Identification and use of the putative *Bacteroides ovatus* xylanase promoter for the inducible production of recombinant human proteins

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The use of genetically modified bacteria to deliver biologically active molecules directly to the gut has become an increasingly attractive area of investigation. The challenge of regulation of production of the therapeutic molecule and colonization of the bowel led us to investigate *Bacteroides ovatus* for the production of these molecules, due to its ability to colonize the colon and xylan utilization properties. Here we have identified the putative xylanase promoter. The 5' region of the corresponding mRNA was determined by 5'RACE analysis and the transcription initiation site was identified 216 bp upstream of the ATG start codon. The putative xylanase promoter was regulated by xylan in a dose- and time-dependent manner, and repressed by glucose. This promoter was subsequently used to direct the controlled expression of a gene encoding the human intestinal trefoil factor (TFF-3) after integration as a single copy into the chromosome of *B. ovatus*. The resulting strain produced biologically active TFF-3 in the presence of xylan. These findings identify the *B. ovatus* xylanase operon promoter and show that it can be utilized to direct xylan-inducible expression of heterologous eukaryotic genes in *B. ovatus*.

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INTRODUCTION

The gut is an immunologically important organ, as it is often the first point of contact for potential pathogens and other foreign antigens. It also contains a large and diverse population of commensal bacteria that in the human colon may number more than 10^{14} bacteria comprising over 1000 species (Egert *et al.*, 2006; Whitman *et al.*, 1998). The gut microbiota has been shown to influence the development and function of the gut mucosal immune system, which becomes tolerant to or fails to mount a response to the gut bacteria (Savage, 1999). A breakdown in immune tolerance to members of the microbiota has been shown to be a contributing factor in the pathogenesis of inflammatory bowel disease (IBD) (Baumgart & Carding, 2007). Altering the microbiota through the use of prebiotics and probiotics to improve or restore gastrointestinal health has become an intense area of research. In particular, the use of genetically modified bacteria to deliver biologically active, immuno-

modulatory molecules directly to the gut has become an increasingly attractive area of investigation in the search for new therapeutic options for chronic intestinal inflammation and IBD. Proof-of-principle studies using *Lactococcus lactis* have shown that this organism can produce and secrete biologically active human cytokines that can be used to successfully treat intestinal inflammation in animal models (Steidler *et al.*, 1995, 1998, 2000). Although effective, these genetically modified probiotics have certain drawbacks such as an inability to regulate the production of the therapeutic molecule, requiring repeated oral administration of the organism to colonize the gut.

To attempt to overcome the drawbacks of the *L. lactis* system, we have utilized *Bacteroides ovatus*, a commensal of the human colon that can utilize xylan as its sole source of carbohydrates, as a potentially improved genetically modified probiotic. Xylans are a major component of plant cell wall polysaccharides that cannot be degraded by digestive enzymes produced by humans and other animals. Commensal colonic bacteria such as *B. ovatus* have a major role in their degradation through the production of xylanolytic enzymes (Salysers *et al.*, 1981; Whitehead & Hespell, 1990). To date, however, only two of the genes

Abbreviations: IL-2, interleukin-2; 5'RACE, rapid amplification of 5' cDNA ends; TFF-3, trefoil factor 3; TIS, transcription initiation site.

The GenBank accession number for the *B. ovatus* promoter and operon is EU334491.

involved in xylan degradation by *B. ovatus* have been cloned and little is known about their regulation. Whitehead & Hespell (1990) identified a region of the *B. ovatus* chromosome encoding a xylanase (*xylI*) and a bifunctional xylosidase–arabinosidase (*xsA*). It was subsequently shown that these two genes are part of an operon that may include other genes and that *xsA* is the last gene in this operon (Weaver *et al.*, 1992; Whitehead, 1995). This operon is under the control of a xylan-inducible promoter, the disruption of which adversely affects the growth of *B. ovatus* on xylan (Weaver *et al.*, 1992). Our previous work utilized this operon to enable the mouse cytokine interleukin-2 (IL-2) to be produced in response to xylan in the culture medium (Farrar *et al.*, 2005). However, insertion of the IL-2 gene into the known part of the xylanase operon disrupted transcription of the xylanase genes. Lack of xylanase production by the recombinant organisms may result in a competitive disadvantage when introduced into the gut.

To generate strains of *B. ovatus* capable of producing therapeutic proteins in a xylan-inducible manner without disruption of the xylanase operon, the promoter of this operon must be used. Therefore, the initial objective of the current study was to clone and sequence all genes within the xylanase operon and the promoter region. We also present data to show that the putative xylanase operon promoter we have identified is regulated by xylan and can be used to drive expression of a heterologous human gene, leading to production of a recombinant, biologically active protein that is important in maintaining gut barrier function.

