Isolation and characterization of putative *Pseudobutyrvibrio ruminis* promoters

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Novel plasmids were constructed for the analysis of DNA fragments from the rumen bacterium *Pseudobutyrvibrio ruminis*. Five previously unidentified promoters were characterized using a novel primer extension method to identify transcription start sites. The genes downstream of these promoters were not identified, and their activity in expression of genomic traits in wild-type *P. ruminis* remains putative. Comparison with promoters from this and closely related species revealed a consensus sequence resembling the binding motif for the RNA polymerase σ^{70} -like factor complex. Consensus –35 and –10 sequences within these elements were **TTGACA** and **ATAATATA** respectively, interspaced by 15–16 bp. The consensus for the –10 element was extended by one nucleotide upstream and downstream of the standard hexamer (indicated in bold). Promoter strengths were measured by reverse transcription quantitative PCR and β -glucuronidase assays. No correlation was found between the composition and context of elements within *P. ruminis* promoters, and promoter strength. However, a mutation within the –35 element of one promoter revealed that transcriptional strength and choice of transcription start site were sensitive to this single nucleotide change.

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INTRODUCTION

DNA binding specificity of bacterial RNA polymerase and the rate of transcription are largely dictated by the sequence of the upstream (UP) element (Estrem *et al.*, 1998), and –10 and –35 motifs, which are common to most bacteria. However, atypical aspects of transcription initiation are still being identified in prokaryotes (Bayley *et al.*, 2000; Gaal *et al.*, 2001; Weiner *et al.*, 2000). Novel structural properties of DNA are also being reported, but their effects on gene expression remain unknown (Petersen *et al.*, 2003; Vanet *et al.*, 2000; Vogel *et al.*, 2003).

Pseudobutyrvibrio ruminis (previously classified as *Butyrivibrio fibrisolvens*) is present in the rumen (Kopečný *et al.*, 2003) and gastrointestinal tract of some animals. *Butyrivibrio*-like organisms may account for as much as

24–30 % of culturable bacteria from the rumen (Forster *et al.*, 1996), although individual species may constitute much lower proportions of total bacterial numbers (Kobayashi *et al.*, 2000). These bacteria are important for projects that aim to alter rumen function using genetically modified bacteria (Brooker *et al.*, 1989; Gregg *et al.*, 1987, 1994, 1998; Gregg & Sharpe, 1991; Mackie & White, 1990; Rogers, 1990; Smith & Hespell, 1983; Teather, 1985; Teather & Forster, 1998). Understanding their transcriptional regulation is essential for controlling the expression of foreign genes within the rumen. Molecular mechanisms governing transcription initiation in *P. ruminis* and closely related species remain poorly understood. To date, only four promoters have been studied using transcriptional analysis in their species of origin: the *flaA* and *flaB* promoters in *P. ruminis* OR77 (Beard *et al.*, 2000), the *Pseudobutyrvibrio* sp. OB156 *thl* promoter (Asanuma *et al.*, 2003), and the *rep* promoter in *B. fibrisolvens* Bu49 (Beard *et al.*, 2000; Hefford *et al.*, 1997). *B. fibrisolvens* Bu49 is closely related to *P. ruminis*, but may be more appropriately classified as *Clostridium proteoclasticum* (Dr Jan Kopečný, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, personal communication). Phylogenetic classification of *Butyrivibrio*-like microorganisms is still in progress.

The aim of this study was to identify consensus DNA-binding motifs for RNA polymerase in *P. ruminis*. For the purpose of this study DNA fragments isolated from *P.*

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Abbreviations: GUS, β -glucuronidase; HEX, hexachlorofluorescein; MCS, multiple cloning site; RT-qPCR, reverse transcription quantitative PCR.

The GenBank/EMBL/DDBJ accession numbers for the promoter 6, promoter 10, promoter 18, promoter 46, pBGT, promoter 38 and pBK6 sequences of *Pseudobutyrvibrio ruminis* are DQ841994–DQ842000.

A supplementary figure showing the relationships between the 16S rDNA sequence of *Pseudobutyrvibrio ruminis* strain 0/10 and closely related species is available with the online version of this paper.

ruminis shown to transcribe a plasmid-borne gene are referred to as promoters. However, these promoters were not shown to initiate transcription from the genome and remain putative promoters in this context.

