

# The NAD(P)H-Utilizing Glutamate Dehydrogenase of *Bacteroides thetaiotaomicron* Belongs to Enzyme Family I, and Its Activity Is Affected by *trans*-Acting Gene(s) Positioned Downstream of *gdhA*†

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Previous studies have suggested that regulation of the enzymes of ammonia assimilation in human colonic *Bacteroides* species is coordinated differently than in other eubacteria. The gene encoding an NAD(P)H-dependent glutamate dehydrogenase (*gdhA*) in *Bacteroides thetaiotaomicron* was cloned and expressed in *Escherichia coli* by mutant complementation from the recombinant plasmid pANS100. Examination of the predicted GdhA amino acid sequence revealed that this enzyme possesses motifs typical of the family I-type hexameric GDH proteins. Northern blot analysis with a *gdhA*-specific probe indicated that a single transcript with an electrophoretic mobility of ~1.6 kb was produced in both *B. thetaiotaomicron* and *E. coli gdhA*<sup>+</sup> transformants. Although *gdhA* transcription was unaffected, no GdhA enzyme activity could be detected in *E. coli* transformants when smaller DNA fragments from pANS100, which contained the entire *gdhA* gene, were analyzed. Enzyme activity was restored if these *E. coli* strains were cotransformed with a second plasmid, which contained a 3-kb segment of DNA located downstream of the *gdhA* coding region. Frameshift mutagenesis within the DNA downstream of *gdhA* in pANS100 also resulted in the loss of GdhA enzyme activity. Collectively, these results are interpreted as evidence for the role of an additional gene product(s) in modulating the activity of GDH enzyme activity. Insertional mutagenesis experiments which led to disruption of the *gdhA* gene on the *B. thetaiotaomicron* chromosome indicated that *gdhA* mutants were not glutamate auxotrophs, but attempts to isolate similar mutants with insertion mutations in the region downstream of the *gdhA* gene were unsuccessful.

*Bacteroidaceae* are gram-negative obligate anaerobes and are predominant members of the microflora associated with the oral cavity and digestive tract of humans and herbivorous animals. The human colonic species have been particularly well studied in relation to the genetics and biochemistry of antibiotic resistance (35, 37), gene transfer (42, 43, 48, 50), and other virulence factors (21). Significant progress has also been made in dissecting the enzymology and regulation of polysaccharide utilization in colonic *Bacteroides* species (2, 10, 12, 23, 36, 38). Most species of human colonic *Bacteroides* are incapable of utilizing amino acids as a nitrogen source, and growth with oligopeptides is restricted to only a few species. Therefore, pathways of ammonia assimilation are critical to the growth and development of these bacteria in the large intestine. Although current understanding of the pathways of nitrogen assimilation and nitrogen regulation in these bacteria is limited, studies which have demonstrated that the synthesis and secretion of specific proteases, which play a role in pathogenicity (17, 21), are subject to nitrogen regulation (28) underscore the importance of further investigation. Moreover, a new precedent in terms of the structure of glutamine synthetases (GS) was established from studies with *Bacteroides fragilis* (46), and when compared to its counterpart in the family *Enterobacteriaceae* evidence suggests that regulation of ammonia assimila-

tion is fundamentally distinct in *Bacteroides*. The *B. fragilis* GlnA product is not subject to adenylation, and *ntrB* and *ntrC* homologs have not been identified. Yamamoto et al. (55, 57) also concluded that the primary means of ammonia assimilation in *B. fragilis* appears to occur via the glutamate dehydrogenase (GDH) pathway. During nitrogen-limited growth, inhibition of GS enzyme activity with methiosulfoxamine did not affect bacterial growth rate or yield, and NAD(P)H-utilizing GDH activity increased. Additionally, it has been proposed that GDH enzyme activity is controlled by a reversible inactivation/activation mechanism, regulated by the ammonia concentration in the growth medium. This hypothesis has been developed from studies which showed that when cells were subjected to ammonia shock, there was little change in the amount of extractable GDH protein but the specific activity of the enzyme was decreased. The underlying mechanism modulating GDH activity, however, remains unclear, but no phosphorylation or adenylation of the GDH enzyme has been detected (57).

To elucidate the biochemical events underpinning the modulation of GDH activity in colonic *Bacteroides* species, we have cloned and sequenced a gene from *B. thetaiotaomicron*, which encodes a NAD(P)H-utilizing GDH enzyme. We have chosen to work with *B. thetaiotaomicron* because it is much easier to manipulate genetically than *B. fragilis*, and the genetic distance between these two species is great enough to make it conceivable that differences in nitrogen regulation and ammonia assimilation might exist. Mutational analysis confirmed that *gdhA* mutants were not glutamate auxotrophs. Evidence is also presented that suggests GDH activity is modulated by genetic

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>E. coli</i> S17-1	Tp <sup>r</sup> Gm <sup>s</sup> <i>recA</i> , IncP RP4 inserted in chromosome	A. A. Salyers (44)
<i>E. coli</i> PA340	<i>gdh-1</i> $\Delta$ ( <i>glhBDF</i> )500	R. A. Bender (8)
<i>B. thetaiotaomicron</i> 5482	Em <sup>s</sup> Gm <sup>r</sup>	A. A. Salyers
<b>Plasmids</b>		
pEcoR251	pBR-328 based positive selection vector Ap <sup>r</sup> $\rho$ <sub>R</sub> - <i>EcoRI</i>	B. A. White (46)
pRK248cI <sub>ts</sub>	Tc <sup>r</sup> cI <sub>ts</sub>	R. A. Bender (9)
pGB2	Spc <sup>r</sup> Str <sup>r</sup> pSC-101 replicon	R. A. Bender (13)
pCQW1	pUC19-based <i>E. coli</i> vector Ap <sup>r</sup> (Em <sup>r</sup> ); promoterless <i>uidA</i> suicide delivery vector in <i>Bacteroides</i> spp.	A. A. Salyers
pANS100	7-kb <i>B. thetaiotaomicron</i> 5482 DNA fragment cloned in pEcoR251; <i>gdhA</i> <sup>+</sup>	This study
pANS101	<i>Bam</i> HI- <i>Kpn</i> I deletion of pANS100; <i>gdhA</i> <sup>+</sup>	This study
pANS102	<i>Bam</i> HI- <i>Sac</i> I deletion of pANS100; <i>gdhA</i> <sup>+</sup>	This study
pANS103	<i>Pst</i> I- <i>Ava</i> I fragment of pANS100 subcloned in pBluescript II; <i>gdhA</i>	This study
pANS104	<i>Kpn</i> I- <i>Ava</i> I fragment of pANS100 subcloned in pBluescript II; <i>gdhA</i>	This study
pANS105	<i>Eco</i> RI deletion of pANS100; <i>gdhA</i>	This study
pANS106	<i>Eco</i> RI- <i>Hind</i> III fragment of pANS100 subcloned in pGB2; <i>gdhA</i>	This study
pANS107	<i>Ava</i> I-directed point mutation in pANS100; <i>gdhA</i>	This study
pANS108	<i>Pvu</i> I- <i>Ava</i> I fragment of pANS100 subcloned in pGB2; <i>gdhA</i>	This study
pANS900	<i>Sma</i> I- <i>Sac</i> I deletion of pCQW1	This study
pANS901	800-bp <i>Eco</i> RI- <i>Hind</i> III fragment of pANS100 subcloned in pANS900	This study
pANS902	1.6-kb <i>Nde</i> I- <i>Ava</i> I fragment of pANS100 subcloned in pANS900	This study
pBluescript II	KS <sup>+</sup> or SK <sup>+</sup> , Ap <sup>r</sup>	Stratagene

