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Structural Studies of Malate Dehydrogenases (MDHs): MDHs in *Brevundimonas* Species Are the First Reported MDHs in *Proteobacteria* Which Resemble Lactate Dehydrogenases in Primary Structure

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The N-terminal sequences of malate dehydrogenases from 10 bacterial strains, representing seven genera of *Proteobacteria*, were determined. Of these, the enzyme sequences of species classified in the genus *Brevundimonas* clearly resembled those malate dehydrogenases with greatest similarity to lactate dehydrogenases. Additional evidence from subunit molecular weights, peptide mapping, and enzyme mobilities suggested that malate dehydrogenases from species of the genus *Brevundimonas* were structurally distinct from others in the study.

NAD-dependent L-malate dehydrogenase (L-malate-NAD-oxidoreductase, EC 1.1.1.37) (L-MDH) is an integral enzyme in several metabolic processes, including the tricarboxylic acid and glyoxylate cycles. In higher organisms, L-MDH isoenzymes are found in mitochondria, peroxisomes, and chloroplasts (NADP-dependent enzyme), in addition to the cytosol. The reaction catalyzed is the reversible oxidation of L-malate to oxaloacetate with NAD as the H acceptor. Although the oxidation of L-malate is in the usual physiological direction, in *Chlorobium* spp., which are obligate phototrophs, the reaction proceeds in the opposite direction as part of the reductive tricarboxylic cycle which fixes CO_2 (7).

A stretch of glycine (G) residues has been implicated in the binding of NAD by most dehydrogenases with a requirement for this coenzyme (33). Three Gs lie separated as follows: GXGXXG, where X is any residue. This motif is, however, absent from the majority of L-MDHs investigated. It has been found only in L-MDHs from four eubacterial phototrophs, representing three phyla (2, 19, 25, 30), and in two species of *Bacillus* (13, 38). Complete gene sequences are available for these L-MDHs (13, 19, 30, 38), all of which show greater similarity in primary structure to L-lactate dehydrogenases (L-LDHs) than to other L-MDHs. This similarity includes the possession of the putative NAD-binding configuration GXGXXG by both dehydrogenases. Using data derived from empirical methods of protein sequence analysis and existing X-ray crystallography data, we have previously proposed an alternative G motif from that employed by most L-MDHs, i.e., GAXGXXG/A, where A is alanine (2). L-MDH shares many structural and catalytic features with L-LDH which are suggestive of a common ancestry (1, 10, 17). The existence of L-MDHs which most resemble L-LDHs in primary structure, and their distribution in the bacterial phyla, may offer clues about the ancestral links between these two classes of dehydrogenase enzymes. The NAD-binding motif of L-MDHs is located close to the N terminus. Consequently, sequencing of this region of the enzyme provides data of particular value for comparing L-MDHs with each other and with L-LDHs. Relatively few sequences have been determined for species of *Proteobacteria* (16, 26, 32), and to date, all those investigated possess the GAXGXXG/A motif.

Isolation of L-MDH from select *Proteobacteria*. Two liters of Luria broth were seeded with two to three bacterial colonies (strains used in the study are listed in Table 1), and cultures were grown at 32°C with shaking (150 rpm). Cells were harvested in the late log phase of growth, washed twice with 10 mM potassium phosphate buffer (pH 7.5) (buffer), and resuspended in 10 ml of buffer. Cell suspensions were disrupted, on ice, by sonication (three 1-min bursts) with intermittent cooling. Thereafter, intact cells and cell debris were removed with a short preliminary centrifugation. The supernatant was ultracentrifuged (200,000 × g for 90 min) at 4°C in a Beckman 18-55M ultracentrifuge fitted with an SW-50.1 rotor. The resulting clear supernatant was the cell-free extract (CFE). L-MDH activity was measured as the reaction in the direction of oxaloacetate reduction by monitoring the oxidation of NADH at A_{340} (2). An open glass column (inner diameter, 5 mm) containing approximately 1 ml of DyeMatrex gel Red-A (Amicon, Inc., Beverly, Mass.) was equilibrated by washing with 20 ml of buffer at 4°C. CFE was added dropwise to the column and allowed to run through under gravity. The eluate was collected and reapplied a total of three times. Unbound protein was removed from the column, as determined by the A_{280} of the emerging liquid, after the gel was washed with 30 ml of buffer. L-MDH was specifically eluted by the following protocol. The gel bed was first allowed to drain (sufficient buffer is retained in the column to prevent damage), after which the outlet valve was closed. One milliliter of 5 mM NAD and 10 mM L-malate in buffer was mixed carefully into the gel, and elution was allowed to proceed for 2 min, whereupon the outlet was reopened and the L-MDH-containing sample was collected. The process was repeated twice to maximize recovery. Enough enzyme was obtained by this approach for several micro-sequencing reactions. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that preparations obtained after affinity chromatography contained L-MDH in a highly concentrated and relatively pure form. Electrophoresis typically gave an intense band of L-MDH and a number of much fainter bands well separated

