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.
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 enzymatic 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* BFI (Mossie et al., 1979), isolated from human feces, was used to study GDH production and for the preparation of a gene library using plasmid pEcoR251 (Zabeau & Stanley, 1982). Plasmid pMT104 (Wehntert et al., 1990) was used as a negative control for cloned GDH activity. *B. fragilis* BFI 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 mg⁻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, 50 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-Horowiz & Burke (1981) or with the Nuclease Bond 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 (Wen & Morrison). Plasmid DNA was used as a control in Southern hybridization analysis. The medium was supplemented with 0.5, 50 or 50 mM NH4Cl (low, medium and high ammonia, respectively), or 0.25 or 1.25% tryptone (low and high peptides, respectively).

**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_{412} 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 oxaloacetate and 0.15 mM NADPH and were initiated by the addition of 30 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 Mighty-Small system (Hoeffer Scientific Instruments). The stained rRNA bands by ethidium bromide staining. The 1.0 kb Xmnl–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 ExodiI nested deletion method of Henikoff (1984) using pBluescript KS(+)(Stratagene). The nucleotide sequence was determined by the fluorescent dyeoxy 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 sequencing (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, DNASAN 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 = 0.5) (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 pGDHI5 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 Bg/I1I deletion, resulting in complete loss of the phenotype. Southern hybridization of the Xmnl–BglII fragment of

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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 ± SD. One unit is defined as µmol cofactor oxidized min⁻¹ (mg protein)⁻¹. ND, not detectable.

<table>
<thead>
<tr>
<th>Organism and nitrogen source</th>
<th>Specific activity</th>
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<tr>
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<td>NADPH-GDH (U)</td>
<td>NADH-GDH (U)</td>
<td>Ratio (NADH:NADPH)</td>
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<td>B. fragilis Bf1</td>
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<tr>
<td>NH₄Cl (0.5 mM)</td>
<td>1348 ± 69.9</td>
<td>185 ± 4.6</td>
<td>0.137</td>
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<tr>
<td>NH₄Cl (5.0 mM)</td>
<td>784 ± 22.2</td>
<td>120 ± 6.1</td>
<td>0.153</td>
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<tr>
<td>NH₄Cl (50.0 mM)</td>
<td>291 ± 18.2</td>
<td>80 ± 5.0</td>
<td>0.27</td>
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<tr>
<td>BHI</td>
<td>107 ± 7.2</td>
<td>180 ± 16.0</td>
<td>1.68</td>
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<tr>
<td>Tryptone (0.25%)</td>
<td>406 ± 14.7</td>
<td>146 ± 7.2</td>
<td>0.35</td>
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<tr>
<td>Tryptone (125%)</td>
<td>85 ± 9.9</td>
<td>172 ± 12.3</td>
<td>2.02</td>
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<tr>
<td>E. coli MX3004(pGDH15)</td>
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<tr>
<td>YT</td>
<td>28 ± 0.0</td>
<td>610 ± 31.2</td>
<td>21.78</td>
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<tr>
<td>YT + glucose (0.5%)</td>
<td>20 ± 0.0</td>
<td>366 ± 17.6</td>
<td>18.30</td>
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<tr>
<td>E. coli MX3004(pMT104)</td>
<td>ND</td>
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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 the 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.

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

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).
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 NAD-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 NH₄Cl 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 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 \([\Delta G = -21.4 \text{ kcal mol}^{-1} \{89.5 \text{ kJ mol}^{-1}\}]\), similar to \(\rho\)-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 233 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 \(\alpha\)-ketoglutarate and the catalytic activity of the enzyme (reviewed by Smith et al., 1975). Three putative consensus GTP/ATP-binding motifs were evident, GIDIKGTK (223–230), GPDD (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 \(B.\) thetaotaomicron \(gdhB\) (90\%; Mark Morrison, personal communication). The identity to \(Porphyromonas gingivalis\) was 79\% (Joe et al., 1994), followed by \(B.\) thetaotaomicron \(gdhA\) (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\%; Lyrly et al., 1991) and \(Halobacterium salinarium\) (34\%; Benachouen & 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 \(XmnI-BglII\) internal fragment of the \(gdhB\) gene. Lanes: 1, \(B.\) fragilis cells were grown in 0.5 mM \(\text{NH}_4\text{Cl}\) (lane 1), 5.0 mM \(\text{NH}_4\text{Cl}\) (2), 50 mM \(\text{NH}_4\text{Cl}\) (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 (Benachouen-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% optimum 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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can be attributed to one enzyme (GdhA), and gdhA expression is regulated in response to the nitrogen source available for growth. Appl Environ Microbiol 62, 3826–3833.


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