Extremophiles (1997) 1:52–60


ORIGINAL PAPER

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# Glutamate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima:* molecular characterization and phylogenetic implications

Received: July 15, 1996 / Accepted: November 12, 1996

**Abstract** The hyperthermophilic bacterium *Thermotoga maritima*, which grows at up to 90°C, contains an L-glutamate dehydrogenase (GDH). Activity of this enzyme could be detected in *T. maritima* crude extracts, and appeared to be associated with a 47-kDa protein which cross-reacted with antibodies against purified GDH from the hyperthermophilic archaeon *Pyrococcus woesei*. The single-copy *T. maritima gdh* gene was cloned by complementation in a glutamate auxotrophic *Escherichia coli* strain. The nucleotide sequence of the *gdh* gene predicts a 416-residue protein with a calculated molecular weight of 45852. The *gdh* gene was inserted in an expression vector and expressed in *E. coli* as an active enzyme. The *T. maritima* GDH was purified to homogeneity. The $NH_2$-terminal sequence of the purified enzyme was PEKSLYEMAVEQ, which is identical to positions 2–13 of the peptide sequence derived from the *gdh* gene. The purified native enzyme has a size of 265 kDa and a subunit size of 47 kDa, indicating that GDH is a homohexamer. Maximum activity of the enzyme was measured at 75°C and the pH optima are 8.3 and 8.8 for the anabolic and catabolic reaction, respectively. The enzyme was found to be very stable at 80°C, but appeared to lose activity quickly at higher temperatures. The *T. maritima* GDH shows the highest rate of activity with NADH ($V_{max}$ of 172 U/mg protein), but also utilizes NADPH ($V_{max}$ of 12 U/mg protein). Sequence comparisons showed that the *T. maritima* GDH is a member of the family II of hexameric GDHs which includes all the GDHs isolated so far from hyperthermophiles. Remarkably, phylogenetic analysis positions all these hyperthermophilic GDHs in the middle of the GDH family II tree, with the bacterial *T. maritima* GDH located between that of halophilic and thermophilic euryarchaeota.

**Key words** Glutamate dehydrogenase · Gene cloning and expression · Sequence analysis · Thermostability · Phylogenetic analysis

Communicated by G. Antranikian

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## Introduction

During the last decade, research on extremophiles has undergone an explosive development, notably in the field of hyperthermophilic prokaryotes that have optimal growth at or above 80°C (Stetter et al. 1990; Stetter 1996). All of them belong to the domain of *Archaea*, with exception of the bacterial orders of the Thermotogales (Huber et al. 1986) and the recently discovered Aquificales (Huber et al. 1992). The latter ones represent the lowest branches in the bacterial line of descent, supporting the suggestion that *Bacteria* have arisen from a thermophilic ancestor (Achenbach-Richter et al. 1987). Deeper insight into the early stages of evolution may be revealed by characterizing the biomolecules present in the most ancient *Bacteria*.

*Thermotoga maritima* is a strictly anaerobic, heterotrophic organism, which was isolated from geothermally heated sea floors in Italy by Huber et al. (1986). The organism is rod-shaped and has an unusual outer membrane structure. It grows optimally at 80°C and degrades carbohydrates to lactate, acetate, $CO_2$, and $H_2$. Several enzyme-

encoding genes from *T. maritima* have been cloned, sequenced, and expressed in *E. coli*, e.g., the genes for glutamine synthetase (Sanangelantoni et al. 1992), glyceraldehyde-3-phosphate dehydrogenase (Tomschy et al. 1993), and lactate dehydrogenase (Ostendorp et al. 1993).

For evolutionary studies, L-glutamate dehydrogenase (EC 1.4.1.3, GDH) seems an excellent candidate, since it is one of the most studied enzymes at the biochemical level (Smith et al. 1975). In addition, several GDHs have been crystallized successfully and high-resolution structures are now available from different microorganisms including hyperthermophilic *Pyrococcus furiosus* (Yip et al. 1995; Rice et al. 1996). So far, biochemical and genetic studies of GDHs from extremophiles have been restricted to species belonging to the *Archaea*, including the halophile *Halobacterium salinarium* (Benachenhou and Balducci 1994) and hyperthermophiles such as *Sulfolobus solfataricus* (Maras et al. 1992; Consalvi et al. 1991a), *S. shibitae* (Benachenhou-Lahfa et al. 1994), *P. furiosus* (Consalvi et al. 1991b; Eggen et al. 1993, 1994; Robb et al. 1992; Lebbink et al. 1995), and the isolates ES4 (Diruggiero et al. 1993) and AN1 (Hudson et al. 1993). However, no GDH sequences have yet been characterized from the most ancient, hyperthermophilic *Bacteria*.

In this report, we describe the cloning, sequencing, and expression of the *gdh* gene from the hyperthermophilic *T. maritima* belonging to the domain of *Bacteria*, report on the biochemical characterization of the encoded GDH from an overproducing *Escherichia coli*, and discuss the phylogenetic implications that originate from comparisons of the deduced GDH amino acid sequence.

