A reporter gene vector to investigate the regulation of glutamine synthetase in Bacteroides fragilis Bf1

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Introduction

Bacteroides fragilis is a strictly anaerobic, pathogenic bacterium found in the human digestive tract (Salyers, 1984). It has been proposed that the production of certain proteases may play a role in the pathogenicity of this organism (Macfarlane et al., 1992). Furthermore, Gibson & Macfarlane (1988) have shown that the levels of nitrogen in the growth medium influence protease activity. The mechanisms used by B. fragilis to assimilate ammonia are, therefore, of interest.

Studies on the pathways of nitrogen assimilation in B. fragilis are still at an early stage. Yamamoto et al. (1984, 1987) have demonstrated the activities of both NADPH- and NADH-linked glutamate dehydrogenase (GDH: EC 1.4.1.3) and glutamine synthetase (GS: EC 6.3.1.2) in extracts of B. fragilis ATCC 23745 cells, but very low activity of NADPH-dependent glutamate synthase and no activity of alanine dehydrogenase. Both GDH and GS were regulated by the levels of ammonia in the growth medium, with higher expression of both being detected under nitrogen-limiting conditions than when excess nitrogen was present. This indicated a novel mechanism of nitrogen assimilation in B. fragilis differing from that of other organisms, where GS is expressed under low, and GDH under high nitrogen conditions.

The GS gene (glnA) from the clinical isolate B. fragilis Bf1 (Mossie et al., 1979) has been cloned (Southern et al., 1986), the nucleotide sequence determined (Hill et al., 1989), and the GS protein characterized (Southern et al., 1987). The glnA gene and its corresponding protein were shown to possess certain novel structural and molecular characteristics differing from the GS enzymes of prokaryotes, eukaryotes and archaeobacteria (Hill et al., 1989). Because of these differences, Hill et al. (1989) proposed that the GS of B. fragilis Bf1 belonged to a new class of GS enzymes termed GSIII.

It is not possible to study the regulation of GS in B. fragilis Bf1 by assaying GS enzyme activity directly since Southern et al. (1987) showed that GS activity was specifically inhibited in permeabilized B. fragilis Bf1 cells and cell extracts. Addition of B. fragilis cell-free extract to the cloned B. fragilis glnA gene product expressed in the GlnA+ E. coli strain YMC11 completely inhibited GS activity, whereas it had no effect on the GS activity obtained from the GlnA+ wild-type E. coli strain YMC10. However, the presence and regulation of GS in B. fragilis Bf1 cells was observed by Southern et al. (1987), who used Western blot analysis to detect the enzyme, and showed that the levels of GS enzyme varied depending on the nitrogen content of the growth medium. The level of GS enzyme was higher under conditions of nitrogen...
Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are shown in Table 1. B. fragilis Bf1 was described by Mossie et al. (1979) and was used by Southern et al. (1986, 1987) for the cloning of the glnA gene. All Bacteroides strains were maintained anaerobically (Abratt et al., 1985) at 37 °C on supplemented brain heart infusion (BHI) agar (Holdeman et al., 1977), with the addition of thymine (Em) (100 μg ml\(^{-1}\)) for the maintenance of transconjugants. Pre-cultures for nitrogen regulation studies were grown for 16-18 h in BHI broth. Escherichia coli strains were grown at 37 °C on Luria-Bertani (LB) agar (Maniatis et al., 1982) supplemented with ampicillin (Ap) (100 μg ml\(^{-1}\)), trimethoprim (Tp) (200 μg ml\(^{-1}\)) or tetracycline (Tc) (10 μg ml\(^{-1}\)) for the maintenance of plasmids. Pre-cultures of Bacteroides and E. coli strains for conjugation experiments were grown for 16-18 h in trypticase yeast glucose (TYG) broth (Valentine et al., 1988), pre-reduced when required for anaerobic growth, and conjugation was carried out on TYG agar with anaerobic selection of transconjugants on TYG containing Em (10 μg ml\(^{-1}\)) and gentamicin (Gm) (200 μg ml\(^{-1}\)). Nitrogen regulation studies were carried out in minimal medium (MM) broth (Varel & Bryant, 1974) modified as follows: volatile fatty acids and casitone were omitted; vitamin Bi was the only vitamin B added; higher final concentrations of menadione (0.5 mg ml\(^{-1}\)), haemin (5 mg ml\(^{-1}\)), Na₂CO₃ (1 mg ml\(^{-1}\)) and CoCl₂·6H₂O (0.01 mg ml\(^{-1}\)) were used; phosphate buffer (0.1 M; pH 7.0) was used instead of KH₂PO₄. The medium was supplemented with 5-7.5 mm (NH₄)₂SO₄ (high or low nitrogen, respectively).