METHODS

Bacterial strains, culture media and general DNA manipulations. *Escherichia coli* DH5 α was grown in LB medium supplemented with ampicillin (100 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹) when required. Cultures of *E. coli* J53/R751 were supplemented with trimethoprim (200 μ g ml⁻¹). *Bacteroides ovatus* V975 was grown anaerobically at 37 °C in brain heart infusion (BHI; Oxoid) medium supplemented with 0.001 % (w/v) haemin. For cultures with defined carbon sources, *B. ovatus* was grown in routine growth medium (RGM; Hespell *et al.*, 1987) supplemented with 0.001 % (w/v) haemin. Glucose and xylan were added as required. When xylan was required, a hot-water-soluble fraction of oat spelt xylan (Sigma) was prepared as described by Hespell & O'Bryan (1992). *B. ovatus* transconjugants were selected on BHI-haemin agar containing gentamicin (200 μ g ml⁻¹) and tetracycline (5 μ g ml⁻¹). Suicide plasmids pBT2 (Tancula *et al.*, 1992) and pCQW1 (Feldhaus *et al.*, 1991) were used to transfer DNA into *B. ovatus* from *E. coli* J53/R751 and *E. coli* HB101, respectively, by conjugation as described by Valentine *et al.* (1992). pCR-Blunt (Invitrogen) was used for cloning blunt-ended PCR products and pUC18 for cloning other DNA fragments. *E. coli* was transformed by the method of Hanahan (1983). *B. ovatus* genomic DNA was isolated by the NaCl/CTAB method described by Wilson (1997). General DNA manipulations were carried out as described by Sambrook *et al.* (1990). Growth of bacteria on different media was monitored by measuring OD₆₀₀.

Cloning of the xylanase operon promoter by inverse PCR. A fragment of *B. ovatus* genomic DNA containing the xylanase operon

promoter was identified and cloned as follows. First, *B. ovatus* genomic DNA was digested with various restriction endonucleases, separated by agarose gel electrophoresis and transferred to a nylon membrane by the method of Southern (1975). To generate a probe, a 600 bp *EcoRI*–*KpnI* fragment of the incomplete xylanase operon was isolated from plasmid pOX1 (Whitehead & Hespell, 1990) and labelled with digoxigenin (DIG)-11-UTP using the DIG High Prime Labelling and Detection Starter kit (Roche). The probe was then hybridized with the digested *B. ovatus* genomic DNA and visualized according to the manufacturer's instructions. The xylanase operon promoter was cloned by digesting *B. ovatus* genomic DNA to completion with *HindIII*, which cuts within a 3.8 kb region of the *B. ovatus* xylanase operon previously sequenced (Whitehead & Hespell, 1990). Digested DNA was diluted 200-fold then self-ligated. Ligation products were purified by phenol/chloroform extraction and used as template DNA in a PCR with primers BOPRO1 (GAATAGCAAAA-CCAGTCAGCGG) and BOPRO2 (AGTTCGCGTAAGGATGT-CGCA) using the Expand Long Template PCR System (Roche) according to the manufacturer's instructions. The PCR product was gel-purified, treated with T4 DNA polymerase and T4 polynucleotide kinase in a one-step blunting and 5' phosphorylation procedure, then cloned into pCR-Blunt for sequencing. The GenBank accession number for the sequence is EU334491.

Rapid amplification of 5' cDNA ends (5'RACE). Total RNA was extracted from *B. ovatus* cells grown in RGM with 0.1 % (w/v) glucose and 0.2 % (w/v) xylan, using the SV Total RNA Isolation System (Promega). cDNA synthesis was performed using 1–2 μ g of total RNA template, 12.5 pmol of primer SP1 (CAATCCATATTCG-ACTGTCCC), 2 mM of each dNTP and 20 U M-MuLV reverse transcriptase (New England Biolabs) according to the manufacturer's instructions. The reaction was carried out for 1 h at 42 °C, followed by heat inactivation. cDNA was purified using the QiaQuick PCR Clean-up kit (Qiagen) then 3' polyadenylated using 80 U terminal transferase (Roche) according to the manufacturer's instructions. Polyadenylated cDNA was used as a template in a PCR with the anchor d(T) (CCACGCGTGAATTCGTGCGACT₁₆V) and SP1 primers, and the resulting product subjected to a second and third round of PCR with an anchor forward primer (CCACGCGTGAATTCGT-CGAC) and nested reverse primers SP2 (GAACATACCCAAACATC-GCCATAAG) and SP3 (CTTTAATTCGATATCATTAATGGCCA-TC), respectively. The PCR product was cloned and sequenced to identify the transcription initiation site (TIS).

