon Ultra 10 000 MWCO centrifugal filter devices from Millipore.

**Nuclease SDS-PAGE assay.** Nuclease detection in renatured SDS-PAGE gels containing nucleic acids was performed essentially as described elsewhere (Rosenthal & Lacks, 1977; Blank *et al.*, 1982). Ten micrograms of salmon testes DNA (Fluka) and 40 μg 16S and 23S rRNA from *E. coli* (Roche) per ml of the resolving gel were used.

**Isolation of the 6×His-tagged NucB from the *P. bryantii* TC1-1 carrying pRH3nuc3d.** First, 150 ml of culture supernatant was concentrated using Amicon Ultra 10 000 MWCO Millipore centrifugal filter devices. Buffer was then exchanged with Ni-NTA binding buffer from Ni-NTA buffer kit (Novagen) and NucB was purified under native conditions on Ni-NTA His bind resin (Novagen) as recommended by the manufacturer. Mercaptoethanol (5 mM) was added to all buffers used in purification. The elution fractions showing DNase activity were concentrated and the elution buffer was exchanged with buffer containing 20 mM Tris pH 7.5, 50 mM NaCl and 1 mM mercaptoethanol. The enzyme of activity preparation was tested with λ and pUC18 DNA as well as with *P. bryantii* TC1-1 RNA in the reactions, which contained 1 μg nucleic acids, 5 mM MgCl₂ as well as 20 mM Tris pH 7.5, 50 mM NaCl and 1 mM mercaptoethanol. NucB cation requirement was tested in reactions where MgCl₂ was replaced with CaCl₂, CoCl₂ or MnCl₂.

**RNA isolation.** The total RNA was extracted from 8 ml *P. bryantii* TC1-1 cultures during exponential growth at OD₅₆₀ 0.3 using the Trizol reagent (Gibco-BRL), following the manufacturer’s guidelines. Strains were grown in modified M330 medium unless stated otherwise.

**Reverse transcription and real-time PCR.** Reverse transcription was performed using the primers RT16sr and RTNucBr, which are specific for *P. bryantii* 16S rDNA and nucB genes, respectively. The RNA was DNase I (Fermentas) treated and RevertAid H minus M-MuLV reverse transcriptase (Fermentas) was used at 42 °C. Control reactions lacking the reverse transcriptase were also performed. Portions of 1 μl of 20 μl reverse transcription reactions were used as templates for standard PCR, using the primer pairs RT16Sr/RT16Sr and RTNucBr/RTNucBr for amplification of 16S rRNA and nucB gene fragment, respectively. The reactions yielded PCR products of 86 and 62 bp. The amount of 16S rRNA and nucB CDNA was quantified with real-time PCR using the above-mentioned primers, Sybr Green PCR Master Mix from Applied Biosystems and the ABI PRISM 7900HT sequence detection system. Relative quantification of nucB CDNA was performed using the standard curve method, and the 16S rRNA amplicon was used as endogenous control for normalization. The samples were amplified in triplicate with the standard thermal cycling profile, and the results were analysed using SDS 2.2 software. RNA was isolated twice from the separate batches of modified M330 medium, and real-time measurement of each cDNA amount was repeated at least once. The amount of nucB mRNA present in the wild-type *P. bryantii* TC1-1 was used as a calibrator. Reverse transcription of RNA samples was repeated in order to check its reproducibility.

**Western blot and immunodetection.** Following the standard SDS-PAGE, the proteins were transferred onto a PVD/F membrane (Bio-Rad) using the semi-dry Multiphor II NovaBlot apparatus (Pharmacia Biotech) with pieces of filter paper soaked in the cathode (25 mM Tris, 40 mM glycine, 10%, v/v, methanol, pH 9.4) and anode buffers 1 and 2 (0.3 M Tris, 10% methanol, pH 10.4, and 25 mM Tris, 10%, v/v, methanol, pH 10.4, respectively). The membrane was blocked with 5% (w/v) skimmed milk powder (Merck) in TBST (50 mM Tris/HCl, 150 mM NaCl, 0.1%, v/v, Tween 20, pH 7.5), and immunodetection was performed using Anti-mouse primary monoclonal antibody (Roche) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). The ECL Western blotting detection reagents (Amersham Biosciences) were used to generate chemiluminescence, which was recorded with the Chemigenius² bio imaging system (Syngen).

