Cloning and nucleotide sequence of the *Butyrivibrio fibrisolvens* gene encoding a type III glutamine synthetase

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A *Butyrivibrio fibrisolvens* *glnA* gene encoding glutamine synthetase (GS) was cloned on a recombinant plasmid pGS4 which enabled *Escherichia coli* *glnA* deletion mutants to utilize $(\text{NH}_4)_2\text{SO}_4$ as a sole source of nitrogen. The nucleotide sequence of a 2423 bp DNA segment containing the GS-coding region of *B. fibrisolvens* was determined and the complete amino acid sequence (701 residues) was deduced. Comparisons of the derived *B. fibrisolvens* GS protein sequence with the amino acid sequences of GS from other bacteria indicate that it is the second reported example of a type III GS, originally identified in the obligate anaerobe *Bacteroides fragilis*. The presence of GS in *B. fibrisolvens* cells and the regulation of the cloned GS in *E. coli* cells was demonstrated by Western blot analysis.

Introduction

The genus *Butyrivibrio* is composed of Gram-negative anaerobic bacteria that ferment a wide variety of carbohydrates with the production of large amounts of butyric acid. *Butyrivibrio fibrisolvens* is a ubiquitously distributed bacterial species in the gastro-intestinal tracts of ruminants and other mammals.

Ammonia is the major and often a required source of nitrogen for most rumen bacteria (Bryant & Robinson, 1962). Enzymes involved in the assimilation of ammonia are essential for the growth of most rumen organisms. In prokaryotes, glutamate dehydrogenase (GDH) and the dual enzyme system, glutamine synthetase (GS) and glutamate synthetase (GOGAT), are the two most important routes by which ammonia may be assimilated (Hespell, 1984).

There is a paucity of information concerning the pathways and regulation of ammonia assimilation in rumen organisms. Studies with $^{15}\text{NH}_4^+$ have shown that bacterial amide is the most rapidly labelled nitrogen pool in the bacterial population of the rumen over a range of

ruminal ammonia concentrations. These results imply that GS is an important route of ammonia assimilation in the bacterial population of the rumen (Smith *et al.*, 1981).

Until recently, it appeared that prokaryotes had two forms of GS termed GSI and GSII. The majority of bacteria investigated have GSI-type enzymes but members of the *Rhizobiaceae*, *Frankiaceae* and *Streptomyces* contain both GSI- and GSII-type enzymes (Darrow & Knotts, 1977; Kumada *et al.*, 1990; Rochefort & Benson, 1990). GSI is the typical prokaryotic GS (Streicher & Tyler, 1980) whereas GSII is similar to eukaryotic GS enzymes (Carlson & Chelm, 1986). The GS gene from the clinical isolate *Bacteroides fragilis* Bf-1 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 GS from *B. fragilis* differs markedly from the GS enzymes of prokaryotes, eukaryotes and archaeobacteria. The holoenzyme is a single disc of six identical subunits with an M_r of approximately 490 000. The GSI and GSII holoenzymes are dodecamers and octamers, respectively. The *B. fragilis* GS subunit is large and has a calculated M_r of 82 827. It contains 729 amino acids, and is approximately 270 and 400 amino acids longer than the GSI and GSII subunits, respectively, of other prokaryotes and eukaryotes. Because of these differences, Hill *et al.* (1989) suggested that GS enzymes with large subunits arranged as hexamers should be grouped together to form a third class of GS enzyme termed

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Abbreviations: GS, glutamine synthetase; ORF, open reading frame.