Construction of *B. ovatus* XylE reporter strains. The promoter-less *xylE* gene (encoding catechol 2,3-dioxygenase) of the *Pseudomonas putida* TOL plasmid was isolated from pLEC23 (Coyne *et al.*, 2003; Zukowski *et al.*, 1983) by digestion with *Bam*HI and *Pst*I, and cloned into *Bam*HI/*Pst*I-digested pUC18. The putative *B. ovatus* xylanase operon promoter was PCR-amplified using primers XYP-f (GCGGATCCTGGGGAGTATCGGACAATG) and XYP-r (GAGGATCCTCTGCTTTCTTTATATGCTTTA-TTTC) (*Bam*HI sites in bold), digested with *Bam*HI and cloned into the *Bam*HI site upstream of *xylE* in pUC18 in both the forward (ON) and reverse (OFF) orientations. Orientations were confirmed by sequencing. The promoter-*xylE* fragment was isolated by digestion with *Hind*III and *Kpn*I and cloned into pBT2. The construct was then transferred by conjugation to *B. ovatus* and integration into the genome confirmed by PCR. Screening for XylE enzyme activity in individual colonies was performed by spraying plates with 50 mM catechol, with a yellow product indicating XylE activity. XylE mRNA expression was detected by RT-PCR using primers XYLE-f (ATTCACCATCCGGAAGG) and XYLE-r (GAGAATGCGGTC-GTGGTAA).

Generation of the *B. ovatus* xylanase operon disruption mutant Ω G1. A 509 bp region from the first gene in the xylanase operon was

PCR amplified using primers GENE1F1 (TTACTTCCGGCTGGG-CAACAAA) and GENE1R1 (GACATTCGATTCTCCCTGATACCA), and the PCR product was recovered using the TA cloning kit and cloned into the vector pCR2.1 (Invitrogen) according to the manufacturer's instructions. The resultant plasmid was then digested with *Bam*HI and *Sst*I, and the genomic insert recovered and ligated into pCQW1 (Feldhaus *et al.*, 1991) digested with the same enzymes. The suicide vector was transferred to *B. ovatus* by conjugation from *E. coli* HB101 and transconjugants were selected as previously described (Weaver *et al.*, 1992).

Construction of trefoil-factor-secreting *B. ovatus* (BO-TFF3).

The coding region of human trefoil factor 3 (TFF-3) was PCR-amplified from a cDNA clone (IMAGE clone ID 4696566, MRC Geneservices) using *Pfu* DNA polymerase (Promega) and primers TFF-f (GACATATGAAGAATGTAAAGTTACTTTTAATGCTAGGA-ACCGCGGCATTATTAGCTGCAGAGGAGTACGTGGGCCTC; *Nde*I site in bold) that contained the *Bacteroides fragilis* enterotoxin secretion signal coding sequence (underlined) and TFF-r (GCAAGCTTTCAGAAGGTGCATTCTGCTTC; *Hind*III site in bold). The *B. ovatus* xylanase operon promoter was PCR-amplified using primers XYLP-f and XYLP-rn (GTCATATGGTCTTTCTTTTATATGTCTTTATTT-CATG; *Nde*I site in bold). The promoter and TFF-3 PCR products were cloned into pCR-Blunt to create plasmids pCR-Pro and pCR-TFF-3 respectively. The TFF-3 coding region was removed from pCR-TFF-3 by digestion with *Nde*I and *Hind*III then ligated into *Nde*I/*Hind*III-digested pCR-XYLP. The putative xylanase promoter-TFF-3 fragment was then isolated by double digestion with *Hind*III and *Xba*I and cloned into a suitable site in pBT2 to produce pBT-TFF. pBT-TFF was transferred to *B. ovatus* by conjugation from *E. coli* J53/R751 and integration of the plasmid into the genome confirmed by PCR.

Reverse transcription (RT)-PCR to confirm transcription of intergenic regions.

Total RNA was isolated using the SV Total RNA isolation System (Promega) according to the manufacturer's protocol. Purified RNA was treated with TURBO DNase (Ambion). cDNA synthesis was performed using 1–2 µg of total RNA, 4 µM random nonamers, 0.5 mM of each dNTP and 200 U M-MuLV reverse transcriptase (New England Biolabs) according to the manufacturer's instructions. The reaction was carried out for 1 h at 42 °C followed by heat inactivation. Two microlitres of the cDNA was used in the subsequent PCR along with the following primers: JUNC1-F, CGAGTGGCAGCAGCTATCTT; JUNC1-R, ACCAGAGGATCCCC-AAAGGAC; JUNC2-F, CTTTGGCCGAACAGGGAATA; JUNC2-R, CAAAGCCTTTTTCAGCGAAG; JUNC3-F, ATCGCAATTATCAG-CCGAAG; JUNC3-R, ACCGTTTCATCATCATCA; XYLP, GATT-AAAGAAGGGGAGAGTG, TIS, CCCTTTCCTCTTGTTTATCGGTG.