## Materials and methods

### Bacterial strains and growth conditions

*Thermotoga maritima* MSB8 (DSM 3109) was grown anaerobically as static cultures in MMS medium (Huber et al. 1986) with yeast extract (0.05% w/v) and additional NaCl (2.6% w/v). *E. coli* TG1 (Sambrook et al. 1989) and *E. coli* XL1-Blue (Stratagene, LaJolla, CA, USA) were used as hosts for cloning and expression, respectively. Both strains were grown in Luria Bertani medium in the presence of 100 µg/ml ampicillin (Sambrook et al. 1989). The glutamate auxotrophic strain *E. coli* PA340 (Berberich 1972) was used for screening of a gene library and cultivated in M9 minimal medium (Miller 1972), supplemented with glucose (0.4% w/v) and threonine, arginine, leucine, histidine, and thiamine (1 mM each).

### Cloning and sequencing

Isolation of genomic DNA and construction of a gene library from *T. maritima* using the plasmid pUN121 (Nilsson et al. 1983) were carried out as described previously (Ostendorp et al. 1993). The gene library was introduced into *E. coli* PA340, followed by selection for complementation of the biosynthetic defect. Partial sequence analysis of the selected plasmid revealed that the *T. maritima gdh* gene was present on a 3.1-kb *Sau*3A fragment. The plasmid, consisting of pUN121 and the *Sau*3A fragment, was designated pLUW490. The *gdh* gene and flanking regions were sequenced in both directions according to the dideoxy chain termination method (Sanger et al. 1977) using the T7-sequencing kit (Pharmacia, Uppsala, Sweden). Sequencing was performed with single-stranded DNA obtained by cloning specific DNA fragments from pLUW490 into the bacteriophages M13mp18/19 or the phagemids pTZ18R/19R (Sambrook et al. 1989). Universal and gene-specific oligonucleotides were used as primers.

### Southern blotting

Genomic DNA from *T. maritima* and pLUW490 DNA was digested with *Asp*718. The DNA fragments from the two samples were separated on a 1% agarose gel and transferred to a positively charged nylon membrane (Boehringer, Mannheim, Germany). A 1.9-kb *Sst*I fragment containing the *T. maritima gdh* gene was used as a probe. Labeling and detection was done as described previously (Engler-Blum et al. 1993) with the nonradioactive dioxigenine-11-dUTP labeling kit (Boehringer).

### The GDH assay

GDH activity was determined at 60°C by measuring the time course of oxidation of NADH at 340 nm. Substrate concentrations for reductive amination were 200 µ*M* NADH, 100 m*M* $NH_4Cl$, and 5 m*M* α-ketoglutarate; and for oxidative deamination, 100 µ*M* NAD and 10 m*M* glutamate. The buffer was 20 m*M* sodium phosphate, pH 8. One unit was defined as 1 µmol of α-ketoglutarate converted per minute.

### Expression of the *gdh* gene

A 2.1-kb *Stu*I–*Bsp*HI fragment from pLUW490 was made blunt using the Klenow fragment of DNA polymerase I and inserted into the *Sma*I-linearized, 5′-dephosphorylated $P_{tac}$ expression vector pJF118ut, a derivative of pJF118eh (Binder 1987; Fürste et al. 1986). The resulting construct, designated pLUW492, that contained the *tac* promoter 90 bps upstream from the initiation codon of the *gdh* gene, was introduced into *E. coli* XL1-Blue and *E. coli* TG1.

### Purification of GDH

GDH was purified from 6 g (wet weight) of recombinant *E. coli* XL1-Blue cells harboring pLUW492, in the following way. The cell-free extract (32 ml, protein concentration 20.4 mg/ml) was prepared by lysis of a 18% (w/v) cell suspension in 20 mM sodium phosphate, pH 8, using a French pressure cell press (American Instrument Company, Beun

de Ronde, Abcoude, The Netherlands) at a pressure of 6.9 MPa. The cell-free extract was incubated at 70°C for 20 min and cleared from the precipitate by centrifugation. The heat-treated extract was loaded on a Q-Sepharose HR 16/10 column (Pharmacia) equilibrated with 20 mM sodium phosphate, pH 8.0. The enzyme was eluted with a linear salt gradient (0–1 M NaCl in the same buffer). GDH activity eluted when 0.65 to 0.71 M NaCl was applied to the column. Fractions were screened for activity by a spot assay (Kesters 1967) and active fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Removal of salt from the pooled active fractions and buffer exchange to 20 mM sodium phosphate, pH 6.5, was done with centrifugal concentrators (Macrosep, Filtontechn, Northborough, MA, USA). Chromatography on a mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM sodium phosphate, pH 6.5, was performed twice (7.5 mg of protein was loaded each time). The enzyme was eluted with a linear salt gradient (0–0.5 M NaCl). GDH activity eluted when 0.23–0.3 M NaCl was applied to the column. The buffer of the pooled active fractions was exchanged to 20 mM sodium phosphate, pH 8, by centrifugation through a 10-kDa cut-off membrane (Amicon Inc., Beverley, MA, USA). Finally, the enzyme was loaded on a mono Q column equilibrated with 20 mM sodium phosphate, pH 8 and eluted with a linear salt gradient (0–0.5 M NaCl). GDH activity eluted when 0.29–0.34 M NaCl was applied to the column. After removal of salt from the purified GDH, it was stored at –20°C.