<sup>a</sup> Abbreviations: r, resistance; s, sensitivity; Ap, ampicillin; Em, erythromycin; Gm, gentamicin; Spc, spectinomycin; Str, streptomycin; Tc, tetracycline; Tp, trimethoprim. For plasmid pCQW1, the phenotype in parentheses is expressed in *B. thetaiotaomicron* 5482 only. Plasmid phenotypes not contained in parenthesis are expressed only in *E. coli*.

material located downstream of *gdhA*, which can function in *trans*.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** Bacterial strains and plasmids used in this study are described in Table 1. All cultures of *Bacteroides* were grown anaerobically at 37°C in either Trypticase-yeast extract-glucose (TYG) medium (23) or defined salts medium (DBM; 51). When necessary, the media were supplemented with gentamicin (200  $\mu$ g ml<sup>-1</sup>) or erythromycin (10  $\mu$ g ml<sup>-1</sup>). *Escherichia coli* strains were cultured at 37°C in either Luria-Bertani broth or minimal A medium (32). When required, amino acids were added to minimal A medium to a final concentration of 1 mM, and ampicillin (100  $\mu$ g ml<sup>-1</sup>), tetracycline (10  $\mu$ g ml<sup>-1</sup>), trimethoprim (100  $\mu$ g ml<sup>-1</sup>) or kanamycin (50  $\mu$ g ml<sup>-1</sup>) was included in the growth medium. Plate medium was prepared by adding agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 1.5% (wt/vol).

**GDH enzyme assays.** Cultures (10 ml) were grown to mid-logarithmic phase (optical density at 600 nm, 0.3 to 0.5) and harvested by centrifugation (10,000  $\times$  g for 10 min at 4°C). The cells were washed with 1% (wt/vol) KCl, pelleted, and resuspended in 1 ml of the same solution. The GDH activity of whole-cell preparations was determined similarly to previously described methods (11). The oxidation of NAD(P)H at 37°C was continuously monitored at 340 nm with a computerized spectrophotometer (DU-650; Beckman Industries, La Jolla, Calif.). Corrections for nonspecific oxidation of the cofactors were determined simultaneously from reactions without added ammonia. Protein was determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. One unit of GDH activity is defined as 1 nmol of cofactor oxidized min<sup>-1</sup> mg of protein<sup>-1</sup>.

**General recombinant DNA procedures.** Unless otherwise stated, standard procedures were used throughout (39), and all DNA-modifying enzymes were obtained from either Promega (Madison, Wis.) or Gibco BRL (Gaithersburg, Md.). DNA fragments for cloning were extracted from agarose gels with the Gene Clean II kit (Bio101, Vista, Calif.) or from polyacrylamide gels by the crush-and-soak method (29). Electroporations were performed with the Gene Pulser apparatus (Bio-rad Laboratories, Hercules, Calif.) as specified by the manufacturer.

**Construction and screening of recombinant DNA library.** Chromosomal DNA was constructed by the method of Ausubel et al. (3) from a 500-ml culture of *B. thetaiotaomicron* cells grown on TYG broth. The genomic DNA was partially digested with *Sau*3AI, and fragments were fractionated by sucrose density gradient centrifugation. Fragments in the size range of 6 to 12 kb were ligated with *Bgl*II-digested pEcoR251 (46, 47), and the recombinant plasmids were transformed into *E. coli* PA340 by electroporation. Expression of GDH activity was

screened via mutant complementation, by plating transformed cells onto minimal A-ampicillin medium and selecting for both glutamate prototrophy and antibiotic resistance.

**Cotransformation experiments.** In preliminary experiments, *E. coli* PA340/pANS100 transformants (Table 1) were cotransformed by electroporation with pRK248cI<sub>ts</sub> (9). The latter plasmid encodes a temperature-sensitive version of the lambda cI repressor protein, which represses  $\rho$ <sub>R</sub>-directed transcription at the permissive temperature.

**Southern and Northern blot analyses.** For Southern blot analyses, digested DNA was transferred to a charged-nylon membrane (Zetaprobe GT; Bio-Rad Laboratories) by vacuum blotting. Probes for *gdhA* (800-bp *Eco*RI-*Hind*III fragment from pANS100) and *ermF* (1.6-kb *Eco*RI-*Hind*III *ermF* fragment from pCQW1) were prepared with a random-primer DNA-labelling kit from Pharmacia Biotech (Piscataway, N.J.). Hybridizations were performed overnight at 43°C, and the membranes were washed in 2 $\times$  SSC (1 $\times$  SSC is 0.1 M NaCl plus 0.015 M sodium citrate)-0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature for 15 min and then twice in 0.1 $\times$  SSC-0.1% (wt/vol) SDS at 65°C, before being exposed to X-ray film.

For Northern blot analysis, *B. thetaiotaomicron* was cultured in 10 ml of DBM containing either 0.5, 1.0, 10.0, 20.0, or 50.0 mM NH<sub>4</sub>Cl. *E. coli* PA340 and PA340 possessing various plasmids were cultured in Luria-Bertani medium plus ampicillin. RNA was isolated from cultures grown to midlogarithmic phase (optical density at 600 nm of  $\approx$ 0.5) by pipetting the culture directly into 0.5 volume of boiling 0.3 M sodium acetate (pH 4.5) containing 30 mM EDTA and 1.5% (wt/vol) SDS. The mixture was boiled for 1 min and then extracted successively with phenol (pH 4.5; preheated to 60°C), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). The final aqueous phase was precipitated with 2 volumes of ethanol. RNA was recovered by centrifugation, washed once with 70% (vol/vol) ethanol, and dissolved in diethylpyrocarbonate-treated H<sub>2</sub>O. RNA concentration was determined spectrophotometrically.

