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Characterization of alanine and malate dehydrogenases from a marine psychrophile strain PA-43

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Abstract Alanine dehydrogenase (AlaDH: EC 1.4.1.1), malate dehydrogenase (MDH: EC 1.1.1.37), and glutamate dehydrogenase (EC 1.4.1.2), all NAD^+ dependent, were detected in extracts from a psychrophilic bacterium, strain PA-43, isolated from a sea urchin off the Icelandic coast. Characterization tests suggested that the strain had a close relationship to *Vibrio*, but sequencing of part of the 16S rDNA gene placed the bacterium among *Shewanella* species in a constructed phylogenetic tree. The bacterium had an optimum growth temperature of 16.5°C, and maximum dehydrogenase expression was obtained in a rich medium supplemented with NaCl. Both AlaDH and MDH were purified to homogeneity. AlaDH is a hexamer, with an approximate relative molecular mass of 260,000, whereas MDH is dimeric, with an apparent relative molecular mass of approximately 70,000. Both enzymes were thermolabile, and the optimum temperatures for activity were shifted toward lower temperatures than those found in the same enzymes from mesophiles, 37°C for MDH and approximately 47°C for AlaDH. The pH optima for AlaDH in the forward and reverse reactions were 10.5 and 9, respectively, whereas those for MDH were 10–10.2 and 8.8, respectively. Partial amino acid sequences, comprising approximately 30% of the total sequences from each enzyme, were determined for N-terminal, tryptic, and chymotryptic fragments of the enzymes. The AlaDH showed the highest similarity to AlaDHs from the psychrotroph *Shewanella* Ac10 and the mesophile *Vibrio proteolyticus*, whereas MDH was most similar to the MDHs from the mesophiles *Escherichia coli* and *Haemophilus influenzae*, with lower identity to the psychrophilic malate dehydrogenases from *Vibrio* 5710 and *Photobacterium* SS9.

Key words Psychrophilic · Cold-adapted · *Vibrio* · *Shewanella* · Malate dehydrogenase · Alanine dehydrogenase · Glutamate dehydrogenase

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Introduction

The study of extremophilic organisms has yielded a great deal of information about the mechanisms that enable organisms to survive extreme conditions. Thermostable enzymes from thermophilic and hyperthermophilic microorganisms have been studied in detail to ascertain their thermal adaptation mechanisms (for review, see Scandurra et al. 1998). In recent years, interest has turned to the psychrophilic and psychrotolerant organisms (Russell 1993). Cold-adapted enzymes have been isolated from deep-sea or polar bacteria and fish that inhabit the vast cold habitats of the Earth. Typically, such cold-adapted enzymes share three main characteristics: they are often more catalytically potent than their mesophilic counterparts at lower temperatures (0°–30°C), their temperature optima are shifted to lower temperatures, and they display only limited thermal stability (Feller et al. 1996). These properties have been attributed to enhanced structural flexibility (Feller et al. 1996), and they make psychrophilic enzymes potentially useful in industry, offering a saving of energy in reactions currently carried out at higher temperatures. Other possible applications include food processing, bioremediation, and biotransformation of fragile substrates that would be unstable at higher temperatures. Furthermore, studies of cold-

adapted enzymes are also likely to improve our overall understanding of enzyme catalysis and of the factors affecting thermostability.

A range of psychrophilic enzymes has been characterized from Arctic and Antarctic organisms at the level of functional properties and gene or protein sequences, some of the more detailed studies being those on the extracellular enzymes subtilisin (Davail et al. 1994; Narinx et al. 1997), β-lactamase (Feller et al. 1995, 1997), and α-amylase (Feller et al. 1992, 1994). Various *Vibrio* species have featured prominently as sources of psychrophilic enzymes for structural and functional studies. Recent kinetic studies were performed using a cold-active *Vibrio* alkaline phosphatase (Hauksson et al. 2000), and a comparative study was carried out using *Vibrio* PA-44 serine proteinase and homologous mesophilic and thermophilic serine proteinases (Kristjánsson et al. 1999). Gene sequences were obtained and, in some cases, molecular modeling studies were carried out for *Vibrio* triose phosphate isomerases (Adler and Knowles 1995; Alvarez et al. 1998) and aspartate transcarbamylase (Xu et al. 1998). Malate dehydrogenase was examined at the level of primary sequence (Ohkuma et al. 1996; Welch and Bartlett 1997), and crystallographic data have been obtained for the malate dehydrogenase from *Aquaspirillum arcticum* (Kim et al. 1999). Primary structures have also been determined for cold-adapted enzymes from *Shewanella* species, including phosphatase I (Tsuruta and Aizono 2000) and alkaline proteinase (Kulakova et al. 1999).

In recent years, significant structural information has begun to emerge on psychrophilic dehydrogenases at the level of primary structure and three-dimensional structure. Only a handful of investigations have been devoted to cold-adapted amino acid dehydrogenases: these include the alanine dehydrogenases from *Shewanella* strain Ac10 and *Carnobacter* sp. (Galkin et al. 1999), glutamate dehydrogenase from the Antarctic icefish *Chaenocephalus aceratus* (Ciardiello et al. 1997), and, most recently, glutamate dehydrogenase from the Antarctic bacterium TAD1 (Di Fraia et al. 2000).

Little is known about the metabolism of any psychrophilic microorganism in detail, and to date there has been no study of the metabolism and the properties of the enzymes found in a single psychrophilic microorganism. This lack prompted us to investigate strain PA-43, a psychrophilic eubacterium isolated from a sea urchin in the North Atlantic off the coast of southwest Iceland. In particular, we have focused on dehydrogenases, screening this organism for dehydrogenase activity to identify which dehydrogenases it contains, as well as considering some of its dehydrogenases as a first step toward more detailed structural studies.

We report here the identification of L-alanine dehydrogenase (AlaDH, EC 1.4.1.1), L-glutamate dehydrogenase (GDH, NAD^+-dependent EC 1.4.1.2), and L-malate dehydrogenase (MDH, EC 1.1.1.37) in strain PA-43. We have purified and characterized AlaDH and MDH from this organism, with particular emphasis on their thermostability and activity at different temperatures. The catalytic activities of these enzymes from other organisms have been well characterized, and structural data have been obtained for MDH from porcine heart (Gleason et al. 1994), *Escherichia coli* (Hall et al. 1992), and the psychrophile *Aquaspirillum arcticum* (Kim et al. 1999). The first X-ray crystallographic structure has also become available for AlaDH from the cyanobacterium *Phormidium lapideum* (Baker et al. 1998). The existence of X-ray crystallographic data for comparison make cold-adapted AlaDH and MDH good candidates for analysis of the differences between their structures and the homologous structures from mesophiles. We have obtained partial amino acid sequences from AlaDH and MDH, and have compared these to those of other organisms as a first step toward determining the structural characteristics required for cold adaptation in these enzymes.

Materials and methods

Materials

Grade II NAD^+ (98%, free acid), grade II NADH (98%, disodium salt), 2-oxoglutarate, and M_r markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Boehringer Mannheim (Lewes, UK). Amino acids, M_r markers for gel filtration chromatography, Q-Sepharose Fast Flow, and Sephacryl S-300 were obtained from Sigma-Aldrich (Dublin, Ireland). Sepharose CL-6B and a fast performance liquid chromatography (FPLC) prepacked gel filtration column, Superose 6 HR 10/30, were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals were of analytical grade and were commercially available.