### Characterization of GDH

All characteristics were determined using the *T. maritima* GDH purified from *E. coli*. The size of the denatured GDH was estimated by SDS-PAGE using prestained molecular weight marker proteins as a standard (Bio-rad, Hercules, CA, USA). The molecular mass of the native GDH was estimated by gel filtration using a HiLoad 16/60 Superdex 75 prep grade column (Pharmacia) equilibrated with 20 mM sodium phosphate buffer, pH 8, containing 100 mM NaCl. A set of molecular weight marker proteins ranging in size from 12.5 to 670 kDa (Bio-rad) was used to determine the calibration curve.

The temperature optimum of GDH (final concentration 2 µg/ml) was determined by measuring NADPH oxidation in 100 mM glycine-NaOH buffer, pH 8.4, at different temperatures. The pH of the buffer was adjusted at each temperature. As a control for thermal breakdown of substrates and cofactor, tubes were incubated containing all reagents except for the enzyme. The pH optima of both the anabolic and catabolic reactions were determined at 60°C using 100 mM citrate/phosphate, Tris-HCl, and glycine-NaOH buffers for the pH ranges 5.5–7.4, 7.6–9.3, and 8.6–10.5, respectively. The thermostability of the enzyme was determined by measuring residual activity after pre-incubating GDH (0.1 mg/ml) in 20 mM sodium phosphate buffer, pH 8.0, at 70°C, 80°C, and 90°C. Kinetic studies of the reductive amination reaction were performed at 60°C. Substrate concentrations ranged from 5–500 mM for $NH_4Cl$, 0.1–10 mM for α-ketoglutarate, 10–200 µM for NADH, and 25–400 µM for NADPH. Two µg of enzyme was used for the NADPH-dependent reaction and 0.5 µg of enzyme was used for the NADH-dependent reaction. Substrate specificity was determined using 10 mM of one of the following substrates: D-glutamate, glutamine, aspartate, alanine, or leucine.

Purified enzyme (16 µg) was rebuffered in 1% acetic acid and concentrated to a final volume of 40 µl. Subsequently, the $NH_2$-terminal sequence was determined using an Applied Biosystems 477A Protein Sequencer (Foster City, CA, USA).

### Western blotting

Cell-free extracts from *T. maritima* (30 µg) and pure GDH samples from *T. maritima* (0.5 µg, purified from *E. coli*) and *P. woesei* (0.5 µg) were separated by SDS-PAGE followed by electrophoretic transfer to nitrocellulose. Immunological analysis was performed as described by the supplier (Promega, Madison, WI, USA). Rabbit anti-*P. woesei* GDH (Eggen et al. 1993) was used as the primary antibody and anti-rabbit alkaline phosphatase conjugate was used for detection.

### Phylogeny

A previous multiple alignment of 23 GDH sequences (Benachenhou-Lahfa et al. 1993, 1994) was extended by including the deduced GDH sequences from *T. maritima*, reported here, *Thermococcus litoralis* (Britton et al. 1995), and *Bacillus subtilis* (Glaser et al. 1993). The multiple sequence alignment was used for the reconstruction of phylogenetic trees by applying the distance programs FITCH and NEIGHBOR or the parsimony program PROTPARS, that are included in the PHYLIP package (version 3.5c developed by J. Felsenstein). To construct the trees we used all the prokaryotic sequences from Family II, the human sequence as a vertebrate sequence, and three sequences (*E. coli*, *C. symbosium*, and *S. cerevisiae*) from Family I as an outgroup to root the trees. The trees obtained were further ascertained by bootstrap analysis (programs SEQBOOT and CONSENSE from the PHYLIP package) as previously described (Benachenhou-Lahfa et al. 1993).

### Nucleotide sequence accession number

The nucleotide sequence was submitted to GenBank (Los Alamos, NM, USA) and was given the accession number Y09925.

## Results

### Cloning and sequencing of the *T. maritima gdh* gene

To select for the gene encoding GDH, a *T. maritima* genomic library was introduced into the glutamate aux-

