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Glutamate Dehydrogenase Activity Profiles for Type Strains of Ruminal *Prevotella* spp.†

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The glutamate dehydrogenase (GDH) activities for the type strains of *Prevotella ruminicola* (strain 23), *Prevotella brevis* (strain GA33), and *Prevotella bryantii* (strain B_14) were assessed by a combination of enzyme assays and analysis of migration patterns of GDH proteins following nondenaturing polyacrylamide gel electrophoresis. Unlike results with most other prokaryotes, but similar to results with other members of the family *Bacteroidaceae*, NADPH-utilizing specific activity was greatest in all species following ammonia-limited growth. Similar also to previous findings with *P. bryantii*, the NAD(P)H-utilizing GDH activity of *P. ruminicola* can be attributed to a single protein. However, *P. brevis* produces an additional GDH protein(s) in response to growth with peptides. These results conclusively demonstrate that all type strains of the ruminal *Prevotella* sp. grouping possess GDH activity.

Prevotella spp. are a numerically predominant group of ruminal bacteria considered to occupy a central role in carbohydrate and protein degradation in the gut (12, 15). However, little is known about nitrogen metabolism and regulation in these bacteria. One interesting feature is their inability to utilize single amino acids or small peptides as a nitrogen source, although large oligopeptides (and ammonia) are readily utilized. However, since the original description of the nitrogen requirements of the *Prevotella ruminicola* type strains by Pittman and Bryant (11), others have reported that strain GA33 is incapable of ammonia assimilation, presumably due to the absence of glutamate dehydrogenase (GDH) enzyme activity (10). Russell (13) also showed that ruminal *Prevotella* spp. respond differently to a combination of relatively high concentrations of glucose (50 mM) and low concentrations of ammonia (3.6 mM) in growth media. Several strains produced measurable amounts of methylglyoxal, and in strain B_14, the type strain of *Prevotella bryantii*, methylglyoxal production was correlated with a dissipation of the transmembrane K^+ gradient and decreased cell viability (glucose toxicity). However, unlike with enteric bacteria (4), methylglyoxal accumulation in *P. bryantii* occurred independently of phosphate limitation and appeared to be related to other aspects of carbon and/or nitrogen metabolism (13). Because GDH is an enzyme which can play a key role in linking carbon and nitrogen metabolism, the differences among *Prevotella* spp. in relation to methylglyoxal production and glucose toxicity might be explained, at least in part, by variations in GDH enzyme activity and expression. We report here the features of GDH enzyme activities for the type strains of *P. ruminicola* (strain 23) and *Prevotella brevis* (strain GA33), as well as some additional features of GDH enzyme activity from *P. bryantii* (strain B_14).

GDH activity and its response to a nitrogen source. All bacterial strains were kindly provided by M. A. Cotta, USDA-National Center for Agricultural Utilization Research, Peoria, Ill. The bacteria were grown in glucose minimal medium (7) prepared to contain either 1 or 10 mM ammonium chloride or 1.5% (wt/vol) Trypticase (lot 1000 K9DHCR; Becton Dickinson Laboratories, Cockeysville, Md.) as the sole nitrogen source. GDH activity was measured by monitoring the oxidation of NAD(P)H at 340 nm as described previously (16). In consideration of the fact that *P. ruminicola* strains generally maintain a cytoplasmic potassium (K^+) pool in excess of 200 mM (13), a series of assays were also conducted with addition of KCl (final concentration, 0.2 M) to the reaction mixtures. The results of these assays are shown in Table 1. Similar to previous findings with *P. bryantii* B_14 (16), both strain 23 and strain GA33 possess measurable amounts of both NADPH- and NADH-utilizing GDH activities. In the presence of 0.2 M KCl, NADPH-utilizing activity was maximal following growth with 1 mM ammonia, and there was a marked reduction in this enzyme activity in all three strains when peptides were provided as the sole nitrogen source. The NADH-utilizing enzyme activity was negatively affected in all three strains by KCl addition, with the exception of strain GA33 following growth on peptide-nitrogen. Interestingly, the NADH-utilizing activity was consistently higher in strain GA33 following growth on peptides.