Real-time RT-PCR analysis of xylanase operon transcription.

B. ovatus was grown in RGM supplemented with various concentrations of glucose and/or xylan. Total RNA was isolated and prepared for cDNA synthesis as described above. RNA was reverse transcribed using random hexamers and M-MuLV reverse transcriptase (New England Biolabs) according to the manufacturer's instructions. Real-time PCR was carried out using iQ SYBR Green supermix (Bio-Rad) and the iCycler thermal cycler (Bio-Rad); 400 nM primers were used in 25 µl reaction volumes. Primers were QPCR2-F (AATTCC-AGTGTCGGAGGTTC) and QPCR2-R (CAGCGACTCCATCAAA-TCGT) for the ORF1 gene of the xylanase operon, and GYRA.F (TTCCGGATGTTAGAGATGGA) and GYRA.R (CCAAGTACCTCA-CCCAGAT) for the housekeeping gene *gyrA*. Quantification of mRNA was performed using the standard curve method, with the quantity of ORF1 normalized to the amount of *gyrA* product for each condition to determine gene expression relative to *gyrA*.

TFF-3 quantification. A direct ELISA was used to quantify levels of TFF-3 produced by recombinant strains of *B. ovatus*. Briefly, 96-well

ELISA plates (Nunc Maxisorp) were coated overnight at 4 °C with 100 µl of twofold serial dilutions of bacterial culture supernatants. Plates were washed three times with PBS/0.05 % (v/v) Tween 20 then blocked with 300 µl 10 % (w/v) BSA in PBS at 20 °C for 1 h. After three washes with PBS/Tween, 100 µl TFF-3 monoclonal antibody (1:250; clone 3G11, Abnova) was added to each well and the plate incubated at room temperature for 2 h. The plate was washed five times with PBS/Tween, then 100 µl of a 1:1800 dilution of biotinylated goat anti-mouse IgG (Vector Lab) and 1:250 streptavidin-HRP added and incubated at 20 °C for 1 h. The plate was washed seven times, then peroxidase substrate [1:1, v/v, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate)] added and incubated for 30 min at 20 °C. A standard curve was obtained from serial dilutions of recombinant TFF-3 (Abnova). Samples were assayed in duplicate.

TFF bioassay. TFF-3 bioactivity was assessed using the colorectal cancer epithelial cell line HT-29 in an epithelial cell migration assay as previously described (Jeffers *et al.*, 2002). Briefly, confluent monolayers of HT-29 cells were grown in 12-well plates in RPMI containing 10 % (v/v) fetal calf serum at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂ in air. Cells were washed with PBS and cultured for a further 24 h in serum-free medium. Wounds were made by scraping the cell monolayer with a disposable pipette tip. Monolayers were then washed with PBS and incubated in serum-free medium in the presence of recombinant TFF-3 (Abnova) or culture supernatants of *B. ovatus* V975 and BO-TFF3 grown with xylan. The rate of wound closure was calculated by measuring the size of the wound opening at 0 and 8 h.

Statistical analysis. SPSS statistical software (v.14) was used to analyse data. Groups were compared using one-way ANOVA. Where $P \leq 0.05$, the difference was considered significant.

RESULTS

Cloning of the putative *B. ovatus* xylanase operon

Digestion of *B. ovatus* genomic DNA with various restriction endonucleases followed by Southern transfer and hybridization to a *xyII* probe identified a 6.5 kb *Hind*III fragment containing the *xyII* gene (data not shown). The *Hind*III-digested genomic DNA was then circularized, PCR-amplified and cloned into pCR-Blunt to generate plasmid pPRO. The PCR-amplified insert was fully sequenced using a primer-walking strategy. The *xyII* and *xsA* genes previously described by Whitehead (1995) were identified, as was the ORF2 upstream of *xyII* that had previously been partially sequenced. This ORF2 comprised 1413 bp, encoding a protein of 471 aa with a predicted molecular mass of 52.4 kDa. This protein was identified as a putative sodium/sugar symporter with 64 % identity to a putative sodium symporter gene of *Prevotella bryantii* (Fig. 1a). A second ORF (ORF1) of 1656 bp was identified further upstream, encoding a protein of 552 aa with a predicted molecular mass of 62.1 kDa and a predicted 58 % homology with sialic-acid-specific 9-*O*-acetyl esterase of *Bacteroides vulgatus*, and 17.3 % homology with the putative arylesterase/lipase of *Prevotella bryantii* (Fig. 1a). Functional assays are required to confirm the identity of these xylanase operon genes. As no further ORFs were identified, the xylanase operon of *B. ovatus* was determined to comprise four genes (Fig. 1a). To confirm that all four genes were part of the same operon, the