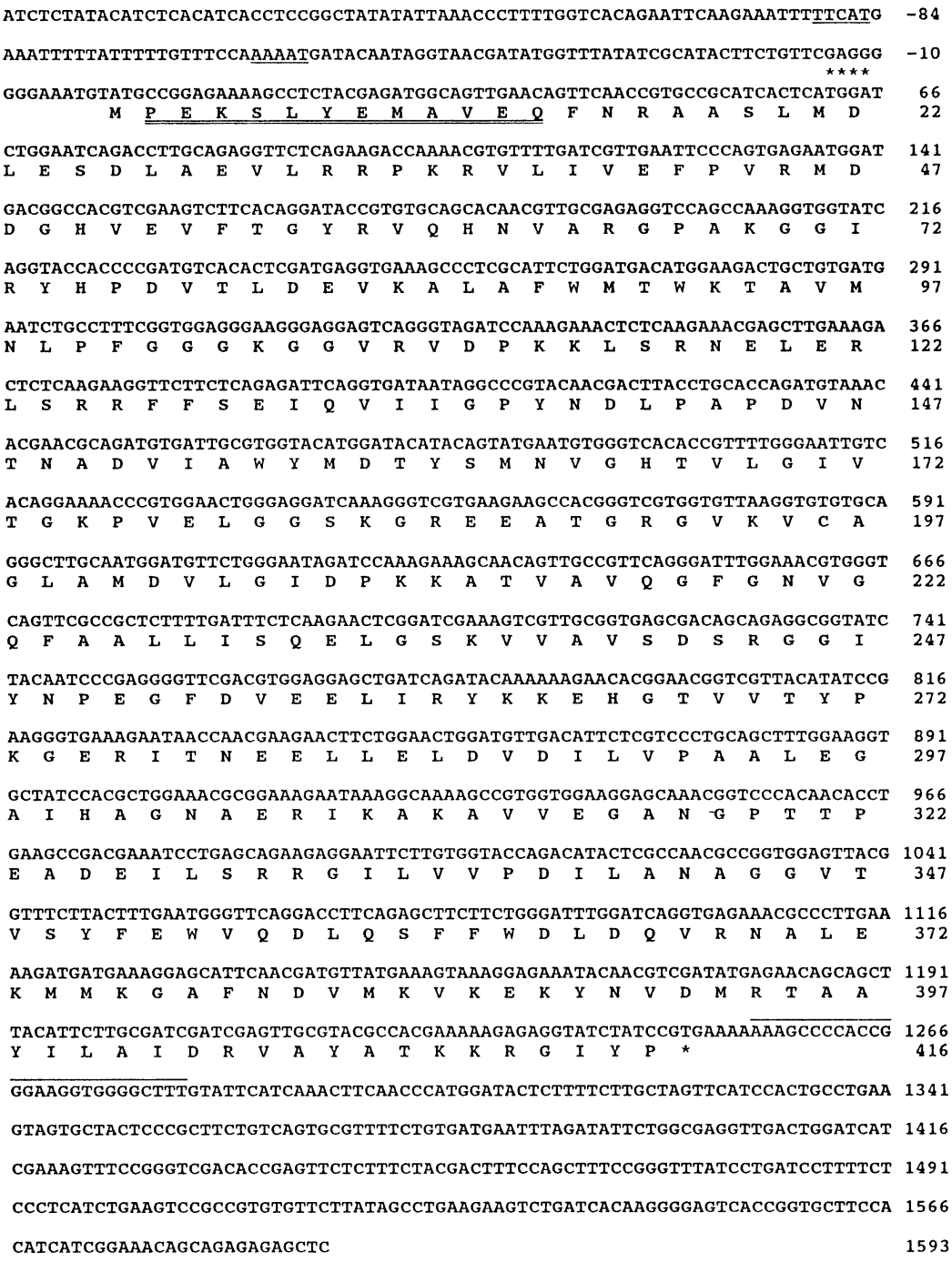


**Fig. 1.** The nucleotide sequence of the *T. maritima gdh* gene and its flanking regions. The deduced amino acids are indicated by single-letter code and aligned with the first nucleotide of each codon. Putative –35 and –10 promoter elements are *underlined*, the putative Shine and Dalgarno sequence is *underlined with stars*, and the putative terminator is *overlined*. Amino acids, which are determined by $NH_2$-terminal sequencing, are *double underlined*

otrophic mutant *E. coli* PA340, followed by selection for complementation of the biosynthetic defect. Two plasmids with inserts of about 3 kb, designated pLUW490 and pLUW491, were isolated from two independent clones. Restriction fragment analysis showed that both inserts were almost identical. Fragments from the pLUW490 insert were subcloned and sequenced, allowing the identification and localization of the *gdh* gene on a 1.9-kb *Sst*I fragment. Hybridization of the digested genomic DNA from *T. maritima* with the 1.9-kb *Sst*I fragment showed a single band, indicative of a single copy of the *gdh* gene in the *T. maritima* genome (results not shown). The nucleotide sequence of the *T. maritima gdh* gene and its flanking regions are shown in Fig. 1. The gene consists of an open reading frame of 1248 nucleotides, encoding a polypeptide of 416 residues with a calculated molecular weight of 45852. All the amino acid

**Table 1.** Purification of glutamate dehydrogenase from *Thermotoga maritima*

| Step | Activity (U) | Protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| 1. Crude extract | 942 | 653 | 1.5 | 100 | 1 |
| 2. Heat precipitation | 519 | 61 | 8.5 | 55 | 6 |
| 3. Q Sepharose | 257 | 14 | 18 | 27 | 12 |
| 4. Mono Q, pH 6.5 | 217 | 4.4 | 49 | 23 | 33 |
| 5. Mono Q, pH 8.0 | 131 | 1.5 | 87 | 14 | 58 |

sequences encoded by open reading frames in the flanking regions of the *gdh* gene showed no significant homology with amino acid sequences in the SwissProt database. The *gdh* gene is preceded by AT-rich regions including a putative Shine–Dalgarno sequence, GAGG, at position –14 to –11, and putative promoter sequences, TTCAT and AAAAAT, at positions –85 and –59, that show homology with the consensus *T. maritima* –35 and –10 elements (Liao and Dennis 1992), respectively. Downstream from the gene, at position 1255, a palindromic structure was located, with the sequence *AAAGCCCCACC*GGGAA*GGTGGGGC-TTT*, that could act as a rho-independent terminator.