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1 TACTAAGGAGTCACTGTTATACAGAGAAATATTTGAAAAATACTATCCGGGCCAGTCAGATATGGTTATTGATTCTGGATGCCCTAACAGAGCTGGAGGGCTGCAATGTAACAGATCCTTCAG
 126 CAAGAGTTTTATCGAATTATGGAGCCAGCGGTCAAGGCTCATAAATAAAGAAAAACGGAGGAGATGGCAATGATCGAAGCGAGCAAATTAACAACAGAAATTGGAACTCTAGTATTTAA
 M I E A S K L T T E F G S L V F N
 251 CGACAAGATTATGAAGAACGCCTTCCCAAGGATATCTACAAGGCAGTTCATAAGACAATTGAAAAGGAACACATCTTGAGCCTGGATGTAGCTACTCGGTAGCAGCAACCATGAAGGAGTGG
 D K I M K E R L P K D I Y K A V H K T I E K E P H L E P G C S Y S C S S N H E G V G
 376 GCAATCGAGAACAGTGTACCACTTCACCCACCTGGTCCAGCCCAATGACAGGTCTTACAGCTGAAAAGCAGCAGTTCATTCTCCAACAGAAAGATGGCAGATCATCATGGAGTTCTCAG
 N R E Q C Y H F T P P G S S P N T G L T A E K H D S F I S P T E D G R S S W S S Q
 501 GAAAAGAGCTGGTTAAGGGCGAACCTGATGCATCAAGCTTCCAGTGGTGGTCTTCGTGTCACAATTCGAGCATCGGAGTTATCAGCATGGGACTCTTCATCACCATGCATTATCAAGGATGG
 E K S W L R A N L M H Q A S Q W W S S C H N S S M R G Y Q H G I L H H A F I K D G
 626 TTCCTTTTACTTCCAACAGCTTTCTGCTCATACGGCGGAGAAGCTCTTGATAGAGACTCACTTCTCGTTCAATGGAGGCTCTTCAAATGAAGCGGTAAAGATGATGCGTCTTCTGGATATG
 S L L L P T A F C S Y G G E A L D R D S L L R S M E A L S N E A V K M M R L L G Y E
 751 AGGATGTTAACCGGTAATACTACAATCGGTTCAAGACAGGAATATTTCCCTTATCGACAAGGATTCTTAAGAAGAGGAAGATTGCTTCTTACAGCGAGAACACTTATCGGTGCTCCAGCC
 D V N R V N T T I G S E Q E Y F L I D K D F Y K K R K D L L L T G R T L I G A P A
 876 AGCAAGGGTCAGGAATGGAAGATCACTACTTCGAGTTATTAAGCCTAAGGTATCTGCATACATGCATGACCTTGATGAAGAGCTCTGGAAGCTTGGAAATCCCTGCTAAGACCAAGCACAACGA
 S K G Q E M E D H Y F G V I K P K V S A Y M H D L D E E L N R L G I P A K T K H N E
 1001 GGTTCACCTTCTCAGCAGAGCTGGCTCCTGTATTTGAGACAGCAACATCGCTGTAGATCACACCAGCTCACAATGGAAGTTATGAAGAAGGTTGCTGACAAGCACAACATATGCATGTCTTC
 V A P S Q K E L A P V F E T A N I A V D H N Q L T M E V M K K V A D K H N Y A C L L
 1126 TTCACGAGAAGCATTGAGGGAGTTAAGGATCTGGTAAGCACAATAACTGGTCAATCTGCACAGATACAGGAATCAACCTTCTTGATCCAGGTAAAGAACCCAGGCGAGAATCCTTTTCTT
 H E K P F E G V N G S G K H N N W S I C T D T G I N L L D P G K N P G E N I P F L
 1251 GTCTTTCTTATGCTGTTATCGCAGCAGTTGATGAGTATGCACCAATCCTCAGACTTTCAGTAGCATCTGCCGGAACGACCACAGACTTGGAGGAAACGAGGCACCTCCAGCAATCATCTCTAT
 V F L M S V I A A V D E Y A P I L R L S V A S A G N D R R L G G N E A P P A I I S I
 1376 CTTGCTGGTGATGAGCTTGCAAGTACTCAAGGCTGTTGAAGCGGAGAAGCTTACAAGGCAGGCAAGTCTCAGATGACATGGGAGCAACAGTACTTCACCTTCAACAGGACAACACAG
 F V G D E L A E V L K A V E A G E A Y K A A G K S Q M T W E Q Q Y F T F T K D N T D
 1501 ACAGAAACAGAACATCACCTTTTCTTCACTGGTAACAAGTTCAAGTCAAGTGGTGTGACAGTCTGTTGCTAACGGTAAATATGGTCTCAACACATGCACTTGCAAGGAAGTAGCAACG
 R N R T S P F A F T G N K F E S D G G H S S V A N G K Y G P Q H M Q L Q K E V A T
 1626 CTTAATGCAAAAGCTTTCAGCATACAGCGAGATGAGCTTAAAGAGAAAGTTAAGGAAGTTCTTAAAGAGACACTTCTTGACATAAGAGAGTTCTTTTCAACGGAAACGGATATACAGACGAGTG
 L N A K L S A Y S G D E L K E K V K E V L K E T L L A H K R V L F N G N G Y T D E W
 1751 GGTTCAGGAAGCTGCTAAGAGAGGCTTCCAAACCTTAAGGCTCTCCAGATTGCATGCCATAGTGGATCAGCGATGAGTCAATAGATCTTTTCAACAGACACGGTATCTTCACCAAGGAAGAGA
 V E E A A K R G L P N L K A L P D C M P Y W I S D E S I D L F T R N G I F T K E E I
 1876 TTTACTCAAGATATGAGATCCTCCTTGAGAACTATTCTAAGTCAATTCATATTGAGTCTCTCACAAATGAGGAGATGATCAGAAAAGACTTAACAGAAGGCTTGTAGCATATGAAAAAGATCTG
 Y S R Y E I L L E N Y S K S I H I E S L T M Q E M I R K D L T E G L V A Y E K D L
 2001 TCAAAAGAGATTGTTCAAGAAAAGAGCCTTCTCGATGGAGACTGCTGTGCACTTGAGCTGGGAGTTCTTAAGTCTCTTGATAAGTCCAGCGCAGAGATGGGCAAGGCACCTTCAAGCTCTTCGA
 S K E I V Q K K S L L D G D C C A L E L G V L K S L D K S S A E M G R A L S K L F E
 2126 GGATACCAAGAAGGCAGAGGGCATGACAGAAGCTCTTGAGACAGCAAGTTACTATGAGAGCACAGTCTTGACAGATATGGATGAGCTTAGAAAAGTACGCTGATGAGGCAGAGGCACCTTATCCAG
 D T K K A E G M T E A L E T A S Y Y E S T V L A D M D E L R K Y A D E A E A L I P E
 2251 AGAAGTACCTTAGCTATCCAACATACGGAGAGATGCTGTTCTCACTTAGATAATCTGAGTGCCGCTAAATATCATAAATGATTAGGGCGATTATTGCGGATCCAAATACAATTATCGCCCAT
 K Y L S Y P T Y G E M L F S L R *
 2376 AATTGCATATTTAGTTACAAGGGAAGGAGATACATCCACATGGAAC