Fig. 1. *B. ovatus* xylan utilization operon. (a) Schematic representation of the xylan utilization genes in *B. ovatus* V975 and the related genes in *P. bryantii* B₁₄, with estimated sizes indicated in bp. The percentage values shown represent the degree of amino acid homology between the four related genes in *B. ovatus* and *P. bryantii*. *xyII*, xylanase I; *xsA*, xylosidase/arabinosidase; *xynE*, putative arylesterase/lipase; *xynD*, putative sodium-sugar symporter; *xynA*, endoxylanase; *xynB*, β -xylosidase/exoxylanase. The promoter is indicated by a black arrow. (b) Growth of the parental *B. ovatus* strain (V975) and a mutant strain in which the first gene in the xylanase operon was mutated (Ω G1) was assessed using RGM supplemented with 0.001 % (w/v) haemin and either 0.05 % (w/v) xylan (+X) or 0.1 % (w/v) glucose (+G) as a carbon source. Data shown are from one of two separate experiments (* P < 0.05). (c) RT-PCR was used to confirm that in *B. ovatus* V975 the two genes identified upstream of *xyII* are transcribed from the same promoter. Lanes: M, DNA marker; 1, amplification of sequences between the junction of ORF1 and ORF2 (primers, JUNC1-F and JUNC1-R); 2, amplification of sequences between the junction of ORF2 and *xyII* (primers, JUNC2-F and JUNC2-R); 3, amplification of sequences between the junction of *xyII* and *xsA* (primers, JUNC3-F and JUNC3-R); 4, *gyrA* amplification product (positive control); 5, no cDNA control.

first gene of the operon (putative acetyltransferase) was inactivated by insertional disruption. The resulting mutant (Ω G1) demonstrated reduced growth on xylan, similar to that seen with disruption of the *xyII* gene (Weaver *et al.*, 1992). Growth in medium containing glucose was unaffected (Fig. 1b). Furthermore, RT-PCR analysis of Ω G1 showed that the *xsA* gene of the xylanase operon was not expressed when the mutant was grown on xylan (data not shown), and RT-PCR amplification of the junctional region sequences between the ORF1 and ORF2, ORF2 and *xyII*, and *xyII* and *xsA* genes of the wild-type organism generated amplification products of the expected sizes (Fig. 1c). Together, these results confirm that four genes constitute the xylanase operon, which appears to be under the control of a single promoter. *xsA* was considered the last gene in this operon, as shown previously (Weaver *et al.*, 1992).

Identification of the putative xylanase operon promoter

A region of non-coding DNA upstream of the first gene (ORF1) in the xylanase operon was predicted to contain the xylanase operon promoter. A possible ribosome-binding site (ATATAA) was located 12 bp upstream of the first ATG initiation codon of the operon (Fig. 2a), which was homologous to the first 5 bp of the putative consensus sequence of *P. bryantii* (Miyazaki *et al.*, 2003). A similar AT-rich sequence was also observed 4–12 bp upstream of the start codon of all four xylan utilization genes (Fig. 2b). Cloning of the putative promoter region upstream of the promoterless *xyle* gene in the forward orientation resulted in catechol 2,3-dioxygenase activity in *E. coli* whereas no enzyme activity was detected when the

Fig. 2. Identification of the TIS of the *B. ovatus* xylanase operon promoter. (a) The TIS was identified by 5'RACE analysis using total bacterial cellular RNA. RACE products were amplified in three nested RT-PCR products and visualized by agarose gel electrophoresis (left panel). Lanes: M, 1 kb DNA ladder. 1, 5' end DNA amplification product; 2, PCR product of DNA in lane 1 using nested internal primer SP2 and non-specific primer (anchor); 3, repeat PCR on DNA in lane 2 using nested internal primer SP3 and non-specific primer (anchor). The amplicon in lane 3 was excised, cloned and sequenced to identify the related TIS (right panel). The stop codon of the upstream gene and start codon of the first gene in the xylanase operon is in bold in the first line of the sequence, and the TIS and putative ribosome-binding site (RBS) are in bold and underlined. (b) Putative RBS (bold underlined) upstream of the *B. ovatus* xylan utilization genes. This sequence is similar to the first 5 bp of the *P. bryantii* putative RBS consensus sequence (Miyazaki *et al.*, 2003). (c) RT-PCR of *B. ovatus* V975 RNA confirming the TIS. RT-PCR was used to attempt to identify an additional TIS upstream of the TIS identified in (a). Primer design is depicted in the schematic; the photograph shows PCR products obtained using different primer combinations. Lanes: M, DNA ladder; 1, forward primer upstream of putative TIS (XYLP) with QPCR2-R reverse primer; 2, forward primer complementary to putative TIS (TIS) with QPCR2-R reverse primer; 3, ORF1-specific forward and backward primers (QPCR2-F and QPCR2-R); 4, *gyrA* RT-PCR product (positive control); 5, no cDNA control.