RNA samples (10  $\mu$ g) were denatured with glyoxal, separated electrophoretically through 1% (wt/vol) agarose gels in 10 mM sodium phosphate (pH 7) (39), and then transferred to Zetaprobe GT membranes by vacuum blotting. The blot was hybridized with a <sup>32</sup>P-labelled *gdhA*-specific probe, prepared as described above. As an internal control for integrity and amounts of RNA, an oligonucleotide (5'-TACCGCGCTGCTGGCAG) which corresponds to a universally conserved region of 16S rRNA was end labeled with [ $\gamma$ -<sup>32</sup>P]dATP by using T4 polynucleotide kinase and also used as a probe.

**Construction of *gdhA* mutants by using the suicide delivery vector pANS900.** To insert ionally inactivate the *gdhA* gene in *B. thetaiotaomicron*, we used a strategy based on the integration vector pANS900, which encodes for Em<sup>r</sup> in *Bacteroides* but is incapable of replication in this genus. Plasmid pANS901 was constructed by subcloning the 800-bp *Eco*RI-*Hind*III *gdhA* gene fragment from

TABLE 2. GDH specific activity measured from whole-cell preparations of *B. thetaiotaomicron* 5482

Nitrogen source <sup>a</sup>	GDH sp act (nmol of cofactor oxidized min <sup>-1</sup> mg of protein <sup>-1</sup> )	
	NADPH	NADH
1.0 mM NH <sub>4</sub> Cl	877 ± 23.3	467 ± 32.5
10.0 mM NH <sub>4</sub> Cl	573 ± 66.3	437 ± 57.1
50.0 mM NH <sub>4</sub> Cl	253 ± 18.5	184 ± 14.1
TYG	206 ± 29.7	119 ± 21.2

<sup>a</sup> Cultures were grown in either DBM (51) containing the indicated amounts of ammonium or in TYG medium (23). Values are the means ± standard deviations determined from at least independent cultures.

pANS100 into pANS900 (Table 1); then the plasmid was transferred to *E. coli* S17-1 by electroporation. Bacterial matings were carried out as described previously (50) with wild-type *B. thetaiotaomicron* 5482 as recipient. Control matings were also carried out with *E. coli* S17-1 containing the shuttle vector pVAL-1 (50). Mating mixtures were resuspended in 2 ml of anaerobically prepared TYG broth, and aliquots were spread on selective plates (TYG plus gentamicin and erythromycin) and incubated under anaerobic conditions at 37°C for 36 to 48 h. Putative insertion mutants were restreaked on TYG plus gentamicin and erythromycin plate medium and then checked to confirm that they were *Bacteroides* strains by incubating plates under both aerobic and anaerobic conditions. The locations of the disruptions in the *B. thetaiotaomicron* chromosome were determined by Southern blot analysis, and the phenotypes of *gdhA* mutants were confirmed by enzyme assay. Nondenaturing PAGE activity gels were prepared as previously described (53) to ascertain whether the *gdhA* mutants might produce a truncated GdhA protein, which could still account for ammonia assimilation and growth.

**Nucleotide sequencing.** DNA sequence analysis of the GDH gene was performed by dideoxy sequencing of recombinant plasmids with modified T7 polymerase (Sequenase 2.0; U.S. Biochemical Corp., Cleveland, Ohio). Reaction mixtures were analyzed in 6% (wt/vol) polyacrylamide gels containing 8 M urea, and sequence information was analyzed with the University of Wisconsin Ge-

netics Computer Group DNA sequence analysis software (version 8) (16). The entire sequence spanning the *gdhA* locus was determined for both strands.

## RESULTS

### Effect of nitrogen source upon growth and GDH activity.

Growth of *B. thetaiotaomicron* 5482 in defined minimal medium and modulation of GDH enzyme activity in response to ammonia concentration were similar to those previously observed in studies with *B. fragilis*. The doubling times of *B. thetaiotaomicron* cultures used for GDH assays were calculated to be 180, 120, and 114 min during growth in defined medium containing 1, 10, and 50 mM ammonium chloride, respectively, and 100 min in TYG medium. Therefore, 1 mM ammonium chloride appears to be a growth-limiting concentration of nitrogen for this bacterium. The results of GDH assays with cells cultured in the above media and harvested during the mid-log phase of growth are listed in Table 2. Typical of the colonic *Bacteroides* spp., both NADPH- and NADH-dependent activities were measurable for all growth conditions, and the highest specific activities were observed when the bacterium was grown with a growth-limiting concentration of ammonia. The ratio of NADPH to NADH specific activities ranged between 1.3 (cells cultured with 10 mM ammonium chloride) and 1.9 (1 mM ammonium chloride), and growth in the presence of peptides did not have a major impact upon NAD(H)-dependent GDH activity. Therefore, *B. thetaiotaomicron* seems an appropriate choice for more detailed studies of the mechanism(s) modulating GDH enzyme activity in human colonic *Bacteroides* species.

**Cloning and characterization of the *gdhA* gene.** *E. coli* PA340 carries a mutation in the *gdh* gene, and the *gltBD* genes,