## Purification and characterization of *T. maritima* GDH produced in *E. coli*

Heterologous expression of the *gdh* gene was obtained by inserting it as a 2.1-kb *Stu*I–*Bsp*HI fragment in the expression vector pJF118ut, followed by introduction of the resulting plasmid pLUW492 into *E. coli*. Approximately 11 mg of GDH was present in cell-free extract derived from 6 g of recombinant *E. coli* cells. From this extract, GDH was purified 58-fold in five steps, resulting in approximately 1.5 mg pure protein, as illustrated by SDS-PAGE (Table 1, Fig. 2). The sequence of the first 12 $NH_2$-terminal residues of the purified enzyme was determined and corresponded to residues 2–13 of the peptide sequence derived from the gene, indicating that the initiator methionine is cleaved off in *E. coli*. The native enzyme had an apparent molecular weight of 265000, as estimated by gel filtration on a calibrated Hiload 16/60 Superdex 75 column, and a subunit molecular weight of 47000 as estimated by SDS-PAGE. These results are consistent with the calculated molecular weight (45852) derived from the amino acid sequence deduced from the gene and suggest that *T. maritima* GDH is a homohexameric enzyme.

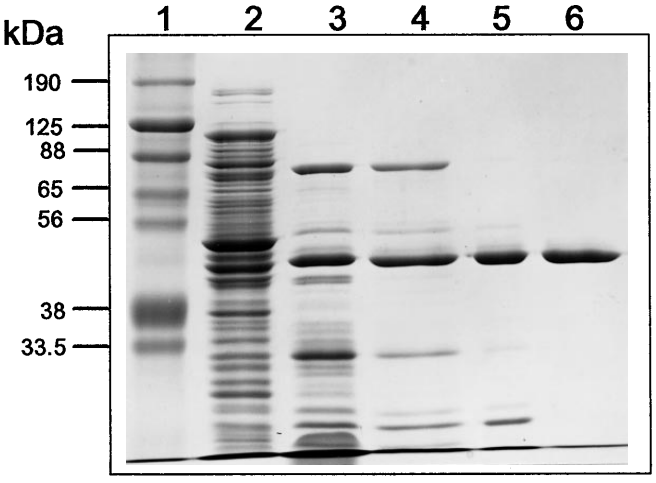


**Fig. 2.** Sodium dodecyl sulfate polyacrylamide gel electropharesis (SDS-PAGE) illustrating the purification of *T. maritima* GDH produced in *E. coli*. *Lane 1*, prestained molecular weight standard mixture; *lane 2*, cell-free extract of *E. coli* XL1-Blue/pLUW492 (28 µg); *lane 3*, supernatant after heat precipitation (21 µg); *lane 4*, pooled fractions after Q Sepharose column (9 µg); *lane 5*, pooled fractions after Mono Q column, pH 6.5 (8 µg); and *lane 6*, pooled fractions after Mono Q column, pH 8.0 (4 µg)

Immunological studies with antiserum raised against the *P. woesei* GDH (Eggen et al. 1993) were performed to compare the GDH in *E. coli* and *T. maritima*. The antiserum cross-reacted with the *T. maritima* GDH purified from *E. coli* and with a similar-sized 47-kDa protein in a *T. maritima* cell-free extract (results not shown).

Optimal activity of the *T. maritima* GDH was obtained at 75°C. The pH optima were 8.3 and 8.8 for the reductive amination and oxidative deamination, respectively. This result is in agreement with the shift to higher pH values for the deamination reaction of other GDHs (Smith et al. 1975).

The thermostability of GDH was determined by preincubation of GDH samples at 70°C, 80°C, and 90°C and subsequent measurements of the residual activity using the reductive amination assay (data not shown). The enzyme showed a half-life of approximately 16 h at 70°C and 10 h at 80°C. At 90°C the enzyme lost all its activity within 30 minutes.

The substrate specifity of the enzyme appeared to be very narrow and no significant NADP-dependent oxidation of D-glutamate, glutamine, aspartate, alanine, or leucine was observed. In contrast to the observations with the GDH from the archaeal isolate AN1 (Hudson et al. 1993), no NADH or NADPH oxidizing activity was detected with the *T. maritima* GDH after replacing $NH_4^+$ by L-glutamine.

Kinetic studies of the reductive amination were performed with the substrates NADPH, NADH, α-ketoglutarate, and $NH_4^+$. The $V_{max}$ obtained with NADH was 172 U/mg and that with NADPH was 12 U/mg purified GDH protein. The apparent $K_m$ values for the substrates $NH_4^+$ and α-ketoglutarate were 40 mM and 0.7 mM, respectively, while α-ketoglutarate inhibited enzyme activity at