Because the greatest change in GDH enzyme activity appears to be in response to growth in the presence of peptides, all three *Prevotella* type strains were grown to early log phase in defined medium containing 10 mM ammonium chloride and then shocked by the addition of a prereduced, anaerobically sterilized solution of Trypticase to give a peptide concentration of 1.5% (wt/vol). Control cultures received a similar volume of prereduced, anaerobically sterilized distilled water. Cultures were harvested at either 20 min or 1 doubling time (~80 min) after the addition of Trypticase, and GDH specific activities were determined. Addition of Trypticase to cultures of all three strains already growing on ammonia resulted in substantial reductions in NADPH-utilizing GDH activity (Table 2), although the times required for the decreases in enzyme activity were different among strains. In strain B_14, significant reductions were not observed until 1 doubling time after the addition of Trypticase (72 min). In contrast, NADPH-utilizing GDH specific activity decreased 60 and 90% in strains 23 and GA33, respectively, within 20 min of the addition of peptides.

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TABLE 1. GDH activities in three *Prevotella* type strains in response to ammonia concentration and nitrogen source

Cofactor, assay condition	GDH sp act (nmol/min/mg) with indicated addition to reaction mixture[a]								
	P. bryantii $B_1$4			*P. ruminicola* 23			*P. brevis* GA33		
	1 mM NH_4Cl	10 mM NH_4Cl	1.5% Trypticase	1 mM NH_4Cl	10 mM NH_4Cl	1.5% Trypticase	1 mM NH_4Cl	10 mM NH_4Cl	1.5% Trypticase
NADPH, no KCl	681.8	333.9	66.3	101.2	39.3	35.0	65.0	63.8	86.1
NADH, no KCl	82.3	64.3	25.0	148.9	58.9	59.7	97.6	90.0	103.0
NADPH, 0.2 M KCl	1,343.7	759.1	167.2	557.8	216.7	26.1	370.1	299.5	99.0
NADH, 0.2 M KCl	<10	<10	<10	27.0	22.4	32.0	20.9	19.1	66.3

[a] NADPH- and NADH-utilizing specific activities are expressed as nanomoles of NAD(P)H oxidized per minute per milligram of protein. Data represent means of results from no less than four separate observations from two different experiments, and the coefficients of variation were less than 10%.

Interestingly, the NADH-utilizing GDH specific activity in strains GA33 and 23 appeared to increase once peptides had been added to the growth medium and the cells were allowed to grow for 1 doubling time (Table 2).

Southern blot analysis and GDH enzyme migration patterns in nondenaturing polyacrylamide gel electrophoresis (PAGE). Chromosomal DNA was isolated from all three strains by following standard procedures (16), digested with *Eco*RI, and subjected to agarose gel electrophoresis. After electrophoresis, the DNA fragments were depurinated and denatured and then transferred to a Zeta-probe GT membrane with a vacuum blotting apparatus (Bio-Rad Laboratories, Hercules, Calif.). A 1.3-kb *Hin*dIII fragment from strain $B_1$4 GDH structural gene *gdhA* (pANS701) (16) was radiolabelled with [α-^{32}P]dCTP by random primer labelling and allowed to hybridize with the membrane overnight at 43°C as described previously (16). Although the DNA probe hybridized strongly with chromosomal DNA from strain $B_1$4 as expected, no cross-hybridization was seen with chromosomal DNA isolated from either strain 23 or strain GA33 (data not shown). We interpret these results as demonstrating that the *gdh* structural genes among these *Prevotella* strains are not highly homologous.