METHODS

Bacterial culture, strains and plasmids. *P. ruminis* strains 0/10 and OR38 were cultivated in rumen fluid medium (Klieve *et al.*, 1989) at 39 °C and transformed by electroporation (Beard *et al.*, 1995). *Escherichia coli* strain PMC112 (Gibson, 1984) was grown in Luria-Bertani (LB) medium at 37 °C and also transformed by electroporation (Dower *et al.*, 1988). Plasmid DNA was extracted from *E. coli* and *P. ruminis* using the Wizard Plus SV Minipreps DNA purification system; chromosomal DNA was extracted from *P. ruminis* as described previously (Woods *et al.*, 1989). *E. coli* transformants were selected with ampicillin (100 µg ml⁻¹) or kanamycin (30 µg ml⁻¹) and *P. ruminis* transformants were selected with erythromycin (100 µg ml⁻¹).

P. ruminis cultures were prepared as follows. A 2.5 % (v/v) inoculum from a frozen glycerol stock was used to start a culture that was grown for approximately 2 days. From this culture, a 2.5 % (v/v) inoculum was added to pre-warmed medium to create a starter culture. After approximately 20 h growth, a 2.5 % (v/v) inoculum was added to pre-warmed medium, and the culture was grown for 6–10 h before harvesting.

Construction of promoter rescue plasmid pBK6. The construction of plasmid pBK6 is shown in Fig. 1. In summary, the ampicillin resistance gene was removed from pBHE and replaced with the kanamycin resistance gene from pUK21. A ribosome-binding site (RBS) was inserted upstream of the erythromycin resistance gene, *ermAM* (pBK2). To avoid read-through transcription from other promoters, a fragment containing the transcription terminator of bacteriophage T4D was inserted upstream of the promoter insertion point (pBK5). Finally, a multiple cloning site (MCS) was inserted downstream of the T4 terminator, upstream of the RBS, to generate plasmid pBK6.

Construction of promoter reporter plasmid pBGT. The construction of plasmid pBGT is shown in Fig. 2. Plasmid pBK2 was ligated to the *gusA* gene from pFUS1 (Reeve *et al.*, 1999) to produce plasmid pBG. The *Pst*I/*Nde*I fragment of pBK6 that contains the T4 terminator and MCS was ligated to the major fragment of similarly digested pBG to produce pBGT.

Construction of *P. ruminis* candidate promoter library. Chromosomal DNA from *P. ruminis* was partially digested with *Sau*3AI, and fragments of 400–800 bp were purified from agarose gel using the UltraClean 15 kit (Mo Bio Laboratories). Fragments were ligated to *Bam*HI-cut and dephosphorylated forms of plasmids pBK5 or pBK6. Plasmids were transferred to *E. coli* and recombinants were selected by PCR-based screening.

Recombinant plasmid pBK5 or pBK6, containing chromosomal DNA fragments from *P. ruminis*, were pooled into groups of four and transferred to *P. ruminis*. Plasmids that contained active promoters conferred the erythromycin-resistant (Erm^r) phenotype. Promoter 38 was isolated similarly from *P. ruminis* OR38 using promoter rescue plasmid pBHE (Beard *et al.*, 2000). Promoter-active DNA fragments were excised and ligated into pBK6.

Confirmed promoter sequences were excised from pBK6 using *Xho*I and *Nde*I and were ligated to *Xho*I/*Nde*I-digested pBGT to measure transcription and translation levels.

Fig. 1. Incorporation of the kanamycin resistance gene into pBHE. The pUC118 segment of pBHE was removed using *Bgl*I and *Sac*I. The resulting fragments were end-filled using DNA polymerase I Klenow fragment and the remainder of pBHE was ligated to pUK21, which had been digested with *Ac*I and end-filled. The pUK21 multiple cloning site (MCS) was excised with *Spe*I, and the remaining 6.8 kb fragment circularized by ligation. A ribosome-binding site (RBS) was synthesized as two complementary oligonucleotides RBSF and RBSR (Table 1), which were annealed to produce a double-stranded fragment with *Bam*HI-compatible overhanging termini. This was inserted into the *Bam*HI site downstream of the insertion site for potential promoters and directly upstream of the promoter selection gene (*ermAM*) to produce plasmid pBK3. The gene 32 transcription/translation terminator (T4 terminator of bacteriophage T4D; Prentki & Krisch, 1984) was excised from plasmid pHP45 (Prentki & Krisch, 1984), digested with *Sph*I, end-filled and digested with *Bam*HI. This was inserted upstream of the promoters' insertion site. Plasmid pBK3 was similarly prepared, although initially cleaved with *Xba*I and then *Bam*HI. DNA containing the terminator and backbone of pBK3 were ligated to produce pBK4. The *Sal*I site of pBK4 was inactivated by cleavage of the *Sal*I site, end-filled and self-ligated to produce plasmid pBK5. To insert a MCS downstream of the transcription terminator and upstream of the promoter selection gene (*ermAM*), pBK5 was digested with *Nde*I and *Bam*HI and ligated with the MCS, which was constructed as complementary oligonucleotides MCSF and MCSR to provide cleavage sites for *Nde*I and *Bam*HI. The final construct was termed pBK6.