GSIII. We report the cloning and characterization of the *glnA* gene from the rumen anaerobe *B. fibrisolvens*. This *glnA* gene encodes a GS which is similar to the *B. fragilis* GS and is the second example of a GS which belongs to the GSIII class.

Methods

Bacterial strains, vectors, and growth conditions. Chromosomal DNA from *B. fibrisolvens* H17c (Dehority, 1966) was used for the cloning of the *B. fibrisolvens glnA* gene. The *E. coli* host strain used for the original cloning experiment was the YMC11 *glnA* deletion mutant (Backman *et al.*, 1981). Subcloning and other genetic manipulations were carried out in *E. coli* LK111 (Zabeau & Stanley, 1982). The *E. coli*-*Bacillus subtilis* shuttle vector pEB1, used for the construction of the *B. fibrisolvens* genomic library, has been described previously (Lin *et al.*, 1990). Subcloning for sequencing was done using the high copy number pUCBM20 and -21 vectors (Boehringer Mannheim). *B. fragilis* strain Bf-1 was used by Southern *et al.* (1986, 1987) and Hill *et al.* (1989) for the cloning and molecular analysis of the *B. fragilis glnA* gene. *B. fibrisolvens* H17c was grown in M10 medium as described by Strydom *et al.* (1986). *E. coli* strains were grown in Luria-Bertani (LB) medium or glucose minimal medium (Miller, 1972) with 1 g (NH₄)₂SO₄ l⁻¹ (GMMN) as the sole nitrogen source. Glucose minimal medium for nitrogen regulation studies contained 15 mM-glutamine (GMMML) or 15 mM-glutamine and 13 mM-(NH₄)₂SO₄ (GMMH). Ampicillin (100 µg ml⁻¹) was added to media for plasmid selection and maintenance. *B. fragilis* Bf-1 was grown on supplemented brain heart infusion (BHI) agar (Holdeman & Moore, 1972) as described by Hill *et al.* (1989).

Preparation of DNA and recombinant DNA techniques. Construction of the *B. fibrisolvens* H17c genomic library in pEB1 has been described (Lin *et al.*, 1990). Plasmid DNA prepared from pools of clones containing *B. fibrisolvens* DNA was used to transform the *E. coli* YMC11 *glnA* deletion strain. An *E. coli* YMC11 GlnA⁺ ampicillin resistant transformant was isolated on GMMN medium. DNA fragments for subcloning were purified from 0.8% (w/v) agarose Tris/acetate gels by adsorption onto glass beads (Gene Clean). The preparation of plasmid DNA and restriction endonuclease mapping of the clones was done using standard techniques (Sambrook *et al.*, 1989). *B. fibrisolvens* chromosomal DNA was prepared as described by Berger *et al.* (1989) and Southern hybridization was used to confirm that the insert DNA originated from *B. fibrisolvens*.