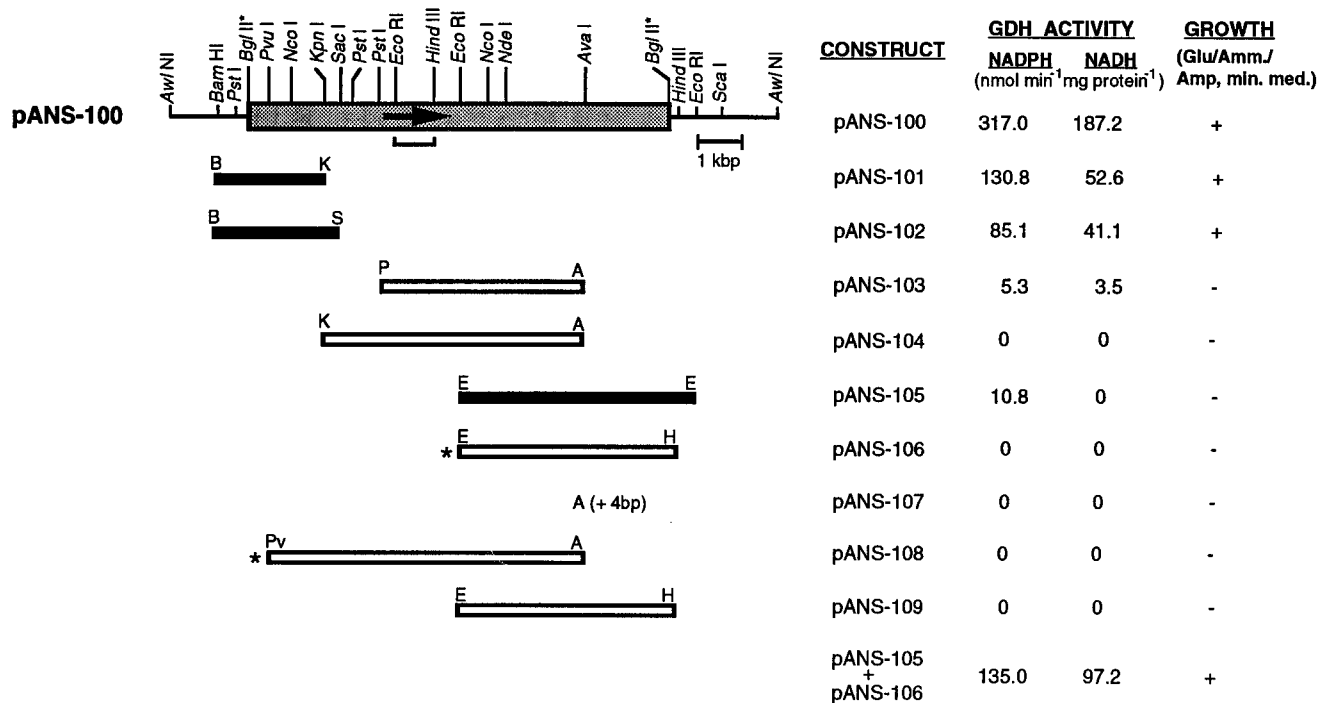


FIG. 1. Restriction enzyme and subcloning/deletion analysis of the recombinant plasmid pANS100. The extent of the ORF and the direction of transcription of the *gdhA* structural gene in pANS100 are indicated by the solid arrow. The open bars represent DNA fragments which were subcloned into a different vector, and the solid bars represent DNA fragments which were deleted from pANS100. The 800-bp *EcoRI*-to-*HindIII* fragment which was used as a probe in Northern and Southern blot analyses is indicated by the horizontal bracket. Fragments were subcloned into pBluescript KS<sup>+</sup> or SK<sup>+</sup> except those indicated by an asterisk, which were subcloned into pGB2. GDH specific activity and ability to transform *E. coli* PA340 to glutamate prototrophy are shown to the right of each construct. The coefficients of variation for those clones with measurable GdhA enzyme activity did not exceed 15%. Glu, glucose; Amm., ammonium; min. med., minimal medium.

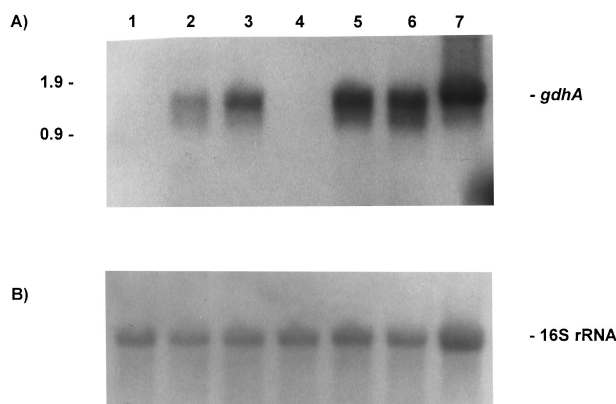


FIG. 2. Transcription of the *B. thetaiotaomicron gdhA* gene in *E. coli* does not require DNA sequences located downstream of this gene. Total RNA was extracted from either *E. coli* PA340 alone (lane 1) or *E. coli* PA340 transformed with pANS100 (lane 2), pANS105 (lane 3), or pANS106 (lane 4) or cotransformed with pANS105 and pANS106 (lanes 5 and 6). Total RNA from *B. thetaiotaomicron* cultured in DBM containing 10 mM ammonium chloride is shown in lane 7. (A) RNA was hybridized with a  $^{32}\text{P}$ -labelled probe for the *EcoRI*-to-*HindIII* fragment of the *gdhA* structural gene from pANS100; (B) the same membrane was stripped and rehybridized with a  $^{32}\text{P}$ -end-labelled oligonucleotide probe specific for a universally conserved region in 16S rRNA. The sizes of RNA markers and their migration distance (Promega Corp.) are indicated to the left in panel A.

which encode the large and small subunits of glutamate synthase (GOGAT), respectively, are also deleted, giving rise to a phenotype of glutamate auxotrophy. This strain therefore provides a potential background for cloning genes encoding enzymes involved with both ammonia assimilation and glutamate biosynthesis. When a plasmid DNA library of *B. thetaiotaomicron* DNA was transformed into PA340, two types of colonies were apparent following growth on the selective medium lacking glutamate. Type A colonies developed within 24 h to a diameter of approximately 2 to 3 mm, while the second class of transformants (type B) took longer to develop (~48 h) and were approximately half the size of those classified as type A. Fifteen transformants of each class were selected and restreaked for single colonies, and plasmid DNA was prepared from each transformant. Within each class, the plasmid restriction fragment patterns obtained from each transformant were very similar (data not shown), and a representative clone from a type A transformant, pANS100, is shown in Fig. 1. The plasmid pANS100 was subsequently used to retransform *E. coli* PA340, and the transformation efficiencies, measured by either  $\text{Ap}^r$  or glutamate prototrophy, were the same. The origin of the 7-kb DNA insert present in pANS100 was confirmed by Southern blot analysis of *B. thetaiotaomicron* chromosomal DNA digested with various restriction enzymes, and the hybridization patterns were found to be consistent with predictions from restriction sites present in cloned DNA (data not shown).

The *E. coli* PA340/pANS100 transformant was also characterized enzymatically by measuring GDH specific activity. Both NADH- and NADPH-dependent GDH activities were measurable, and the specific activities obtained were not greatly affected by ammonia concentrations between 1 and 50 mM. The NADPH-dependent GDH specific activities ranged between 386 and 520 nmol of cofactor oxidized  $\text{min}^{-1}$  mg of protein $^{-1}$ , while NADH-dependent activities ranged between 89 and 211 nmol of cofactor oxidized  $\text{min}^{-1}$  mg of protein $^{-1}$  at higher ammonia concentrations.