Fig. 2. Vector pBG was constructed with the promoterless *gusA* gene located directly downstream of a promoter insertion site. Plasmids pBK2 and pFUS1 (Reeve *et al.*, 1999) were digested with *Pst*I and *Xba*I. Dephosphorylated pBK2 plasmid backbone and the section of pFUS1 containing the *gusA* gene were ligated together. Vectors pBK6 and pBG were digested with *Pst*I and *Nde*I. The backbone fragment of pBG was ligated to the T4 transcriptional terminator–MCS fragment from pBK6 to produce the promoter reporter plasmid pBGT.

RNA extraction. RNA was extracted from *E. coli* and *P. ruminis* using hot, acidic phenol (Kalkmoff *et al.*, 1999). *E. coli* were grown to OD₆₀₀ ~1. *P. ruminis* cultures were harvested after growth for 6–10 h. To reduce the amount of extracellular polysaccharide in the extracts, *P. ruminis* were rinsed with diethyl pyrocarbonate (DEPC)-treated, chilled saline (0.89 %, w/v, NaCl). RNA was stored at –80 °C, in ethanol or RNase-free water. All solutions were treated with 0.1 % (v/v) DEPC to inactivate nucleases. Where necessary, to remove residual DNA, samples were treated with RQ1 RNase-free DNase as described by the manufacturer (Promega).

DNA sequencing and PAGE. For calibration and primer extension studies hexachlorofluorescein (HEX)-labelled DNA fragments were ethanol precipitated with Big Dye V3.1 or rhodamine-based sequencing products. Sequencing was by the dideoxy-dye-termination process (Applied Biosystems Sequencing Technical Manual; Perkin Elmer). The precipitate was redissolved in water and analysed on an ABI PRISM 377XL sequencer. Using ABI 377 sequencer software, HEX-labelled product was represented by a green peak, the same as for adenosine. To ensure the differentiation of HEX-labelled product from a coinciding adenosine base, at least two dilutions of this product were analysed.

Calibration of primer extension method. The migration of HEX-labelled DNA standards was calibrated against that of rhodamine- or Big Dye-based TSS1-primed sequencing products. HEX-labelled PCR products of precisely known size were amplified from pUC18 using *Pfx* DNA polymerase and HEX-labelled primer TSS1 in combination with primers Std1 (product size 100 bp), Std2 (200 bp), Std3 (300 bp), Std4 (391 bp), Std5 (472 bp) or Std6 (595 bp; Table 1).

Primer extension. Extension reactions (30 µl) contained 5–35 µg of total bacterial RNA, 3 µM HEX-labelled primer, 10 units AMV reverse transcriptase and the manufacturer's buffer (Promega), 1.7 mM each of the four deoxynucleotide triphosphates and 20–40 units RNasin RNase inhibitor (Promega), and were incubated at 42 °C for 60 min. Primer extension products were purified using the UltraClean 15 kit (Mo Bio Laboratories), redissolved in water and co-electrophoresed with sequencing products, primed using an unlabelled version of the extension primer.

Primer extension analysis of the *blaA* promoter used primers complementary to three locations within *blaA* in pUC18 (Brosius *et al.*, 1982). HEX-labelled primers TSS1, TSS2 and TSS3 (Table 1) were used to initiate reverse transcription with the primers' 5' terminal bases at positions 118, 269 and 519 bp downstream of the *blaA* transcription start site respectively.

Primer extension reactions were performed on RNA extracted from *P. ruminis* containing pBK6 recombinants harbouring active promoters. Reverse transcription and rhodamine-based sequencing reactions were primed using HEX-labelled TS1 and unlabelled TS1 respectively. Correcting observed primer extension product sizes using the equation described in Table 2 identified transcription start sites.

Quantitative β-glucuronidase (GUS) assay. *P. ruminis* cells were harvested after 6 h growth, resuspended in saline, and adjusted to OD₆₀₀ 2.0. Aliquots were snap-frozen at –80 °C for use in GUS and protein assays.