Characterization tests

The following tests were performed on strain PA-43: growth characteristics on general media with salt, growth and colonial appearance on cholera medium TCBS (thiosulfate–citrate–bile–sucrose) (Oxoid CM333; Basingstoke, UK), Gram stain reaction, KOH test, morphology of cells and their grouping, oxidase test, catalase test (bioMérieux, Lyon, France), sensitivity to the vibriostatic agent O129 (10 μg and 150 μg; Oxoid DD14 and Oxoid DD15), salt requirement, oxidative–fermentative attack on glucose in a salt-containing oxidative–fermentative medium (ZOF), luminescence test, ADH, leucine decarboxylase (LDC), and ornithine decarboxylase (ODC) tests, a test for indole production, Voges-Proskauer test, and methyl red test. Similarity to several known low-temperature, salt-requiring *Vibrio* sp. was estimated from test results of the BIOLOG bacteria identification system. The BIOLOG test results were coded as binary characters and subjected to numerical taxonomy analysis using the program NTSYS-pc 4.0 (Applied Biostatistics, Exeter Software, Setauket, NY, USA).

DNA extraction, PCR amplification, and 16S rDNA sequencing

DNA was extracted with a DNA extraction kit (Dynabeads; Dynal AS, Oslo, Norway). The polymerase chain reaction

(PCR) amplifications were performed as described by Skirnisdottir et al. (2000). About 1 µl was used as template in PCR amplifications with universal eubacterial primer sets, forward F9 and reverse R1544. PCR products from isolates were sequenced with an ABI 377 DNA sequencer using the Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK). The following bacterial specific primers were used: F9, F338, F515, R805, and R1195 for sequencing of the 16S rRNA gene (Skirnisdottir et al. 2000).

Phylogenetic analysis

Sequences were manually aligned with closely related sequences obtained from the Ribosomal Database Project (RDP) after BLAST searches. Sequence alignment and phylogenetic analysis of the PA-43 strain was carried out with the ARB program (http://www.mikro.biologie.tu-muenchen.de), omitting regions of sequence ambiguity. The phylogenetic trees were constructed using 668 bp of the 16S rRNA gene. Distance trees were constructed using neighbor-joining algorithms with the Jukes and Cantor correction, and maximum-likelihood trees were constructed by the fastDNAml software included in the ARB package. Homologous nucleotide positions, based on the filter of the ARB database, were included in the alignment and used for the comparison analysis.

Determination of optimum temperature for growth

The optimum temperature was determined by growing in a clear liquid medium (CHYSS) at five different temperatures, i.e., 10°, 13°, 15°, 17.5°, and 21°C. This medium was composed (per liter) of yeast extract (Oxoid L21), 2 g; casein, enzymatic hydrolysate (Sigma C0626), 5 g; and sea salts (Sigma S9883), 2 g, all dissolved in distilled water and autoclaved for 15 min at 121°C. The volume of medium used was 20 ml in a 100-ml Erlenmeyer flask. Aeration was carried out by shaking at 160 rpm at the respective temperatures, and the inoculum was 1% of the medium volume using a 24-h CHYSS culture grown at 15°C. The growth rate constant (µ) for each temperature was determined and then plotted against the temperature, expressed as 1,000/K.

Media and growth conditions for biomass production

In the initial search for the best medium for maximum biomass production, the strain was grown at 15°C in small Erlenmeyer flasks (20 ml medium in a 100-ml flask) in a refrigerated incubator shaker (G-25LC; New Brunswick, Edison, NJ, USA) at a shaking speed of approximately 160 rpm. Many different complex media and minimal salts media were tested. Additional NaCl or sea salts were used to fulfill the salt requirements of the strain, which is moderately halophilic. Initially, large Erlenmeyer flasks (5 l) were used for production of smaller quantities of biomass (40–50 g). Growth tests were also performed in a 3-l fermentor (Applicon, Schiedam, Netherlands; 3 l, flat-bottom glass vessel with ADI 1020 control unit). Larger amounts of biomass were also prepared at 15°C in a 150-l fermentor (Bioengineering, Wald, Switzerland) using approximately 85 l of medium in each run. The cells were harvested by centrifugation in a continuous flow centrifuge (Cepa, New Brunswick). The cell mass was frozen in suitable small vials and jars.

Measurement of enzyme activity

The activity of the dehydrogenases was routinely assayed spectrophotometrically (Uvikon 941 Plus; Kontron Instruments, Watford, UK) by measuring the initial rates of NAD^+ reduction at 340 nm at 25°C. One unit is defined as the quantity of enzyme that reduces 1 µmol NAD^+ per minute. All assays were performed in a final volume of 1 ml and were carried out over a period of 1 min, during which time the rates were linear.

AlaDH assay

Forward reaction: 50 mM sodium pyrophosphate, pH 8.8, 1 mM NAD^+, 40 mM L-alanine, and enzyme. Reverse reaction: 0.1 M Tris/HCl, pH 8.5, 0.2 mM NADH, 160 mM $(NH_4)_2SO_4$, 2.5 mM pyruvate, and enzyme.

MDH assay

Forward reaction: 50 mM sodium pyrophosphate, pH 8.8, 1 mM NAD^+, 20 mM L-malate, and enzyme. Reverse reaction: 0.1 M Tris/HCl, pH 8.0, 0.2 mM NADH, 5 mM oxaloacetate, and enzyme.

GDH assay

Forward reaction: 50 mM sodium pyrophosphate, pH 8.8, 1 mM NAD^+, 40 mM L-glutamate, and enzyme. Reverse reaction: 0.1 M Tris/HCl, pH 8.0, 0.2 mM NADH, 160 mM $(NH_4)_2SO_4$, 5 mM 2-oxoglutarate, and enzyme.

The assay mixtures for lactate and alcohol dehydrogenase contained 50 mM sodium pyrophosphate, pH 8.8, 1 mM NAD^+, and 40 mM DL-lactate or ethanol, respectively. Glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase were assayed as described by Bergmeyer et al. (1983) and Ciardiello et al. (1995), respectively.

Purification of alanine dehydrogenase

Cells (100 g wet weight) were suspended in 75 ml potassium phosphate buffer, pH 7.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM β-mercaptoethanol. The cells were then disrupted by sonication on ice (4 × 20-s bursts) with a sonicator (Misonix, Farmingdale, NY,

USA). The cell lysate was centrifuged at 100,000 *g* for 45 min, and the cell-free supernatant (150 ml) was applied to a Procion Red HE-3B Sepharose column (200 ml) equilibrated with the same buffer. The column was then washed with 3 column volumes of buffer and eluted with 1 column volume of 0.5 mM NADH in buffer. The collected fractions were assayed for AlaDH activity at 25°C as described next.

The pooled fractions were applied to a Q Sepharose column (20 ml) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM β-mercaptoethanol. The column was washed with 200 ml buffer, followed by 100 ml 275 mM KCl in the same buffer. AlaDH was eluted by washing with 340 mM KCl in the same buffer at a flow rate of 60 ml h^{-1}. The fractions with AlaDH activity were pooled and concentrated to 4 ml using Centriplus 100 concentrators (Amicon, Cork Ireland). The solution was loaded on a Sephacryl S-300 column (1.6 × 76 cm), equilibrated with the same buffer, and eluted at a flow rate of 12 ml h^{-1}. The active fractions were concentrated to 1 ml and stored frozen at –20°C. Activity was maintained by the addition of glycerol to the purified enzyme (final concentration, 50%).

Purification of malate dehydrogenase

The procedure for purification of MDH was the same as that described for AlaDH up to the Procion Red HE-3B Sepharose step, as the NADH eluate contained both MDH and AlaDH, and both enzymes could be obtained from the same cell extract. The NADH eluate was applied to a Q Sepharose column as previously described, washed with 200 ml buffer, and washed with 100 mM KCl, followed by 140 mM KCl, which eluted MDH. Subsequent washings, as described, eluted AlaDH. The fractions with MDH activity were pooled, concentrated with Centriplus 50 concentrators, and loaded on the same Sephacryl S-300 column as used for AlaDH. The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Fractions containing MDH activity were concentrated to approximately 1 ml with a Centriplus 50 concentrator and stored frozen at –20°C.

Determination of protein concentration

The absorbance at 280 nm was used to monitor protein elution patterns. Exact protein concentrations were measured by the method of Bradford (1976) using the Bio-Rad protein assay reagent with bovine serum albumin as standard.