Preparation and analysis of nucleic acids. Unless otherwise stated, all constructs were derived and analysed in E. coli HB101 prior to conjugation into Bacteroides. Plasmid DNA was isolated according to the method of Ish-Horowiz & Burke (1981), and cloning and transformation in E. coli were carried out as described by Maniatis et al. (1982). Restriction endonucleases were used according to the manufacturers’ instructions. Total cellular RNA was extracted from 10 ml or 100 ml cultures of early exponential phase E. coli or B. fragilis cells, respectively (Aiba et al., 1981).

Conjugation procedures. Bacterial matings were carried out as described by Valentine et al. (1988), using E. coli HB101( R751) or E. coli S17.1 as donors, and Bacteroides thetaiotaomicron 5482, B. fragilis 638 and B. fragilis Bf1 as recipients. A donor-to-recipient ratio of 1:5 was used throughout.

Plasmid construction. The construction of the reporter gene shuttle vector, pEGL1, and its derivatives is shown in Table 1, and Figs 1 and 2. The SspI-PstI fragment of the plasmid pHZ117 (Zappe, 1988) containing the C. acetobutylicum endoglucanase structural gene (eglA) and 21 bp upstream of the TTG start codon (including the ribosome-binding site, but excluding the major promoter region as determined by

limitation. It was not, however, known whether the enzyme was being regulated at the level of transcription or translation.

To study the regulation of GS in B. fragilis Bf1, we have developed a reporter gene vector using the promoterless β-1,4-endoglucanase gene (eglA) of Clostridium acetobutylicum P262 (Zappe et al., 1986, 1988). This vector has been used to identify the promoter region of the B. fragilis Bf1 GS involved in its regulation by nitrogen in various Bacteroides species.

### Methods

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids are shown in Table 1. B. fragilis Bf1 was described by Mossie et al. (1979) and was used by Southern et al. (1986, 1987) for the cloning of the glnA gene. All Bacteroides strains were maintained anaerobically (Abratt et al., 1985) at 37 °C on supplemented brain heart infusion (BHI) agar (Holdeman et al., 1977), with the addition of thymine (Em) (100 μg ml\(^{-1}\)) for the maintenance of transconjugants. Pre-cultures for nitrogen regulation studies were grown for 16-18 h in BHI broth. Escherichia coli strains were grown at 37 °C on Luria-Bertani (LB) agar (Maniatis et al., 1982) supplemented with ampicillin (Ap) (100 μg ml\(^{-1}\)), trimethoprim (Tp) (200 μg ml\(^{-1}\)) or tetracycline (Tc) (10 μg ml\(^{-1}\)) for the maintenance of plasmids. Pre-cultures of Bacteroides and E. coli strains for conjugation experiments were grown for 16-18 h in trypticase yeast glucose (TYG) broth (Valentine et al., 1988), pre-reduced when required for anaerobic growth, and conjugation was carried out on TYG agar with anaerobic selection of transconjugants on TYG containing Em (10 μg ml\(^{-1}\)) and gentamicin (Gm) (200 μg ml\(^{-1}\)). Nitrogen regulation studies were carried out in minimal medium (MM) broth (Varel & Bryant, 1974) modified as follows: volatile fatty acids and casitone were omitted; vitamin Bi was the only vitamin B added; higher final concentrations of menadione (0.5 mg ml\(^{-1}\)), haemin (5 mg ml\(^{-1}\)), Na₂CO₃ (1 mg ml\(^{-1}\)) and CoCl₂·6H₂O (0.01 mg ml\(^{-1}\)) were used; phosphate buffer (0.1 M; pH 7.0) was used instead of KH₂PO₄. The medium was supplemented with 5-7.5 mm (NH₄)₂SO₄ (high or low nitrogen, respectively).