GUS assays were performed as described by Reeve *et al.* (2002), with the exceptions that 10 µl 17.5 mg ml^{–1} *p*-nitrophenyl β-D-glucuronide (pNPG) was added to start the reaction and absorbance measurements were taken at 405 nm every 2 min for 2.5 h using a Bio-Rad model 3550-UV microplate reader pre-warmed to 37 °C.

Table 1. Oligonucleotides

Name	Nucleotide sequence (5'–3')
MCSF	GATCGTCGACAGGCCTCGAGGATCCGAGCTCGTT-AACA
MCSR	TATGTTAACGAGCTCGGATCCTCGAGGCCTGTGCG-AC
RBSF	GATCCATATGAAGAGGAGA
RBSR	GATCTCTCCTCTTCATATG
SBRF	ATCAGGAAGTGATGGAGCAT
SBRR	TTGTTACACAAACGGTGAT
Std1	ATAACACTGCGGCCAACTTA
Std2	TCTCAGAATGACTTGGTTGA
Std3	ACGTTTTCCAATGATGAGCA
Std4	AAGATGCTGAAGATCAGTTG
Std5	ATTTCCGTGTCGCCCTTATT
Std6	ATGTGCGCGGAACCCCTATT
TSS1	CGATCAAGCGAGTTACATG
TSS2	ATACCGCGCCACATAGCAGA
TSS3	CAGCGTTTCTGGGTGAGCAA

Table 2. Comparison between actual and observed length of HEX-labelled PCR products based on electrophoretic co-migration with rhodamine- or Big Dye-based sequencing products

These results allowed the derivation of equations to correct the observed size of HEX-labelled reverse transcripts. 1 (rhodamine-based): $y=0.9894x+0.1221$; 2 (Big Dye-based): $y=0.9821x+5.836$, where x =observed size and y =corrected size of HEX-labelled DNA molecules.

HEX-labelled primer	Predicted length of HEX-labelled DNA (nt)	Observed migration (rhodamine-based)	Observed migration (Big Dye-based)
Std1	100	101	95.5
Std2	200	202	198
Std3	300	303	300
Std4	391	395	392
Std5	472	477	474.5

Reverse transcription quantitative PCR (RT-qPCR).

Quantification of plasmid copy number and of plasmid-derived transcript numbers used the Finnzymes DyNAmo SYBR GREEN qPCR kit and the PE Applied Biosystems International Prism-7700 Sequence Detection System 1.7. Plasmid copy numbers were measured in the cultures from which RNA was extracted for RT-qPCR studies. Bacteria were resuspended in saline and adjusted to OD₆₀₀ 4.0. Aliquots were snap-frozen at -80°C for use in protein assays and plasmid extraction.

A two-step RT-qPCR method was used to measure *gusA* transcript levels in *P. ruminis*. Total RNA was extracted from *P. ruminis* cultures after 6 h growth, diluted to $1\ \mu\text{g}\ \mu\text{l}^{-1}$, treated with DNase (Promega) and $0.5\ \mu\text{g}$ was added to a $25\ \mu\text{l}$ reverse transcription reaction. Reactions contained $0.2\ \mu\text{M}$ primer SBRR (Table 1), Stratascript reaction buffer, $0.2\ \text{mM}$ of each dNTP, $10\ \text{U}$ RNasin and $50\ \text{U}$ Stratascript reverse transcriptase. Reactions were incubated for 1 h at 42°C and $2\ \mu\text{l}$ of the mixture was added to each qPCR reaction.

Primers for RT-qPCR were designed as described by Bustin (2000), using the online primer design program Primer3 (Misener & Krawetz, 2000), and melting temperatures were calculated using the online Oligonucleotide Properties Calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>), based on the nearest neighbour method (Breslauer *et al.*, 1986). To measure plasmid and transcript copy numbers, primers SBRF and SBRR were used to amplify a section of the *gusA* gene. qPCR was performed as described by the kit manufacturer (Dynamo SYBR green qPCR kit instruction manual, Finnzymes) using 40 cycles at 95°C for 15 s, 60°C for 20 s, 72°C for 8 s, and 80°C for 5 s. Data were acquired at the last step of each cycle. Results were analysed using Sequence Detector V1.7 software (Applied Biosystems). Baseline start and stop values were 3 and 6 respectively. Threshold value was defined as 0.023. Quantification of transcript was calculated from C_T values (number of cycles required for a detectable signal) using a standard curve derived from spectrophotometrically measured concentrations of plasmid pBGT.