Nucleotide sequence determination and analysis. The Exo-III mung bean nuclease technique was used to create a deletion series for sequencing (Henikoff, 1984). The nucleotide sequence of the *glnA* gene was determined by the dideoxy-chain termination method using the Sequenase 2.0 kit (US Biochemical Corp.) according to the manufacturer's specifications. The nucleotide and deduced amino acid sequences were analysed using the Genetics Computer Group Inc. software package (version 7.0). All the current databases accompanying the GCG package were screened for related nucleotide and amino acid sequences.

Western immunoblotting. Western blotting from SDS-PAGE gels onto nitrocellulose membranes was done by the method of Towbin *et al.* (1979). *B. fragilis* GS antibody binding and the development of bands using a goat anti-rabbit serum conjugated to horse-radish peroxidase were done as described previously (Southern *et al.*, 1987).

Results and Discussion

Cloning and expression of a GS gene from *B. fibrisolvens* in *E. coli*

The *E. coli glnA* deletion strain YMC11 was transformed with the genomic library of *B. fibrisolvens*. An *E. coli* YMC11 GlnA⁺ transformant was isolated on GMMN agar plates containing ampicillin. The resident plasmid was designated pGS4 and was shown to harbour a 6.1 kb insert of *B. fibrisolvens* DNA. A 5.3 kb fragment of pGS4 insert DNA was subcloned in both orientations using the pUCBM20 and -21 sequencing vectors (pGS20 and pGS21, respectively). Both subclones retained GS activity. Hybridization of *Hpa*I and *Bgl*II fragments internal to the pGS20 insert to *B. fibrisolvens* H17c chromosomal fragments of the same size confirmed the origin of the cloned DNA fragment (data not shown).

Nucleotide sequence of the *B. fibrisolvens glnA* gene

The nucleotide sequence of a 2423 bp region of *B. fibrisolvens* DNA containing the *glnA* gene was determined (Fig. 1). The DNA sequence contained an open reading frame (ORF) of 2103 nucleotides, which began with an ATG codon at position 201 and ended with a TAA codon at position 2303. The predicted size of the polypeptide encoded by this ORF was 701 amino acids, which has a calculated *M_r* of 79645. A potential ribosomal binding sequence (GGAGGAGAG) was situated 6 bp upstream of the ATG initiation codon. No significant sequence homology was found upstream of the initiation codon with the canonical *E. coli* σ70 promoter sequence or with the promoter consensus sequence for genes under *ntr* control (σ54) (Dixon, 1984; Reitzer & Magasanik, 1985). The *B. fibrisolvens glnA* regulatory region was scanned for the presence of a consensus sequence similar to the consensus established for the binding of the *ntrC* gene product to the *glnA* promoter region in enteric bacteria (Dixon, 1984). Such a sequence could not be found. A 13 bp inverted repeat sequence capable of forming a stem-loop structure was located 33 bp downstream of the putative *glnA* stop codon.

Comparison of the translated amino acid sequence with sequences of GS enzymes from other organisms

The degree of amino acid sequence homology between the *B. fibrisolvens* GS and those of other prokaryotes was determined on the basis of identical amino acid sequence

Fig. 1. (facing page). Nucleotide and deduced amino acid sequences of the *B. fibrisolvens glnA* gene and flanking regions. The initiation codon ATG is at nucleotide 200 and the termination codon TAA is at nucleotide 2301. The derived amino acid sequence of 701 amino acids is given in the one letter code. The putative ribosome binding site and the inverted repeat sequence are underlined.

Table 1. Comparison of the identity of the amino acid sequences of GS enzymes from various bacteria

The results are given as the percentage identity of the amino acid residues in the GS enzymes.

