Isolation and characterization of putative *Pseudobutyrivibrio ruminis* promoters

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Novel plasmids were constructed for the analysis of DNA fragments from the rumen bacterium *Pseudobutyrivibrio 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 \(\sigma^{70}\)-like factor complex. Consensus –35 and –10 sequences within these elements were TTGACA and ATAAATA 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 \(\beta\)-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 region of one promoter revealed that transcriptional strength and choice of transcription start site were sensitive to this single nucleotide change.

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 \textit{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 \textit{et al.}, 2000; Gaal \textit{et al.}, 2001; Weiner \textit{et al.}, 2000). Novel structural properties of DNA are also being reported, but their effects on gene expression remain unknown (Petersen \textit{et al.}, 2003; Vanet \textit{et al.}, 2000; Vogel \textit{et al.}, 2003).

\textit{Pseudobutyrivibrio ruminis} (previously classified as Butyrivibrio fibrisolvens) is present in the rumen (Kopecky \textit{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 \textit{et al.}, 1996), although individual species may constitute much lower proportions of total bacterial numbers (Kobayashi \textit{et al.}, 2000). These bacteria are important for projects that aim to alter rumen function using genetically modified bacteria (Brooker \textit{et al.}, 1989; Gregg \textit{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 \textit{et al.}, 2000), the *Pseudobutyrivibrio* sp. OB156 thy promoter (Asanuma \textit{et al.}, 2003), and the rep promoter in *B. fibrisolvens* Bu49 (Beard \textit{et al.}, 2000; Hefford \textit{et al.}, 1997). *B. fibrisolvens* Bu49 is closely related to *P. ruminis*, but may be more appropriately classified as \textit{Clostridium proteoclasticum} (Dr Jan Kopecky, 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.
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 PMCl12 (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 pUK21 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 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 Sau3AI, and fragments of 400–800 bp were purified from agarose gel using the UltraClean 15 kit (Mo Bio Laboratories). Fragments were ligated to BamHI-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⁺) 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 XhoI and NdeI and were ligated to XhoI/NdeI-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 BglII and SacI. 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 AcI and end-filled. The pUK21 multiple cloning site (MCS) was excised with SpeI, 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 BamHI-compatible overhanging termini. This was inserted into the BamHI 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 pH451 (Prentki & Krisch, 1984), digested with SphI, end-filled and digested with BamHI. This was inserted upstream of the promoters’ insertion site. Plasmid pBK3 was similarly prepared, although initially cleaved with XbaI and then BamHI. DNA containing the terminator and backbone of pBK3 were ligated to produce pBK4. The SacI site of pBK4 was inactivated by cleavage of the SacI 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 NdeI and BamHI and ligated with the MCS, which was constructed as complementary oligonucleotides MCSF and MCSR to provide cleavage sites for NdeI and BamHI. The final construct was termed pBK8.
RNA extraction. RNA was extracted from E. coli and P. ruminis using hot, acidic phenol (Kalmokoff et al., 1999). E. coli were grown to OD_{600} ~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 TSS1 and unlabelled TSS1 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 OD_{600} 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>MCSF</td>
<td>GATGTTGCAGCTGGCAGAGTCCAGGCAGTCTGTT-ACCA</td>
</tr>
<tr>
<td>MCSR</td>
<td>TATGTTACAGGCTCGAGATCCTCGAGGCTCGTT-AC</td>
</tr>
<tr>
<td>RBSF</td>
<td>GATCCATATGAGAAGGAGGAG</td>
</tr>
<tr>
<td>RBSR</td>
<td>GATGCTTCCCTTCTCATGAG</td>
</tr>
<tr>
<td>SBRF</td>
<td>ATCACAGAAGTATGATGAGCAT</td>
</tr>
<tr>
<td>SBRR</td>
<td>TTGTTCACACAAACGGTGAT</td>
</tr>
<tr>
<td>Std1</td>
<td>ATACAACCTGGCGCAACACTT</td>
</tr>
<tr>
<td>Std2</td>
<td>TCTCAGAATGACTTGGTTGA</td>
</tr>
<tr>
<td>Std3</td>
<td>ACAGTTTCCAATAGTATGAGGCA</td>
</tr>
<tr>
<td>Std4</td>
<td>AAGATGCTGAGATGAGCATGAG</td>
</tr>
<tr>
<td>Std5</td>
<td>ATTTCCGTTCGCCCTTATT</td>
</tr>
<tr>
<td>Std6</td>
<td>ATGTGCCGGGAACCCCTTATT</td>
</tr>
<tr>
<td>TSS1</td>
<td>CGCAACAGCAGGAGTTACATG</td>
</tr>
<tr>
<td>TSS2</td>
<td>ATACCCGCCCCACATGACG</td>
</tr>
<tr>
<td>TSS3</td>
<td>CAGCGTTTCTGGGTAGCAA</td>
</tr>
</tbody>
</table>
Phylogenetic analyses. Phylogenetic analyses were performed as described by Kopecny 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 Phyloendendron.

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 Tфиtescan online analysis form (http://www.ifti.org/cgi-bin/ifti/Tfitescan.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), Pseudoobutyrivibrio sp. OR77 flaA (Beard et al., 2000) and flaB (Kalkmoff et al., 2000), Pseudoobutyrivibrio sp. OB156 thi (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 1–1.5 µm in length) and on 97% nucleic acid identity to 16S rRNA sequences of previously studied species of Pseudoobutyrivibrio 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.
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 ± 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 ≥75% of promoters showed hexamer motifs for the −10 and −35 elements, corresponding to the DNA-binding motifs for RNA polymerase complexed with the $\sigma^{70}$ subunit (Wosten, 1998). Consensus sequences for −10 and −35 elements, TTGWMA and ATAAATW, 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 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: ATAAATW. 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...
et al., 2003). In all promoters studied here at least four positions within the 235 element, TTGWMA, matched the E. coli consensus, TTGACA (Lisser & Margalit, 1993). The 235 element was less well conserved than the 210 element. The spacer between the 210 and 235 elements (16–18 bp) showed a narrow range compared to those of species such as Bacteroides fragilis (10–20 bp; Bayley et al., 2000), 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 σ70 factor (Ishihama, 2000) and a similar situation is likely to apply to promoters of other species.

Promoters 38a, 38b and 46 contained an extended 210 element (EX210), 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 (TRTG; Helmann, 1995; Voskuil & Chambliss, 2002) was not apparent in the species studied here. The frequency of the EX−10 element in P. ruminis

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 (α<0.05). Promoters with the same superscript numbers had significantly different activities (Student’s t test; P<0.05).

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<table>
<thead>
<tr>
<th>Promoter</th>
<th>gusA mRNA level</th>
<th>β-Glucuronidase level</th>
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<tbody>
<tr>
<td>10</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>18</td>
<td>B1,2</td>
<td>A</td>
</tr>
<tr>
<td>46</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>54</td>
<td>B1</td>
<td>B3,4</td>
</tr>
<tr>
<td>None*</td>
<td>B2</td>
<td>B4,5</td>
</tr>
</tbody>
</table>

*Negative control, plasmid pBGT containing a promoterless 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 & Ricke, 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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REFERENCES


OB156-derived recombinant. Butyrivibrio fibrisolvens polymerase. Annu Rev Microbiol
Environ Microbiol 65
http://mic.sgmjournals.org 3079
The expression signals of the
Householder, T. C., Belli, W. A., Lissenden, S., Cole, J. A. & Clark, V. L.
Control of transcription termination in prokaryotes. Annu Rev Genet 30, 35–57.