Measuring protein concentration. Protein concentration was measured by the BCA protein assay kit (Pierce) microtitre plate protocol, using a Bio-Rad model 3550-UV microplate reader (595 nm) and a standard curve constructed using BSA.

Phylogenetic analyses. Phylogenetic analyses were performed as described by Kopečný *et al.* (2001). To classify *P. ruminis* 0/10, 16S rRNA sequences from GenBank and EMBL were analysed using CLUSTAL_X (Thompson *et al.*, 1997) and PHYLIP (Felsenstein, 1989). Sequence data for distance matrices were bootstrapped using SEQBOOT (resampled 1000 times). The DNADIST program was used to analyse distances using the Kimura–Nei method (Kimura, 1980) with the following settings: transition:transversion ratio=2.0, empirical base frequencies, and coefficient of variation 1. Trees were produced from distance matrices using the neighbour-joining method (Saitou & Nei, 1987) and a consensus tree was generated using CONSENSE (Felsenstein, 1989). Bar: 10 substitutions per 100 nt. A consensus tree was drawn using the online program Phylo dendron.

Computational analyses of DNA and protein sequences.

Nucleotide and amino acid sequences were compared to those in GenBank using BLAST analysis (Tatusova & Madden, 1999). Promoter sequences were searched for transcription factor binding sites using the Tfsitescan online analysis form (<http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>) and the object-orientated transcription factor database tfsites (Ghosh, 1998, 2000). To identify consensus sequences among promoters, those in which transcription start sites have previously been mapped, including *B. fibrisolvens* Bu49 *rep* (Hefford *et al.*, 1997), *Pseudobutyrvibrio* sp. OR77 *flaA* (Beard *et al.*, 2000) and *flaB* (Kalmokoff *et al.*, 2000), *Pseudobutyrvibrio* sp. OB156 *thl* (Asanuma *et al.*, 2003), and those isolated in this study, were examined using the program MEME (Bailey & Gribskov, 1998). Regions extending from -200 to $+100$ bp were examined. Due to the lack of available sequence, promoters 46 and *flaB* were examined between -200 and $+26$ and -152 and $+100$ respectively.

Statistical analyses. Results from RT-qPCR and GUS assays were analysed using the two-tailed Student's *t* test. ANOVA (Box *et al.*, 1978) was used to ensure comparable copy numbers among different constructs. Grouping of promoter activities was determined using the Tukey–Kramer honestly significant difference (HSD) comparisons of means test. For all tests it was assumed that the populations were normal and variances were equal.

RESULTS

Phylogenetic classification of *P. ruminis* strain 0/10

P. ruminis 0/10 was isolated from sheep rumen by Linda Kennedy (Murdoch University, Western Australia) on RF agar. Classification was based on morphology (i.e. a narrow, curved rod with a polar flagellum, approximately $1\text{--}1.5\ \mu\text{m}$ in length) and on 97% nucleic acid identity to 16S rRNA sequences of previously studied species of *Pseudobutyrvibrio* such as strain OR35. Supplementary Fig. S1, available with the online version of this paper, shows the relationships between the 16S rDNA sequence of *P. ruminis* strain 0/10 and closely related species, some of which were used for analyses in this study.

Isolation of promoter fragments from *P. ruminis*

Plasmid pBK6 (Fig. 1) transforms both *E. coli* and *P. ruminis*. Of the 173, 0.4–0.6 kb fragments of *P. ruminis* genomic DNA (total 88.8 kb) screened for promoter activity, promoters 6, 10, 18, 38 and 46 were identified

by the conversion of transformants to the Erm^r phenotype. Replicate experiments resulted in the isolation of promoter 54, which differed by one base pair from promoter 18. The effectiveness of the bacteriophage T4D gene 32 transcription/translation terminator (Prentki & Krisch, 1984) in preventing significant read-through transcription of the selectable marker gene (*ermAM*) was shown by the absence of Erm^r transformants from the plasmid in the absence of 'promoter' inserts.

Validation of primer extension analysis: identification of *blaA* transcription start site

To correct migration anomalies caused by the presence of different fluorescent moieties on reverse transcripts compared to DNA sequencing products, the electrophoretic migration of precisely defined products amplified from pUC18 was used to derive equations 1 and 2 (Table 2). To validate these equations, primer extension analysis of the well-defined *blaA* promoter was performed (Brosius *et al.*, 1982). Priming sites within the *blaA* gene, selected to produce reverse transcripts of 119, 270 and 520 bp (Fig. 3), showed that results from either sequencing system could be corrected to map the 5' base of *blaA* mRNA to within $\pm$ one base.