Determination of relative molecular mass

The M_r values for the purified enzymes were determined by analytical gel filtration on a prepacked FPLC column, Superose 6 HR 10/30, equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M KCl. The column was equilibrated with the following marker proteins: apoferritin, 443 kDa; *Clostridium symbiosum* GDH, 300 kDa; β-amylase, 200 kDa; bovine serum albumin, 66.2 kDa; cytochrome *c*, 12.4 kDa. Samples (100 µl; 1 mg ml^{-1}) were loaded separately. The flow rate was 0.5 ml min^{-1}.

Electrophoretic methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) and native PAGE were performed using a Mini-Protean II apparatus (Bio-Rad, Dublin, Ireland). The subunit M_r was determined by SDS-PAGE on 10% gels. The following proteins were used for calibration: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; aldolase, 39.2 kDa; triose phosphate isomerase, 26.6 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Protein bands were visualized by staining with Coomassie brilliant blue R-250, and gels were destained with methanol/water/acetic acid (3:6:1, v/v/v). Native gels (7.5% acrylamide) were stained for dehydrogenase activity with a stain containing 50 mM sodium pyrophosphate buffer, pH 8.8, 1 mM NAD^+, 0.1 mM phenazine ethosulfate, 0.6 mM *p*-iodonitrotetrazolium violet, and 40 mM L-malate, L-alanine, L-glutamate, DL-lactate, or ethanol. The stain for glucose-6-phosphate dehydrogenase contained 1 mM $NADP^+$ instead of NAD^+ and 20 mM glucose-6-phosphate.

Determination of amino acid sequence

N-terminal sequence data were obtained by carrying out SDS-PAGE of the purified enzyme on 12.5% gels and Western blotting the protein bands onto polyvinylidene fluoride (PVDF) membranes (Sequi-Blot; Bio-Rad, 0.2 µm). Internal sequences were obtained by digestion of the native enzyme by trypsin or chymotrypsin (Sigma) at 20°C for 20 min, with a 1:20 ratio of protease to enzyme, followed by SDS-PAGE and blotting. Automated Edman-type sequence analysis was conducted using a Perkin-Elmer (Norwalk, CT, USA) Procise Model 494 sequencer with Model 610 software for data analysis.

Thermostability

Thermostability was measured by incubating the enzymes in 50 mM potassium phosphate buffer, pH 7.0, in water baths set to different temperatures. Aliquots were removed at intervals and assayed at 25°C, as described.

Results

Strain characteristics

The strain PA-43 was isolated from a sea urchin off the coast of Southwest Iceland. It is a short gram-negative rod, oxidase- and catalase positive, a facultative anaerobe that appears to break down glucose by oxidation and fermenta-

tion [(+/+) for O/F test], forms 2-mm green, flat colonies on TCBS agar after 2 days incubation at 15°C, and is sensitive to the vibriostatic agent O129 (10 μg). It is moderately halophilic, growing optimally in approximately 2% NaCl and more slowly in 6% NaCl, but it is unable to grow in 8% NaCl.

Studies on other morphological, physiological, biochemical, and various other characteristics of this strain (to be reported later), as well as the NT analysis of the BIOLOG results, suggested that this marine strain (PA-43) was likely to belong to the genus *Vibrio*, and our initial report on these enzymes called it *Vibrio* PA-43 (Irwin et al. 1998). However, partial sequencing of the 16S rRNA gene showed that strain PA-43 was placed with members of the genus *Shewanella* (Fig. 1). A generated similarity matrix showed that it was 98.5% similar to *Shewanella* sp. KMM3299 (AF 145921), 97.8% similar to *Shewanella baltica* (AF 173966), 96.7% similar to *Shewanella* sp. ACEM-9 (AF 295592), and 96.6% similar to *Vibrio* sp. OS53 (AB 038028).

Optimum temperature for growth

The optimum growth temperature for strain PA-43, which comes from a permanently cold environment, is between 16° and 17°C (Fig. 2). It can grow at 0°C, and also grows very slowly at 21°C but not at 22°C. This observation means the strain is very close to being a psychrophile as defined by Morita (1975), but it is certainly not an extreme psychrophile or hyperpsychrophile (Feller et al. 1996). It is now recognized that there is a continuum in cardinal temperature characteristics among cold-adapted bacteria, and some are known that do not conform exactly to the definition for the psychrophile group (Russell 1993).

Production of biomass for enzyme analysis

To maximize the biomass yield, many different media, both complex and minimal, were devised and tested in shake cultures at 15°C. As the strain required approximately 1.5%–2% NaCl for growth, media for all subsequent growth contained additional NaCl or sea salts. The rich complex medium designated NB2+1% NaCl gave the greatest amount of biomass in shake flask cultures after about 30 h incubation at 15°C. This medium contained nutrient broth no. 2 (Oxoid CM 67) with 1% NaCl added, so that the final concentration of NaCl was approximately 1.5%, or 0.26 M; it was used to produce biomass for enzyme purification and characterization. Smaller amounts of biomass were produced in large shake flasks, in which about 7 l of medium gave approximately 40 g wet cell mass per run. Biomass was also produced in a 150-l fermentor using 85 l medium in each run to produce approximately 1,000 g of wet cell mass.

Enzyme content

Figure 3 illustrates the yield of enzyme obtained in crude extract when strain PA-43 was grown in four different media. There was some variation from batch to batch, and the sonication conditions affected the yield, but in general

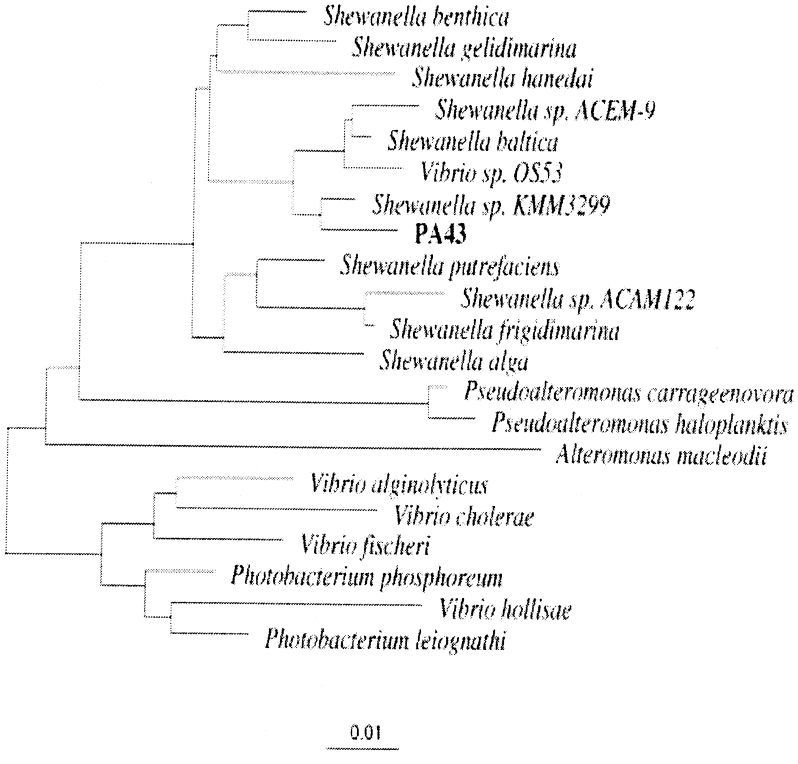

Fig. 1. A phylogenetic tree determined by neighbor-joining analysis. Phylogenetic analysis was based on 668 nucleotide positions in the bacteria 16S rRNA gene. *Pasteurella volantium* was used as an out-group. The *bar* indicates the estimated number of base changes per nucleotide sequence position

