The NADH-dependent glutamate dehydrogenase enzyme of *Bacteroides fragilis* Bf1 is induced by peptides in the growth medium

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***Bacteroides fragilis* Bf1 possesses two enzymes having glutamate dehydrogenase (GDH) activity. One is dual cofactor NAD(P)H-dependent, while the other has NADH-specific activity. The gene encoding the NADH-GDH (*gdhB*) was cloned by complementation of the glutamate auxotrophic mutant *Escherichia coli* MX3004 and the recombinant protein was characterized with respect to the GDH activities present in the parental organism grown under different nitrogen conditions. The NAD(P)H-dependent GDH of *B. fragilis* was confirmed to be most active under high ammonia conditions, but the NADH-specific GDH levels were increased by high peptide concentrations in the growth medium and not regulated by the levels of ammonia. Northern blotting analysis showed that *gdhB* regulation was at the transcription level, with a single transcript of ~1.6 kb being produced. GDH activity was demonstrated by zymography of the parental and recombinant enzymes. The recombinant GDH was NADH-specific and co-migrated with the equivalent enzyme band from *B. fragilis* cell extracts. The *gdhB* structural gene comprises 1335 bp and encodes a protein of 445 aa (49 kDa). Comparisons of the derived protein sequence with that of GDH from other bacteria indicated that significant sequence homology and conservation of functional domains exists with enzymes of Family I-type hexameric GDH proteins.**

Keywords: *Bacteroides fragilis*, nitrogen metabolism, glutamate dehydrogenase

INTRODUCTION

Bacteroides fragilis is an important anaerobic opportunistic pathogen of humans. It occurs normally in the digestive tract, but can cause severe abscess formation and sepsis when outside this environment. One of the factors thought to be involved in the pathogenicity of this bacterium is the production of proteases, which are, in turn, regulated by the levels of nitrogen available (Gibson & MacFarlane, 1988a, b).

In prokaryotes, glutamate dehydrogenase (GDH) and the dual enzyme system, glutamine synthetase (GS) and

glutamate synthase (GOGAT), are the most important routes by which ammonia may be assimilated (Reitzer & Magasanik, 1987). GDH is an extremely important enzyme which links carbohydrate and nitrogen metabolism (Smith *et al.*, 1975) and the physiological roles of GDHs as anabolic and/or catabolic enzymes are generally defined by the nature of their cofactor specificity (Joe *et al.*, 1994). NADP(H)-specific GDH enzymes usually catalyse the assimilation of ammonia by reductive amination of α -ketoglutarate to form L-glutamate (anabolic), while NAD(H)-dependent GDH enzymes catalyse the reverse reaction (catabolic).

Yamamoto *et al.* (1984) have suggested that GDH activity may play the primary role in nitrogen assimilation in *B. fragilis*, since the levels of GDH were very much higher than GS and cells which had their GS activity inhibited with methiosulfoxamine did not show a decrease in cell growth rate.

Abbreviations: CFE, cell-free extract; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase.

The GenBank accession number for the sequence reported in this paper is U78108.

Nitrogen metabolism in *B. fragilis* has been shown to have several unusual features when compared to the *Enterobacteriaceae*. Ammonia is not replaced by amino acids as the source of nitrogen for growth (Varel & Bryant, 1974), while peptides can also be utilized. The enzymes involved in the assimilation of nitrogen are a structurally novel form of GS (GSIII) (Southern *et al.*, 1986, 1987; Hill *et al.*, 1989), two GDHs (Yamamoto *et al.*, 1984, 1987a, b; Saito *et al.*, 1988) and GOGAT. These enzymes have been shown to be regulated by the levels of ammonia available. GS is induced under low ammonia conditions and is regulated at the level of transcription (Southern *et al.*, 1986, 1987; Abratt *et al.*, 1993).

The two GDH enzymes were first identified by Yamamoto *et al.* (1984) who concluded that one had dual specificity for pyridine nucleotides [NAD(P)H], while the other was NADH-specific. Characterization of the activities of these enzymes showed that the NAD(P)H-dependent GDH was induced by low concentrations of ammonia, whereas high ammonia concentrations were thought to partially induce the NADH-dependent GDH. Further characterization of the former enzyme at the protein level showed that the activity was controlled through a reversible activation/inactivation mechanism which was regulated by ammonia (Yamamoto *et al.*, 1987a, b; Saito *et al.*, 1988).

