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

VALERIE R. ABRATT,¹ HAROLD ZAPPE² and DAVID R. WOODS^{1*}

¹Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

²Virginia Polytechnic and State University, Blacksburg, Virginia 24061, USA

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The *Clostridium acetobutylicum* *eglA* gene, encoding a β -1,4-endoglucanase (EG), was shown to be a useful reporter gene for the study of gene expression in *Bacteroides fragilis*. The *eglA* reporter gene has the advantages that it can be easily identified in both *Escherichia coli* and *B. fragilis* on agar media containing carboxymethylcellulose, and EG production can be rapidly quantified in liquid medium. Since the *B. fragilis* glutamine synthetase (GS) is inactivated in permeabilized cells and cell extracts, the *eglA* reporter gene was used to study the regulation of GS production in *B. fragilis*. Gene fusions containing the GS *glnA* promoter region fused to the promoterless *eglA* gene showed that *glnA* expression was regulated by nitrogen in *B. fragilis* at the transcriptional level. A *glnA* upstream region containing a near-perfect direct repeat sequence was essential for efficient GS expression and for regulation by nitrogen.

Introduction

Bacteroides fragilis is a strictly anaerobic, pathogenic bacterium found in the human digestive tract (Salysers, 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

* Author for correspondence. Tel. (021) 650 3270; fax (021) 650 4023; e-mail sec@micro.uct.ac.za.

Abbreviations: DIG, digoxigenin; EG, endoglucanase; GDH, glutamate dehydrogenase; GS, glutamine synthetase.

Table 1. *Bacterial strains and plasmids*

Strain	Relevant phenotype*		Description*	Reference/source
	<i>E. coli</i>	<i>Bacteroides</i>		
<i>E. coli</i>				
HB101	Gm ^s			Boyer & Roulland Dussoix (1969)
S17.1	Gm ^s RP4-2			Simon <i>et al.</i> (1983)
<i>Bacteroides</i>				
<i>B. fragilis</i> Bf1		Em ^s Gm ^r		Mossie <i>et al.</i> (1979)
<i>B. fragilis</i> 638		Em ^s Gm ^r		Privitera <i>et al.</i> (1979)
<i>B. thetaiota-omicron</i> 5482		Em ^s Gm ^r		A. A. Salyers, University of Illinois, Urbana, USA
Plasmids				
pHZ117	Ap ^r EG ⁺		<i>Ca eglA</i> gene in pUC19	Zappe (1988)
pJS139	Ap ^r GS ⁺		<i>Bf glnA</i> gene in pEcoR251	Southern <i>et al.</i> (1986)
R751	IncB(eta)			Shoemaker <i>et al.</i> (1985)
	Tra ⁺ Mob ⁺			
pVAL-1	Mob ⁺ Rep ⁺	Mob ⁺ Rep ⁺		Valentine <i>et al.</i> (1988)
	Tc ^r Ap ^r	Em ^r		
pEGL117	Mob ⁺ Rep ⁺	Mob ⁺ Rep ⁺	Complete <i>eglA</i> from pHZ117 in pVAL-1	This work
	Tc ^r Ap ^s EG ⁺	Em ^r EG ⁺		
pEGL1	Mob ⁺ Rep ⁺	Mob ⁺ Rep ⁺	Reporter gene: promoterless <i>eglA</i> gene from pHZ117 in pVAL-1	
	Tc ^r Ap ^s EG ⁺	Em ^r EG ⁺	Reporter gene in pEGL1 fused to 508 bp upstream region of <i>glnA</i>	
pEGL3	Mob ⁺ Rep ⁺	Mob ⁺ Rep ⁺	Reporter gene in pEGL1 fused to 584 bp upstream region of <i>glnA</i>	
	Tc ^r Ap ^s EG ⁺	Em ^r EG ⁺		
pEG106	Mob ⁺ Rep ⁺	Mob ⁺ Rep ⁺		
	Tc ^r Ap ^s EG ⁺	Em ^r EG ⁺		

* Abbreviations used: r, resistance; s, sensitivity; Ap, ampicillin; Tc, tetracycline; Em, erythromycin; 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.

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 (Holderman *et al.*, 1977), with the addition of erythromycin (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 B₁₂ 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 7.5 or 0.05 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-Horowitz & 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

Fig. 1. Construction and restriction endonuclease maps of pKS100, pUCp1, pUCpΔ, pHZ117, pHZ117Δ, pEGL1 and pEGL106. (a) *B. fragilis glnA* deletion plasmids; (b) *C. acetobutylicum eglA* reporter gene and fusions. Abbreviations: E, *EcoRI*; H, *HindIII*; X, *XhoI*; Sa, *Sall*; Sm, *SmaI*; BE, *BstEII*; Ss, *SspI*; P, *PstI*; K, *KpnI*; EV, *EcoRV*. Single lines, vector regions; hatched bars, *glnA*; solid bars, *eglA*; arrows, orientation of the *glnA* and *eglA* promoter regions.

primer extension; V. Abratt, unpublished results) was subcloned between the *SmaI* and *PstI* restriction sites of pUC19 (pHZ117Δ). The *EcoRI*–*PstI* fragment of pHZ117Δ was then cloned between the *EcoRI* and *PstI* sites of pVAL-1 to form the promoterless reporter gene shuttle vector pEGL1.