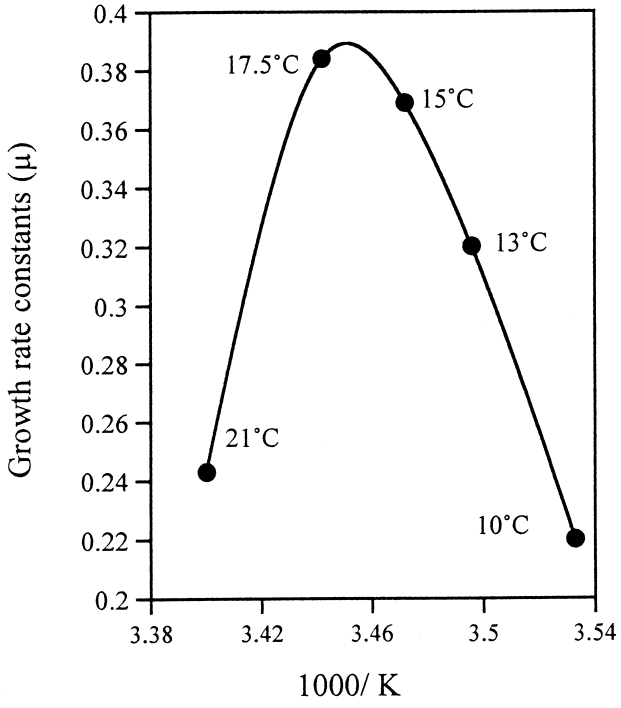

Fig. 2. Arrhenius plot of the growth rate constants (μ) as a function of temperature (expressed as 1,000/K) The cells were grown in CHYSS medium (hydrolyzed casein, yeast extract, and sea salts) at the temperatures indicated

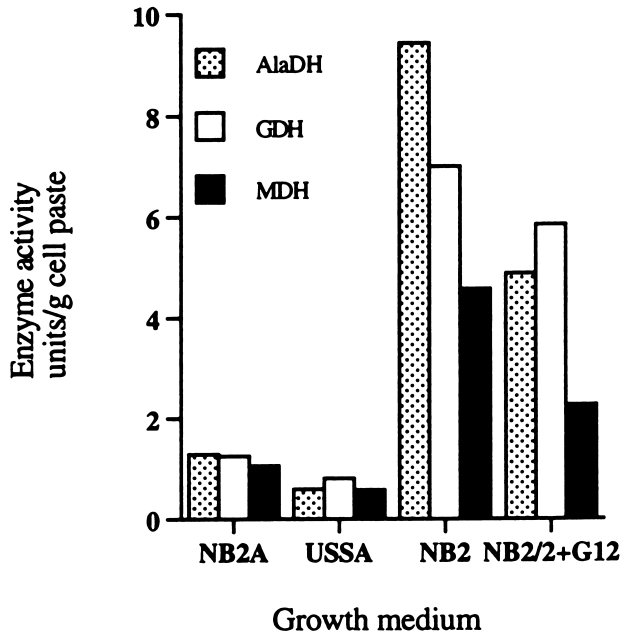

Fig. 3. Yield of L-alanine dehydrogenase (*AlaDH*), L-glutamate dehydrogenase (*GDH*), and L-malate dehydrogenase (*MDH*) from PA-43 grown in different media. The organism was grown in the following media at 15°C and the cell mass was harvested in early stationary phase from the following liquid media: *NB2A*, nutrient broth no. 2 (Oxoid CM 67) with 1% NaCl and agar added; *USSA*, skim milk agar and added sea salts (2%); *NB2*, nutrient broth no. 2 with 1% (w/v) NaCl added; *NB2/2+G12*, Oxoid nutrient broth no. 2 (50% strength) with 1.1% (w/v) NaCl and 1.25% (w/v) sodium glutamate added. The cells were lysed as described in Materials and methods, and enzyme activity in the supernatants was assayed using the standard assays at 25°C in the forward reaction

the highest specific activities for AlaDH, GDH, and MDH were always obtained when the organism was grown in NB2 medium containing 1% NaCl: average specific activities in the supernatant were 0.33, 0.28, and 0.26 units (mg protein)$^{-1}$ respectively. Glutamate (1.25% w/v) was added to the NB2 growth medium (at 50% of the usual concentration) to see if this would induce GDH production in preference to AlaDH, but this was not the case. In NB2 medium with 1% NaCl, AlaDH, MDH, and GDH constituted approximately 0.5%, 0.2%, and 0.1% or less, respectively, of the total soluble protein. A glyceraldehyde-3-phosphate dehydrogenase assay of the NB2 supernatant showed a specific activity of only 0.016 units mg^{-1}.

Activity staining of supernatant run on a 7.5% native polyacrylamide gel with a stain containing $NADP^+$ and glucose-6-phosphate detected a faint band with glucose-6-phosphate dehydrogenase activity, but no activity was detected by spectrophotometric assay. No lactate or alcohol dehydrogenase activity was detected by either activity staining or spectrophotometric assay. Purified alanine dehydrogenase showed some activity toward glutamate (approximately 2.5% of that toward L-alanine) when assayed with 40 mM L-glutamate, but only one band was seen on gels that were activity stained with L-glutamate as the added substrate. Preliminary data suggest that PA-43 GDH is an enzyme of unusually high molecular weight and may belong to a newly discovered class of large GDHs, the first of which to be discovered was the NAD^+-dependent GDH from *Streptomyces clavuligerus* (Miñambres et al. 2000).

Purification of AlaDH and MDH

The purifications of AlaDH and MDH are summarized in Table 1 and Table 2 , respectively. A purification of approximately 124 fold was obtained for AlaDH, with 36% recovery; this varied from 100 fold to 160 fold and from 25% to 50% recovery from preparation to preparation. Procion Red HE-3B Sepharose affinity chromatography gave 55-fold and 54-fold purification for AlaDH and MDH, respectively, in a typical preparation, and was the most effective step for purifying both enzymes, as only about 2% of the total protein applied was susceptible to affinity elution with 0.5 mM NADH. MDH was purified to homogeneity with 26% recovery; in some preparations, this was as low as 19%.

The purity of both enzymes was confirmed by SDS-PAGE (Fig. 4). The M_r of AlaDH as deduced by SDS-PAGE was 42,300 ± 1,200, and the value estimated by analytical gel filtration was 260,000, implying that the native enzyme comprises six identical subunits. The subunit M_r of MDH estimated by SDS-PAGE was 36,500±2,500, and analytical gel filtration of the native enzyme gave a value of approximately 70,000, showing that strain PA-43 MDH was dimeric.

Table 1. Summary of purification procedure for L-alanine dehydrogenase (AlaDH) from PA-43

Procedure	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)	Purification (fold)
Crude extract	149	1043	420	0.402	100	1
Procion Red chromatography	119	19	418	22.0	99	55
Q Sepharose chromatography	75	4.8	215	44.8	51	111
Sephacryl S-300 chromatography	13.9	3.0	150	50.0	36	124

The activity was measured in the direction of oxidative deamination of L-alanine, as described in the Materials and methods section; starting material was 100 g (wet weight) of PA-43 cell mass

Table 2. Summary of purification procedure for L-malate dehydrogenase (MDH) from PA-43

Procedure	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)	Purification (fold)
Crude extract	170	803	739	1.09	100	1
Procion Red chromatography	133	7.8	401	51.1	54	47
Q Sepharose chromatography	78	1.9	288	152	39	139
Sephacryl S-300 chromatography	17.4	0.84	194	231	26	212

The activity was measured in the direction of oxidation of L-malate, as described in the Materials and methods; starting material was 100 g (wet weight) of PA-43 cell mass