Sequence data of the proteins were taken from Hill *et al.* (1989) for *B. fragilis* (BFRA), Bozouklian & Elmerich (1986) for *Azospirillum brasilense* (ABRA), Colonna-Romano *et al.* (1987) for *Rhizobium leguminosarum* (RLEG), Colombo & Villafranca (1986) for *E. coli* (ECOL), Janson *et al.* (1986) for *Salmonella typhimurium* (STYP), Toukdarian *et al.* (1989) for *Azotobacter vinelandii* (AVIN), Cardy & Murrell (1990) for *Methylococcus capsulatus* (MCAP), Rawlings *et al.* (1987) for *Thiobacillus ferrooxidans* (TFER), Tumer *et al.* (1983) for *Anabaena* sp. (ANAB), Nakano *et al.* (1989) for *Bacillus cereus* (BCER), Strauch *et al.* (1988) for *B. subtilis* (BSUB), Janssen *et al.* (1988) for *Clostridium acetobutylicum* (CACE), Possot *et al.* (1989) for *Methanococcus voltae* (MVOL) and *B. fibrisolvens* (BFIB) (this paper). The sequence data for *Neisseria gonorrhoeae* (NGON) were obtained from the GenBank databank (accession no. M84113).

	BFRA	ABRA	RLEG	ECOL	STYP	AVIN	MCAP	TFER	NGON	ANAB	BCER	BSUB	CACE	MVOL
BFIB	40	22	25	24	25	21	22	21	22	24	26	24	22	22
BFRA		23	22	22	21	24	20	20	24	25	23	24	20	22
ABRA			68	62	63	64	63	62	62	58	43	44	39	41
RLEG				64	64	64	63	58	57	56	41	41	39	39
ECOL					97	69	68	65	63	55	44	43	42	39
STYP						69	69	65	63	55	44	44	42	40
AVIN							71	67	65	53	42	43	42	41
MCAP								70	63	54	41	40	37	38
TFER									64	55	42	41	39	37
NGON										54	39	40	38	39
ANAB											35	38	36	38
BCER												86	62	51
BSUB													64	52
CACE														52

comparisons (Table 1). The highest amino acid sequence identity was obtained with the GS subunit sequence from *B. fragilis* (40% identity). An analysis of Table 1 places the GS enzymes from Gram-negative and Gram-positive organisms in separate clusters based on homology, with the GS enzymes from *B. fibrisolvens* and *B. fragilis* showing a greater divergence in sequence similarity.

Alignment of the predicted GS polypeptide sequences from *B. fibrisolvens* and *B. fragilis* revealed considerable homology in a number of highly conserved blocks, particularly in the central regions of the proteins (Fig. 2). The *B. fibrisolvens* GS is 28 amino acid residues shorter than the *B. fragilis* GS enzyme, with the majority of the missing amino acid residues being located at the N-terminal end (residues 1–20) of the *B. fragilis* GS protein. Nevertheless, the two sequences share 40% amino acid identity when optimally aligned.

Five major regions of amino acid similarity between the prokaryotic type I and the eukaryotic type II GS enzymes have been identified (Rawlings *et al.*, 1987). The active site of the type I GS is formed between enzyme subunits, and it has been shown that the five conserved amino acid regions are associated with the proposed GS active site (Almassy *et al.*, 1986). Regions II to V are β -strands associated with two Mn^{2+} cations of one subunit while region I contains the tryptophan residue which is thought to complete the active site formed between adjacent subunits.

Hill *et al.* (1989) reported that these five regions were not strongly conserved in the *B. fragilis* GS protein, but

they were able to identify regions I, II, III and V which showed some similarity to the corresponding regions in the GSI and GSII proteins (Fig. 2).

In a comparison with the GSI and GSII enzymes, only two of the five major regions of homology (regions II and III) could be identified in the GS enzyme from *B. fibrisolvens* (Fig. 2). Hill *et al.* (1989) reported that region II of all GSI and GSII enzymes contained four highly conserved amino acids which are not contiguous [EV(X₃₋₄)Q(X₁)E]. These four amino acids are also conserved in region II of the *B. fibrisolvens* GS. Region III is considered to be the ATP-binding site because the sequence resembles the sequence KXXXXGXXGXGKT found in several ATP-binding proteins (Walker *et al.*, 1982; Tischer *et al.*, 1986). In region III of the *B. fibrisolvens* GS enzyme, an ATP-binding sequence can be identified. Furthermore, region III contains seven amino acid residues (KPXXXXNGXGXHXXS) which are conserved in the ten eukaryote and prokaryote GS enzymes investigated (Hill *et al.*, 1989), as well as in the GS enzymes from *Azospirillum brasilense* (Bozouklian & Elmerich, 1986), *Azotobacter vinelandii* (Toukdarian *et al.*, 1990), *Bacillus cereus* (Nakano *et al.*, 1989), *E. coli* (Colombo & Villafranca, 1986), *Methylococcus capsulatus* (Cardy & Murrell, 1990) and *Rhizobium leguminosarum* (Colonna-Romano *et al.*, 1987). In all GS enzymes investigated, the spacing between regions II and III is also highly conserved and is approximately 42 amino acid residues.