The presence of two GDH enzymes [a dual NAD(P)H- and an NADH-dependent enzyme] has recently been reported in another colonic *Bacteroides* species, *B. thetaiotaomicron* (Baggio & Morrison, 1996). Although both enzyme activities were detectable under all growth conditions tested, the highest specific activities were observed at limiting ammonia concentrations. The *gdhA* gene has been cloned from this organism and shown to encode the NAD(P)H-utilizing GDH enzyme. This gene required downstream DNA sequences (or the sequence supplied *in trans*) for activity, but not transcription, in *Escherichia coli*. Insertional inactivation of the gene in *B. thetaiotaomicron* did not generate a glutamate auxotrophic mutant, indicating the presence of a second enzymic pathway catalysing ammonia assimilation and glutamate biosynthesis. The NADH-dependent GDH enzyme from this organism has also been cloned (*gdhB*) and has been shown to be most active in a medium containing peptides (Mark Morrison, personal communication).

The anaerobic rumen bacteria *Ruminococcus flavefaciens* and *Prevotella* spp. have been shown to possess NAD(P)H-dependent GDH activity, induced under ammonia limitation conditions (Duncan *et al.*, 1992; Wen & Morrison, 1996, 1997). In *Prevotella* spp. it is repressed by the presence of peptides. *Prevotella brevis*, however, has an additional NADH-dependent GDH enzyme, which is induced by growth in peptides as sole nitrogen source.

Neither of the GDH enzymes of *B. fragilis* has been cloned or characterized at the molecular level, nor have the mechanisms underlying the control of GDH activity

and the distinctive roles of the two enzymes in nitrogen assimilation in this organism been elucidated. In this work we report the cloning and molecular characterization of the NADH-specific GDH and its response to nitrogen sources as compared to the NAD(P)H-dependent GDH enzyme in *B. fragilis*.

METHODS

Bacterial strains, plasmids and growth conditions. *B. fragilis* Bf1 (Mossie *et al.*, 1979), isolated from human faeces, was used to study GDH production and for the preparation of a gene library using plasmid pEcoR251 (Zabeau & Stanley, 1982). Plasmid pMT104 (Wehnert *et al.*, 1990) was used as a negative control for cloned GDH activity. *B. fragilis* Bf1 was grown routinely at 37 °C in brain heart infusion (BHI) broth (Difco) and agar (1.5%), supplemented according to Abratt *et al.* (1985), in a Forma Scientific anaerobic chamber (atmosphere 85% N₂, 10% H₂ and 5% CO₂). *E. coli* strains were grown aerobically at 37 °C in YT broth (Sambrook *et al.*, 1989) and agar (1.5%). For anaerobic growth, sodium nitrate (0.2%) and glucose (0.5%) were added to the YT broth and the cells grown in the anaerobic chamber. Screening for *gdh* genes was carried out in *E. coli* MX3004 (Castano *et al.*, 1992) on glucose minimal medium (GMM; Sambrook *et al.*, 1989) containing 13 mM NH₄Cl as sole nitrogen source (GMMN). Ampicillin (Ap) and chloramphenicol (Cm) were used at final concentrations of 100 and 50 µg ml⁻¹, respectively, in the medium when required.

Nitrogen regulation studies in *B. fragilis* were conducted in GMM broth (Varel & Bryant, 1974), modified as described by Abratt *et al.* (1993). The medium was supplemented with 0.5, 5.0 or 50 mM NH₄Cl (low, medium and high ammonia, respectively), or 0.25 or 1.25% tryptone (low and high peptides, respectively).