**RESULTS**

**DNase and RNase activity of *P. bryantii* TC1-1 and B4**

Using the nuclease SDS-PAGE assay, a DNase was observed in the concentrated culture supernatants and concentrated cell-wash extracts of M2-grown *P. bryantii* TC1-1 with the same apparent molecular mass as the *P. bryantii* B4 DNase. The latter was shown previously to have an apparent molecular mass of 45 kDa (Accetto & Avgustin, 2001). The RNase activity of the *P. bryantii* B4 cell-wash extracts was also assayed and RNase activity was observed at 45 kDa.

**Sequence analysis of *P. bryantii* B4 nucB**

Its 33.5 kDa predicted protein product belongs, according to PFAM, in the DNA/RNA non-specific endonuclease family, contains a predicted signal peptide and shares 49% amino acid identity with the protein coded in *P. intermedia* 17 locus PINA1511. Upstream of the nucB start codon, a 130 bp sequence is located, containing a putative transcriptional terminator of the preceding ribosomal protein L31 gene, leaving only approximately 50 bp for a putative promoter sequence. The length of the upstream region and putative terminator location is also conserved in *P. intermedia* 17. Promoter sequences typical for *Bacteroides fragilis* (Bayley *et al.*, 2000) or *P. bryantii* (Peterka, 2002) were not found in this region. The aforementioned L31 ribosomal protein shares 90% amino acid sequence identity with its *P. intermedia* 17 counterpart. The *P. bryantii* TC1-1 nucB gene was subsequently successfully amplified with the primers pstmnuw and pstmnuuc, which were designed on the basis of the *P. bryantii* B4 genomic
sequence. It was found out that the genes are highly similar in the upstream region, as well as in structural part, with a total of only 21 nucleotide substitutions, 18 of them in the structural part (2% difference), leading to 11 (3.6%) amino acid differences between them.

**Expression of nucB in P. bryantii TC1-1**

Three nucB variants were produced from *P. bryantii* B14 genomic DNA by PCR, using the forward primers pstnnucf, pstnnucf1 and pstnnucf2, annealing at different positions in the nucB upstream region (Fig. 1), and the pstnnuc reverse primer. The PCR products were ligated to the shuttle vector pRH3, transferred first to *E. coli* TOP10 in order to check the orientation of the inserts, and then transferred to *P. bryantii* TC1-1. The recombinant plasmids containing 108, 77, and 16 bp of *nucB* upstream region in addition to the structural gene were named pRH3nuc3a, pRH3nuc3b and pRH3nuc3c, respectively. The DNase activity of *P. bryantii* TC1-1 containing pRH3nuc3a culture supernatant was slightly higher than control after growth for 1 day on conventional M2 medium for rumen bacteria. However, when this recombinant strain was grown on modified DSMZ medium M330, relatively strong non-specific DNase activity was observed in the culture supernatants (Fig. 2). This activity resulted in cleavage of plasmid as well as λ DNA, and was EDTA inhibited. When concentrated supernatants of modified M330-grown pRH3nuc3a bearing *P. bryantii* TC1-1 were analysed on SDS-PAGE nuclease gel, three zones of DNA degradation were observed: one coinciding with the already known major *P. bryantii* nuclease, and two other zones corresponding to the proteins of approximately 36 and 34 kDa. The same zones were present also in wild-type *P. bryantii* TC1-1. However, in the recombinant Strain TC1-1 bearing pRH3nuc3a, the intensity of DNA degradation was highest at 36 kDa, whereas the major activity of the wild-type strain was at 45 kDa (Fig. 3). When concentrated supernatants, or cells of *P. bryantii* B14 grown in modified M330 were analysed. An additional nucB variant, carrying a 6×His-tag at its C terminus, was amplified from *P. bryantii* B14 genomic DNA with the primers pstnnucf and pstnucBrhis, cloned as described above, yielding pRH3nuc3d, and transferred to *P. bryantii* TC1-1. When the concentrated culture supernatants of modified M330-grown *P. bryantii* TC1-1 carrying a 6×His-tag variant of nucB were analysed by Western blotting, using anti 6×His antibodies, two chemiluminescent bands were observed at 37 kDa and 42 kDa (Fig. 4).