An important difference between the prokaryotic GSI

Fig. 2. Alignment of amino acid sequences of GS proteins from *B. fibrisolvens* (BFIB; this paper) and *B. fragilis* (BFRA; Hill *et al.*, 1989). The underlined regions are the regions identified in the *B. fragilis* polypeptide sequence by Hill *et al.* (1989) showing homology with type I and type II GS subunits.

and the prokaryotic GSIII enzymes is that the former contains a tryptophan residue, or the functionally similar phenylalanine residue, at the position corresponding to position 139 in region I of the *B. fibrisolvens* GS, which is associated with the active site (Almassy *et al.*, 1986; Hill *et al.*, 1989). In the GSIII enzymes, the tryptophan is replaced by either valine, in the *B. fragilis* GS protein, or isoleucine in the *B. fibrisolvens* GS protein. These two amino acids are themselves functionally similar, but they show no functional similarity with tryptophan.

Region IV is thought to be the glutamate-binding site, but neither the *B. fragilis* nor *B. fibrisolvens* GS enzymes show convincing similarity with the other GS enzymes in region IV and region IV cannot be identified in the *B. fragilis* or *B. fibrisolvens* GS enzymes. Region V was identified in the *B. fragilis* GS by relatively high similarity with region V of the *Streptomyces coelicolor* GS (Hill *et al.*, 1989). However, the *B. fibrisolvens* GS shows no convincing similarity with the *B. fragilis* or other GS enzymes in region V (Fig. 2).

The GSIII enzyme from *B. fragilis* is not regulated by adenylation (Southern *et al.*, 1987) and does not

contain an 18-amino-acid sequence containing a tyrosine residue which has been shown to be the site of adenylation in GSI enzymes (Shapiro & Stadtman, 1968; Almassy *et al.*, 1986; Hill *et al.*, 1989). An 18-amino-acid sequence containing tyrosine which even remotely resembles the GSI adenylation region could not be identified in the *B. fibrisolvens* GS. The *B. fibrisolvens* GS appears to be the second example of a GS from a Gram-negative bacterium which is not regulated by adenylation.

Detection of GS in crude cell extracts by Western blotting

Since the *B. fragilis* GS showed homology to the *B. fibrisolvens* GS, an antiserum raised against the purified *B. fragilis* GS was tested to determine whether it reacted with the *B. fibrisolvens* GS. The *B. fragilis* GS antiserum detected a single polypeptide with an apparent M_r of approximately 75000 in crude cell extracts of *B. fragilis* cells, *B. fibrisolvens* cells and *E. coli* YMC11(pGS4) cells (Fig. 3a,b). Previously, we showed that the *B. fragilis*

Fig. 3. Western blots of crude extracts from *B. fibrisolvans*, *B. fragilis* and *E. coli* YMC11(pGS4) cells. The SDS-PAGE-fractionated proteins were transferred to nitrocellulose paper and reacted with antiserum prepared against purified samples of the cloned *B. fragilis* GS. (a) Crude extracts (5 µg protein) of *B. fibrisolvans* (lane 1) and *B. fragilis* (lane 2) cells. (b) Crude extracts (25 µg protein) of *E. coli* YMC11(pGS4) grown in GMML (lane 1) and GMMH (lane 2) medium.

antiserum did not react with any SDS-PAGE fractionated proteins from *E. coli* YMC11 cells (Southern *et al.*, 1987). It appears that the production of the cloned *B. fibrisolvans* GS is regulated in *E. coli* YMC11 cells since the production of the *B. fibrisolvans* GS was induced by growth of *E. coli* YMC11(pGS4) cells in nitrogen-limiting GMML medium (Fig. 3b). Although *E. coli* YMC11 is a heterologous host, the fact that the production of the *B. fibrisolvans* GS appears to be regulated in *E. coli* YMC11 will be helpful in studying the regulation of expression of the *B. fibrisolvans* *glnA* gene. Studies on the regulation of GS activity in *B. fibrisolvans* are not feasible at present, since a suitable nitrogen-limiting medium which supports the growth of *B. fibrisolvans* is not available.