General recombinant DNA procedures. All DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Chromosomal DNA preparations from *B. fragilis* were prepared by the method of Wehnert *et al.* (1990) and *E. coli* JM109 chromosomal DNA, prepared according to Sambrook *et al.* (1989), was used as a control in Southern hybridization analysis. Plasmid DNA preparations were isolated by the alkaline lysis method of Ish-Horowitz & Burke (1981) or with the Nucleobond AX DNA isolation kit (Macherey-Nagel). Plasmid DNA was introduced into *E. coli* by the transformation of competent cells (Sambrook *et al.*, 1989) with strain MX3004 as the recipient for GDH selection and strain JM109 (Yanisch-Perron *et al.*, 1985) for routine DNA cloning and manipulation procedures. All endonuclease restriction enzymes were purchased from Amersham or Boehringer Mannheim and were used as specified by the manufacturer. Electrophoresis was performed on 0.8–1.5% agarose gels according to standard procedures. DNA fragments were purified from agarose gels with the GeneClean kit (Bio101).

Construction and screening of the recombinant DNA library. The construction of the *B. fragilis* genomic library has been described by Southern *et al.* (1986). Recombinant plasmids were transformed into competent *E. coli* MX3004 cells. Colonies were screened for expression of GDH or GOGAT activity by plating transformed cells onto GMMN medium containing Ap and Cm and screening for glutamate prototrophy after 2 d incubation.

Preparation of cell-free extracts (CFEs). Cultures were grown to early stationary phase and 100 ml was harvested by

centrifugation (10000 *g* for 10 min at 10 °C). The cells were washed three times with 50 mM Tris/HCl (pH 8.0) and resuspended in 5 ml of the same buffer. Cells were disrupted by sonication (4 °C) at 95 W using five 30 s bursts with 30 s cooling intervals (VirSonic Digital 475 Cell Disruptor), centrifuged at 15000 *g* for 15 min at 4 °C and the CFE used for GDH assays. For experiments to measure the activity of the enzyme under anaerobic conditions, cells were harvested under anaerobic conditions, washed with anaerobic 50 mM Tris/HCl (pH 8.0) buffer and disrupted using a French pressure cell (220800 kPa; SIM, Aminco). The disrupted cell material was collected in a nitrogen-filled tube which was then centrifuged and the CFE removed under anaerobic conditions. Assays were conducted under strict anaerobic conditions in the anaerobic chamber.

GDH enzyme assays. GDH activity was assayed spectrophotometrically (DU650 spectrophotometer, Beckman) by following the decrease in A_{340} during oxidation of NADPH or NADH (Yamamoto *et al.*, 1984). The reactions were conducted at 25 °C in 1.0 ml reaction mix containing 100 mM Tris/HCl (pH 8.0), 40 mM NH_4Cl , 5 mM α -ketoglutarate and 0.15 mM NADPH and were initiated by the addition of 50 μl CFE (75 μg total protein). When NADH was used as a cofactor, Tris/HCl (pH 7.4) was used. Enzyme activity was determined as the difference in oxidation rates of NADPH in the presence and absence of substrate. Enzyme specific activities are expressed as units (mg protein^{-1}) where one unit is defined as the amount of enzyme required to oxidize 1 μmol cofactor min^{-1} at 25 °C. The protein concentrations of the CFEs were determined using the Bio-Rad dye reagent with bovine serum albumin as a standard.

GDH zymograms. CFEs (45 μg total protein) were subjected to discontinuous non-dissociating PAGE (Laemmli, 1970), modified by omitting SDS and β -mercaptoethanol, using the Mighty-Small system (Hoeffer Scientific Instruments). The acrylamide concentrations used were 7.5 and 3.5% in the stacking and resolving gels, respectively. Bromophenol blue was used as the tracking dye. Following electrophoresis, the gels were incubated in staining solution consisting of 0.5 mM NADP^+ or NAD^+ , 20 mM L-glutamate, 0.3 mg nitro blue tetrazolium ml^{-1} , 0.05 mg phenazine methosulfate ml^{-1} and 50 mM Tris/HCl (pH 8.0). GDH activity appeared as dark purple bands against a clear background.

SDS-PAGE analysis. Cell-free coupled transcription and translation (Amersham) were carried out on plasmid pGDH15 and pEcoR251 and the resultant [^{35}S]methionine-labelled polypeptides compared using 10% SDS-PAGE (Laemmli, 1970).