promoter was in the reverse orientation. The *xylE* reporter construct was introduced into *B. ovatus* and integrated into the genome by homologous recombination in the promoter region. It was not possible, however, to detect XylE enzyme activity among any of the recombinants using optimal culture conditions. Therefore, *xylE* reporter gene expression was analysed by RT-PCR. As seen in Fig. 3, *xylE* transcripts were only detected in *B. ovatus* containing the promoter-*xylE* reporter in the forward orientation, confirming that this region of DNA was able to drive transcription in *B. ovatus*.

Functional analysis of the xylan utilization promoter

Real-time RT-PCR was used to quantify expression of the xylanase operon in wild-type *B. ovatus* V975 and to determine the growth conditions under which the xylanase promoter is active. The amount of transcript increased with increasing concentrations of xylan up to 0.05 % (w/v) (Fig. 4a). At higher xylan concentrations promoter activity was reduced. Maximum levels of xylanase operon expression occurred within 2 h of exposure to xylan and expression persisted for up to 24 h (Fig. 4b). Glucose suppressed xylan-induced promoter activity at concentrations as low as 0.025 % (w/v) (Fig. 4c).

Identification of the transcription initiation site (TIS)

The xylanase operon TIS was localized by 5'RACE. A single 5'RACE reaction product was visualized on ethidium-bromide-stained agarose gel after two rounds of nested

Fig. 3. The xylanase promoter drives *xylE* expression in *B. ovatus*. The putative *B. ovatus* xylanase operon promoter was integrated into the *B. ovatus* chromosome in either the forward or reverse orientation and RT-PCR was used to detect transcriptional activation after exposure to xylan (0.2 %, w/v) for 24 h. Lanes: C, no cDNA (no RT step) control; 1, BO-XylE (reverse); 2 and 4, *gyrA* expression; 3, BO-XylE (forward); M, DNA ladder.

Fig. 4. Functional characterization of the xylanase operon. The effect of xylan concentration (a), xylan exposure time (b) and presence of glucose (c) on activation of the xylan operon in wild-type *B. ovatus* V975 was determined by quantitative RT-PCR using primers specific for the ORF1 region of the xylan operon and *gyrA* as a reference gene. In (a) bacteria were exposed to increasing concentrations of xylan for 24 h prior to analysis. In (b) bacteria were exposed to 0.05 % (w/v) xylan for the indicated times. The effect of repeated exposure to xylan on expression of the xylan operon was assessed by adding xylan (0.05 %, w/v) to the growth media at 4, 8 and again at 24 h (Cont. X). In (c) bacteria were incubated with 0.05 % (w/v) xylan in the presence of increasing concentrations of glucose for 24 h prior to analysis. For all experiments standard curve method was used to determine the level of ORF1 gene expression relative to *gyrA* as a reference gene. The results shown represent the means ($\pm$ SEM) of triplicate determinations from one of three experiments.

PCRs. The sequence of the RACE product was determined and a TIS identified at a cytosine residue 216 bp upstream of the ATG start codon (Fig. 2a). Additional RT-PCR assays were performed to confirm the identity of the putative TIS and to determine if additional TIS existed upstream. No PCR products were obtained using a primer XYLP (Fig. 2c) upstream of the TIS, which contrasted with the large amount of product detected using the same reverse primer in combination with a forward primer

complementary to the putative TIS (Fig. 2c). The DNA sequence adjacent to the TIS revealed motifs of AT-rich character, including TTCTAA centred on -9 and TGTAAT centred on -23. No inverted repeat sequences indicative of binding sites for one or more regulatory proteins were found in the promoter sequence. Close inspection of the putative xylanase promoter sequence in Fig. 2(a) revealed the presence of a *Bacteroides* consensus promoter (Bayley *et al.*, 2000) at -74 relative to the ATG translational start codon. It is possible that this represents a second TIS; however, as only one TIS was identified by 5'RACE this promoter may not be active under the conditions used in our experiments. Therefore, the TIS identified in this study is likely to be the primary TIS.