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**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>E. coli</th>
<th>Bacteroides</th>
<th>Description*</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HB101</td>
<td>Gm'</td>
<td>Gm' RP-4-2</td>
<td></td>
<td>Boyer &amp; Roulland Dussoix (1969) Simon et al. (1983)</td>
</tr>
<tr>
<td>S17.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides B. fragilis Bf1</td>
<td>Em'Gm'</td>
<td></td>
<td>Ca eglA gene in pUC19</td>
<td>Zappe (1988)</td>
</tr>
<tr>
<td>B. fragilis 638</td>
<td>Em'Gm'</td>
<td></td>
<td>Bf glnA gene in pEcoR251</td>
<td>Southern et al. (1986) Shoemaker et al. (1985)</td>
</tr>
<tr>
<td>B. thetaiotaomicron 5482</td>
<td>Em'Gm'</td>
<td></td>
<td></td>
<td>Valentine et al. (1988)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>pHZ117</td>
<td>Ap' EG'</td>
<td></td>
<td>Reporter gene: promoterless eglA</td>
<td></td>
</tr>
<tr>
<td>pJSl39</td>
<td>Ap' GS'</td>
<td></td>
<td>Reporter gene in pEGL1 fused to</td>
<td></td>
</tr>
<tr>
<td>R751</td>
<td>IncB(eta)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVAL-1</td>
<td>Mob' Rep'</td>
<td></td>
<td>Complete eglA from pHZ117</td>
<td></td>
</tr>
<tr>
<td>pEGL17</td>
<td>Mob' Rep'</td>
<td></td>
<td>in pVAL-1</td>
<td></td>
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<tr>
<td>pEGL1</td>
<td>Mob' Rep'</td>
<td></td>
<td>Reporter gene: promoterless eglA</td>
<td></td>
</tr>
<tr>
<td>pEGL3</td>
<td>Mob' Rep'</td>
<td></td>
<td>Reporter gene in pEGL1 fused to</td>
<td></td>
</tr>
<tr>
<td>pEG106</td>
<td>Mob' Rep'</td>
<td></td>
<td>Reporter gene in pEGL1 fused to</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations used: r, resistance; s, sensitivity: Ap, ampicillin; Tc, tetracycline; Gm, gentamicin; Tp, trimethoprim; Tra, ability to self-transfer; Mob, able to be mobilized by a conjugative element; Rep, able to replicate; Inc, plasmid incompatibility group; EG, endoglucanase (eglA) gene; GS, glutamine synthetase (glnA) gene; Ca, C. acetobutylicum P262; Bf, B. fragilis Bf1.
region of the glnA gene (pUCpΔ). These plasmids were pooled, digested with EcoRI and Smal (blunt) and cloned between the EcoRI and KpnI (blunt) sites of pEGL1. Recombinant plasmids conferring increased endoglucanase (EG) activity on E. coli transformants were selected for sequence analysis and conjugation into the various Bacteroides recipients.

The endoglucanase gene cloned from C. acetobutylicum P262 (eglA and 280 bp of upstream region) was tested for anaerobic expression from its own promoter in Bacteroides by subcloning the 1.4-kb EcoRI–PstI fragment from pHZ117 between the EcoRI and PstI sites of pVAL-1 (pEGL117).