Southern blotting analysis. For Southern blotting analysis, restriction-endonuclease-digested *B. fragilis* Bf1 and *E. coli* JM109 DNA were transferred to a nylon membrane (Hybond-N; Amersham) via the capillary alkali transfer procedure. The *gdhB* probe was generated by random-primed labelling of a 1.0 kb *XmnI*-*BglII* fragment derived from pGDH15, using a non-radioactive digoxigenin labelling and detection kit (Boehringer Mannheim). Hybridization, washing and detection were performed according to manufacturers' instructions.

Northern blotting analysis. Total RNA was isolated from mid-exponential phase cultures (30 ml) of *B. fragilis* Bf1 and *E. coli* MX3004(pGDH15), with *E. coli* MX3004(pMT104) being used as control (Aiba *et al.*, 1981). Separation of RNA on formaldehyde gels, transfer to nylon membranes (Boehringer Mannheim) and hybridization conditions were according to manufacturers' instructions. Equal amounts of RNA (15 μg) were loaded per lane and confirmed by the equal intensity of

rRNA bands by ethidium bromide staining. The 1.0 kb *XmnI*-*BglII* fragment used in Southern hybridization was used as probe. The RNA ladder from USB was used as size marker.

Nucleotide sequence and sequence analysis. Subclones for sequencing were constructed by the *ExoIII* nested deletion method of Henikoff (1984) using pBluescript KS(+) (Stratagene). The nucleotide sequence was determined by the fluorescent dideoxy chain-termination method of Sanger *et al.* (1977) using the Thermo-Sequenase Sequencing kit (USB) and Cy5 fluorescently labelled universal and reverse primers, as per manufacturers' instructions. The sequencing reaction products were separated using the ALFexpress DNA sequencer (Pharmacia) and the nucleotide sequence obtained was analysed using the University of Wisconsin Genetics Computer Group DNA sequence analysis software (Devereux *et al.*, 1984) and CLUSTAL W, DNAMAN and TREEVIEW software packages.

RESULTS

GDH activity of *B. fragilis* under various nitrogen conditions

Initially, all experiments were conducted both aerobically and anaerobically, but since the enzyme activity was not found to be affected by oxygen, all subsequent assays were done aerobically. CFEs of *B. fragilis* grown in minimal or complete media showed both NADPH- and NADH-linked GDH activities. This represented the combined total activity of both the dual cofactor NAD(P)H- and the NADH-specific GDH enzymes. The levels and ratios of the specific activity of each cofactor varied according to the levels of organic or inorganic nitrogen supplied (Table 1). When ammonia was the nitrogen source, the highest NADPH- and NADH-linked specific activities were found at 0.5 mM ammonia (1348 and 185 U respectively) and the least at 50 mM ammonia (291 and 80 U, respectively). When *B. fragilis* was grown in complete medium (supplemented BHI broth), NADH-linked activity exceeded that of NADPH (ratio NADH:NADPH = 1.68). Growth of *B. fragilis* with 0.25% tryptone resulted in an NADH:NADPH activity ratio of 0.35, compared with growth in 1.25% tryptone (ratio = 2.02) (Table 1), reflecting the increased NADH- and reduced NADPH-linked GDH activity in the presence of high levels of organic nitrogen.

Cloning and characterization of a GDH gene from *B. fragilis*

Screening of a *B. fragilis* gene library in *E. coli* MX3004 grown on GMMN resulted in the isolation of plasmid pGDH15 which complemented the glutamate auxotrophy of the host cells. The recombinant plasmid was partially mapped using restriction endonuclease digestion (Fig. 1a) and found to contain a 5.7 kb DNA insert. The region conferring GDH activity was localized by deletion analysis of the cloned DNA, with a 1.9 kb *BglII* deletion, resulting in complete loss of the phenotype. Southern hybridization of the *XmnI*-*BglII* fragment of

Table 1. GDH specific activity in *B. fragilis* Bf1 grown under different nitrogen conditions and in *E. coli* MX3004 transformed with the cloned *gdhB* gene

Values are the means of at least three different experiments $\pm$ SD. One unit is defined as μmol cofactor oxidized min^{-1} (mg protein^{-1}). ND, not detectable.