**Genetic information located downstream of *gdhA* in pANS100 is required for GDH enzyme activity.** Subcloning and

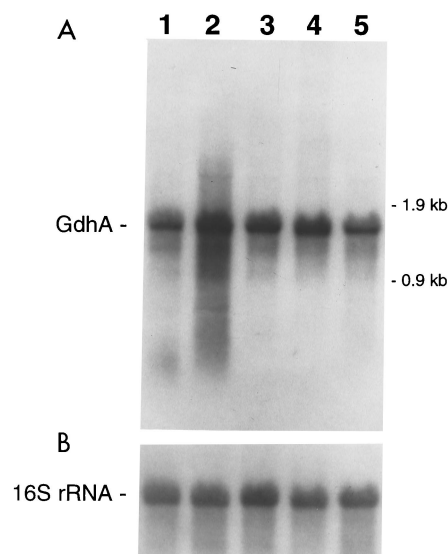


FIG. 3. Northern blot analysis of *B. thetaiotaomicron gdhA* RNA. Total RNA was isolated from cells cultured in DBM supplemented with 0.5, 1.0, 10.0, 20.0, or 50.0 mM ammonia (lanes 1 to 5, respectively). The membrane was hybridized with either the *gdhA*-specific probe (A) or the 16S rRNA-specific oligonucleotide probe (B). The sizes of RNA markers and their migration distance (Promega Corp.) are indicated to the right in panel A.

deletion analyses were used to delimit sequences in pANS100 necessary for GdhA activity in *E. coli* PA340. The transformants were examined for complementation of the *E. coli* PA340 glutamate auxotrophy by an assay of GdhA enzyme activity. A summary of the results obtained is shown in Fig. 1. DNA sequence analysis indicated that *gdhA* was located within the 2-kb *PstI*-to-*EcoRI* fragment of pANS100 (see below). Enzyme activity was maintained when sequences upstream of the *gdhA* gene in pANS100 were removed but was reduced relative to the activity measured from pANS100 transformants (Fig. 1). When sequences encompassing the *gdhA* structural gene in pANS100 were subcloned into a different vector (pBluescript II SK $^+$ , giving rise to pANS103 and pANS104), GDH enzyme activity was virtually eliminated; and when the region downstream of the GdhA coding region was deleted in pANS100 to generate pANS105, greatly reduced levels of GDH activities were also observed (Fig. 1). However, when pANS105 and a plasmid containing the region immediately downstream of *gdhA* (pANS106) were cotransformed into *E. coli* PA340, both NADPH- and NADH-dependent GDH activities were restored. In an attempt to further delineate the essential downstream region, plasmid pANS100 was digested with *AvaI*, the DNA overhangs were filled in with Klenow fragment, and the blunt ends were religated (pANS107). This modification resulted in the loss of the *AvaI* restriction site and should result in a frameshift mutation within any potential open reading frames (ORFs) spanning this region. While no GdhA activity could be detected in the PA340 pANS107 transformants, glutamate prototrophy was restored in *E. coli* PA340 cotransformed with both pANS106 and pANS107. These data suggest that sequences immediately downstream of *gdhA* are necessary for the expression or activity of GdhA in *E. coli*.

***gdhA* transcript and sequence analyses.** *E. coli* PA340 was cotransformed with pANS100 and pRK248cI $_{\text{ts}}$ , and aliquots of the electroporated cells were plated onto minimal A medium supplemented with ampicillin and tetracycline and then incubated at either 30 or 42°C. Selection for glutamate prototrophy