Endoglucanase activity assays. Colonies grown on LB(Tc) agar (E. coli transformants) or BH(LEm) agar (Bacteroides transconjugants) containing 0.1% medium- viscosity carboxymethylcellulose (CMC) were tested for CMCase activity by the Congo Red method (Teather & Wood, 1982). Quantitative determination of promoter expression in Bacteroides cells was done by inoculating cells from a 16–18 h BHI pre-culture into pre-warmed MM (50-fold dilution) supplemented with high (7.5 mm) or low (0.05 mm) concentrations of (NH₄)₂SO₄. Cell-free extracts were prepared by harvesting samples (100 ml) of cells by centrifugation at various times during the growth cycle. The cells were washed with phosphate/citrate (PC) buffer (50 mm-K₂HPO₄, 14 mm-citric acid, pH 6.0; 100 ml), resuspended in the same buffer (2 ml), and disrupted by sonication. EG activity was determined by the dinitrosalicic acid (DNS) assay for the release of glucose equivalents from 1% (w/v) CMC during 30 min incubation at 50 °C (Ghose, 1987). Protein concentrations were determined using the biuret protein assay (Gornall et al., 1949). The repression ratio relating EG activity under high-/low-nitrogen conditions was calculated.

Sequence analysis and primer extension studies. DNA sequencing was done by the method of Sanger et al. (1977). Synthetic oligonucleotides (5′-ATAGCTCTTGTAGTGCG-3′ for glnA and 5′- CATAACTAATGTTGC-3′ for egfA) were used as internal primers. Transcriptional start points of the C. acetobutylicum egfA gene and the B. fragilis glnA gene were determined using E. coli HB101 (pPH171) and B. fragilis Bl1 total RNA respectively (Hill et al., 1989) (Fig. 2).

RNA hybridization analysis. Equal amounts of total RNA (10 mg ml⁻¹) from B. fragilis Bl1(pEGL106), grown under conditions of high (7.5 mm) or low (0.05 mm) (NH₄)₂SO₄, were fixed to Hybond-N' hybridization membrane (Amersham) according to the manufacturer’s instructions, using a dot-blotting apparatus. Gel-purified DNA containing the EcoRI–PstI fragment of pHZ117 or the EcoRI–BstEII fragment of pKS100 (Fig. 1a,b) were prepared as probes for egfA or glnA mRNA respectively by the DNA random-priming digoxigenin (DIG) method (DNA Nonradioactive Labelling Kit, Boehringer Mannheim Biochemica). The labelled probes were hybridized (68 °C) to the mRNA. Hybridization and chemiluminescent detection were carried out according to the manufacturer’s instructions (DIG Luminescent Detection Kit, Boehringer Mannheim Biochemica). Graphical representation of the data was obtained by scanning the autoradiographs using a densitometer, and the peak area was calculated by triangulation of the peaks.

Results

Expression of the C. acetobutylicum P262 endoglucanase gene (eglA) in Bacteroides

Plasmids pVAL-1 (shuttle vector), pEGL117 (shuttle vector carrying intact egfA), and pEGL1 (shuttle vector carrying the promoterless egfA reporter gene) (Table 1) were conjugated from E. coli HB101(R751) or S17.1...
(a) B. fragilis Bfi glnA promoter sequence

\[
\begin{align*}
&\text{XhoI} & -127 & -108 (\text{pEGL3}) & -82 \\
&\text{CTCAGG-N478-AGTCTTTCGCCCCCAACCACAAATAGGGGTTGAAAGAAGAAGAAAAACATTTTTGTTGCCAAAATAC} \\
&\text{mRNA} & \text{-31 (pEGL106)} & -18 \\
&\text{CCCTATATAAATAGGGGTATATTAGAAAAAACATATAATTTTGTGCC~~C~T~~~~C~T~}
\end{align*}
\]