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TABLE 1. Bacterial strains

Species (*Proteobacteria* subclass[a])	Taxonomic status	Reference or source
E. coli[b,c] (gamma)	Laboratory strain	Origin unknown
K. pneumoniae[b,c] (gamma)	Laboratory strain	Obtained as culture contaminant
S. maltophilia[b] (gamma)	Type strain	21
Brevundimonas diminuta[b] (alpha)	Type strain	27
Brevundimonas vesicularis[b] (alpha)	Type strain	27
Burkholderia pseudomallei[b] (beta)	Type strain	39
Burkholderia cepacia[b] (beta)	Type strain	39
V. indigofera[b] (beta)	Type strain	11
C. acidovorans[b] (beta)	Type strain	31
A. delafieldii[b] (beta)	Clinical isolate, CCUG 12929[d]	Human joint aspirate (34)
Acidovorax facilis (beta)	Type strain	40
Ralstonia solanacearum (beta)	Type strain	39
Burkholderia carophylii (beta)	Type strain	39
Alcaligenes xylosoxidans subsp. *xylosoxidans* (beta)	Type strain	4, 14
P. alcaligenes (gamma)	Type strain	12, 18

[a] Based on 16S rRNA cataloging described in references 33–35.
[b] Strains for which the N-terminal sequence of L-MDH was determined.
[c] Identity confirmed with the api 20E identification system for *Enterobacteriaceae* (Bio Mérieux).
[d] CCUG, Culture Collection of the University of Gothenburg.

from that of L-MDH (results not shown). The L-MDH subunit was recognizable by its position in the gel. All L-MDHs examined to date are composed of identical subunits with molecular masses between 30 and 38 kDa (2). L-LDH activity was not detected in the enzyme preparations. Subunit sizes varied from 33 to 37 kDa for the 10 strains examined (Fig. 1). The subunits from *Brevundimonas vesicularis* and *Brevundimonas diminuta* subunits were of equal size and were the smallest in the study. The largest subunit was that of *Burkholderia cepacia.*

Microsequencing of membrane-immobilized L-MDH. L-MDHs were Western blotted from SDS-PAGE gels onto Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation Ltd., Bedford, United Kingdom) and stained and destained according to the protocols of the manufacturer. The N-terminal sequence of membrane-bound protein was determined in an Edman degradation reaction (6) by using an Applied Biosystems protein sequencer (model 477A) fitted to a 120A analyzer. An amount of enzyme between 10 and 100 pmol ($\approx$ 0.35 to 3.5 μg) was sufficient for a single analysis. Only a single N terminus was detected for each enzyme, which is consistent with a high degree of purity (Fig. 1). Eight of the 10 strains examined possessed an L-MDH with an N terminus which had features typical of the majority of these enzymes. In addition to the NAD-binding motif, GAXGXXG/A, several other residues were common to all sequences (Fig. 1). By contrast, L-MDHs from *Brevundimonas* species were most similar to those L-MDHs which resemble L-LDHs in primary structure. These sequences contain a GXGXXG motif and also have a number of other residues which are absolutely conserved. However, the residues which are conserved in this class of L-MDHs are distinct from those conserved in L-MDHs of the majority class (Fig. 1). A further difference between the two L-MDH sequence classes was seen in their subunit molecular masses (Fig. 1). Subunits from L-MDHs which resemble L-LDHs show remarkably little variation in size and are smaller than those from the majority of other L-MDHs.

L-MDH sequences for the two species from the *Enterobacteriaceae* family that were analyzed were identical and showed no differences from the sequence reported for *Escherichia coli* (32). Identical sequences were seen among the respective species from the genera *Burkholderia* and *Brevundimonas*. *Comamonas acidovorans* and *Acidovorax delafieldii* were grouped together based on their level of rRNA homology, as measured by the competition technique in rRNA-DNA hybridization experiments (20). L-MDHs from these two species had identical sequences. Some agreement between the enzyme sequences and the taxonomic placement of individual strains of the *Proteobacteria* was thus obtained. Regarding the above discussions on L-MDH sequence classes, it is noteworthy that the genus *Brevundimonas* is located in the alpha subclass of the *Proteobacteria* (35), whereas the other genera examined belong to the beta (36) or gamma (37) subclasses (Table 1). As more L-MDH sequences become available, it will be interesting to see if any convincing relationship between the distribution of L-MDH types and the subclasses of the *Proteobacteria* emerges.