Identification of transcription start sites and consensus promoter sequences

Transcription start sites were identified within promoters 10, 18, 38, 46 and 54 (Fig. 4). Promoter 38 contained two transcription start sites, corresponding to promoters 38a and 38b. A primer-extension product was not detected from promoter 6. When aligned by transcription start sites, consensus sequences at which the nucleotide identity was consistent in $\geq 75\%$ of promoters showed hexamer motifs for the -10 and -35 elements, corresponding to the DNA-binding motifs for RNA polymerase complexed with the σ^{70} subunit (Wosten, 1998). Consensus sequences for -10 and -35 elements, **TTGWMA** and **ATAATATW**, respectively, were interspaced by 15–16 bp. The -10 consensus was extended one nucleotide either side of the standard hexamer (indicated in bold), and was 5–17 bp upstream of the transcriptional start. In four of the nine promoters examined a TG dinucleotide adjacent to the 5' A residue of the expanded -10 element may correspond to a further extended -10 or -16 motif (Burr *et al.*, 2000; Wosten, 1998).

Analysis using MEME identified a number of statistically significant, common motifs (POSITION *P* value < 0.0001). However, none were common to all promoters or in a consistent position relative to a transcription start site.

Quantitative measurement of expression from cloned promoters in *P. ruminis*

Results from RT-qPCR for *gusA* transcript and GUS activity assays for promoters 6, 10, 18, 46 and 54 showed

Fig. 3. Identification of the pUC18 *blaA* transcription start site. Columns: predicted size of cDNA (bp) primed using TSS1 (119), TSS2 (270) and TSS3 (520). (A) pUC18 sequence from primers TSS1, TSS2 or TSS3, (B) pUC18 sequence from the same primers, co-precipitated and electrophoresed with HEX-labelled primer extension products. Annotations show predicted (●) and observed (■) migration positions of HEX-labelled DNA molecules.

that transcript levels were not proportional to enzyme activity (Table 3). Relative values for *gusA* mRNA levels and β -glucuronidase activity, by promoter number, were $10 > 6 > 18 > 46 > 54$ and $10 > 18 > 46 > 6 > 54$ respectively (Fig. 5). RT-qPCR showed that plasmid copy number did not differ significantly among *P. ruminis* transformants harbouring variants of pBGT that contained different promoters (ANOVA; $P > 0.05$; $n = 2$).

DISCUSSION

The data reported here show -10 elements in *P. ruminis* and closely related species to be expanded from the conventional hexamer to an octamer: **ATAATATW**. This consensus can also be represented as **AtAaTata** (capitals show $> 85\%$ conservation). Expansion of -10 elements has been observed in other eubacteria, although their composition may differ from those identified here (Barnell *et al.*, 1992; Bayley *et al.*, 2000; Malakooti *et al.*, 1995; Patek

Prom.	UP	-35	interspacer	EX-10	-10	DIS
46	82% A/T ttaacaa	T T G T T A	ttt--ttcaaggtctttg	A T A A T A T A	tgataag	
18	74% A/T atacata	T T G C A A	cctgaatgagaatcattt	A C A A T A T A	gcctt	
10	78% A/T aaaatca	A A G A C A	gggactctcaaaatatat	A T A A T A A A	tatcga	
38A	78% A/T aaagtgc	T T G A C A	aag--caagctgctcatg	A T A A T A T A	aacgagt	
38B	76% A/T aaacctg	T T G A C T	ttg--tggtagtgggatg	A T A A T A T A	ctattcgtt	
rep	86% A/T aattaaa	T T A T A A	caaa-tggttgtaatctgt	A T T A T A A T	ttgttatagtccttgta	
flaA2	60% A/T ttgtacc	T T T A C A	atttaataaggtataacct	A C A T A A T T	ccaccttg	
thl	72% A/T atacaca	T T G A A A	agaa--aatgtagtgtg	G T A G T A T T	ttcac	
CONSENSUS		T T G W M A		A T A A T A T W		
		<u>7</u> 8	<u>7</u> 8	<u>6</u> 8	<u>7</u> 8	<u>7</u> 8
		<u>7</u> 8	<u>7</u> 8	<u>6</u> 8	<u>7</u> 8	<u>6</u> 8
54	69% A/T aacctga	A T G A G A	atca-tttacaatatagc	C T T A T G A A	atttgaacgtag	