(b) C. acetobutylicum P262 eglA reporter gene

\[
\begin{align*}
&\text{SmaI/SpeI} & -7 \\
* & \text{gcatggtacc} & \text{T} \text{ATAATAGGGGGTATT} & \text{iA} & \text{TTT~} \\
\end{align*}
\]

Fig. 2. Construction of glnA promoter fusions to the eglA reporter gene. Upstream sequences of the glnA gene promoter region (a) were fused to the promoterless eglA reporter gene (b). Fusion pEGL3 contained the XhoI to -108 bp region, and pEGL106 the Smal to -31 bp region fused upstream of the ribosome-binding site of the eglA reporter gene. Abbreviations and symbols: Lower case, vector sequence; N, nucleotide; single underline, direct repeat sequences -31 to -73 bp and -82 to -127 bp upstream of the glnA translation start; double underline, ribosome binding site; dashed underline, restriction endonuclease cloning sites; box, transcription start point; mRNA, transcription start points; *, position at which the glnA promoter sequences are fused to the promoterless eglA reporter gene.

Table 2. Production of EG in high- and low-nitrogen media by B. fragilis Bfi cells containing p VAL-1, pEGL117, pEGL1, pEGL3, or pEGL106

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVAL-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pEGL117</td>
<td>48.3</td>
<td>54.4</td>
<td>0.88</td>
</tr>
<tr>
<td>pEGL1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pEGL3</td>
<td>8.3</td>
<td>20.1</td>
<td>0.41</td>
</tr>
<tr>
<td>pEGL106</td>
<td>29.1</td>
<td>59.3</td>
<td>0.49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth stage*...</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium†...</td>
<td>H</td>
<td>L</td>
<td>H/L‡</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H/L‡</td>
<td>25.7</td>
<td>23.0</td>
<td>1.10</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>7.7</td>
<td>8.2</td>
<td>0.93</td>
</tr>
<tr>
<td>H/L‡</td>
<td>17.5</td>
<td>6.5</td>
<td>2.69</td>
</tr>
<tr>
<td>H</td>
<td>8.3</td>
<td>20.1</td>
<td>0.41</td>
</tr>
<tr>
<td>L</td>
<td>8.7</td>
<td>23.2</td>
<td>0.37</td>
</tr>
<tr>
<td>H/L‡</td>
<td>1.6</td>
<td>32.1</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Bacteria were harvested at early (4–5 h), mid (6–7 h) and late (9–10 h) exponential growth as described in Methods.
† MM was supplemented with 7.5 mM (H) or 0.05 mM (L) (NH₄)₂SO₄.
‡ Repression ratio of EG expression under high: low nitrogen conditions.

Donor cells into B. thetaiotaomicron 5482, B. fragilis 638, and B. fragilis Bfi. Em-resistant transconjugants were analysed for conjugation frequency, the presence of the plasmid in the cytoplasm, and endoglucanase activity on BHI-CMC and MM-CMC agar plates.

The conjugation frequency (number of transconjugants/number of recipients) was higher for B. thetaiotaomicron (5 x 10⁻⁵) than for the B. fragilis strains (3 x 10⁻⁷). All transconjugants carried the shuttle vector in the cytoplasm, indicating that insertion into the chromosome had not occurred. None of the Bacteroides strains containing pVAL-1 produced EG after growth on BHI- or MM-CMC agar. Both B. fragilis 638(pEGL117) and B. fragilis Bfi(pEGL117) produced EG on BHI- and MM-CMC, while B. thetaiotaomicron 5482(pEGL117) produced very low levels of EG as detected by the size of the zones in the Congo red agar plate assay. The Bacteroides strains containing pEGL1 showed markedly less EG activity than those containing pEGL117. The residual low EG activity conferred on B. fragilis by pEGL1 was thought to be due to a weak promoter situated 7 bp upstream from the eglA gene TTG start codon (Fig. 2). These results indicated that the C. acetobutylicum eglA gene was expressed from its own promoter in B. fragilis.