Peptide mapping and starch gel electrophoresis (SGE). The N-terminal sequences and subunit molecular weights of L-MDHs from the two species of *Brevundimonas* were identical, and they were different from those of the other strains of *Proteobacteria*. Peptide mapping and enzyme mobility studies were performed in order to obtain more data for purposes of comparison.

Following SDS-PAGE, gels were stained briefly (Coomassie) and destained, and the band corresponding to the L-MDH subunit was excised with a scalpel. Gel slices (containing about 5 μg of protein) were equilibrated by gentle shaking (2× for 1 h each) in 10 ml of equilibration buffer (125 mM Tris-HCl [pH 6.8], 0.1% SDS, 10 mM Na_2EDTA [pH 7.0]). Partial digestion with V8 protease (catalog no. P-2922, Sigma) and electrophoretic separation of generated peptides were performed as previously described (9) with the following specifications. A total of 0.075 μg of protease was used per 5 μg of L-MDH. Electrophoresis was performed at a constant current of 10 mA until the tracking dye had reached the interface with the separating gel. The power was then shut off for 30 min, allowing digestion to proceed, and the run was subsequently completed at 20 mA. Mapping gels were stained with silver nitrate (24). Peptide maps of L-MDHs from *Proteobacteria* generated by limited digestion with V8 protease are shown in Fig. 2. Structurally related enzymes would be expected to give similar maps. L-MDHs from the two *Brevundimonas* species had several peptides in common, suggestive of substantial similarity at the whole-enzyme level. L-MDHs from *Stenotrophomonas maltophilia*, *Burkholderia pseudomallei*, and *C. acidovorans*, representing the majority class of L-MDHs, were also analyzed. These enzymes, which showed slight variations in their N-terminal sequences (Fig. 1), produced very different peptide maps, and no sequence-class-specific peptides were generated. Peptide mapping did not, therefore, provide direct evidence for the existence of two distinct groups of L-MDHs among the *Proteobacteria*. However, it did show that enzymes of species of *Brevundimonas* possess structural similarity extending beyond their N termini and that in primary structure they are unlike any other L-MDHs tested.

In preparation of analyses using SGE, bacteria were grown in 100 ml of Luria broth at 32°C with shaking (200 rpm). Cells

	N-terminal Sequence			mol wt (kDa)	Reference
Majority class of L-MDHs	1 10		20		
B. pseudomallei	?**K**PAKR**VAVT**	**GAAG**Q**I**A	YS**L**LFKI?NGKL	36.00	ps
B. cepacia	A**K**PAKR**VAVT**	**GAAG**Q**I**A	YS**LL**	37.00	ps
V. indigofera	A**K**PAKR**VAVT**	**GAAG**Q**I**A	YS**LL**	36.00	ps
C. acidovorans	?**K**KPV?**VAVT**	**GAAG**Q**I**G	YA	35.00	ps
A. delafieldii	?**K**KPVR**VAVT**	**GAAG**Q**I**G		34.50	ps
S. maltophilia	M**K**APVR**VAVT**	**GAAG**Q**I**G	YA**LL**F	35.75	ps
E. coli	M**K**----**VAV**L	**GAAG**G**I**G	QA**L**ALLLKTQLPS	32.60	ps
K. pneumoniae	M**K**----**VAV**L	?**AAG**G**I**G	QA**L**ALLLKT	32.60	ps
S. typhimurium	M**K**----**VAV**L	A**AAG**G**I**G	QA**L**ALLLKN	32.40	14
P. immobile	S**K**TPIR**VAVT**	**GAA**GN**I**G	YH**L**LFRIA	No data	24

	N-terminal Sequence			mol wt (kDa)	Reference
L-MDHs resembling L-LDHs in primary structure	1 10		20		
Bacillus subtilis	MGNTR**K**KVSVI	**GAG**FT**G**	**A**T**TA**F	33.49	11
Bacillus sp.	MAIAR**K**KISVI	**GAG**FT**G**	**A**T**TA**F	33. 64	36
Br. vesicularis	A?A--**K**IALI-	**GAG**MI**G**	G**T**L**AA**	33.00	ps
Br. diminuta	A?A--**K**IALI-	**GAG**MI**G**	G**T**L**AA**	33.00	ps
Chlorobium vibrioforme	M----**K**ITVI-	**GAG**NV**G**	**A**T**TA**F	33.11	17
Chlorobium tepidum	M----**K**ITVI-	**GAG**NV**G**	**A**T**TA**F	33.27	17
Chloroflexus aurantiacus	MR---**K**KISII	**GAG**FV**G**	S**T**T**A**H	32.72	28
Heliobacterium gestii	MT---**K**KITII	**GAG**NV**G**	**A**T**A**??	No data	2

