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

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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 establishment (Kolenbrander *et al.*, 2002), periodontitis (Paster *et al.*, 2001), advanced caries (Martin *et al.*, 2002), oro-pharyngeal abscesses (Brook, 2004) and noma, a grotesque childhood orofacial 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 (Vingadassalom *et al.*, 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 pRRI2 (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 (Rangarajan & 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*

The GenBank/EMBL/DDBJ accession numbers for the *P. bryantii* B₁₄ and TC1-1 *nucB* sequences are EF472956 and EF472957, respectively.

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* B₁₄ 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 B₁₄ (the type strain) (Bryant *et al.*, 1958) and TC1-1 (Van Gylswyk, 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 omitted, 1 g l⁻¹ each of maltose, cellobiose and soluble starch were added, and the glucose content was increased from 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 & Avguštin, 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* B₁₄ genomic sequence, extending from the above-mentioned cloned region, was retrieved with inverse PCR and sequenced. The gene order between *P. bryantii* B₁₄, TC1-1 and *P. intermedia* 17 was found to be conserved. Thus the sequence of a *P. bryantii* B₁₄ *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* B₁₄ genomic DNA was cleaved with *Pst*I,

Table 1. Primers used in the study

Name	Sequence
inv1	CTTCTCGGTGCAACGTCATTC
inv2	AGCATCCTGACCAGTAG
inv3	GTACCAGCTCCTTATACATTG
inv4	GGTGAAGATACCAGTTACATC
pstnnucf	GCCGCTGCAGTTAAGTCAAGAGGGTACGTTT
pstnnucr	GCCGCTGCAGCTTCGTATGGTGTCTTGAGTC
pstnnucf1	GCCGCTGCAGGCATATGCGGTCGGTCGTAC
pstnnucf2	GCCGCTGCAGTAACTAACTTCACCTATGCACAC
RTnucBf	TGGTTGCTTGCGGCAGTAG
RTnucBr	AGAAGCGGAAGACCCAGATGT
RT16Sf	AAATGTCCGGGCTCAACCT
RT16Sr	ACCGCTACACCACGAATTCC
kontrnnuc	GGTTAAGCCATGCGGGTATC
pstnucBrhis	GCCGCTGCAGTTATTAATGATGATGATGATGATGAGAACCCCGTTAAGTTT- CCAGGCTCTTAG

religated and amplified with the primers inv1 and inv2. For the second inverse PCR, the *P. bryantii* B₁₄ 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* B₁₄ *nucB* gene variants were *Pst*I digested, ligated to the *Pst*I 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*TI 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 Ultra4 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 Ultra4 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 activity of enzyme 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 OD₆₀₀ 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 rRNA 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 RT16Sf RT16Sr and RTnucBf 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 PVDF 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-his₆ 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 (Syngene).

RESULTS

DNase and RNase activity of *P. bryantii* TC1-1 and B₁₄

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* B₁₄ DNase. The latter was shown previously to have an apparent molecular mass of 45 kDa (Accetto & Avguštin, 2001). The RNase activity of the *P. bryantii* B₁₄ cell-wash extracts was also assayed and RNase activity was observed at 45 kDa.

Sequence analysis of *P. bryantii* B₁₄ *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 pstnnucf and pstnnucr, which were designed on the basis of the *P. bryantii* B₁₄ 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 pstnnucr 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). These additional DNA degradation zones were not seen when supernatants, concentrated supernatants, or cells of *P. bryantii* B₁₄ grown in modified M330 were analysed. An additional *nucB* variant, carrying a 6 $\times$ 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 $\times$ His-tag variant of *nucB* were analysed by Western

Fig. 2. Culture supernatant DNase activity of *P. bryantii* TC1-1 recombinant strains grown in M330 medium. Bacteriophage λ was used as the substrate. Lane 1, 1 kb standard (Fermentas); lane 2, bacteriophage λ DNA; lane 3, TC1-1 wild-type strain; lanes 4, 5 and 6, strain TC1-1 harbouring pRH3nuc3a, pRH3nuc3b and pRH3nuc3c, respectively.

blotting, using anti 6 $\times$ His antibodies, two chemiluminiscent 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 $\times$ 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 $MgCl_2$ was replaced with $CoCl_2$ or $MnCl_2$. Activity was not observed when $CaCl_2$ 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 $MgCl_2$ was also not required for activity.