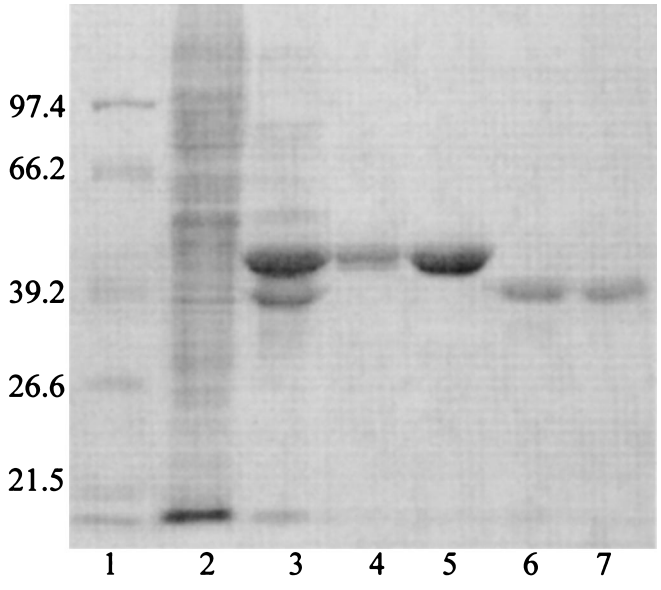

Fig. 4. SDS-PAGE (10%) of the fractions obtained during the purification of AlaDH and MDH from PA-43: *lane 1*, marker proteins with relative molecular masses indicated on the *left*; *lane 2*, supernatant; *lane 3*, Procion Red HE-3B eluate; *lane 4*, Q Sepharose AlaDH eluate; *lane 5*, Sephacryl S-300 purified AlaDH; *lane 6*, Q Sepharose MDH eluate; *lane 7*, Sephacryl S-300 purified MDH

Stability of MDH and AlaDH under different storage conditions

MDH lost about 50% of its activity when stored for 3 weeks in potassium phosphate buffer, pH 7, at –20°C. It showed some sensitivity to freeze–thawing, retaining 44% of the initial activity after four cycles of freeze–thawing, compared to 55% for a sample subjected to one cycle. AlaDH was far less stable to freezing, losing most of its activity in less than 2 weeks at concentrations <0.2 mg ml^{-1} at 4°C or –20°C. The enzyme was very sensitive to freeze–thawing; even at higher concentrations (>1 mg ml^{-1}), it lost more than 75% of its activity after two freeze-thaw cycles and storage at –20°C for 1 week. The enzyme was stabilized by the addition of 50% glycerol and lost activity rapidly unless β-mercaptoethanol was included in the buffers during its purification. In the presence of 50% glycerol, AlaDH activity was retained for up to 5 months at –20°C.

AlaDH (>0.05 mg ml^{-1}) is stable for at least 3 days in 50 mM phosphate buffer at pH 6–8 and 4°C. It loses 75% of its activity in 0.1 M Tris/Cl buffer after 3 days at pH 7–10 at 4°C, but retains 87% of its activity after 3 days in 0.1 M glycine/KOH, pH 10, at the same temperature. MDH at concentrations greater than 0.1 mg ml^{-1} was stable at pH 7–8 in phosphate buffer. Dilute MDH (<0.01 mg ml^{-1}), however, lost activity within minutes, even in phosphate buffer at this pH, but it was stable on ice or at room temperature for 2–3 h in 50 mM sodium pyrophosphate, pH 8.8, suggesting that optimum stability is found in this buffer.

Effect of pH on enzyme activity

AlaDH and MDH activities were determined in buffers of different pH. The optimum pH for the oxidative deamination of L-alanine was 10.5, and it was 9.0 for the reductive amination of pyruvate (Fig. 5A). For MDH, the optimum pH for malate oxidation was 10–10.2, and the optimum pH for oxaloacetate reduction was about 8.8 (Fig. 5B).

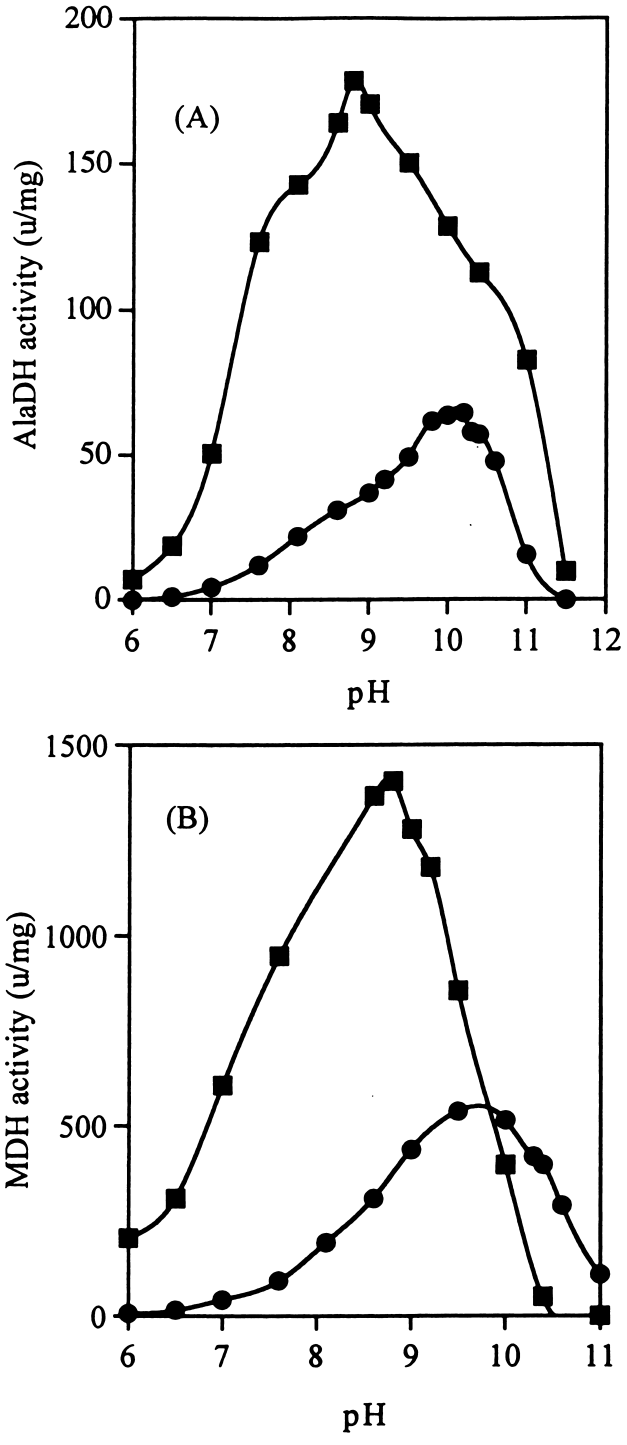

Fig. 5. Activity of AlaDH (**A**) and MDH (**B**) as a function of pH. Assays were performed in 50 mM potassium phosphate buffer/50 mM glycine. pH values below 7 were adjusted by adding K_2HPO_4; KOH was added to adjust pH values above 7. The forward reaction (NAD^+ reduction) is denoted by *circles* and the reverse reaction (NADH oxidation) by *squares*