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1 GCATTTTCTTTTATAAAAATGTTTGTCTAGTTCAATTTTTATCTTTACTTTTGGCAACCCGATAAAAGAAATAGTAAATCGAAATTTAATCAACCAATAAAAG 100
101 AAAAAAGATTATGAATGCAGCAAAGGTATTAGACGATCTGAAAAGACGGTTCGCCAACGAAACCGGAGTATCATCAGGCAGTAGAAGAAGTACTTTCTAC 200
      M N A A K V L D D L K R R F P N E P E Y H Q A V E E V L S T
201 TATTGAAGAAGAAATACAAACAAACACCCGGAGTTTGACAAAGCAAACCTAATCGAACCGCTTGTGTATTCCTGATAGAGTATTTTCAGTTCCGTGTAACCTGG 300
      I E E E Y N K H P E F D K A N L I E R L C I P D R V F Q F R V T W
301 ATGGACGACAAAGGTAATATTCAGACAAACATGGGTTACCGTGTTCAGACACAACACCGCTATCGGCCCTACAAGGCGGTATCCGTTTCCACGCATCTG 400
      M D D K G N I Q T N M G Y R V Q H N N A I G P Y K G G I R F H A S
401 TAAACCTTCCATCCTGAAATTCCTTGCCTTTGAGCAGACATTCAAAAACCTCACTGACTACGCTGCCTATGGGTGGTGAAGGGTGGTCCGACTTCTC 500
      V N L S I L K F L A F E Q T F K N S L T T L P M G G G K G G S D F S
501 TCCACGTGTAAAGTCAAACCGCTGAAGTAATGCGTTTCGTACAGGCATTATGCTGGAACCTGTGGCGTCACATCGGTCCCGAAACTGACGTACCTGACG 600
      P R G K S N A E V M R F V Q A F M L E L W R H I G P E T D V P A G
601 GATATCGGTGTAGGTGGCCGAAAGTAGTTTCATGTTCCGGTATGTACAGAAGCTGGCTCACGAAATTTACCGGCACATTCACTGGTAAAGCCGCGAAT 700
      D I G V G G R E V G F M F G M Y K K L A H E F T G T F T G K G R E
701 TCGGTGGTTCAGTATTCGTCCGGAAGCTACCGTTACCGTAAACATCTACTTCTGATGGAATGTGAAAACCAAGTACTGACCTGAAAGTAAAGT 800
      F G G S L I R P E A T G Y G N I Y F L M E M L K T K G T D L K G K V
801 TTGTCITGTTCCGGTTCGGGTAACGTAGCTCAATATACAATCGAAAAGTAATCGAACTGGGTGTAAGTAGTTACTTGTCTCCGACTCTGACGGTTAC 900
      C L V S G S G N V A Q Y T I E K V I E L G G K V V T C S D S D G Y
901 ATCTACGATCCGGACGGTATCGACCGGAAAAGCTGGATTACATCATGGAACCTGAAGAACCTGTACCGTGGCCGATCCGCGAATACCGAGAGAAATATG 1000
      I Y D P D G I D R E K L D Y I M E L K N L Y R G R I R E Y A E K Y
1001 GTTGCAAAATGTAGAAGGAGCTAAGCCTTGGGTGAAAAATCGCATATCGCACTGCCTTCTGCCACTCAGAACGAATGAAACGGGACACGCCCCGTCA 1100
      C C K Y V E G A K P W G E K C D I A L P S A T Q N E L N G D H A R Q
1101 GTTGTAGCAAACGGTGTATAGCTGTATCTGAGGGTGCAAATATGCCTTCTACTCCGGAAGCTGTCCGTGTAATCCAAGACGCTAAGATTCTGTACGGT 1200
      L V A N G C I A V S E G A N M P S T P E A V R V F Q D A K I L Y A
1201 CCGGTAAAGCAGCTAATGCAGGTGGTGTATCAGTATCAGGTCCTGAAATGACTCAGAATCCATCAAGTTGAGCTGGAGCGCTGAAGAAGTAGACGAAA 1300
      P G K A A N A G G V S V S G L E M T Q N S I K L S W S A E E V D E
1301 AGCTGAAGAGCATCATGAAGAATATCCACGAAGCTTCCGTTTCAGTATGGTACTGAAGCCGACGGATATGAAACTATGTAAGAGGTGCTAACGTAGCCGG 1400
      K L K S I M K N I H E A C V Q Y G T E A D G Y V N Y V K G A N V A G
1401 ATTTATGAAGGTTGCCAAGCTATGATGGCTCAGGGTATCGTATAATCAGCAAAATATAAGAAAGCTATAGTCATTTTAAAGATAAAGGCTCCCGCCTTC 1500
      F M K V A K A M M A Q G I V *
1501 CGAAAGGAAGGACGGGAGTTTTTTTATGTTGTACCCGAGTGGTGA AAAACAAGTAAAGAGTGCAGGC AAACATATAAAAAGAGTGAGTGACAGGAAGTAC 1600
1601 TTCACTCTGGCAAATGATGATAGAAGAAAAAGTAAAGAAGTACATGACTTGGATGAAAGATGTACTTGAATAAAAAAATATCGGAGGTTTTAAAGATTG 1700
1701 TTCATAGTATTCTCTATCTTTAGATAGAAATATCAGTTTGACAAGCAAGCAAAATCGGTCTATCTTGTTCACATATTATAAACCCGGACAGAGAATA 1800
1801 AAAAAGACTTCGGTTTATTAAACTTGCCTCCGACTAACATCTATTTTATGGAAGAAAAAAAATAACTGCCGGAACGGTCTGCCATTTGCCGACA 1898

```

FIG. 4. Nucleotide sequence of the *gdhA* gene in pANS100. The predicted amino acid sequence is shown in single-letter code below the coding sequence. The translation stop codon is indicated by an asterisk.

was not affected by growth of *E. coli* cotransformants at either the permissive or nonpermissive temperature for *cI* binding to DNA (data not shown), confirming that *gdhA* gene expression in pANS100 was not dependent upon  $p\lambda_R$ -directed transcription. DNA sequence analysis confirmed that the orientation of *gdhA* transcription would occur opposite  $p\lambda_R$ -directed transcription.

Although measurable GdhA enzyme activity in *E. coli* appears to be dependent upon the DNA sequence located downstream of the *gdhA* structural gene, *gdhA* transcription is not (Fig. 2). Northern blot analysis showed that the relative abundance of a *gdhA*-specific transcript was similar in *E. coli* PA340 strains containing either the GdhA<sup>+</sup> plasmid pANS100 or the GdhA<sup>-</sup> plasmid pANS105, as well as in pANS105/pANS106 cotransformants (GdhA<sup>+</sup>). Moreover, with the exception of RNA prepared from *E. coli* PA340 and a PA340/pANS106 transformant, the *gdhA*-specific fragment from pANS100 hybridized to a single transcript of ~1.6 kb in all samples including *B. thetaiotaomicron*, indicating that *gdhA* transcription was not part of a larger, polycistronic transcript. When RNA prepared from *B. thetaiotaomicron* cells grown in DBM with different ammonia concentrations was probed with the *gdhA*-specific probe, there appeared to be no visible differences in

the relative abundance of the *gdhA*-specific transcript from the different cell preparations (Fig. 3).

The entire nucleotide sequence of a 1,898-bp segment of pANS100 containing the *gdhA* gene was obtained for both strands, and an ORF with no more than two possible translation start sites was identified (Fig. 4). Translation initiation from the first Met codon would encode a polypeptide of 444 amino acids with a predicted molecular mass of 48 kDa, while translation initiated from the second Met codon would result in a polypeptide of 381 amino acids with a predicted molecular mass of 42 kDa. The consensus motif involved with NADP(H) binding (Gly-X-Gly-X-X-Ala) was also apparent in the nucleotide sequence. GDHs are thought to be a good model for a molecular study of evolutionary relationships, because of their ubiquitousness in nature and their slow mutation rate (1.8 amino acid substitutions 100 residues<sup>-1</sup> 10<sup>8</sup> years<sup>-1</sup> [54]). Previous multiple-sequence alignments of hexameric *gdh* sequences led to the hypothesis that two closely related but already different *gdh* genes were present in the last common ancestor of eubacteria, archaeobacteria, and eukaryotes (7) and that each of these enzyme families is distinguishable by the presence of highly conserved amino acid motifs. However, GDH sequences from the *Bacteroidaceae* were not available