Conclusion

Although the establishment of the novel type-III class of GS is justified on the characterization of a single GS from *B. fragilis*, the demonstration that this GS type is present in other bacteria is important in confirming and

extending the relevance and importance of GSIII in prokaryotes. The GS from *B. fibrisolvans* can be assigned to the GSIII class because of its very large subunit size, and amino acid sequence and antigen homology with the *B. fragilis* GS. Size and amino acid sequence homology differentiate the *B. fragilis* and *B. fibrisolvans* GSIII enzymes from enzymes belonging to the GSI and GSII classes. The GSIII enzyme has been demonstrated in two diverse members of the family *Bacteroidaceae* which inhabit similar environments. It will be interesting to determine whether other members of the *Bacteroidaceae* have the GSIII enzyme and whether this different GS is a distinguishing characteristic of this bacterial family.

References

- ALMASSY, R. J., JANSON, C. A., HAMLIN, R., XUONG, N. H. & EISENBERG, D. (1986). Novel subunit-subunit interactions in the structure of glutamine synthetase. *Nature, London* **233**, 304-309.
- BACKMAN, K., CHEN, Y.-M. & MAGASANIK, B. (1981). Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli* chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 4767-4771.
- BERGER, E., JONES, W. A., JONES, D. T. & WOODS, D. R. (1989). Cloning and sequencing of an endoglucanase (*endI*) gene from *Butyrivibrio fibrisolvans* H17c. *Molecular and General Genetics* **219**, 193-198.
- BRYANT, M. P. & ROBINSON, I. M. (1962). Some nutritional characteristics of predominant culturable ruminal bacteria. *Journal of Bacteriology* **84**, 605-614.
- BOZOUKLIAN, H. & ELMERICH, C. (1986). Nucleotide sequence of the *Azospirillum brasilense* Sp7 glutamine synthetase structural gene. *Biochimie* **68**, 1181-1187.
- CARDY, D. L. & MURRELL, J. C. (1990). Cloning, sequencing and expression of the glutamine synthetase structural gene (*glnA*) from the obligate methanotroph *Methylococcus capsulatus* (Bath). *Journal of General Microbiology* **136**, 343-352.
- CARLSON, T. A. & CHELM, B. K. (1986). Apparent eukaryotic origins of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. *Nature, London* **322**, 568-570.
- COLOMBO, G. & VILLAFRANCA, J. J. (1986). Amino acid sequence of *Escherichia coli* glutamine synthetase deduced from the DNA nucleotide sequence. *Journal of Biological Chemistry* **261**, 10587-10591.
- COLONNA-ROMANO, S., RICCIO, A., GUIDA, M., DEFEZ, R., LAMBERTI, A., IACCARINO, M., ARNOLD, W., PRIEFER, U. & PUHLER, A. (1987). Tight linkage of *glnA* and a putative regulatory gene in *Rhizobium leguminosarum*. *Nucleic Acids Research* **15**, 1951-1964.
- DARROW, R. A. & KNOTTS, R. R. (1977). Two forms of glutamine synthetase in free-living root nodule bacteria. *Biochemical and Biophysical Research Communications* **78**, 554-559.
- DEHORITY, B. A. (1966). Characterization of several bovine rumen bacteria isolated with a xylan medium. *Journal of Bacteriology* **91**, 1724-1729.
- DIXON, R. (1984). Tandem promoters determine regulation of the *Klebsiella pneumoniae* glutamine synthetase (*glnA*) gene. *Nucleic Acids Research* **12**, 7811-7830.
- HENIKOFF, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351-359.
- HESPELL, R. B. (1984). Influence of ammonia assimilation pathways and survival strategy on rumen microbial growth. In *Herbivore Nutrition in the Subtropics and Tropics*, pp. 346-358. Edited by F. M. C. Gilchrist & R. I. Mackie. Craighall: The Science Press.
- HILL, R. T., PARKER, J. R., GOODMAN, H. J. K., JONES, D. T. & WOODS, D. R. (1989). Molecular analysis of a novel glutamine synthetase of the anaerobe *Bacteriodes fragilis*. *Journal of General Microbiology* **135**, 3271-3279.