Fig. 4. Alignment of eight promoters identified in *P. ruminis* and closely related species. Promoters 10, 18, 38, 46 and 54 were isolated from *P. ruminis* strain 0/10 or OR38. Promoter 38 contained two transcription start sites, corresponding to promoters 38a and 38b. For comparison, transcription start sites are shown (below the line) for promoters of *B. fibrisolvens* Bu49 rep (Hefford *et al.*, 1997), *Pseudobutyrvibrio* sp. OR77 flaA-2 (Beard *et al.*, 2000) and *Pseudobutyrvibrio* sp. OB156 thl (Asanuma *et al.*, 2003). Transversion in the -35 element from a T in promoter 18 to an A in promoter 54 is shown by shading. Transcription start sites are underlined. The -10 and -35 elements are in bold capitals. Possible extensions of the -10 element are in bold, italic capitals and have been incorporated into the consensus sequence. Possible further extensions to -10 elements (EX -10) are in bold and incorporate the first base of the conserved (capitalized) -10 region. The A/T-rich region 50 bp upstream of the -35 element is shown. Consensus sequences are shown at the bottom of the figure, with the frequencies of consensus nucleotides displayed beneath their respective positions.

et al., 2003). In all promoters studied here at least four positions within the -35 element, TTGWMA, matched the *E. coli* consensus, TTGACA (Lisser & Margalit, 1993). The -35 element was less well conserved than the -10 element. The spacer between the -10 and -35 elements (16–18 bp) showed a narrow range compared to those of species such as *Bacteroides fragilis* (10–20 bp; Bayley *et al.*, 2000),

Table 3. Comparison of mean promoter activities in *P. ruminis* after 6 h growth using RT-qPCR to measure mRNA levels, and GUS assay to measure β -glucuronidase levels

Groups with the same letter are not significantly different using the Tukey–Kramer (TK) Test ($\alpha < 0.05$). Promoters with the same superscript numbers had significantly different activities (Student's *t* test; $P < 0.05$).

Promoter	<i>gusA</i> mRNA level	β -Glucuronidase level
10	A	A
18	B ^{1,2}	A
46	B	A
6	B	B ^{3,4}
54	B ¹	B ^{3,5}
None*	B ²	B ^{4,5}

*Negative control, plasmid pBGT containing a promoterless *gusA* gene.

Helicobacter pylori (19–23 bp; Vanet *et al.*, 2000), although it was similar to that of others such as *Corynebacterium glutamicum* (16–18 bp; Patek *et al.*, 2003) and *Lactobacillus sp.* (16–19 bp; McCracken *et al.*, 2000). Using this shotgun approach, the isolation of promoters that bind the primary sigma factor of *P. ruminis* was considered to be likely as it has been estimated that over 80 % of promoters in *E. coli* are regulated by the primary σ^{70} factor (Ishihama, 2000) and a similar situation is likely to apply to promoters of other species.

Promoters 38a, 38b and 46 contained an extended -10 element (EX -10), with a TGN motif upstream of the standard hexamer. This motif is present in approximately 20 % of *E. coli* promoters (Burr *et al.*, 2000; Sanderson *et al.*, 2003) and in up to 60 % of genes from Gram-positive bacteria such as *Streptococcus pneumoniae*, *Clostridium pasteurianum* and *Bacillus subtilis* (Agarwal & Tyagi, 2003). In *E. coli* the EX -10 is generally associated with lack of an identifiable -35 element (Belyaeva *et al.*, 1993; Chan & Busby, 1989; Keilty & Rosenberg, 1987). However, in this and other studies (Sabelnikov *et al.*, 1995; Voskuil & Chambliss, 1998), the TG dinucleotide directly upstream of the -10 element was associated with highly conserved -10 and -35 elements. The expanded EX -10 motif observed in *B. subtilis* (TRTGN; Helmann, 1995; Voskuil & Chambliss, 2002) was not apparent in the species studied here. The frequency of the EX -10 element in *P. ruminis*

Fig. 5. Promoter strengths expressed from variants of vector pBGT in *P. ruminis* O/10. Grey columns, *gusA* transcript copy number (shown on left axis). Error bars represent SD ($n=2$). White columns, β -glucuronidase activity (shown on right axis). Error bars represent SEM ($n=3$). g, control variant of pBGT, without a promoter directly upstream of the *gusA* gene.

and closely related species cannot be inferred from this study, because it may vary between species of the same genus. For example, it has been shown that the TG motif was present in 54% of promoters in some species of *Lactobacillus*, but was not significantly represented in others (McCracken *et al.*, 2000).