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TAAATATATCCTTTATAAGTATATATTAAGTCAAGAGGGTACGTTTCGAGC
-130                                     -108 pstnnucf
GTAGTTGCATATGCGGTCTGGTCTGACCTTTATCGTTTTAAACGAAATT
-77 pstnnucf1
TCAATAGGTAATTCTATATAACTAACTTCACTTATGCACACAATTAAAT
-16 pstnnucf2
CTTTATTTTGTAGCCATGGGCGTATTAGCTCTGGTTGCTTGCGGCAGTAG

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Fig. 1. The *nucB* upstream sequence. Primer annealing sites are shown in bold, the putative transcription terminator is shown in italics, and the *nucB* and ribosomal protein L31 gene start and stop codons, respectively, are underlined.

Fig. 3. Concentrated culture supernatant DNase activity of *P. bryantii* TC1-1 harbouring *nucB* in pRH3nuc3a analysed on renatured SDS-PAGE gel containing DNA. Lanes 1 and 2, TC1-1 wild-type strain with and without mercaptoethanol in the loading buffer; lanes 3 and 4, strain TC1-1 containing pRH3nuc3a with and without mercaptoethanol in the loading buffer.

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).

Fig. 4. Western blot analysis of concentrated culture supernatants of the *P. bryantii* strain TC1-1, harbouring pRH3nuc3d coding for His-tagged NucB. Lane 1, TC1-1 wild-type strain; lane 2, Precision plus protein standard (Bio-Rad); lane 3, TC1-1 harbouring pRH3nuc3d, tetracycline added; lane 4, TC1-1 harbouring pRH3nuc3d, tetracycline omitted.

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 $\pm$ 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