Organism and nitrogen source	Specific activity		
	NADPH-GDH (U)	NADH-GDH (U)	Ratio (NADH:NADPH)
<i>B. fragilis</i> Bf1			
NH ₄ Cl (0.5 mM)	1348 $\pm$ 69.9	185 $\pm$ 4.6	0.137
NH ₄ Cl (5.0 mM)	784 $\pm$ 22.2	120 $\pm$ 6.1	0.153
NH ₄ Cl (50.0 mM)	291 $\pm$ 18.2	80 $\pm$ 5.0	0.27
BHI	107 $\pm$ 7.2	180 $\pm$ 16.0	1.68
Tryptone (0.25 %)	406 $\pm$ 14.7	146 $\pm$ 7.2	0.35
Tryptone (1.25 %)	85 $\pm$ 9.9	172 $\pm$ 12.3	2.02
<i>E. coli</i> MX3004(pGDH15)			
YT	28 $\pm$ 0.0	610 $\pm$ 31.2	21.78
YT + glucose (0.5 %)	20 $\pm$ 0.0	366 $\pm$ 17.6	18.30
<i>E. coli</i> MX3004(pMT104)	ND	ND	—

Fig. 1. (a) Partial restriction map of the *B. fragilis* Bf1 DNA insert in pGDH15. The thick line represents the insert DNA and the dotted line the beginning of pEcoR251 vector DNA. The position and orientation of the *gdhB* gene is shown by the arrow and the *XmnI*–*BglII* fragment used as probe for Southern and Northern hybridization analysis is indicated by the open bar. The numbers mark the positions of the endonuclease restriction sites in the insert. (b) Partial nucleotide sequence of the 1.8 kb section of the pGDH15 insert DNA containing the *B. fragilis* Bf1 *gdhB* gene. In the upstream region, the putative promoter regions are underlined. The ATG start codon and TGA stop codon are shown in bold, with the intervening coding region designated by dots. Arrows show the directions of the direct and indirect repeat sequences in the upstream and downstream regions. (c) The deduced amino acid sequence of *gdhB* showing the following features: conserved hexameric Family I GDH regions, bold and dashed underlined; GDH active site, bold underlined; NADPH binding site, bold; ATP/GTP binding site, underlined.

the insert to the equivalent band in *B. fragilis* DNA digested with the same enzymes and not to *E. coli* DNA confirmed that the insert DNA originated from *B. fragilis* (data not shown).

Activity of recombinant GDH in *E. coli*

The activity of the cloned gene product was assayed in CFE of *E. coli* MX3004 transformed with pGDH15 and

Fig. 2. Activity staining of (a) NADPH- and (b) NADH-linked GDH activity on acrylamide gels. The extracts were prepared from *B. fragilis* cells grown under different nitrogen conditions and from *E. coli* MX3004 transformed with the cloned *gdhB* gene or a control plasmid, with 45 µg total protein run in each lane as described in Methods. *B. fragilis* cells were grown in 0.5 mM NH_4Cl (lane 1), 5.0 mM NH_4Cl (2), 50 mM NH_4Cl (3), BHI (4), 0.25% tryptone (5) or 1.25% tryptone (6). *E. coli* MX3004(pGDH15) was grown in YT (lane 8) or YT+0.5% glucose (10). Lane 7, blank; lane 9, *E. coli* MX3004(pMT104) control.

compared to the GDH activity of the strain transformed with the control plasmid pMT104 (Table 1). During aerobic growth in YT broth, the cloned gene product was found to have NADH-specific GDH activity (610 U) while no activity could be detected in the control system. The cloned gene product had very low levels of NADPH-linked activity (28 U) (Table 1). An approximately twofold reduction in GDH activity was detected in *E. coli* MX3004(pGDH15) when it was grown anaerobically in YT supplemented with nitrate and glucose. The GDH repression effect was found to be caused by the addition of glucose, since the NADH-dependent activity of the recombinant enzyme was reduced to 366 U when the cells were grown aerobically in YT supplemented with glucose.