Analysis of the glutamine synthetase promoter fusions

Production of EG by two recombinant plasmids containing different regions of the glnA upstream region fused to the reporter gene was studied (Fig. 2, Table 2). Plasmid pEGL106 contained 584 bp of the glnA up-
stream region from position –31 from the ATG start to the upstream XhoI site. This included the nearly perfect direct repeat sequences (positions –31 to –127 bp from the ATG start) observed by Hill et al. (1989). Plasmid pEGL3 contained 508 bp of the glnA upstream region from position –108 from the ATG start to the upstream XhoI site.

These plasmids were conjugated into the Bacteroides recipients and EG was assayed qualitatively by activity on BHI-CMC agar plates, and quantitatively in MM broth under high and low nitrogen conditions (Table 2). pEGL106 conferred significant EG activity on the Bacteroides recipients as indicated by the Congo red plate assay method. The level of activity was greatest in B. fragilis Bfl and B. fragilis 638 (largest zones), and very low in B. thetaiotaomicron 5482. B. fragilis strains containing pEGL3 produced small but detectable zones on agar plates stained with Congo red, but no activity was detected in B. thetaiotaomicron 5482(pEGL3).

The activity of EG was quantified as a measure of eglA gene expression under the control of the glnA promoter. All three Bacteroides strains containing the two putative promoter fusions were grown in MM broth cultures from early to late exponential phase under conditions of high and low nitrogen. Bacteroides strains containing pVAL-1, pEGL117 or pEGL1 were used as controls. Maximum expression of the reporter gene in B. fragilis Bfl(pEGL106) occurred at early exponential phase (Table 2). B. fragilis Bfl(pEGL106) produced more EG in the low-nitrogen medium than in the high-nitrogen medium. Regulation by nitrogen was greatest at late exponential phase, and repression ratios of EG activity were 0.49, 0.37 and 0.05 for early, mid- and late exponential phase, respectively (Table 2). Production of EG by B. fragilis Bfl(pEGL3) in the early exponential phase was lower than that of B. fragilis Bfl(pEGL106), and it showed no consistent regulation by the levels of nitrogen in the growth medium. EG expression in B. fragilis Bfl(pEGL3) varied in the low- and high-nitrogen media such that in early exponential phase more EG was produced in cells grown in the low-nitrogen medium whereas in mid-exponential phase the opposite was observed. This result indicates that, while the glnA promoter region present in pEGL3 is still functional during strong exponential growth of the cells, an essential region involved in nitrogen regulation has been disrupted and is no longer present. B. fragilis Bfl(pEGL1) produced low levels of EG, presumably from the weak C. acetobutylicum mRNA transcription start (Fig. 2) still present in the reporter gene. Similar amounts of EG were produced in high- and low-nitrogen media in mid- and late exponential phase cells. No EG activity was detected in B. fragilis Bfl(pVAL-1). B. fragilis Bfl(pEGL117) cells produced relatively high levels of EG activity which were not markedly affected by growth in high- or low-nitrogen medium and which reached a maximum in early exponential phase (Table 2). Similar results were obtained with B. fragilis 638 containing the various plasmids, but very low levels of activity with no regulation by nitrogen occurred in B. thetaiotaomicron 5482 containing the various plasmids (results not shown).

**RNA analysis**

RNA isolated from B. fragilis Bfl(pEGL106) at early exponential phase was used to determine whether the observed regulation of EG activity was occurring at the level of transcription, and whether the reporter gene was being co-regulated with the chromosomal glnA gene. Hybridization of equal amounts of total RNA, extracted from B. fragilis Bfl(pEGL106), to both eglA and glnA internal DNA probes showed that both the eglA and glnA mRNA transcripts occurred at higher levels under low-nitrogen conditions than when there was excess nitrogen present (Fig. 3). The nitrogen repression ratios (high:low) at the mRNA level, as calculated from the peak absorbances of the scans, were 0.52 and 0.43 for glnA and eglA, respectively.