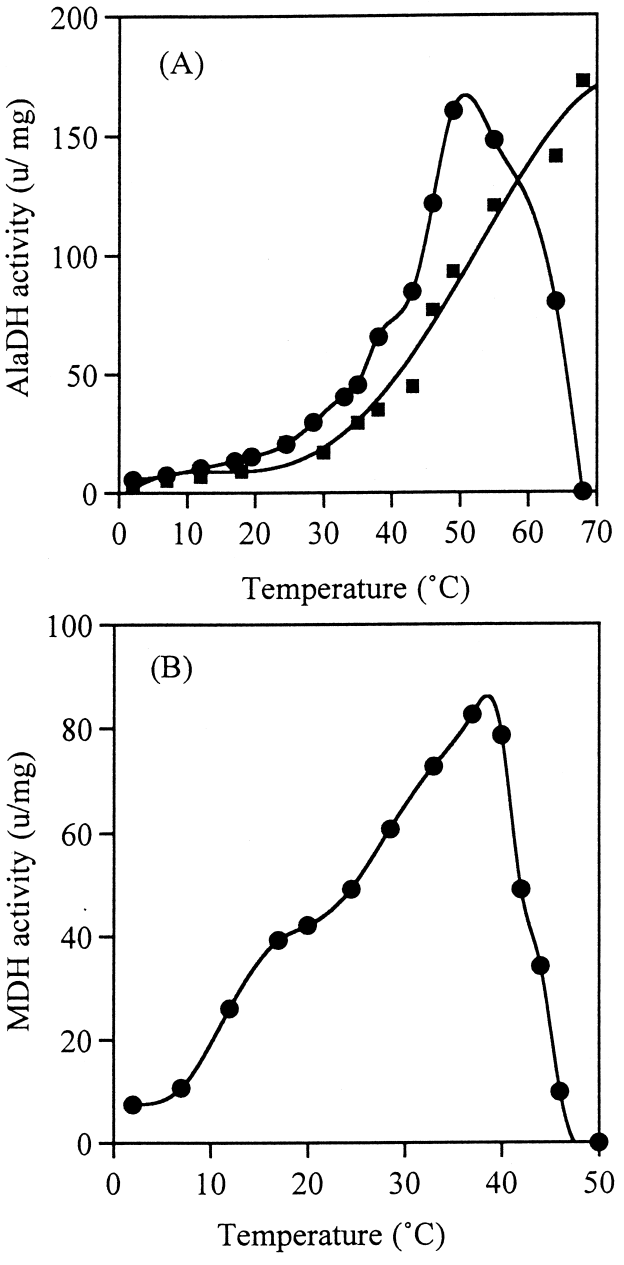

Fig. 6. Temperature dependence of AlaDH (**A**) and MDH (**B**) activity. The enzymes were assayed in the forward direction as described in Materials and methods at the temperatures indicated. In **A**, AlaDH from the mesophile *Bacillus subtilis* was assayed for comparison (*squares*)

Optimal temperature of AlaDH and MDH activity and thermostability

Figure 6A,B shows the effect of temperature on the catalytic activity of AlaDH and MDH, respectively. The apparent optimum temperature for AlaDH activity in the direction of NAD^+ reduction was 45°–50°C and about 37°C for MDH. In the case of AlaDH, the enzyme from *Bacillus subtilis*, used as a mesophilic comparison, had not reached its maximum activity even at 68°C.

The thermostability of both enzymes was investigated at 25°, 37°, and 50°C. The heat stability of AlaDH was also examined at 42°, 56°, and 60°C. AlaDH was considerably more thermostable than MDH, retaining 100% of initial activity after 90 min at 37°C and 42°C. Semilogarithmic plots of AlaDH and MDH residual activity as a function of incubation time (Fig. 7A and 7B, respectively) show that the

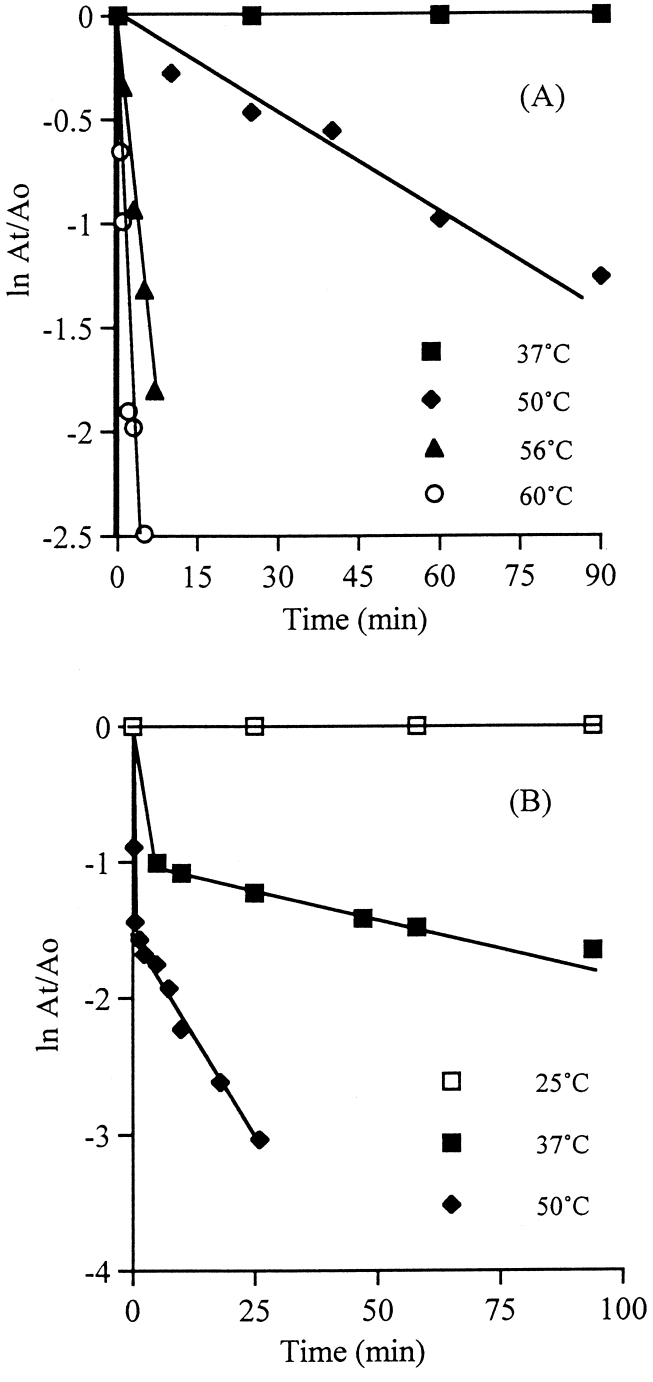

Fig. 7. Semilogarithmic plots of AlaDH (**A**) and MDH (**B**) activity as a function of time at different temperatures. The enzymes were incubated in potassium phosphate buffer, pH 7.0, at the temperatures shown. The concentration of AlaDH was 0.5 mg ml^{-1}; for MDH, the concentration was 0.1 mg ml^{-1} for the 25°C and 37°C experiments and 0.5 mg ml^{-1} for the 50°C experiment. Aliquots were withdrawn at timed intervals and assayed under standard conditions in the forward reaction at 25°C

inactivation of AlaDH is monophasic, whereas that for MDH is biphasic, with an initial rapid loss of activity followed by a slower inactivation. The half-life values for AlaDH were as follows: 50°C, 42 min; 56°C, 2.25 min; 60°C, about 15 s. In contrast, the half-life values for MDH (fast phase of inactivation) at 37°C and 50°C were 6 min and approximately 15 s, respectively.

Thee stability of the psychrophilic enzymes was compared to that of the same enzymes from mesophilic organisms (AlaDH from *B. subtilis,* MDH from porcine heart). *B. subtilis* AlaDH retained 100% of its initial activity after incubation at 60°C for 1 h. The half-life of porcine heart MDH at 50°C was approximately 3 min.

Sequence data for AlaDH and MDH

Protein sequencing of five fragments of AlaDH, one N-terminal and four tryptic and chymotryptic fragments, produced the sequences displayed in Fig. 8. A BLAST search was used to compare these sequences to those in the EMBL/Gen Bank database, and they were aligned with sequences of six other AlaDHs. A total of 103 residues (approximately 30% of the total sequence) were obtained, showing 94% identity to the AlaDH from *Shewanella* sp. Ac10, 78% identity to that from *Vibrio proteolyticus*, and 62% of that of *Phormidium lapideum*. However, these identity percentages may turn out to be slightly overstated for the full sequence, as the region compared includes residues 90–99, which are highly conserved across all the AlaDHs studied.

The partial primary structure of MDH was compared to that of five other bacterial malate dehydrogenases. Figure 9 shows a multiple sequence alignment for six MDHs, including that from PA-43, and two other psychrophilic MDHs, from *Vibrio* 5710 and *Photobacterium* SS9. Approximately 34% of the sequence was obtained, the 46 N-terminal residues by N-terminal analysis and residues 88–157 (approximately) by sequencing of a 25-kDa tryptic fragment. The N-terminal 46 residues containing part of the cofactor binding domain (Ohkuma et al. 1996) were very well-conserved (45/46 residues were identical to *Vibrio* 5710, 41/46 to *Photobacterium* SS9, 44/46 to *E. coli.*). When the 106 available residues were examined, the sequence identities were as follows: *E. coli*, 91%; *Haemophilus influenzae*, 90%; *Salmonella typhimurium*, 87%; *Vibrio* 5710, 86%; and *Photobacterium* SS9, 82%.