GDH zymograms

The activities of the cloned GDH gene product in *E. coli* MX3004 and the resident GDH enzymes in *B. fragilis* were further characterized using non-denaturing PAGE and visualization by activity staining. Cell lysates from cells grown under all the conditions described above were investigated. Duplicate acrylamide gels were run with an equivalent amount of protein (45 µg) loaded in each lane. After electrophoresis, the gels were stained for GDH activity, with either NADPH or NADH being added as the cofactor (Fig. 2a, b). The two *B. fragilis* GDH enzymes were clearly distinguishable, both in their electrophoretic mobilities and in their responses to the various cofactors and nitrogen sources supplied. Under low ammonia conditions (0.5 mM), dual cofactor NAD(P)H-dependent GDH was most active and only very low levels of the NADH-linked enzyme were detected. With increasing ammonia concentrations (5 and 50 mM), the NAD(P)H-dependent GDH was progressively less active, but still easily detectable, while the NADH-specific enzyme remained at very low basal levels. The NADH-specific activity detected in assays of the *B. fragilis* CFE at 0.5 mM NH_4Cl was, therefore, being contributed predominantly by the dual cofactor enzyme. However, the situation was very different when organic nitrogen was present in the growth medium. During growth of *B. fragilis* in supplemented BHI broth, the NAD(P)H-dependent enzyme was not detected on

the gels and high NADH-specific GDH activity was observed. Under conditions of low tryptone (0.25%), the NAD(P)H-dependent enzyme was active, but not to the same extent as under low ammonia conditions. No NADH-linked activity was detected. However, when high levels of tryptone were present in the growth medium, the NAD(P)H-linked GDH activity was no longer present and increased activity of the NADH-dependent GDH was seen. The activity of the cloned GDH gene product in *E. coli* was confirmed to be NADH-specific by the appearance of a single band of equivalent mobility to the enzyme in lysates of *B. fragilis*. No activity was detected in cells transformed with the control plasmid pMT104. Addition of glucose to the YT growth medium of *E. coli* MX3004(pGDH15) caused a reduction in NADH-dependent GDH activity.

Nucleotide sequence analysis of the *gdhB* gene

A 1640 bp fragment containing the *gdhB* gene was sequenced. Computer analysis of this region revealed an ORF of 1335 nt from a presumptive start codon (ATG) at position 327 to a stop codon (TGA) at position 1664. A second in-frame ATG was found further upstream (position 276) that would yield a protein 17 aa larger. The most probable initiation codon, however, is the former, since the N terminus of the deduced polypeptide translated from ATG at position 327 shows homology with the N termini of the majority of GDHs sequenced so far. It generates a polypeptide of 445 aa with a deduced molecular mass of 49 kDa. This is in agreement with the results of SDS-PAGE analysis where a protein product of this size was encoded by the *gdhB* gene (results not shown).

No conventional ribosome-binding site (Shine & Dalgarno, 1974) was observed immediately upstream of the ORF, but further analysis of the upstream region showed the presence of putative promoter sequences (Fig. 1b) with homology to the -10 and -35 *E. coli* RNA polymerase binding consensus sequences. This may be recognized as a heterologous promoter in *E. coli*. It is not known whether this promoter is functional in *B. fragilis*. A perfect inverted repeat sequence was located in the promoter region. A 10 bp direct repeat was identified upstream of the ORF and two inverted repeats

were found downstream of the stop codon between positions 1721 and 1760, and 1761 and 1775 (Fig. 1b). At the RNA level, these inverted repeats would be capable of forming a stem-loop structure [$\Delta G = -21.4$ kcal mol⁻¹ (-89.5 kJ mol⁻¹)], similar to ρ -independent transcriptional terminators (Rosenberg & Court, 1979).

The calculated G+C content of the *gdhB* gene was 46.5%, which corresponds well with the range of 42–44% determined for chromosomal DNA from strains of *B. fragilis* (Holdeman & Moore, 1972).