<i>B. thetaiotaomicron</i> GdhA	ASVNL	KFL A F E Q	R P EATGY
<i>P. gingivalis</i> Gdh	PSVNL	KFL G F E Q	R P ESTGF
Gdh Family I Consensus	PSVNL	KFL(AG)F(EG)(EQ)	R(PT)EATGY

FIG. 5. Comparison of the predicted amino acid sequences for the *gdhA* gene of *B. thetaiotaomicron* and the *gdh* gene of *Porphyromonas gingivalis* to the consensus sequence motifs of family I GDHs.

for inclusion in these studies, and while the dual cofactor specificity of GdhA is not unique, it is still relatively rare (18). The predicted GdhA sequence was found to possess the highly conserved motifs typical of GDH enzyme family I (7), although the predicted ORF possessed a novel amino acid substitution within one of these motifs (Fig. 5). The *gdh* sequence of *Porphyromonas (Bacteroides) gingivalis* was also recently deposited in the databases; it possesses motifs typical of the family I hexameric GDH proteins but with several novel amino acid substitutions (Fig. 5). For these reasons, the presumptive Gdh sequences from *B. thetaiotaomicron* and *P. gingivalis* (30), as well as other *gdh* sequences available in the databases, were examined by using the FastA and "Growtrees" programs available through the Wisconsin Genetics Program (version 8.0 [4,

16]). Both sequences were divergent from all other family I sequences, with the exception of the *Clostridium symbiosum* Gdh sequence (Fig. 6). Moreover, the analysis indicated that the *B. thetaiotaomicron* sequence was more closely related to but preceded all of the family II type sequences, which include archaeobacteria (14).

**Analysis of *gdhA* insertion mutants.** To further establish the role of GdhA activity in ammonia assimilation by *B. thetaiotaomicron*, we constructed a strain in which the chromosomal *gdhA* gene was inactivated by plasmid integration, as described in Materials and Methods. Southern blot analysis of genomic DNA from *B. thetaiotaomicron* and the putative *gdhA* insertion confirmed that the site of insertion was within the *gdhA* gene (data not shown), and GDH enzyme assays showed that both NADPH- and the NADH-specific activities were greatly reduced in the *gdhA* mutant compared to the wild-type parent strain. However, this mutant was not a glutamate auxotroph, and growth in DBM containing 10 mM ammonium chloride was similar to that of the wild-type parent strain (Table 3). Nondenaturing polyacrylamide gel electrophoresis activity gels were used to ascertain whether the *gdhA* insertion mutant produced a truncated GdhA protein, which might account for

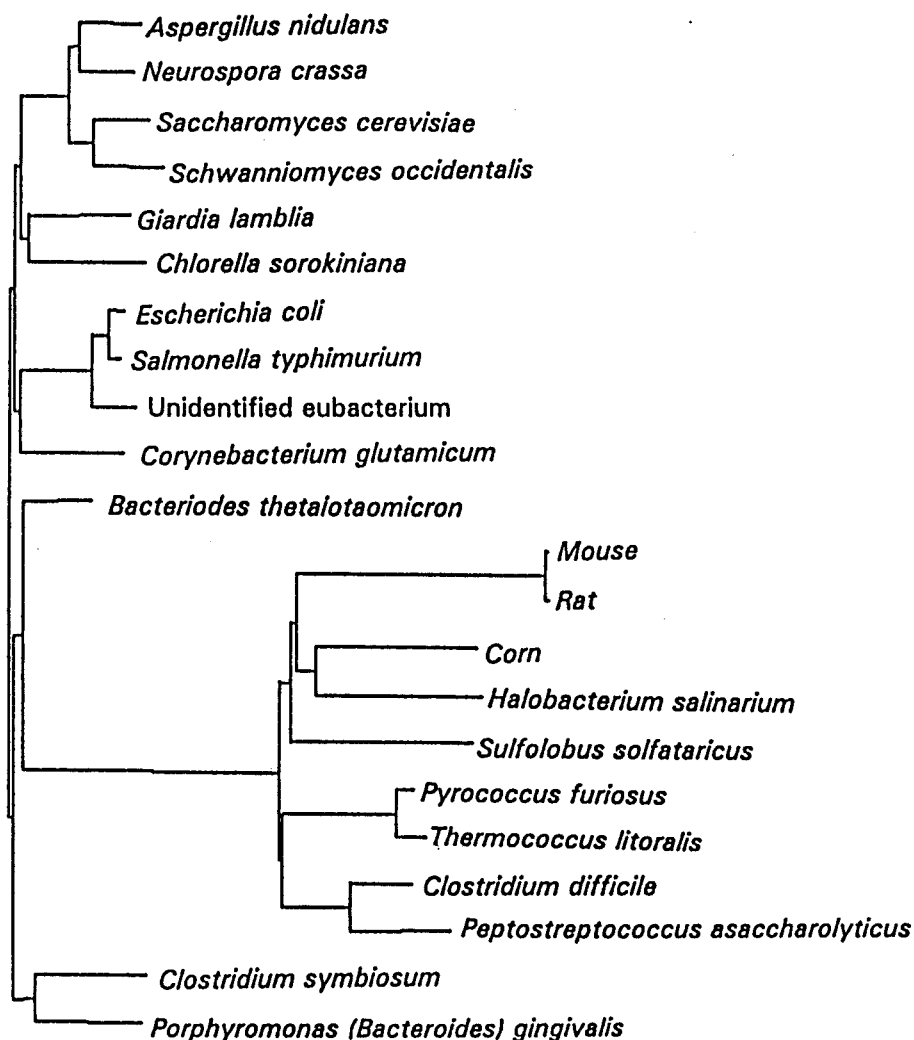


FIG. 6. Multiple alignment of 22 glutamate dehydrogenase sequences by the neighbor-joining method of the "Growtrees" program (Wisconsin Genetics Package, version 8.0).

TABLE 3. GDH specific activities and doubling times measured from whole-cell preparations of *Bacteroides thetaiotaomicron* 5482 and a *gdhA* mutant strain grown in either DBM or TYG broth medium

Strain	GDH sp act (nmol of cofactor oxidized min <sup>-1</sup> mg of protein <sup>-1</sup> )				Doubling time (min) in:	
	NADPH		NADH			
	TYG	DBM	TYG	DBM	TYG	DBM
Wild type	167 ± 20.1	459 ± 24.0	114 ± 6.2	270 ± 3.8	85	85
<i>gdhA</i> mutant	4 ± 7.0	16 ± 26.1	15 ± 12.5	13 ± 15	85	120

residual enzyme activity and ammonia assimilation. Although the wild-type strain possessed a strongly reactive protein, the *gdhA* insertion mutant possessed no such protein (Fig. 7). Thus, it would appear that *B. thetaiotaomicron* possesses a second enzymatic pathway catalyzing ammonia assimilation and glutamate biosynthesis. We also attempted to determine the effects of disruptions in the region downstream of the *gdhA* gene in *B. thetaiotaomicron*. Using the pANS900 suicide vector containing the 1.9-kb *NdeI*-to-*AvaI* fragment from pANS100 (Fig. 1), we have not been able to obtain any colonies following procedures identical to those used to isolate *gdhA* mutants.

## DISCUSSION

As part of the *Cytophaga-Flavobacter-Bacteroides* group, the human colonic *Bacteroides* probably represents an ancient branch in the eubacterial line of descent (52), and phylogenetic analysis indicated that the *gdhA* structural gene from *B. thetaiotaomicron* was distantly related to virtually all other eubacterial glutamate dehydrogenases. Similar to other species of human colonic *Bacteroides* (55), streptococci (19), and other strict anaerobes (34), GdhA enzyme activity in *B. thetaiotaomicron* was greatest during ammonia-limited growth. Subcloning and deletion analysis of pANS100 showed that DNA sequences flanking *gdhA* affect enzyme activity. Additionally, *AvaI* digestion of pANS100, coupled with the generation of blunt ends by Klenow fragment and subsequent religation,

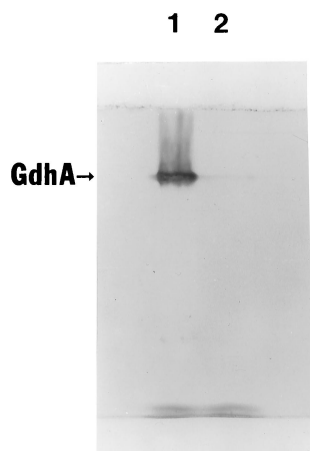


FIG. 7. Nondenaturing PAGE activity gel to detect the presence or absence of GdhA enzyme activity in cell extracts of wild-type *B. thetaiotaomicron* 5482 (lane 1) or a *gdhA* insertion mutant of the same strain (lane 2). The preparation of cell extracts, electrophoresis, and NADP<sup>+</sup> specific activity staining were all performed by previously described procedures (53).

resulted in the loss of measurable GdhA enzyme activity in *E. coli*. DNA sequences downstream from the *gdhA* gene, which could be provided in *trans*, are required for GdhA enzyme activity but not gene transcription. Collectively, these results support the contention that nitrogen regulation and modulation of GDH enzyme activity in human colonic *Bacteroides* species are markedly different from those in other eubacteria.