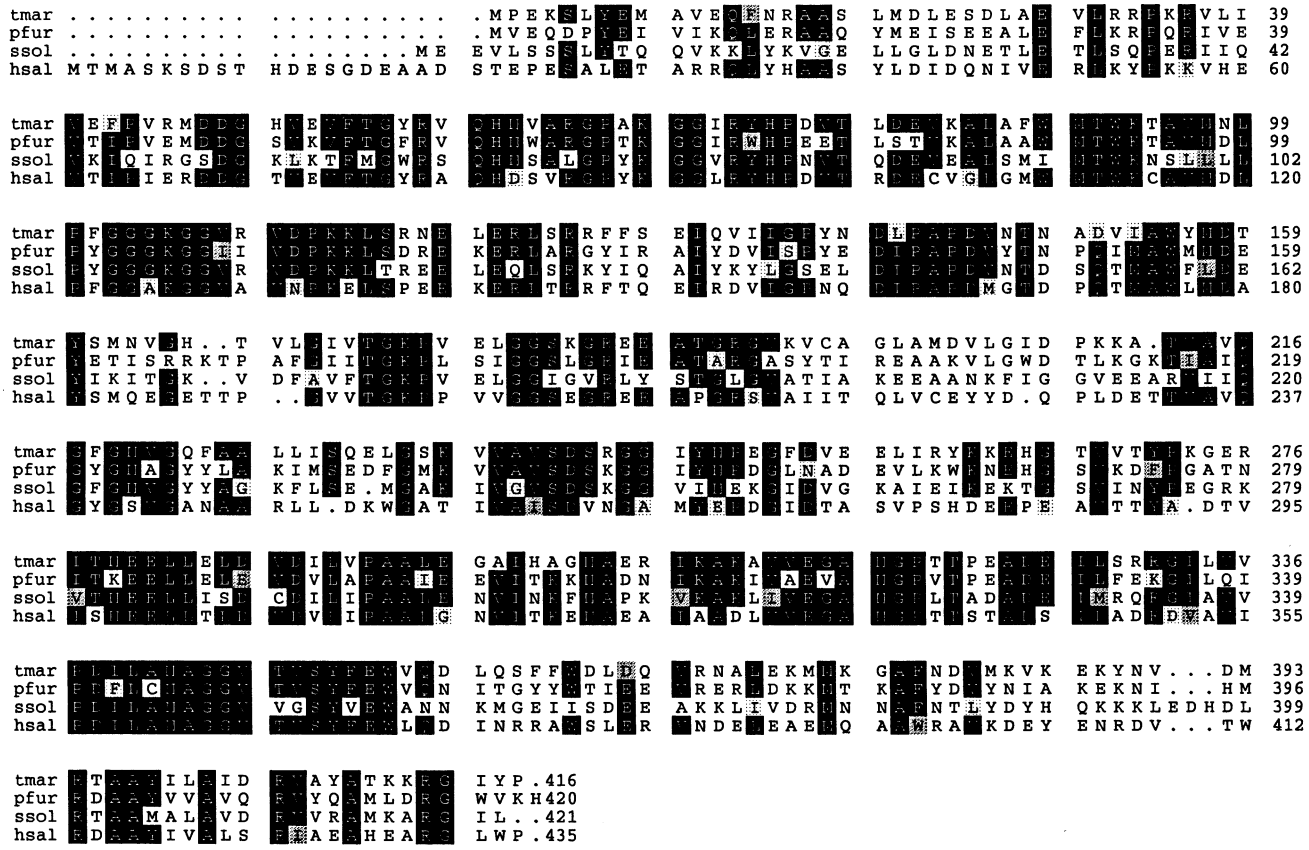


**Fig. 3.** Comparison of the deduced GDH amino acid sequences from *T. maritima* (*tmar*), *P. furiosus* (*pfur*), *S. solfataricus* (*ssol*), and *H. salinarium* (*hsal*). The multiple alignment was made with the programme PILEUP and illustrates identical (*reversed shading*) and similar (*shaded*) residues

concentrations higher than 5mM. The *T. maritima* GDH shows a dual cofactor specificity similar to GDHs from *Archaea* and higher *Eukarya*, since its affinity for NADPH did not differ greatly from that for NADH (apparent $K_m$ of 58μM and 22μM, respectively). However, it should be noted that a concentration of 200μM of cofactor did not give complete saturation. The NADP- and NAD-dependent glutamate oxidation activities were found to be the same (4U/mg protein).

## Multiple alignment and phylogenetic analysis

The deduced sequence from the *T. maritima* GDH was aligned with 26 other sequences of hexameric GDHs, including the newly reported sequences from the hyperthermophilic archaeon *Thermococcus litoralis* and the mesophilic bacterium *B. subtilis*. The GDH from *T. maritima* shows high similarity to GDHs from *P. furiosus*, *S. solfataricus*, and *H. salinarium* (Fig. 3). Hexameric GDHs can be divided into two families (I and II) which are probably paralogous (Benachenhou-Lahfa et al. 1993). The GDHs from *T. maritima*, *Thermococcus litoralis*, and *B. subtilis* are clearly new members of family II, which contains representatives of all three domains of life. Phylogenetic trees were further reconstructed using distance and maximum parsimony methods that yielded essentially the same results (Fig. 4). As expected, the GDH from *T. litoralis* was positioned on a common node with the closely related *P. furiosus*, and that from *B. subtilis* clustered with the GDHs from the two other gram-positive mesophilic bacteria. However, the further addition of the *T. maritima* GDH changed strongly the topology of the previously published trees (Benachenhou-Lahfa et al. 1993, 1994) in two ways. First, GDHs from the two prokaryotic domains, *Bacteria* and *Archaea*, are now mixed together, since *T. maritima* branches between the archaeon *H. salinarium* and the *Euryarchaea* of the order Thermococcales, represented by *T. litoralis* and *P. furiosus*, which have the most homologous GDHs (Britton et al. 1995). Second, mesophilic bacterial GDHs now share a common node with *Crenoarchaea* of the order Sulfolobales. Interestingly, the five sequences of GDHs isolated from hyperthermophilic species are located in the middle of the tree. All these unexpected nodes located in the middle of the tree were tested using both different methods of rearrangement (variation in the number and/or order of input sequences, branch swapping) and bootstrap analysis. In each case, we observed these nodes were not well supported. This is illustrated by the low bootstrap values (Fig. 4) ranging from 46.5% for the branching of the Sulfolobales to as low as 34.8% for the branching of *T. maritima*.