The deduced GdhB polypeptide possessed the three highly conserved domains characteristic of the Family I hexameric GDHs (Benachenhou-Lahfa *et al.*, 1993). These are ASVNL (amino acid positions 95–99), KFLGFEQ (103–109) and RPEATGF (203–209). In addition, a putative NADP-binding motif, GFGNVAWGAATKATELG (binding site residues underlined), was located between residues 235 and 251. The putative active site of the enzyme, GGGKGG (121–126), was also identified in which the critical lysine residue (124) in the glycine-rich region is thought to be involved in the binding of α -ketoglutarate and the catalytic activity of the enzyme (reviewed by Smith *et al.*, 1975). Three putative consensus GTP/ATP-binding motifs were evident, GIDIKGKT (223–230), GPDG (259–262) and VKAD (310–313), where both the sequence and spacing conformed to the required GTP-binding domains. No leader sequence was evident from the deduced polypeptide sequence, but the last 18 amino acids were strongly hydrophobic in character.

The deduced amino acid sequence of the *B. fragilis* *gdhB* gene was aligned with the corresponding sequences of several other previously sequenced GDH enzymes. The highest identity was found with *B. thetaiotaomicron* *gdhB* (90%; Mark Morrison, personal communication). The identity to *Porphyromonas gingivalis* was 79% (Joe *et al.*, 1994), followed by *B. thetaiotaomicron* *gdhA* (73%; Baggio & Morrison, 1996) and *Prevotella ruminicola* (66%; Wen & Morrison, 1996), all of which fall into Family I. Much lower identities were found with Family II enzymes, for example *Clostridium difficile* (37%; Lyster *et al.*, 1991) and *Halobacterium salinarum* (34%; Benachenhou & Baldacci, 1991).

Northern hybridization analysis

Analysis of the levels of mRNA produced from the *gdhB* gene was carried out using total RNA obtained from *B. fragilis* grown under different nitrogen conditions and from *E. coli* transformed with the cloned gene (Fig. 3). The probe used was the *XmnI*–*Bgl*II internal fragment of the *gdhB* gene and, in all cases, the amount of total RNA loaded in the lanes was the same (15 µg). There was a correlation between the level of mRNA transcribed from the *gdhB* gene and the activity seen in the enzyme assays and on the GDH activity gels. The probe hybridized most strongly to the mRNA from *B. fragilis* cells grown in BHI broth and only very low basal levels of hybridization were seen with RNA from cells grown

Fig. 3. Northern blotting analysis of RNA from *B. fragilis* Bf1 and *E. coli* MX3004(pGDH15). Cells were grown to mid-exponential phase in various media as described in the text. The RNA was isolated and 15 µg loaded into each well. The RNA was probed with the internal *XmnI*–*Bgl*II fragment of the *gdhB* gene. Lanes: 1, *B. fragilis* cells were grown in 0.5 mM NH₄Cl (lane 1), 5.0 mM NH₄Cl (2), 50 mM NH₄Cl (3) or BHI (4). Lane 5, *E. coli* MX3004(pMT104); lane 6, *E. coli* MX3004(pGDH15).

under all ammonia conditions. RNA isolated from *E. coli* transformed with the cloned gene hybridized to the probe and *E. coli* transformed with the control plasmid did not. Growth of *B. fragilis* in high peptides yielded higher levels of *gdhB* mRNA than when cells were grown in low peptides (results not shown). These results demonstrate that there is transcriptional regulation of GdhB activity in response to the various nitrogen conditions.

DISCUSSION

The presence of two GDH enzymes in *B. fragilis* was first reported by Yamamoto *et al.* (1984) who proposed that the dual-cofactor-dependent enzyme was responsible for ammonia assimilation under conditions of low ammonia and that the NADH-specific GDH was induced when ammonia was in excess.

In this study, we have cloned the *gdhB* gene for NADH-specific GDH and have characterized the recombinant GdhB enzyme with respect to its activity in the *B. fragilis* parent.

The *gdhB* gene has many structural similarities to previously cloned GDH genes and was shown to be closely related to the hexameric GDH Family I group (Benachenhou-Lahfa *et al.*, 1993). At a regulatory level, however, this enzyme appears to have an unusual role in the nitrogen metabolism of *B. fragilis* through its increased activity in the presence of organic nitrogen. The highest GdhB activity was observed when *B. fragilis* was grown in the presence of high levels of organic nitrogen, either in BHI broth or in minimal medium containing tryptone (1.25%) as sole nitrogen source. Under these conditions, the NAD(P)H-linked activity of

GdhA was repressed. The regulation of GdhB was shown to occur at the transcription level.