**Discussion**

The B. fragilis reporter gene system described in this work facilitated the rapid quantitative and qualitative assay of promoter activity in both E. coli and B. fragilis. Previously described vectors of this type have had certain
drawbacks. The promoterless erythromycin resistance vector pFD214 (Smith, 1987), can be used qualitatively, but activity is difficult to quantify routinely, and promoter constructs cannot be tested in E. coli. The β-glucuronidase reporter gene vectors pMJF-2 and pMJF-3 (Feldhaus et al., 1991) can be used quantitatively, but activity cannot be observed anaerobically in plate assays.

We have used the C. acetobutylicum P262 eglA gene, which encodes CMCase activity, to quantify promoter functions in a range of Bacteroides strains. It was shown that only low levels of CMCase activity could be detected when eglA was functioning off either the C. acetobutylicum eglA promoter or the B. fragilis Bfl1 glnA promoter in B. thetaiotaomicron, and no regulation of the glnA promoter by nitrogen was observed. Good expression of the eglA gene was observed in B. fragilis strains, with repression of the glnA occurring under high-nitrogen conditions. This is of interest since it indicates possible differences in promoter recognition mechanisms in the two species. This supports the theory that the colonic Bacteroides species are genetically distinct from one another (Salyers, 1984).

Very little is known about Bacteroides promoters, and promoter fusion analysis of the B. fragilis Bfl1 glnA upstream region can contribute useful information in this regard. Hill et al. (1989) identified a putative Shine-Dalgarno (Shine & Dalgarno, 1976) ribosome-binding site, mRNA transcriptional starts and an ATG start codon (Fig. 2). In this study it was shown that promoter expression and nitrogen regulation of the glnA gene in B. fragilis Bfl1 appeared to be dependent on the −31 to −107 bp upstream region of the glnA gene, spanning most of the two direct repeat sequences (−31 to −127 bp), since efficient expression and nitrogen regulation of eglA did not occur in B. fragilis Bfl1 (pEGL3) which contained only the −108 to −127 bp portion of this region.

The observed regulation of eglA expression by nitrogen in B. fragilis Bfl1 cells containing pEGL106 is considered to be an accurate reflection of glnA expression in B. fragilis Bfl1 since the production of EG by cells containing the control plasmid, pEGL117, carrying the eglA gene expressed from the C. acetobutylicum P262 promoter was not regulated by variations in the level of nitrogen in the growth medium. Furthermore, hybridization analysis of the mRNA transcripts produced by pEGL106 and the chromosomal glnA gene in B. fragilis Bfl1 showed that they were both regulated by nitrogen at the level of transcription. While the exact levels of EG produced by the multicopy glnA::eglA fusion plasmid pEGL106 cannot be directly compared quantitatively with the GS produced from the chromosomal glnA gene, the nitrogen repression ratios of each gene (high:low) at the mRNA level, as calculated by peak triangulation of the scans, may be directly compared. These were of approximately the same order of magnitude for both the chromosomal glnA gene and the eglA reporter gene functioning off the glnA promoter (pEGL106) (0.52 and 0.39 respectively). This correlated with the repression ratio for EG enzyme activity in B. fragilis Bfl1 (pEGL106) (0.49), assayed from the same cell samples.

These studies on the expression and regulation of the B. fragilis Bfl1 glnA gene, which could not be assayed by conventional methods due to inhibition of GS activity in cell extracts, correlate with the findings of Yamamoto et al. (1984) that GS activity in B. fragilis ATCC 23745 was regulated by nitrogen, and that higher levels of glnA expression occurred under conditions of limiting nitrogen.

The C. acetobutylicum P262 eglA reporter gene shuttle vector is, therefore, a useful tool in the qualitative and quantitative analysis of gene expression in B. fragilis.

References