The UP element is an AT-rich region upstream of the -35 element. No match was found in these ruminal species for the *E. coli* UP element consensus (Estrem *et al.*, 1998). However, there was a marked reduction in GC content directly upstream of the -35 element, which may influence DNA curvature and transcription initiation (Gabrielian *et al.*, 1999). Computational analysis of these promoter regions did not reveal any previously uncharacterized motifs.

The lack of correlation between the relative abundance of mRNA and of gene product seen in this study has previously been reported in other studies (Bannantine *et al.*, 1997; Glanemann *et al.*, 2003; Niehus *et al.*, 2002; Rosado & Gage, 2003). Plasmid pBG constructs all shared a common 103 nt leader sequence upstream of the *gusA* gene and any differences in mRNA stability could not be attributed to different lengths or sequences of the 5' untranslated region. It has been shown that the activity of a single promoter, as reported by various proteins, can vary greatly (Kahala & Palva, 1999; Niehus *et al.*, 2002). Among those studies, only the luciferase system accurately reported the relationship between mRNA and gene product levels. It is possible that disparity between mRNA and β -glucuronidase levels is related to the stability of β -glucuronidase in *P. ruminis*.

No correlation was found between promoter sequence and transcriptional activity. Promoter activity is likely to be affected by the composition and relative positions of elements within promoter regions, and other factors such as gene-specific activators and repressors (Lloyd *et al.*,

2001; Rhodius & Busby, 1998; Rojo, 2001), ppGpp (Barker *et al.*, 2001; Chatterji & Ojha, 2001), termination and anti-termination factors (Henkin, 1996; Henkin & Yanofsky, 2002), anti-sigma factors (Helmann, 1999), NTP concentration (Schneider *et al.*, 2002), transcript cleavage factors (Hsu *et al.*, 1995) and factor-dependent DNA curvature or torsional state (Dai & Rothman-Denes, 1999; Xu & Hoover, 2001). Indeed computational analysis (data not shown) suggests that promoter 46 may be regulated by NarL/NarP (Dong *et al.*, 1992; Householder *et al.*, 1999; Li & Stewart, 1992) and promoter 10 contained five repetitive elements that may act as binding motifs for regulatory proteins.

Due to the promoter-rescue plasmid-based experimental approach employed in this study, it was not possible to identify the genes directly downstream from the promoters, within the *P. ruminis* genome. The activity of these promoters in the *P. ruminis* genome is putative. Their usefulness in expressing newly introduced, plasmid-borne genes is clear from the data presented here. Gene(s) under the control of specific promoter sequences, flanking the promoters, could be identified using techniques similar to those used to identify genomic sequences flanking inserted transposons (Kwon & Rieke, 2000). Identification of these genes would allow genome-derived transcript levels to be measured using RT-qPCR, to confirm that these promoters do indeed initiate transcription from the genome.

A single-base difference between the -35 elements of promoters 18 and 54 resulted in the recognition of different consensus regions by RNA polymerase (Fig. 4) and promoter 54 showed significantly lower activity than promoter 18. The mutation may have reduced recruitment to promoter 54, or may have enhanced recruitment but reduced promoter escape due to strong -35 element–polymerase interactions. Interestingly, elements of promoter 54 did not resemble the consensus sequences derived in this study, and consensus-like promoter architecture is likely to be essential for both optimal RNA polymerase recruitment and escape.

In future studies, investigation of the interplay between transcription and translation could be extended by performing assays of both processes in parallel, allowing direct correlation between reporter protein accumulation and transient mRNA levels (Glanemann *et al.*, 2003). The promoter reporter plasmid constructed in this study is useful for the quick identification of strong promoters for high-level expression of exogenous genes in *P. ruminis*, such as promoter 10. Specifically, such promoters may be used for the high-level expression of fluoroacetate dehalogenase, an enzyme produced by recombinant *B. fibrisolvens* in the rumen to detoxify the plant poison fluoroacetate (Gregg *et al.*, 1998).

The development and implementation of tools for the study of promoters in *P. ruminis* has allowed the characterization of novel promoters and has laid the foundation for future studies of a larger subset of

promoters from this bacterium. The investigation of promoters in a standard context, as provided for by these tools, will benefit future studies of active RNA polymerase DNA-binding motifs to determine whether consensus sequences derived in this study are representative of the species.

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