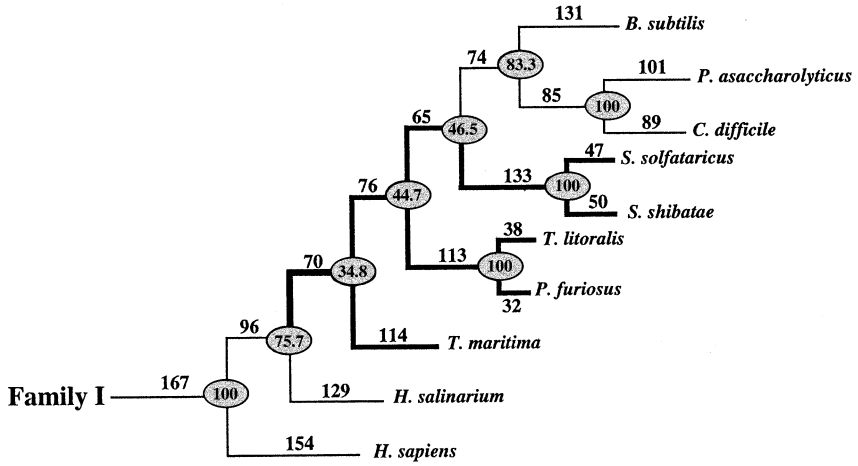


**Fig. 4.** Phylogenetic tree for the GDH sequences from family II based on amino acid sequences. The strengths of the different nodes in the tree based on the amino acid sequences were estimated using the bootstrap analysis, and are shown in *hatched ovals* in each node. The branch lengths are drawn at the scale and indicated by *boldface numbers*. The branchings corresponding to thermophilic species are drawn in *bold*. The organisms are as follows: *B. subtilis*, *Bacillus subtilis*; *P. asaccharolyticus*, *Peptostreptococcus asaccharolyticus*; *C. difficile*, *Clostridium difficile*; *S. solfataricus*, *Sulfolobus solfataricus*; *S. shibatae*, *Sulfolobus shibatae*; *T. litoralis*, *Thermococcus litoralis*; *P. furiosus*, *Pyrococcus furiosus*; *T. maritima*, *Thermotoga maritima*; *H. salinarium*, *Halobacterium salinarium*; *H. sapiens*, *Homo sapiens*

## Discussion

This paper describes the cloning, sequencing, and heterologous expression of the *T. maritima gdh* gene, and the subsequent purification and characterization of the GDH produced in *E. coli*. The *T. maritima* GDH is the first bacterial GDH characterized from a hyperthermophilic source with a low branch of descendance.

GDH is the major cytoplasmatic protein of the thermophilic Archaea *Sulfolobus solfataricus* (Consalvi et al. 1991a), *P. furiosus* (Consalvi et al. 1991b), and the isolates ES4 (Diruggiero et al. 1993) and AN1 (Hudson et al. 1993). For *P. furiosus* it was observed that GDH represented up to 20% of the total soluble protein (Consalvi et al. 1991b) and the enzyme was found to participate in an electron sink reaction leading to the formation of alanine from pyruvate (Kengen and Stams 1994). In a *T. maritima* cell-free extract, NADH- and NADPH-dependent GDH activities of 0.5 U/mg and 0.08 U/mg, respectively, were found (after correction for NAD(P)H oxidase activity) (R. Kort, unpublished observations). Based on the specific activity of the purified recombinant enzyme, it can be calculated that GDH constitutes about 0.3% of the total soluble protein of *T. maritima*, indicating that the enzyme is not as abundant as reported for *P. furiosus*. This is compatible with the finding that *T. maritima* does not produce significant amounts of alanine during its fermentation (R. Kort and W.M. de Vos, unpublished observations).

It has been reported that the *T. maritima* elongation factor (EF)-Tu folded incorrectly when its coding sequence was expressed in *E. coli* (Tiboni et al. 1989). In contrast, the *T. maritima* glyceraldehyde-3-phosphate dehydrogenase was produced in *E. coli*, purified to homogeneity, characterized, and shown to be identical with the enzyme isolated directly from *T. maritima* in all enzymatic and physiological properties investigated (Tomschy et al. 1993). The properties examined so far for the *T. maritima* GDH and the GDH produced in *E. coli* appear to be identical: (a) both proteins reacted with antibodies against the *P. woesei* GDH, (b) the size of the subunit estimated from the Western blot was the same in both hosts, (c) both enzymes were active at 80°C, and (d) both enzymes were able to use both NADH and NADPH as coenzymes, preferring the non-phosphorylated dinucleotide.