REFERENCES

- Accetto, T. & Avguštin, G. (2001). Non-specific DNases from the rumen bacterium *Prevotella bryantii*. *Folia Microbiol (Praha)* **46**, 33–35.
- Accetto, T., Peterka, M. & Avguštin, G. (2005). Type II restriction modification systems of *Prevotella bryantii* TC1-1 and *Prevotella ruminicola* 23 strains and their effect on the efficiency of DNA introduction via electroporation. *FEMS Microbiol Lett* **247**, 177–183.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Avguštin, G., Wallace, R. J. & Flint, H. J. (1997). Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *Int J Syst Bacteriol* **47**, 284–288.
- Avguštin, G., Ramšak, A. & Peterka, M. (2001). Systematics and evolution of ruminal species of the genus *Prevotella*. *Folia Microbiol (Praha)* **46**, 40–44.
- Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S. & other authors (2004). The Pfam protein families database. *Nucleic Acids Res* **32** (Database issue), D138–D141.
- Bayley, D. P., Rocha, E. R. & Smith, C. J. (2000). Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol Lett* **193**, 149–154.
- Bendjennat, M., Blanchard, A., Loufi, M., Montagnier, L. & Bahraoui, E. (1999). Role of *Mycoplasma penetrans* endonuclease P40 as a potential pathogenic determinant. *Infect Immun* **67**, 4456–4462.
- Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**, 783–795.
- Benedik, M. J. & Strych, U. (1998). *Serratia marcescens* and its extracellular nuclease. *FEMS Microbiol Lett* **165**, 1–13.
- Blank, A., Sugiyama, R. H. & Dekker, C. A. (1982). Activity staining of nucleolytic enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergents from gels. *Anal Biochem* **120**, 267–275.
- Brook, I. (2004). Microbiology and management of peritonsillar, retropharyngeal, and parapharyngeal abscesses. *J Oral Maxillofac Surg* **62**, 1545–1550.
- Bryant, M. P., Small, N., Bouma, C. & Chu, H. (1958). *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica*, the new genus and species. *J Bacteriol* **76**, 15–23.
- Daniel, A. S., Martin, J., Vanat, I., Whitehead, T. R. & Flint, H. J. (1995). Expression of a cloned cellulase xylanase gene from *Prevotella ruminicola* in *Bacteroides vulgatus*, *Bacteroides uniformis* and *Prevotella ruminicola*. *J Appl Bacteriol* **79**, 417–424.
- Dubnau, D. (1999). DNA uptake in bacteria. *Annu Rev Microbiol* **53**, 217–244.
- Duncan, W. J., Persson, G. R., Sims, T. J., Braham, P., Pack, A. R. C. & Page, R. C. (2003). Ovine periodontitis as a potential model for periodontal studies. *J Clin Periodontol* **30**, 63–72.
- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638.
- Edwards, J. E., McEwan, N. R., Travis, A. J. & Wallace, R. J. (2004). 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek* **86**, 263–281.
- Enwonwu, C. O., Falker, W. A., Jr & Phillips, R. S. (2006). Noma (cancer oris). *Lancet* **368**, 147–156.
- Focareta, T. & Manning, P. A. (1991). Distinguishing between the extracellular DNases of *Vibrio cholerae* and development of a transformation system. *Mol Microbiol* **5**, 2547–2555.
- Gardner, R. G., Russell, J. B., Wilson, D. B., Wang, G. R. & Shoemaker, N. B. (1996). Use of a modified *Bacteroides-Prevotella* shuttle vector to transfer a reconstructed β -1,4-D-endoglucanase gene into *Bacteroides uniformis* and *Prevotella ruminicola* B₁₄. *Appl Environ Microbiol* **62**, 196–202.
- Hobson, P. N. (1969). Rumen bacteria. *Methods Microbiol* **3B**, 133–149.
- Jarvill-Taylor, K. J., VanDyk, C. & Minion, F. C. (1999). Cloning of *mnuA*, a membrane nuclease gene of *Mycoplasma pulmonis*, and analysis of its expression in *Escherichia coli*. *J Bacteriol* **181**, 1853–1860.
- Kolenbrander, P. E., Anderson, R. N., Bleher, D. S., Egland, P. G., Foster, J. S. & Palmer, R. J., Jr (2002). Communication among oral bacteria. *Microbiol Mol Biol Rev* **66**, 486–505.
- Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrone, R. H., Boye, M. & Møller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* **68**, 673–690.
- Martin, F. E., Nadkarni, M. A., Jacques, N. A. & Hunter, N. (2002). Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. *J Clin Microbiol* **40**, 1698–1704.
- Mercer, D. K., Patel, S. & Flint, H. J. (2001). Sequence analysis of the plasmid pRRI2 from the rumen bacterium *Prevotella ruminicola* 223/ M2/7 and the use of pRRI in *Prevotella/Bacteroides* shuttle vectors. *Plasmid* **45**, 227–232.
- Miyazaki, K., Miyamoto, H., Mercer, D. K., Hirase, T., Martin, J. C., Kojima, Y. & Flint, H. J. (2003). Involvement of the multidomain regulatory protein XynR in positive control of xylanase gene expression in the ruminal anaerobe *Prevotella bryantii* B₁₄. *J Bacteriol* **185**, 2219–2226.
- Paster, B. J., Boches, S. H., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. & Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *J Bacteriol* **183**, 3770–3783.
- Peterka, M. (2002). Characterization of *rrn* loci from bacterial species *Prevotella ruminicola* and *Prevotella bryantii*. PhD thesis, University of Ljubljana, Ljubljana, Slovenia.
- Provedi, R., Chen, I. & Dubnau, D. (2001). NucA is required for DNA cleavage during transformation of *Bacillus subtilis*. *Mol Microbiol* **40**, 634–644.
- Rangarajan, E. S. & Shankar, V. (2001). Sugar non-specific endonucleases. *FEMS Microbiol Rev* **25**, 583–613.
- Rosenthal, A. L. & Lacks, S. A. (1977). Nuclease detection in SDS-polyacrylamide gel electrophoresis. *Anal Biochem* **80**, 76–90.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shoemaker, N. B., Anderson, K. L., Smithson, S. L., Wang, G. R. & Salyers, A. A. (1991). Conjugal transfer of a shuttle vector from the human colonic anaerobe *Bacteroides uniformis* to the ruminal anaerobe *Prevotella (Bacteroides) ruminicola* B₁₄. *Appl Environ Microbiol* **57**, 2114–2120.
- Takahashi, I., Hayano, D., Asayama, M., Masahiro, F., Watahiki, M. & Shirai, M. (1996). Restriction barrier composed of an extracellular nuclease and restriction endonuclease in the unicellular cyanobacterium *Microcystis* sp. *FEMS Microbiol Lett* **145**, 107–111.

Van Gylswyk, N. O. (1990). Enumeration and presumptive identification of some functional groups of bacteria in the rumen of dairy cows fed grass silage-based diets. *FEMS Microbiol Lett* **73**, 243–261.

Vingadassalom, D., Kolb, A., Mayer, C., Rybkine, T., Collatz, E. & Podglajen, I. (2005). An unusual primary sigma factor in the *Bacteroidetes* phylum. *Mol Microbiol* **56**, 888–902.

Walker, N. D., McEwan, N. R. & Wallace, R. J. (2003). Cloning and functional expression of dipeptidyl peptidase IV from the ruminal bacterium *Prevotella albensis* M384. *Microbiology* **149**, 2227–2234.

Wang, Y., Shoemaker, N. B. & Salyers, A. A. (2004). Regulation of a *Bacteroides* operon that controls excision and transfer of the conjugative transposon CTnDOT. *J Bacteriol* **186**, 2548–2557.

Wu, S. I., Lo, S. K., Shao, C. P., Tsai, H. W. & Hor, L. I. (2001). Cloning and characterization of a periplasmic nuclease of *Vibrio vulnificus* and its role in preventing uptake of foreign DNA. *Appl Environ Microbiol* **67**, 82–89.

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