Previous studies of ammonia assimilation by *B. fragilis* led to the conclusion that the GDH-catalyzed pathway was primarily responsible for ammonia assimilation and growth in both ammonia-excess and ammonia-limited environments (55). When the *gdhA* gene of *B. thetaiotaomicron* was disrupted by insertional mutagenesis, NAD(P)H-dependent GDH enzyme activity was virtually eliminated but the growth rate and yield of the mutant strain in defined medium was only slightly affected. Nondenaturing PAGE activity gels have been used to assess whether the *gdhA* mutants lack GdhA or possess a truncated version of the GdhA protein which might still support growth with ammonia as the sole nitrogen source. The results showed that the *gdhA* insertion mutants lack a functional GdhA protein (Fig. 7), suggesting that an alternative pathway of ammonia assimilation exists in this bacterium. The partial characterization of the *E. coli* PA340 type B transformants indicates that the cloned DNA encodes an NAD(H)-dependent GDH enzyme activity (GdhB). That this DNA fragment in type B transformants was derived from *B. thetaiotaomicron* was confirmed by Southern blot analysis (5), but the levels of NADH-dependent GDH activity measured from *gdhA* mutants would not seem to be able to account for the growth rate or yield of the bacterium. Therefore, the role of GdhB activity in ammonia assimilation and glutamate biosynthesis remains unclear. Many prokaryotes utilize the GS-GOGAT-catalyzed pathway for ammonia assimilation, glutamine, and glutamate biosynthesis; and with the cloning strategy used here, we anticipated isolating *ghd(F)* homologs, as well as any other enzymes catalyzing ammonia assimilation and glutamate biosynthesis, if these were expressed in *E. coli*. Measurement of GS enzyme activity in *B. fragilis* cell extracts is problematic (46, 55), and although we have encountered similar problems, *B. thetaiotaomicron* genes which complement an *E. coli* *ghnALG* (*ntrABC*) deletion mutant have been cloned and isolated (6). However, NAD(P)H-dependent GOGAT activity is not detectable in *B. thetaiotaomicron* whole-cell preparations or in cell extracts. Further dissection of the routes of ammonia assimilation and glutamate biosynthesis in human colonic *Bacteroides* species will probably be best accomplished by mutational analyses, using the genetics-based resources developed in recent years that are compatible with this genus.

Nitrogen regulation in many other gram-negative eubacteria is coordinated by the intracellular concentration of glutamine relative to  $\alpha$ -ketoglutarate, and in *Klebsiella aerogenes*, *gdhA* transcription is repressed by the Nac (nitrogen assimilation control) protein in response to nitrogen limitation (27, 41). In *E. coli* K-12 and *S. typhimurium* strains, GDH enzyme levels appear to be unaffected by nitrogen limitation (20, 31), and while the GDH enzyme from *E. coli* D<sub>5</sub>H<sub>3</sub>G<sub>7</sub> can be phosphorylated in vitro (25), the effects on enzyme activity do not appear to have been reported. The NAD(P)H-utilizing GDH enzymes from *B. thetaiotaomicron* (18) and *B. fragilis* (56) have previously been purified, and in both instances it was concluded that the active enzyme was comprised of an identical polypeptide, arranged in a hexameric configuration. Therefore, it would appear that the gene products encoded downstream of GdhA (GdhX) do not constitute a subunit of the active enzyme. Northern blot analyses suggest that *gdhA* and *gdhX* are not cotranscribed and that *gdhX* transcription is mediated by

its own promoter. Whether this promoter is recognized in *E. coli* or whether transcription in *E. coli* relies on a fortuitous promoter element remains unclear. Coupled with the finding that *gdhA* transcription was not affected in a pANS100 deletion (pANS105) but GdhA enzyme activity was not measurable, this also argues against a role for the GdhX protein in regulating *gdhA* transcription.

We believe, instead, that the GdhX protein modulates GdhA enzyme activity. Based on our current observations, we propose that the GdhX protein(s) might function in either a posttranslational modification of the GdhA enzyme or its assembly into the active, hexameric configuration. Such a hypothesis is consistent with the reversible inactivation of the *B. fragilis* GDH enzyme observed by Yamamoto et al. (57). Enzyme inactivation was inducible by rapidly increasing the ammonia concentration in the growth medium, even in the presence of chloramphenicol, but the amount of immunoprecipitable GDH protein was not decreased, and neither phosphorylation nor adenylation of the *B. fragilis* GDH protein could be demonstrated. Studies have shown that GTP can inhibit GDH activity in eukaryotes (45), perhaps by interfering with monomer polymerization into the active, hexameric configuration. However, no such scheme of inactivation appears to have been demonstrated to date in eubacteria.

The pathway of  $\alpha$ -ketoglutarate biosynthesis in *Bacteroidaceae* proceeds via a reductive carboxylation of succinate rather than via isocitrate (1). While the human colonic *Bacteroides* species produce relatively large amounts of succinate in pure culture, its fermentation to propionate can also provide additional ATP through electron transport-linked phosphorylation, and such a fermentation scheme is stimulated by coenzyme B<sub>12</sub> (33). Therefore, regulation of GDH activity in the *Bacteroidaceae* may be relevant in coordinating the carbon flux between ATP-yielding and biosynthetic processes. Because attempts so far to isolate *gdhX* insertion mutants have proved to be unsuccessful, it is tempting to speculate that the role of GdhX in regulating growth and metabolism may extend beyond nitrogen regulation. It seems likely that further analyses of *gdhX* and its gene product(s) will reveal new information relevant to *Bacteroides* physiology and regulatory mechanisms in anaerobic prokaryotes.

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