**Isolation of NucB from P. bryantii TC1-1 carrying pRH3nuc3d grown in M330**

6×His-tagged NucB was purified from the culture supernatant on Ni-NTA resin. The DNase-active elution fractions contained two protein bands when analysed by SDS-PAGE, with the molecular masses coinciding with those observed in Western blots. Purified NucB degraded λ and pUC18 DNA, as well as RNA, and the DNase activity was inhibited by 20 mM EDTA. DNase activity was comparable when MgCl2 was replaced with CoCl2 or MnCl2. Activity was not observed when CaCl2 was used or when no divalent cations were added to the reaction mixtures. The addition of 20 mM EDTA did not inhibit the RNase activity significantly, and MgCl2 was also not required for activity.
Analysis of the nucB upstream region

The analysis of the DNase activity of *P. bryantii* TC1-1 recombinant strains described above, grown in modified M330 medium, showed that *P. bryantii* TC1-1 containing pRH3nuc3b (nucB beginning at position −77) had slightly higher activity than the −108 variant (pRH3nuc3a). The strain harbouring pRH3nuc3c, however, which contained only 16 bp of the upstream region, had markedly decreased nuclease activity, which was only slightly higher than in the wild-type strain (Fig. 2).

Quantification of nucB mRNA in *P. bryantii* strains harbouring pRH3nuc3a, pRH3nuc3b and pRH3nuc3c

The amount of nucB mRNA was quantified with two-step real-time RT-PCR. When tetracycline was omitted from the modified M330 medium, the amount of nucB mRNA decreased approximately three- to fivefold in recombinant strains (pRH3nuc3a, 1.7 ± 0.06 vs 5.6 ± 1.8; pRH3nuc3b, 1.6 ± 0.4 vs 8.1 ± 2.8; pRH3nuc3c, 2.4 ± 1.0 vs 8.8 ± 2.1; means ± SD of at least four measurements), but stayed above the control level, i.e. the amount in the wild-type strain TC1-1. Supernatants of cultures grown without tetracycline were also checked for DNase activity, which decreased to levels comparable to wild-type activity (not shown). It was not possible to detect NucB on Western blots when tetracycline was omitted from the growth medium of pRH3nuc3d containing *P. bryantii* TC1-1 (Fig. 4). Plasmids were present in the strains also in the absence of tetracycline, and the plasmid yield was unchanged. The amount of nucB mRNA in *P. bryantii* TC1-1 pRH3nuc3b, grown on modified M330 medium or on M2 medium, was comparable, the latter being 7.2 ± 1.8 times higher than nucB mRNA of *P. bryantii* TC1-1 wild-type grown in modified M330 medium.

RT-PCR analysis of nucB mRNA transcript

In order to determine whether the transcription of nucB mRNA in *P. bryantii* TC1-1 harbouring one of the pRH3nuc3 plasmids started from the tetQ promoter, an RT-PCR experiment was performed with the primers RTnucBr, which was used for reverse transcription and PCR, and kontrnnuc, the later annealing 109 bases upstream of the tetQ stop codon in pRH3. This primer pair should produce an RT-PCR product only when nucB mRNA is part of a larger mRNA transcript starting with tetQ, i.e. 522, 491 and 430 bp products from nucB mRNA of *P. bryantii* TC1-1 harbouring pRH3nuc3a, pRH3nuc3b and pRH3nuc3c, respectively. The RT-PCR product of expected size was in fact observed for all nucB variants carried on plasmids in *P. bryantii* TC1-1 regardless of whether tetracycline was added to the growth medium or not.

DISCUSSION

We recently developed a reasonably fast and efficient gene introduction protocol for *P. bryantii* TC1-1 involving methylase plasmid protection and electroporation (Accetto et al., 2005), which was successfully used in this study for the delivery of plasmids containing nuclease genes. The shuttle vector used was pRH3 (Daniel et al., 1995). It is composed of already sequenced 3.2 kb cryptic plasmid pRRI2, isolated from Prevotella sp. 223/M2/7 (Mercer et al., 2001), the tetQ gene from Bacteroides plasmid pNFD13-2 and *E. coli* cloning vector pBluescript SK+ (Fig. 5). *P. bryantii* TC1-1 possesses a nuclease with the same apparent
molecular mass as the previously described 45 kDa major nuclease of *P. bryantii* B4 (Accetto & Avguštin 2001). However, the nuclease activity of TC1-1 is far weaker than that of other *P. bryantii* strains, thus making it a suitable recipient for nuclease genes. Two additional zones of DNA degradation, corresponding to proteins with molecular masses of approx. 34 and 36 kDa, were discovered when *P. bryantii* TC1-1 was grown in modified M330 medium.