Discussion

We report on the purification to homogeneity of an AlaDH and an MDH from a cold-adapted bacterium. Initial characterization studies placed the microorganism in the genus *Vibrio*, but 16S rRNA gene sequencing revealed that strain PA-43 was most similar to *Shewanella* species and placed it among these in a phylogenetic tree. *Vibrio* and *Shewanella* are closely related genera, both belonging to the γ-subclass of the *Proteobacteria*, and a *Vibrio* sp., OS53, was placed close to PA-43 in the phylogenetic tree among *Shewanella* species (see Fig. 1). The branching order of the tree shows two distinct clusters of *Shewanella*. Strain PA-43 is placed in one branch, which contains known halophilic *Shewanella* species (*S. gelidimarina, S. hanedai, S. benthica*; Ven-

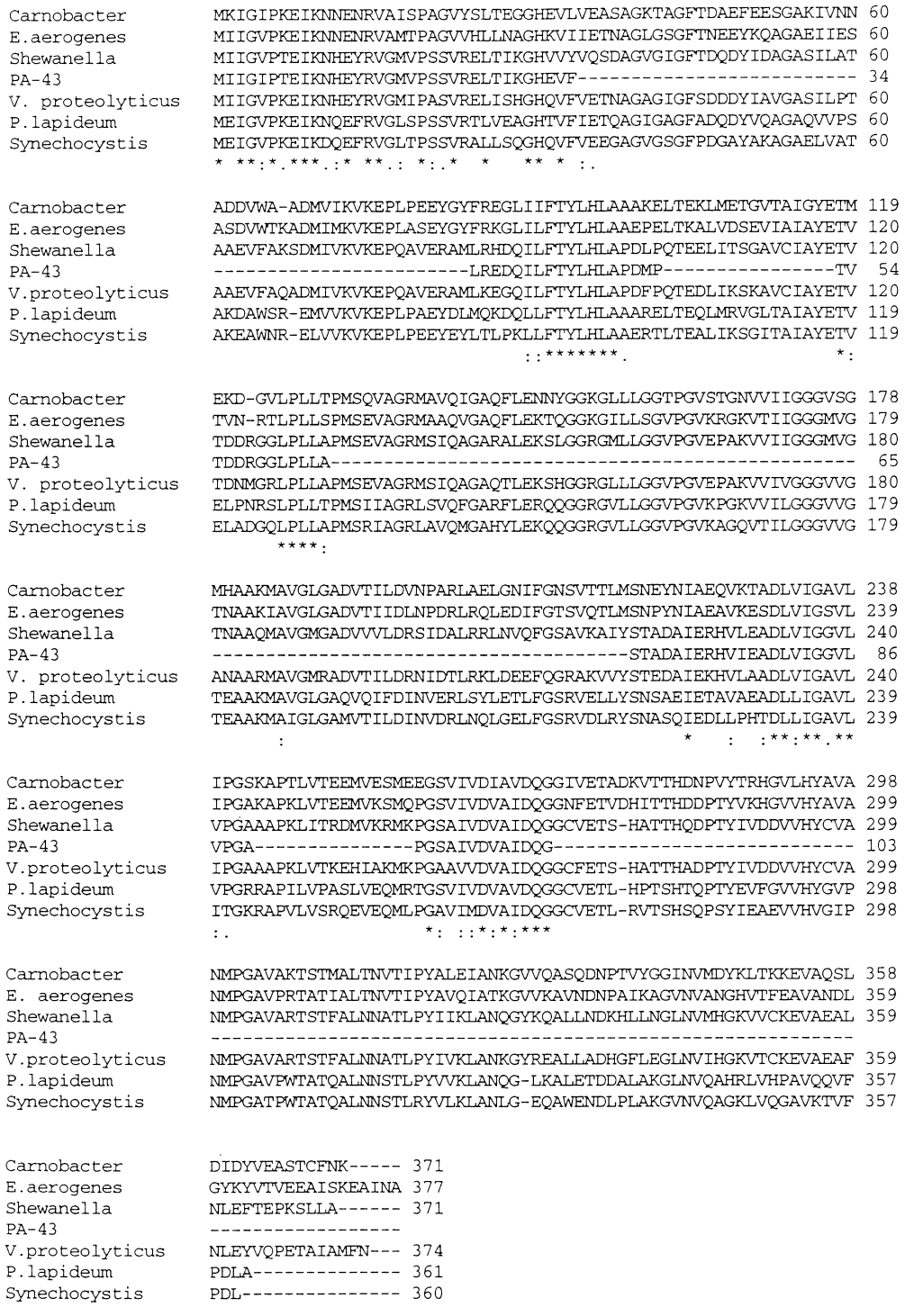

Fig. 8. Alignment between PA-43 AlaDH partial sequence and six other AlaDHs. Amino acid sequences were aligned using the program Clustal W. The following EMBL/Gen Bank accession numbers were used: *Shewanella* sp. Ac10, AF070715; *Vibrio proteolyticus*, AF070716; *Phormidium lapideum*, D37807; *Enterobacter aerogenes*, AB013821; *Carnobacterium* sp. strain St2, AF070714; *Synechocystis*, D90900. An *asterisk* indicates residue identity; a *colon* indicates similarity

kateswaran et al. 1999), and it is closest to *Shewanella* sp. KMM3299 (98.5% similar); it is next closest to *Shewanella baltica* (97.8% similar).

Screening by activity staining and spectrophotometric assay revealed the presence of significant glutamate dehydrogenase activity, but only small amounts of glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase activity, and no lactate or alcohol dehydrogenase activity was detected under the growth conditions used. These enzymes may be inducible when the organism is grown in different media, as the medium supplied here would constitute a rich source of amino acids for energy metabolism with a consequently decreased need for glycolytic enzymes, or, alternatively, the absence of these common housekeeping enzymes may suggest that this organism has unusual energy-yielding metabolism.

It is noteworthy that this organism contains both AlaDH and an NAD^+-dependent GDH, as most microorganisms tend to have either GDH or AlaDH. It would imply that this organism under the growth conditions used has no need of