The mobilities of GDH proteins in PAGE activity gels have previously been used as a means to differentiate among bacteria originally classified as species of the genus *Bacteroides*, which include those bacteria now assigned to the genus *Prevotella* (14). However, in these earlier studies only NAD^+ was used as a cofactor and ruminal isolates representing all major *Prevotella* groups were not compared. The three *Prevotella* type strains examined in this study were harvested at mid-log phase, following growth with either 10 mM ammonium chloride or 1.5% (wt/vol) peptides as the sole nitrogen source. Cells were disrupted by two passages through a French pressure cell (220,000 kPa; SLM Instruments, Inc., Urbana, Ill.), and unbroken cells and large debris were removed by centrifugation at 15,000 × *g* at 4°C for 15 min. Aliquots of the resulting cell extract (20 μg of total protein) were subjected to nondenaturing PAGE. The separated GDH proteins were visualized by previously described procedures, with $NAD(P)^+$ as the cofactor (3, 6, 16), and the migration patterns of the GDH proteins for each strain were distinct (Fig. 1). The $NADP^+$-positive staining bands were most intense in cell extracts prepared from ammonia-grown cells, consistent with such growth conditions giving rise to the highest levels of NADPH-utilizing activity. Of the three type strains evaluated, *P. brevis* GA33 also appeared to produce multiple GDH proteins, and their relative abundance seems to be affected by the N source available for growth. The upper-migrating band appears to be NADP(H) utilizing and in greatest abundance in ammonia-grown cells. The lower-migrating band(s) appears to be more active when NAD^+ is used as a cofactor and in greatest abundance following growth of the strain on peptide-nitrogen. The results presented here unequivocally demonstrate that *P. brevis* does indeed possess GDH enzyme activity. In this regard, *P. brevis* appears to be quite similar to the human colonic *Bacteroides* spp., which also possess multiple GDH enzymes (2, 17), whereas strains $B_1$4 and 23 appear to possess only single enzymes.

As might be expected, there were similarities and differences among the type strains examined in terms of GDH activity. The NADPH-utilizing activities in *P. ruminicola* and *P. bryantii* (but not *P. brevis*) increased twofold in response to ammonia-limited growth, and NADPH specific activities in all strains were reduced in response to growth on peptides (Table 1). Although the latter result is not surprising relative to other prokaryotes, the increase in GDH specific activity in response to ammonia limitation is unusual. Studies of the NAD(P)H-utilizing GDH enzyme of *Bacteroides fragilis* have shown that this enzyme is subject to a reversible activation-inactivation mechanism, apparently in response to the ammonia concentration in the growth medium (18). In *P. bryantii* $B_1$4, it appears that the GDH enzyme is regulated in response to the nitrogen source at the level of transcription (16) and the reduction in specific activity following 1 doubling time after the addition of

TABLE 2. Effect of peptide shock on GDH activities in three *Prevotella* type strains

Treatment	GDH sp act (nmol/min/mg) with indicated cofactor[a]					
	P. bryantii $B_1$4		*P. ruminicola* 23		*P. brevis* GA33	
	NADPH	NADH	NADPH	NADH	NADPH	NADH
Control (no peptides added)	558.2 ± 48.8	<10	214.0 ± 52.9	15.3 ± 3.2	281.7 ± 4.3	28.8 ± 5.3
Harvested at 20 min[b]	452.0 ± 35.5	<10	88.9 ± 25.9	29.5 ± 6.1	32.4 ± 3.1	37.7 ± 5.0
Harvested at 1 doubling time[b]	304.0 ± 13.3	<10	88.9 ± 18.1	64.4 ± 19.5	40.3 ± 2.7	73.0 ± 10.5

[a] Data presented in this table are the results when 0.2 M KCl was included in the reaction mixture and are means ± standard deviations of results from no less than four separate observations from two different experiments.

[b] Relative to the addition of Trypticase to give a peptide concentration of 1.5% (wt/vol).