FIG. 1. N-terminal amino acid sequences and subunit molecular masses of L-MDHs from select *Proteobacteria*. The upper panel shows those sequences which have the NAD-binding motif (boxed) and other conserved residues (boldface type) typical of the majority of L-MDHs examined. The lower panel shows L-MDHs from *Brevundimonas* species aligned with L-MDHs which have the greatest similarity in primary structure to L-LDHs. The NAD-binding motif (boxed) and conserved residues (boldface type) are different than those for other L-MDHs. ps, present study. Dash indicates gap introduced to achieve maximal alignment; question mark indicates residue was not determined.

were harvested in the late log phase of growth, and CFE was prepared by sonication exactly as described previously (3). Preparation of horizontal starch gels (9 by 190 by 210 mm), electrophoresis, and staining for L-MDH activity followed standard procedures for SGE (28). A Tris-citrate buffer system (buffer B in reference 28) was found to be the most applicable for achieving a good focus of L-MDHs in the gel. In brief, electrophoresis was performed at a constant 150 V at room temperature. Following electrophoresis, horizontal 1- to 2-mm slices were cut with a thin wire and incubated at 32°C in enzyme-staining solution. L-MDH activity in the gel appeared as strong, almost black, bands against a white background. As a larger set of *Proteobacteria* was tested than that for which purified L-MDH was available, CFE was used for all strains as a standardizing measure. A sample gel is shown in Fig. 3. (The order in which the 13 native L-MDHs migrated, based on the results of many gels, is given in the accompanying figure legend.) With the exception of the *S. maltophilia* enzyme, L-MDHs representative of the majority class of these enzymes remained in the lower half of the gel (slow migratory enzymes). By contrast, L-MDHs from *Brevundimonas diminuta* and *Brevundimonas vesicularis* were the first and fourth, respectively, most rapidly migrating enzymes and travelled considerably further in the gel than most other L-MDHs. The SGE analyses showed that the combinations of charge and size for L-MDHs from species of the genus *Brevundimonas* were significantly different than those of the majority of other *Proteobacteria* tested. A thin band of activity was obtained for extracts of *Pseudomonas alcaligenes* (Fig. 2, lane 8). *P. alcaligenes* is a member of what is now generally recognized to be the authentic genus *Pseudomonas*, synonymous with the rRNA similarity group I of Palleroni et al. (20), and belongs in the gamma

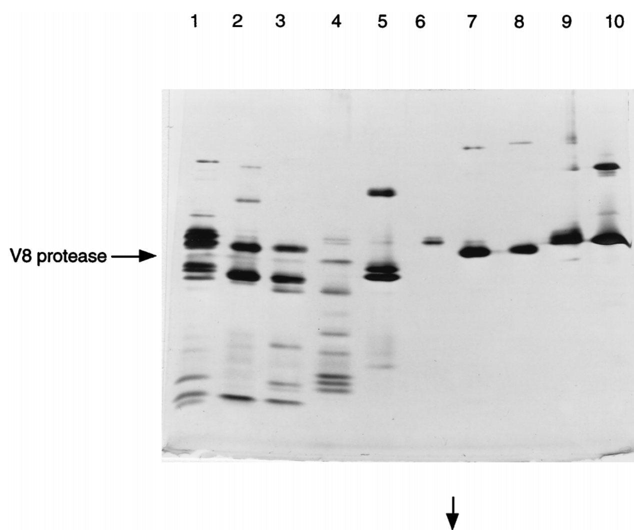

FIG. 2. Electrophoretic separation of peptide products from the limited digestion of L-MDHs with *Staphylococcus aureus* V8 protease. Protease-treated L-MDHs were run in lanes 1 to 5 and untreated L-MDHs were run in lanes 6 to 10 as follows: *S. maltophilia*, lanes 1 and 6; *Brevundimonas diminuta*, lanes 2 and 7; *Brevundimonas vesicularis*, lanes 3 and 8; *Burkholderia pseudomallei*, lanes 4 and 9; and *C. acidovorans*, lanes 5 and 10. Arrows indicate the position of stained V8 protease in the gel and the direction of electrophoresis. Faint high-molecular-weight bands are visible. These bands are of much higher molecular weight than the excised L-MDH band and probably represent enzyme or peptide aggregates.