Utilization of the xylanase promoter for the controlled production of functional human trefoil factor (TFF-3) by *B. ovatus*

A strain of *B. ovatus* capable of producing and secreting human TFF-3 in a xylan-inducible manner was constructed. TFF-3, or intestinal trefoil factor (ITF), was chosen on the basis of its role in protecting and repairing the intestinal mucosal barrier (Dignass *et al.*, 1994; Mashimo *et al.*, 1996) and its potential use as a therapeutic agent in chronic inflammatory disorders of the intestine. The region of the TFF-3 gene encoding the 59 aa mature protein was cloned under the control of the xylanase promoter in pCR-Blunt to generate the plasmid pCR-TFF. The *B. fragilis* endotoxin secretion signal sequence was introduced 5' of the TFF-3 coding sequence to mediate secretion of the recombinant protein. The use of an *NdeI* site for cloning resulted in 3 bp change (AGA to CAT) in the non-coding region, which was outside the predicted RBS of the promoter and was not expected to affect translation. The promoter-TFF fragment was cloned into pBT2 to create pBT-PTFF (Fig. 5a), which was then successfully transferred to *B. ovatus* V975 to create BO-TFF3.

To assess the production of human TFF-3, BO-TFF3 recombinant strain and wild-type *B. ovatus* V975 were grown in medium with or without xylan. Culture supernatants were assayed for human TFF-3 by ELISA and in an intestinal epithelial cell wounding assay. TFF-3 was detected in the culture supernatant of BO-TFF3 grown with xylan (1.24 ng ml^{-1}) at concentrations significantly higher ($P < 0.01$) than the TFF-3 concentration produced by BOTFF3 cultured in the absence of xylan (0.19 ng ml^{-1}) (Fig. 5b).

The functional activity of TFF-3 produced by recombinant strains was tested by its effect on HT-29 intestinal epithelial cell migration (Fig. 5c). Culture supernatant from BO-TFF3 cultured with xylan significantly enhanced HT-29 cell restitution and wound closure ($P = 0.01$) compared to cultures in serum-deprived medium alone or after supplementation with culture supernatant from *B. ovatus* V975 ($P = 0.035$ and 0.017 respectively). Wound closure rates in the presence of *B. ovatus* V975 conditioned

medium or with serum-deprived medium alone were comparable. The closure rate seen with 10-fold diluted BO-TFF3 conditioned medium was comparable to that seen with 15 ng ml^{-1} recombinant TFF-3. These results demonstrate that BO-TFF3 produces biologically active TFF-3 in a xylan-inducible manner, and that modification of the 5' end of the gene encoding the recombinant protein had no adverse affect on the biological activity of TFF-3.

DISCUSSION

This report describes the identification and partial functional characterization of the putative xylanase operon promoter from *B. ovatus*; we believe that this is the first attempt to characterize a promoter in this organism. We have demonstrated that this promoter is functional in *E. coli*, and that it can be used to direct xylan-inducible expression of a human gene in *B. ovatus*.

Two new genes were sequenced that completed the xylanase operon. These encode a putative sodium/sugar symporter and sialic-acid-specific 9-*O*-acetyl esterase, both of which would be expected to complement the activity of the *xylI* and *xsa* gene products in the degradation of xylan. Grass xylans frequently have acetyl groups attached by ester linkages (Hespell & Whitehead, 1990) that would be removed by the esterase, and the symporter would mediate the transport of liberated xylose and arabinose residues into the cell.

The TIS of the xylanase promoter was located well upstream of the translation initiation codon at -216. Such a large distance is not unusual and has been described for bacterial genes in other species, for example *Porphyromonas gingivalis* (Jackson *et al.*, 2000) and *Prevotella loescheii* (Manch-Citron *et al.*, 1999), which is closely related to *Bacteroides* spp. This is important in considering this promoter for the controlled expression of heterologous or homologous genes. The sequences at positions -10 or -35 upstream of the start site did not resemble any known RNA polymerase recognition sequences in *E. coli*, but, given the large difference in consensus sequences that have been identified between *E. coli* and other species, *B. ovatus* may have unique RNA polymerase recognition sequences. This possibility is supported by the unconventional promoter motifs of *B. fragilis* that have been previously identified (Bayley *et al.*, 2000). However, no similarity with *B. fragilis* consensus sequences could be identified in the *B. ovatus* xylanase promoter. This may not, however, be entirely unexpected since the absence of these consensus sequences in the *recA* and *sod* genes of *B. fragilis* (Bayley *et al.*, 2000) suggests that these consensus sequences may not strictly apply to all *Bacteroides* promoters. To define the regulatory region(s) in the promoter a detailed deletion and point mutation analysis is required.