Kinetic studies of the reductive amination reaction catalyzed by the recombinant enzyme showed that the apparent $K_m$ values for NADH and NADPH did not differ greatly (22 μM and 58 μM, respectively). Dual cosubstrate-specific GDHs are rarely found in *Bacteria*, but are common in *Archaea* and vertebrate *Eukarya*. However, the *T. maritima* GDH differs from other NAD/NADP-dependent bacterial GDHs such as those from *Mycoplasma laidlawii* (Yarrison et al. 1972) and *Azospirillum brasilense* (Maulik and Gosh 1986), in that its affinity for the phosphorylated and nonphosphorylated coenzymes is significantly higher. In addition, the GDH of *A. brasilense* showed no NAD-dependent glutamate oxidation, in contrast to the *M. laidlawii* GDH, which showed all four GDH activities, i.e. reductive amination of α-ketoglutarate with NADH, and NADPH, as well as oxidative deamination of glutamate with NAD and NADP as the cosubstrates. The *T. maritima* GDH also showed all four activities, although specific activities for the glutamate oxidation were much lower: NADP- and NAD-dependent glutamate oxidation activities were both 4 U/mg, suggesting that the GDH in *T. maritima* has an anabolic function.

The GDH from *T. maritima* belongs to family II of hexameric GDHs. All prokaryotic family II GDHs previously detected were either from *Archaea* or gram-positive

bacteria. Recently, Gupta and Golding suggested a specific evolutionary relationship between *Archaea* and gram-positive bacteria, based on the phylogeny of the heat shock protein HSP70, as well as other proteins including GDHs (Gupta and Golding 1993). The finding that *T. maritima* GDH is also a member of family II indicates that this putative grouping of Archaea and gram-positive bacteria was a sampling artefact in the case of GDHs.

Phylogenetic analysis of GDHs from family II reveals a complex pattern of putative relationships, since the archaeal GDHs are polyphyletic, and *T. maritima* GDH branches in between euryarchaeal GDHs. We have previously noticed that the unusual position of the *H. salinarium* sequence in GDH trees strongly contradicted the classical phylogeny determined by 16S rRNA sequences (Benachenhou-Lahfa et al. 1993). The present result may question the suitability of GDH as a marker in establishing a phylogeny of organisms. This is illustrated by the low bootstrap values of several nodes inside prokaryotic lineages in the family II GDHs in the parsimony tree. The difficulty in using GDHs as reliable phylogenetic markers is probably due to the high degree of conservation between archaeal and bacterial GDHs from family II. Meyer et al. previously noticed that convergent and back mutations become as frequent as divergent mutations after some time in two diverging proteins (Meyer et al. 1986). Accordingly, if two homologous proteins from different domains are highly conserved, this saturation phenomenon is likely to have had enough time to eliminate all useful phylogenetic information in the limited variable regions of the sequence.

By comparing sequences of GDHs from mesophiles and hyperthermophiles in family II, we have previously identified a few putative amino acid signatures for thermophilic adaptation: a slight decrease in cysteine and asparagine content and a slight increase in isoleucine (Benachenhou et al. 1994). However, most of these signatures disappeared after the addition of the *T. maritima* and *B. subtilis* GDH sequences. Two positions were found with an amino acid common and specific to all thermophilic sequences; they correspond to Lys at position 114 and Gly at position 246 in the *T. maritima* sequence. The first change might be significant, since thermophilic proteins can be stabilized by additional ionic bonds at critical locations and ion-pair networks have been identified as the most important stabilizing factor in the *P. furiosus* GDH (Yip et al. 1995). Another common feature of the five sequences of GDHs from hyperthermophiles is a shorter $NH_2$-terminus compared to GDHs from family I and most GDHs from family II. Blake et al. noted that processing of the $NH_2$-terminal residue observed in *P. furiosus* rubredoxin may contribute significantly to protein hyperthermostability by preventing the protein from unzipping at high temperatures (Blake et al. 1991). The additional $NH_2$-terminal polypeptide segment observed in mesophilic GDHs could trigger the process of thermal denaturation if it is loosely connected to the core of the protein structure.

Another hypothesis explaining the rarity of detectable thermophilic signatures in GDHs from hyperthermophiles may be that family II GDHs, even those isolated from mesophilic organisms, are intrinsically more thermostable than GDHs from family I. Such intrinsic thermostability could have introduced a bias in the distribution of GDHs from the two paralogous families in the different lineages of the two domains, i.e., family II GDHs being selected over family I members in organisms adapted to thermophilic conditions.

Comparative analysis of three-dimensional structures from mesophilic and thermophilic family II GDHs is now required to identify clearly the basis for thermostability in this class of enzymes and to test these hypotheses. Recently, the *T. maritima* GDH purified from *E. coli* has been crystallized, opening the way to such analysis (S. Knapp, W.M. de Vos, D. Rice, and R. Ladenstein, submitted for publication).

**Acknowledgments** We are very grateful to André Dorochevsky, Ans Geerling, Wolfgang Huber, Wilfried Voorhorst, and Christoph Winterhalter for their skillful assistance. Part of this work was supported by contract BIOT-CT93-0274 of the European Union.

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