Into this background a gene from *P. bryantii* B4 strain, named nucB, putatively coding for a 33.5 kDa non-specific nuclease, was introduced on a pRH3 shuttle vector. nucB was cloned into the *Pst*I site of pRH3, downstream and in the same orientation as the tetQ gene, thus placing it in a putative transcriptional fusion with tetQ (Fig. 5). Clearly increased DNase activity was observed in the supernatants of the recombinant TC1-1 strain. The amount of the 36 kDa DNase, as judged from renatured DNase SDS-PAGE gels, was increased too. The DNase activity was only present to a clearly higher degree when the recombinant strain *P. bryantii* TC1-1 was grown in modified M330 medium; the increase in the activity was much lower when the strain was grown in M2 medium. The Western blot analysis confirmed that His-tagged NucB is actually expressed from the plasmid, and migrates as a 37 kDa protein; however, a second, 42 kDa protein, was also detected. The latter could represent post-translationally modified NucB, possibly by glycosylation, an inactive precursor, or the product of an aberrant start/stop codon. His-tagged NucB was also purified from *P. bryantii* TC1-1 culture supernatants and was shown to be an endonuclease that degrades DNA as well as RNA. It appears therefore that nucB actually codes for a minor *P. bryantii* non-specific nuclease.

The experiment with a truncated nucB upstream region suggests that the region between 77 and 16 bp upstream of the translational start site is required for nucB upregulation in modified M330 medium. Unexpectedly, strain TC1-1 bearing the nucB variant with only 16 bp of the nucB upstream region and having only slightly increased DNase activity contained at least the same amount of nucB mRNA as the strains with other two nucB variants. This, together with the observation that the nucB upstream region lacks *Bacteroides* –7 and –33 promoter sequences, led us to consider that the nucB upstream region does not actually function as a promoter. It was therefore possible that nucB was transcribed as part of a larger mRNA starting at the tetQ promoter around 2.5 kbp upstream (Fig. 5). An RT-PCR experiment with primers flanking the presumed junction between nucB and tetQ mRNA confirmed that a single tetQ-nucB mRNA transcript exists. pRH3 is stable in *P. bryantii* TC1-1 in the absence of tetracycline (Accetto et al., 2005) and when tetracycline was omitted from the growth medium, the amount of nucB mRNA fell, which is in accordance with findings in *Bacteroides thetaiotaomicron* (Wang et al., 2004). Along with the drop of nucB mRNA, the DNase activity in the supernatants fell and His-tagged NucB was no longer detected on Western blots. All these observations argue that the nucB upstream region exerts its influence on the nucB expression at the translation level. Consistent with this is the observation that *P. bryantii* TC1-1 harbouring pRH3nuc3b contains roughly the same relative amount of nucB mRNA when grown either on M2 or M330 medium, but has markedly lower DNase activity in the supernatants when grown in M2 medium.

The genome of *P. intermedia* 17 contains, apart from a gene homologous to nucB, another gene in locus PIN0092, coding for a 42 kDa protein, which belongs, like NucB, to the DNA/RNA non-specific nuclease protein family, representing the major nucleases in many bacteria. Given the suspected gene order conservation between *P. bryantii* B4 and *P. intermedia* 17, and their similar genome sizes (Peterka, 2002), *P. bryantii* may also harbour a PIN0092 homologue whose product could actually be the major nuclease. Knockout and analysis of this gene would most likely result in increase of electrotransformation frequency of *P. bryantii* strains with pRH3 and shed light on the function of *P. bryantii* DNases.

The gene introduction system for *P. bryantii* TC1-1 based on the vector pRH3 proved reliable. The possibility of regulating the transcription of a foreign gene cloned into the unique *Pst*I and *Xba*I sites of pRH3 via the tetracycline concentration, together with the stability of the plasmid in *P. bryantii* TC1-1 in the absence of tetracycline, enhances the usability of pRH3 beyond mere gene delivery. The nucB expression in *P. bryantii* TC1-1 is to our knowledge the first instance of successful expression of any gene from artificially constructed plasmid vectors apart from plasmid replication and tetracycline resistance genes in genus *Prevotella*.

**ACKNOWLEDGEMENTS**

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![Fig. 5. Map of the shuttle vector pRH3. nucB was inserted into the PstI site downstream of tetQ and in the same orientation. Location and orientation of Prevotella/Bacteroides promoters is also shown.](image)
REFERENCES


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