Yamamoto *et al.* (1984) noted a higher NADH to NAD(P)H-dependent GDH activity ratio in high as compared to low ammonia. They regarded this as evidence for induction of the NADH-dependent GDH enzyme under high ammonia conditions. We have confirmed that the ratio does increase, but have seen no evidence of regulation of this enzyme by ammonia. Northern hybridization analysis showed that the same basal levels of *gdhB* mRNA were produced under all ammonia conditions. Furthermore, the zymograms showed only faint bands of NADH-specific GDH activity in all the ammonia-grown cultures. The difference in ratio may then be interpreted as a basal constitutive level of the GdhB enzyme being present throughout, with a repression of the dual cofactor GdhA enzyme with increased ammonia concentrations. A possible role for post-translational modification of the protein can not be ruled out at this stage. This is being investigated.

The regulation of *gdhB* by peptides raises some interesting questions about the role of this enzyme in *B. fragilis* during *in vivo* growth. It has been shown in *Clostridium botulinum* (Hammer & Johnson, 1988) that highly proteolytic strains have unusually high levels of NADH-dependent GDH activity relative to non-proteolytic strains. It is proposed that GDH is important in proteolytic strains of this organism to generate α -ketoglutarate as a substrate for transamination reactions. *B. fragilis* has been shown to produce several proteases (Gibson & Macfarlane, 1988a, b) and some protease enzymes from this organism have been cloned and characterized (Van Tassel *et al.*, 1992; Chen *et al.*, 1995; Kling *et al.*, 1997; Franco *et al.*, 1997). It has been suggested that proteases play a role in the pathogenesis of this organism (Riepe *et al.*, 1980; Moncrief *et al.*, 1995) and it has also been demonstrated that the levels of protease production vary with the availability of ammonia (MacFarlane *et al.*, 1993), with less protease activity being evident under excess ammonia conditions. The regulation of *gdhB* by organic nitrogen may be linked to protease activity and the breakdown of proteins *in vivo*, and hence to pathogenesis.

Sequence analysis of the cloned *gdhB* gene indicated that optimal energy codons account for 38.2% of the *B. fragilis* *gdhB* gene, indicating a significant physiological function for the GdhB enzyme. Most high expression systems possess between 15 and 25% optimum energy codons (Gharbia & Shah, 1995). For example, the *Clostridium symbiosum* *gdh* gene contains one of the highest prokaryotic expression systems (30.7% optimal codon usage) and the gene product accounts for 15% of total cellular protein, supporting the fact that optimal codon preference levels reflect the level of expression (Teller *et al.*, 1992).

Sequence analysis of the *B. fragilis* *gdhB* gene showed that it had a very high amino acid identity and similarity

(79 and 90%, respectively) to the NADH-dependent GDH of *Por. gingivalis*, an anaerobic pathogenic organism occurring in the oral cavity. *Por. gingivalis* GDH has been shown to be surface-associated (Joe *et al.*, 1993, 1994; Gharbia & Shah, 1995), but it has been suggested that the enzyme may not be an integral membrane protein (Curtis *et al.*, 1991) but may be loosely anchored in it through interaction of the 20 hydrophobic amino acid residues of the C terminus with the lipid bilayer of the membrane (Joe *et al.*, 1994). The 18-aa C terminus hydrophobic region found in *B. fragilis* is very similar to the structure of *Por. gingivalis* GDH. Work is currently in progress to raise antibodies to the recombinant GdhB protein and to do direct immunodetection studies on undisrupted *B. fragilis* cells.

The work reported here confirms that *B. fragilis* possesses two distinct GDH enzymes which are regulated by different nitrogen conditions. Further elucidation of the regulation of these enzymes will require cloning of the dual cofactor-dependent GDH gene. Site-directed mutagenesis could then be used to establish the roles played by each enzyme in nitrogen assimilation in *B. fragilis* and perhaps in the virulence of this medically important pathogen.

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