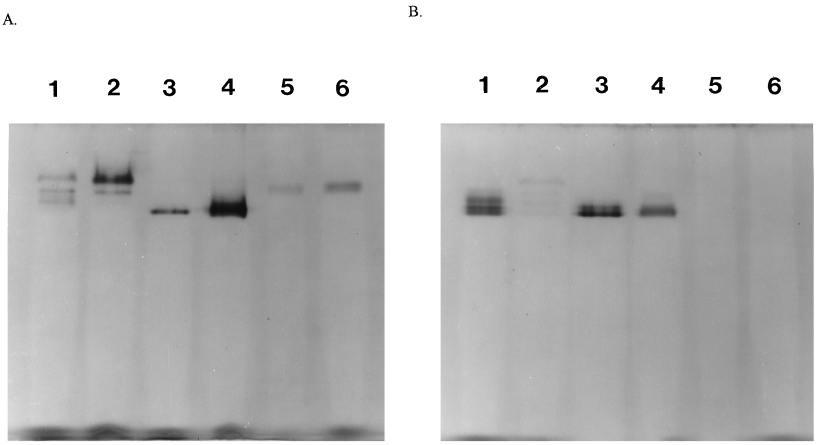

FIG. 1. Electrophoretic mobilities of $NADP^+$-utilizing (A) and NAD^+-utilizing (B) GDH proteins from three *Prevotella* type strains. Lanes 1 and 2 contain cellular proteins from *P. brevis* GA33, lanes 3 and 4 contain cellular proteins from *P. ruminicola* 23, and lanes 5 and 6 contain proteins from *P. bryantii* B_14. Extracts prepared from peptide-grown cultures are loaded in odd-numbered lanes, and extracts from ammonia-grown cells are loaded in even-numbered lanes. Proteins were subjected to nondenaturing PAGE and enzyme activity staining in the presence of 0.2 M KCl, according to previously described procedures (16).

peptides is consistent with this. However, GDH activities in *P. ruminicola* 23 and *P. brevis* GA33 were reduced approximately 60 and 90%, respectively, within 20 min of the addition of peptides (Table 2), suggesting that modulation of enzyme activity, rather than (or in addition to) enzyme synthesis, may occur in these two strains. To address whether the reductions in enzyme specific activities were correlated with changes in the relative abundance of GDH protein, we attempted to use polyclonal anti-GDH antibodies known to cross-react with the GDH proteins from a diverse range of prokaryotes (kindly provided by Frank Robb, Marine Biotechnology Institute, University of Maryland). However the results were inconclusive: the antibodies were only weakly reactive with proteins from *P. ruminicola* 23 and nonreactive with proteins from *P. brevis* GA33 (data not shown).

The glucose toxicity effect due to methylglyoxal production in some *Prevotella* spp. seems to result from an imbalance between the carbon (energy) source and the nitrogen source. Coincident with methylglyoxal accumulation in *P. ruminicola* B_14 is a decline in intracellular K^+ and ultimately cell viability (13). Studies with *Salmonella* spp. and *Escherichia coli* have shown that the intracellular pools of potassium and glutamate are tightly linked (9) and that the potassium (K^+) efflux systems are involved in protection against methylglyoxal toxicity (5). Whether the K^+ efflux observed in *P. ruminicola* B_14 is a reflection of a protective mechanism in response to methylglyoxal production, a reduction in ammonia assimilation and glutamate biosynthesis due to low concentrations of external ammonia (19), or both remains to be determined.

During the final preparation of the manuscript for publication, the strains used here and in an earlier study (16) were proposed to serve as the type strains for the three major species of *Prevotella* of ruminal origin (1). When the GDH structural gene from strain B_14 was used as a probe in Southern blots, no cross-hybridization was seen with chromosomal DNA extracted from the type strains of *P. ruminicola* and *P. brevis*. It seems that the *gdh* genes of the respective isolates are not highly homologous, consistent with the genetic diversity present within this genus (1, 8). The extent of the diversity among isolates was further confirmed by PAGE activity gels; each strain produced a unique GDH activity profile, provided that both $NADP^+$ and NAD^+ were used and the isolates were cultivated in both ammonia- and peptide-containing media.

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