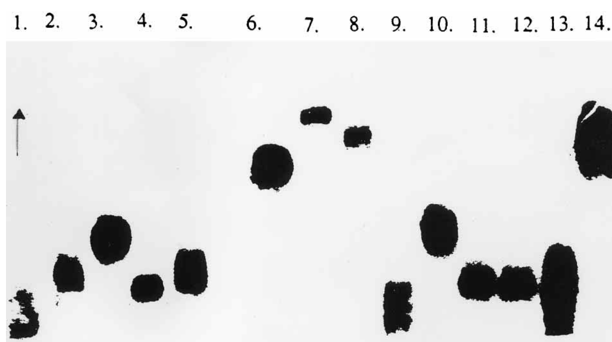

FIG. 3. Mobilities of L-MDHs as revealed by SGE. The species shown are *Ralstonia solanacearum*, lanes 1 and 9; *Burkholderia caryophylii*, lanes 2 and 5; *Burkholderia pseudomallei*, lanes 3 and 10; *A. delafieldii*, lanes 4 and 11; *Brevundimonas diminuta*, lane 6; *Brevundimonas vesicularis*, lane 7; *P. alcaligenes*, lane 8; *Acidovorax facilis*, lane 12; *Alcaligenes xylosoxidans* subsp. *xylosoxidans*, lane 13; and *S. maltophilia*, lane 14. The arrow indicates the direction of electrophoresis. The L-MDHs of the 13 strains of *Proteobacteria* may be arranged in order of decreasing anodal migration as follows: *Brevundiminas vesicularis*, *P. alcaligenes*, *S. maltophilia*, *Brevundimonas diminuta*, *V. indigofera*, *Burkholderia cepacia* and *Burkholderia pseudomallei*, *Burkholderia carophylii*, *C. acidovorans*, *Alcaligenes xylosoxidans* subsp. *xylosoxidans*, *A. delafieldii* and *A. facilis*, and *R. solanacearum*.

subdivision of the *Proteobacteria* (37). Unlike the *Enterobacteriaceae* and *S. maltophilia*, which also belong in the gamma subdivision, it is doubtful that the authentic *Pseudomonas* spp. possess an NAD-dependent L-MDH (8, 23, 29). I did not find, using a spectrophotometric assay, NAD-dependent L-MDH activity in extracts from any members of rRNA homology group I. A thin, atypical single band was obtained for all authentic *Pseudomonas* spp. by using SGE. The band mobility was species dependent, though little variation in mobility was found (unpublished data). The phenomenon has not been investigated further. There are also several other reports of activity staining of NAD-linked L-MDH for authentic *Pseudomonas* spp. (3, 5, 15). It is possible that phenazine methosulfate (used in the staining procedure) can act as an electron acceptor in the oxidation of L-malate under the experimental conditions. The appropriate electron acceptor and the cofactor requirements have been shown to depend upon the degree of purification of a membrane-associated enzyme from *Pseudomonas ovalis* Chester (23). To check that the L-MDH activity detected in CFE of those strains from which the enzyme was sequenced was not an artifact, the SGE analyses were repeated with the enzyme preparations from affinity chromatography. For each preparation, a single band of L-MDH activity was demonstrated, and the order of migration of the enzymes in the gel was the same as that obtained by using CFE.

These analyses more than double the number of L-MDH N-terminal sequences reported for species of genera belonging to the *Proteobacteria*. L-MDHs from members of the genus *Brevundimonas* probably belong to a group of these enzymes which most resemble L-LDHs in primary structure. This study brings to five the number of genera of eubacteria for which these L-MDHs have been documented, and it is the first report of their existence in the *Proteobacteria*. The distribution of L-MDH sequence classes and their evolutionary relationships with L-LDHs are clearly complex. For example, L-MDH from the archaebacterium *Haloarcula marismortui* (1), although most resembling L-LDHs and their sequence-similar L-MDHs in primary structure (1, 10, 13, 19, 30), has the NAD-binding motif of the majority class of L-MDHs. A meaningful interpretation of the ancestral links of these dehydrogenase enzymes will require more sequence data.

Sequence accession numbers. Sequence accession numbers for the L-MDH sequences have been allocated as follows (SWISS-PROT protein sequence database): *E. coli*, P06994; *Klebsiella pneumoniae*, P80535; *Burkholderia pseudomallei*, P80536; *Burkholderia cepacia*, P80537; *Vogesella indigofera*, P80538; *C. acidovorans*, P80539; *A. delafieldii*, P80540; *S. maltophilia*, P80542; *Brevundimonas diminuta*, P80542; and *Brevundimonas vesicularis*, P80543.

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