The reporter gene *xylE* was used successfully in *B. fragilis* in plasmid pLEC23 (Coyne *et al.*, 2003). We therefore tried to use *xylE* to characterize the xylanase promoter. However,

Fig. 5. Utilization of the xylanase promoter for the controlled production of human TFF-3. (a) The xylanase promoter with 5' sequence of the upstream gene and the mature human TFF-3 gene were PCR-amplified, ligated together in pCR-Blunt and subcloned into pBT2 to create pBT-TFF. Only the restriction sites used for cloning are shown. Tetracycline (*tet*) resistance was used for selection in *B. ovatus* and kanamycin (*kan*) resistance for selection in *E. coli*. *oriV*, origin of replication; *repA*, *repB*, *repC* encode replication functions, with *mob* required for mobilization from *E. coli* to *B. ovatus*. (b) Levels of TFF-3 in culture supernatant of *B. ovatus* BO-TFF3 and control strain V975 cultured in the absence (BO-TFF-X) or presence (BOTFF+X) of xylan (0.05%, w/v) for 24 h as determined by ELISA. The data shown are from one of two experiments. (c) An epithelial cell wound closure assay was used to quantify TFF-3 activity in culture supernatants of *B. ovatus* BO-TFF3 and control strain V975 grown in the presence of xylan (0.05%, w/v) for 24 h. As controls, cells were grown in medium alone (Ctrl) or in the presence of 15 ng ml⁻¹ recombinant human TFF-3 (rTFF). The data shown are from one of two experiments.

we were unable to detect Xyle enzyme activity after integration into chromosomal DNA. It is unlikely that Xyle is not functional in *B. ovatus* as it was shown to be functional in *B. fragilis*; however, in that study it was integrated into an expression plasmid vector (Coyne *et al.*, 2003). The lack of Xyle expression in *B. ovatus* recombinants may be a consequence of integration of a single copy into the chromosomal DNA as compared to multiple plasmid-encoded copies in *E. coli* transformants and *B.*

fragilis producing high levels of catechol dioxygenase activity. The xylanase operon promoter was upregulated by xylan and expression increased with increasing xylan concentration up to 0.05% (w/v). Expression was, however, reduced at higher xylan concentrations and by the addition of glucose. Glucose repression has been previously described for the *Streptomyces lividans* xylanase promoter (Chen & Westpheling, 1998). However, the direct repeat or inverted repeat sequences present in the *S.*

lividans promoter are not present in the *B. ovatus* xylanase promoter. Furthermore, the *B. ovatus* xylanase promoter reaches maximal expression 2–4 h after induction. These features may be utilized by *B. ovatus* to improve the efficiency of polysaccharide digestion and maximize the nutritional effect. It is possible that this promoter and the xylan operon could be used to improve xylan utilization by rumen bacteria, especially as xylosidase and not xylanase activity is the rate-limiting step in xylan breakdown by *B. ovatus* (Weaver *et al.*, 1992).

We have previously used the xylan-inducible nature of *B. ovatus* xylan utilization genes to direct the controlled production of murine IL-2 (Farrar *et al.*, 2005). However, integration of the IL-2 gene into the xylanase operon may adversely affect the ability of the engineered *B. ovatus* to colonize the colon (S. R. Carding, unpublished observations). In the work presented here, the integrity of the xylanase operon was maintained, increasing the prospect of using engineered *B. ovatus* strains in probiotic regimens. The feasibility of using the xylanase promoter to control the expression of a human gene is demonstrated here using human TFF-3 as a test protein. To control human TFF-3 expression the mature part of the protein was positioned downstream of the xylanase promoter and translationally fused to the *B. fragilis* enterotoxin secretion signal sequence. This signal sequence was shown previously to mediate secretion of recombinant protein (Farrar *et al.*, 2005). Although the correct folding of the human protein and preservation of the triple loop structural motifs required for biological activity (Thim *et al.*, 1993) was a major concern, high levels of biologically active protein were secreted by BO-TFF3.

In summary, we have developed a novel system for the controlled *in vivo* delivery of biologically active human proteins that may in the future be of use in the controlled delivery of a variety of human proteins directly to the mucosal surface for the treatment of chronic intestinal inflammatory disorders. This system has distinct advantages over other genetically modified bacteria that have been used in phase I clinical trials (Baat *et al.*, 2006), in which cytokine production cannot be regulated (Steidler *et al.*, 2000). For recombinant strains of *B. ovatus* to be effective in patients, the subjects may have to be placed on a xylan-reduced (low-fibre) diet to prevent inappropriate expression of xylan-regulated heterologous gene expression *in vivo*.

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