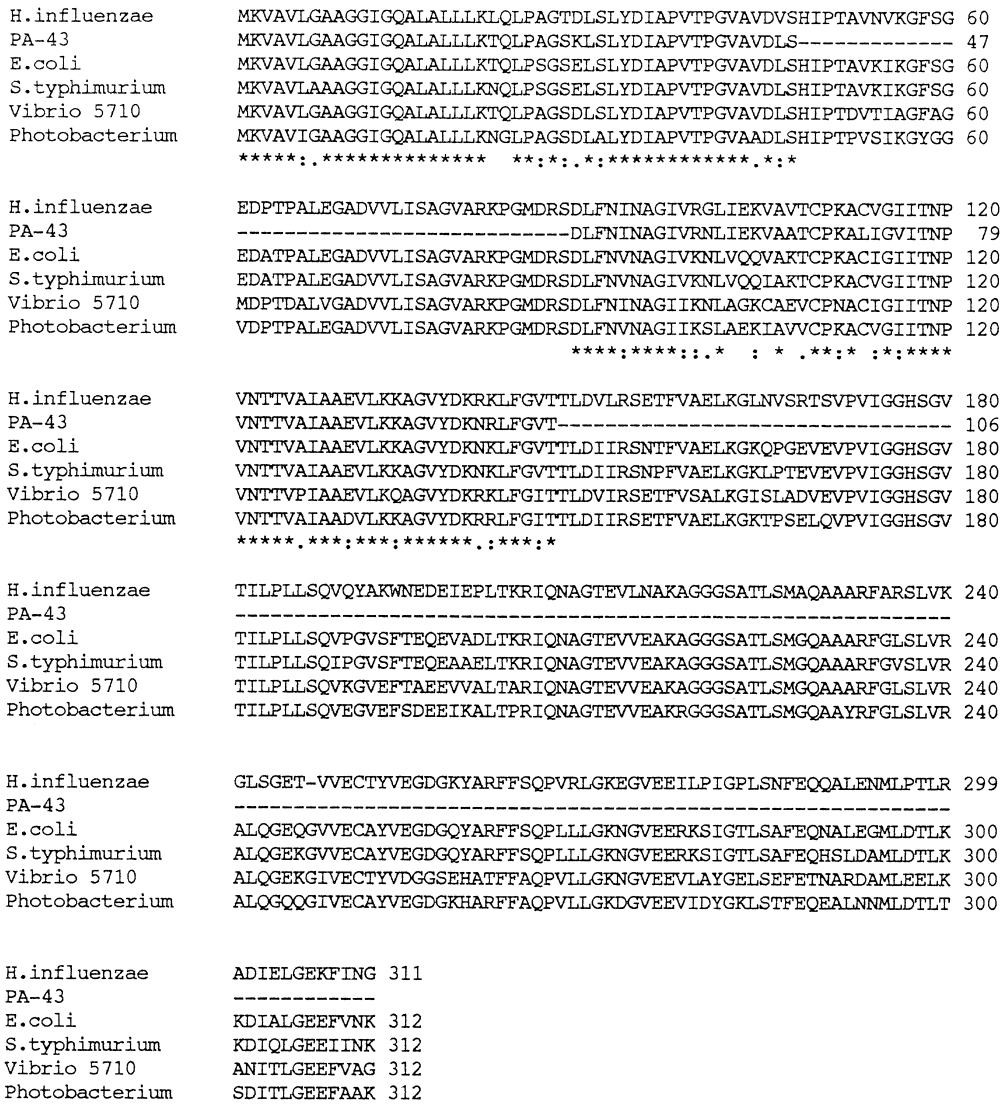

Fig. 9. Alignment between PA-43 MDH partial sequence and five other MDHs. Amino acid sequences were aligned using the program Clustal W. The following EMBL/Gen Bank accession numbers were used: *Vibrio* 5710, D78194; *E. coli* K12, AE000403; *Salmonella typhimurium*, M95049; *Haemophilus influenzae*, P44427; *Photobacterium* SS9, L13319. An *asterisk* indicates residue identity; a *colon* indicates similarity

an $NADP^+$-dependent GDH, which generally functions in a biosynthetic capacity to fix NH_4^+ (Hudson and Daniel 1993). In contrast, the metabolic role of NAD^+-dependent GDH is in the oxidative deamination of glutamate, which would be consistent with the physiological location of the microorganism, an enterobacterium, inside the gut of its host where it could be supplied with a nitrogen source in the form of ready-made amino acids provided by the host's digestive process. The abundance of AlaDH in strain PA-43 grown in the rich medium suggests that the primary function of this enzyme may be to assimilate alanine, which might serve partly as a nitrogen source. Further support for this view of the the nitrogen nutrition of the organism comes from the fact that assays of the supernatant (from bacteria grown in the rich medium) for glutamine synthetase (Sawa et al. 1988) and GOGAT activity (Meers et al. 1970) showed no detectable activity of either of these enzymes, which function in alternative pathways of nitrogen fixation in other free-living microorganisms.

The AlaDH is hexameric and NAD^+ dependent, in common with most AlaDHs examined to date. The pH optima for the forward and reverse reactions are relatively high, in common with those of the *B. subtilis* (Yoshida and Freese 1965) and *Bacillus sphaericus* (Ohashima and Soda 1979) enzymes. The apparent optimum temperature for activity is also relatively high, although temperature optima that are considerably in excess of the normal ambient temperature for the organism are not uncommon (Xu et al. 1998) and the apparent optimum temperature is approximately 20°C less than that of *B. subtilis* AlaDH, which was used as a reference mesophile. PA-43 AlaDH displayed significant activity at 2°C, 5.5 units mg^{-1}, in comparison to 3.2 units mg^{-1} for that of *B. subtilis*, and PA-43 had a higher activity than the mesophile up to 45°C. The ratio of activities at the apparent optimum temperature to 2°C was 29-fold that for the psychrophilic enzyme, compared to 53-fold for the mesophile, suggesting that the enzyme shows some psychrophilic character in that respect, although this is not nearly as pronounced as that shown with the aspartate transcarbamylase from the Antarctic bacterium TAD1, in which the ratio of the activity at the optimum temperature (30°C) to that at 0°C is only 4 (Sun et al. 1998).

Sequence alignment revealed that the AlaDH sequence obtained was closest to that from *Shewanella* sp. Ac 10, which has an optimum growth temperature of 20°C (Galkin et al. 1999) and thus is a psychrotroph by the definition of Morita (1975), and the mesophile *Vibrio proteolyticus*. *Shewanella* Ac10 AlaDH had a maximum activity temperature of 47°–50°C, compared to the slightly lower temperature maximum of 45°–50°C for the PA-43 enzyme. In contrast, the *Shewanella* Ac10 enzyme was considerably more thermally robust, at least under the conditions used (potassium phosphate buffer, pH 7.2), retaining about 52% of its activity after incubation for 30 min at 60°C (Galkin et al. 1999), compared to a half-life of only 15 s for the PA-43 enzyme at the same temperature.

Efforts are under way to clone the genes for both AlaDH and MDH, which will enable us to complete the sequence and make a full comparison to sequences of their psychrophilic, mesophilic, and thermophilic homologues. Galkin et al. (1999) used the three-dimensional (3-D) structure of *Phormidium lapideum* AlaDH (Baker et al. 1998) to carry out homology modeling of both the *Shewanella* enzyme and that of an even more thermolabile AlaDH from the gram-positive bacterium *Carnobacterium* sp. strain St2, as well as modeling the structures of AlaDH from two mesophiles and a thermophile. Galkin et al. found that the total number of salt bridges decreased in the order thermophilic AlaDH to mesophilic AlaDH to psychrotrophic AlaDH when members of the same bacterial subgroups were compared. The *Carnobacterium* enzyme also had a lower total number of salt bridges, particularly those formed by arginine residues. Given that PA-43 AlaDH is less thermostable than the *Shewanella* Ac10 enzyme but appears to have a very similar primary structure, it may be feasible, using homology modeling and possibly X-ray crystallography, to identify which substitutions of amino acid residues and consequent alterations in salt bridges or other factors influence the thermostability.

Interestingly, PA-43 MDH was far less thermostable than the AlaDH, illustrating that thermostability is also an intrinsic property of the protein itself and does not merely depend on the organism in which it resides. No detailed thermostability characterization is available for another psychrophilic MDH, but *Vibrio* 5710 MDH lost all activity after incubation at 45°C for 20 min, and its apparent optimal temperature for activity was 35°C, similar to the 37°C determined for *Vibrio* PA-43 MDH (Ohkuma et al. 1996).

PA-43 MDH appears to be most similar, at least on the basis of the available data, to *E. coli* MDH. A high-resolution 3-D structure is available for *E. coli* MDH, so that when the entire sequence becomes available, it will be an ideal candidate for molecular modeling. A 3-D structure has been determined for one psychrophilic MDH, that from *Aquaspirillum arcticum*, a microorganism which was isolated from Arctic sediment and grows optimally at 4°C (Kim et al. 1999). The primary structure of the *A. arcticum* MDH was 61% identical to that of *Thermus flavus*, a thermophile, and a comparison of these two MDHs showed that the efficient catalytic efficiency of the *A. arcticum* MDH at low temperatures could be attributed to an increased flexibility of active site residues, favorable charge distribution for substrate and coenzyme, and reduced intersubunit ion pair interactions. It will be interesting to see to what extent these tentative generalizations are borne out by data for a second psychrophilic MDH.

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