Promoter fusions were constructed using the *B. fragilis glnA* promoter region. The 2.4 kb *XhoI* fragment of pJS139 (Southern *et al.*, 1986) was cloned into the Bluescript (pKS) (Stratagene) *SaI* site in the same orientation (pKS100). The 1.25 kb *EcoRI*–*SmaI* fragment of pKS100 containing the *glnA* upstream region (615 bp) and part of the structural gene (635 bp) (Fig. 1a) was subcloned between the *EcoRI* and *SmaI* sites of pUC19 (pUCp1). The latter plasmid was subjected to exonuclease III digestion from the *PstI* site (Henikoff, 1984) to create a set of deletion plasmids containing various fragments of the upstream

region of the *glnA* gene (pUCpΔ). These plasmids were pooled, digested with *EcoRI* and *SphI* (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 BHI(Em) 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 $(\text{NH}_4)_2\text{SO}_4$. 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- K_2HPO_4 , 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 dinitrosalicylic 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 *eglA*) were used as internal primers. Transcriptional start points of the *C. acetobutylicum eglA* gene and the *B. fragilis glnA* gene were determined using *E. coli* HB101(pHZ117) and *B. fragilis* Bf1 total RNA respectively (Hill *et al.*, 1989) (Fig. 2).

RNA hybridization analysis. Equal amounts of total RNA (10 mg ml^{-1}) from *B. fragilis* Bf1(pEGL106), grown under conditions of high (7.5 mM) or low (0.05 mM) $(\text{NH}_4)_2\text{SO}_4$, 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 *eglA* 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 *eglA*), and pEGL1 (shuttle vector carrying the promoterless *eglA* reporter gene) (Table 1) were conjugated from *E. coli* HB101(R751) or S17.1

(a) *B. fragilis* Bf1 *glnA* promoter sequence

XhoI -127 -108 (pEGL3) -82
CTCGAG-N₄₇₈-AGTCTCTTCGCCCAACAAACAAATAGGGGTGTACAAAGAAAAACATATTTATTTTGCAAAATAC

-73 -31 (pEGL106) -18 mRNA
CCCTATAAAAAATAGGGGTATATTAGAAAAAACATATAATTTTGTGCCCAACAAACATAAAAGAGACACCATCATG

(b) *C. acetobutylicum* P262 *eglA* reporter gene

(*SmaI*/*SspI*) weak mRNA
-7
***gcatgggtacccATTATAATAGGGGTATTAACTTGTTTTCAAAAA**

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 *Xho*I to -108 bp region, and pEG106 the *Xho*I 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* Bf1 cells containing pVAL-1, pEGL117, pEGL1, pEGL3, or pEGL106

The mean results of at least three separate experiments are shown; the deviation from the mean did not exceed 15%.

EG activity [nmol glucose equiv. (mg protein) ⁻¹ min ⁻¹]									
Growth stage*...	Early			Mid			Late		
Medium†...	H	L	H/L‡	H	L	H/L‡	H	L	H/L‡
Plasmid									
pVAL-1	0	0		0	0		0	0	
pEGL117	48.3	54.4	0.88	25.7	23.0	1.10	32.5	39.4	0.82
pEGL1	0	0		7.7	8.2	0.93	7.2	8.5	0.84
pEGL3	8.3	20.1	0.41	17.5	6.5	2.69	7.9	9.1	0.87
pEGL106	29.1	59.3	0.49	8.7	23.2	0.37	1.6	32.1	0.05

* 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) $(\text{NH}_4)_2\text{SO}_4$.

‡ Repression ratio of EG expression under high:low nitrogen conditions.

donor cells into *B. thetaiotaomicron* 5482, *B. fragilis* 638, and *B. fragilis* Bf1. 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* (5×10^{-5}) than for the *B. fragilis* strains (3×10^{-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* Bf1(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 *Xho*I 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 *Xho*I 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* Bf1 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* Bf1(pEGL106) occurred at early exponential phase (Table 2). *B. fragilis* Bf1(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* Bf1(pEGL3) in the early exponential phase was lower than that of *B. fragilis* Bf1(pEGL106), and it showed no consistent regulation by the levels of nitrogen in the growth medium. EG expression in *B. fragilis* Bf1(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* Bf1(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* Bf1(pVAL-1). *B. fragilis* Bf1(pEGL117) cells produced relatively high levels of

Fig. 3. Production of *glnA* (a) and *eglA* (b) mRNA in early exponential phase cells of *B. fragilis* Bf1(pEGL106). Equal amounts of total mRNA from cells grown in high (7.5 mM) or low (0.05 mM) $(\text{NH}_4)_2\text{SO}_4$ MM were hybridized to DIG-labelled internal DNA fragments of the *eglA* or *glnA* genes and the levels of mRNA transcripts determined by densitometer scans of the chemiluminescent autoradiographs. Representative results from three experiments are shown. I, high nitrogen; II, low nitrogen.

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* Bf1(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* Bf1(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* Bfl *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* Bfl *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* Bfl 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* Bfl (pEGL3) which contained only the –108 to –127 bp portion of this region.

The observed regulation of *eglA* expression by nitrogen in *B. fragilis* Bfl cells containing pEGL106 is considered to be an accurate reflection of *glnA* expression in *B. fragilis* Bfl 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* Bfl 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* Bfl(pEGL106) (0.49), assayed from the same cell samples.

These studies on the expression and regulation of the *B. fragilis* Bfl *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*.

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