- HOLDEMAN, L. V. & MOORE, W. E. C. (1972). *Anaerobe Laboratory Manual*. Blacksburg, USA: Virginia Polytechnic Institute and State University.
- JANSON, C. A., KAYNE, P. S., ALMASSY, R. J., GRUNSTEIN, M. & EISENBERG, D. (1986). Sequence of glutamine synthetase from *Salmonella typhimurium* and implications for the protein structure. *Gene* **6**, 297–300.
- JANSSEN, P., JONES, W. A., JONES, D. T. & WOODS, D. R. (1988). Molecular analysis and regulation of the *glnA* gene of the Gram-positive anaerobe *Clostridium acetobutylicum*. *Journal of Bacteriology* **170**, 400–408.
- KUMADA, Y., TAKANO, E., NAGAOKA, K. & THOMPSON, C. J. (1990). *Streptomyces hygroscopicus* has two glutamine synthetase genes. *Journal of Bacteriology* **172**, 5343–5351.
- LIN, L. L., RUMBAC, E., ZAPPE, H., THOMSON, J. A. & WOODS, D. R. (1990). Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding β -glucosidase. *Journal of General Microbiology* **136**, 1567–1576.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NAKANO, Y., CHIAKI, K., TAKANA, E., KIMURA, K. & HORIKOSHI, K. (1989). Nucleotide sequence of glutamine synthetase gene (*glnA*) and its upstream region from *Bacillus cereus*. *Journal of Biochemistry* **106**, 209–215.
- POSSOT, O., SIBOLD, L. & AUBERT, J. P. (1989). Nucleotide sequence and expression of the glutamine synthetase structural gene, *glnA*, of the archaeobacterium *Methanococcus voltae*. *Research in Microbiology* **140**, 355–371.
- RAWLINGS, D. E., JONES, W. A., O'NEILL, E. G. & WOODS, D. R. (1987). Nucleotide sequence of the glutamine synthetase gene and its controlling region from the acidophilic autotroph *Thiobacillus ferrooxidans*. *Gene* **53**, 211–217.
- REITZER, L. J. & MAGASANIK, B. (1985). Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 1979–1983.
- ROCHFORD, D. A. & BENSON, D. R. (1990). Molecular cloning, sequencing and expression of the glutamine synthetase II (*glnII*) gene from the actinomycete root nodule symbiont *Frankia* sp. strain CpII. *Journal of Bacteriology* **172**, 5335–5342.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SHAPIRO, B. M. & STADTMAN, E. R. (1968). 5'-Adenylyl-O-tyrosine. The novel phosphodiester of adenylylated glutamine synthetase from *Escherichia coli*. *Journal of Biological Chemistry* **243**, 3769–3771.
- SMITH, C. J., HESPELL, R. B. & BRYANT, M. P. (1981). Regulation of ammonia assimilatory enzymes and urease in *Selenomonas ruminantium*. *Applied and Environmental Microbiology* **42**, 89–96.
- SOUTHERN, J. A., PARKER, J. R. & WOODS, D. R. (1986). Expression and purification of glutamine synthetase cloned from *Bacteroides fragilis*. *Journal of General Microbiology* **132**, 2827–2835.
- SOUTHERN, J. A., PARKER, J. R. & WOODS, D. R. (1987). Novel structure, properties and inactivation of glutamine synthetase cloned from *Bacteroides fragilis*. *Journal of General Microbiology* **133**, 2437–2446.
- STRAUCH, M. A., ARONSON, A. I., BROWN, S. W., SCHREIER, H. J. & SONENSHEIN, A. L. (1988). Sequence of the *Bacillus subtilis* glutamine synthetase gene region. *Gene* **71**, 257–265.
- STREICHER, S. L. & TYLER, B. (1980). Purification of glutamine synthetase from a variety of bacteria. *Journal of Bacteriology* **142**, 69–78.
- STRYDOM, E., MACKIE, R. I. & WOODS, D. R. (1986). Detection and characterization of extracellular proteases in *Butyrivibrio fibrisolvens* H17c. *Applied Microbiology and Biotechnology* **24**, 214–217.
- TISCHER, E., DASARMA, S. & GOODMAN, H. M. (1986). Nucleotide sequence of an alfalfa glutamine synthetase gene. *Molecular and General Genetics* **203**, 221–229.
- TOUKDARIAN, A., SAUNDERS, G., SELMAN-SOSA, G., SANTERO, E., WOODLEY, P. & KENNEDY, C. (1990). Molecular analysis of the *Azotobacter vinelandii* *glnA* gene encoding glutamine synthetase. *Journal of Bacteriology* **172**, 6529–6539.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- TUMER, N. E., ROBINSON, S. J. & HASELKORN, R. (1983). Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. *Nature, London* **306**, 337–342.
- WALKER, J. E., SARASTE, M., RUNWICK, M. J. & GAY, N. J. (1982). Distantly related sequences in the α - and β -subunits of ATP-synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1**, 945–951.
- ZABEAU, M. & STANLEY, K. K. (1982). Enhanced expression of cro- β -galactosidase fusion proteins under the control of the P_R promoter of the bacteriophage lambda. *EMBO Journal* **1**, 1217–1224.