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A few amino acid substitutions are responsible for the higher thermostability of a novel NAD$^+$-dependent bacillar alcohol dehydrogenase

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The gene *adh-hT* encoding a thermostable and thermophilic NAD$^+$-dependent alcohol dehydrogenase (ADH) from the novel and more thermophilic *Bacillus stearothermophilus* LLD-R strain was cloned and its nucleotide sequence determined. The deduced protein sequence shows remarkable amino acid substitutions when compared to the sequence of the protein isolated from strain NCA1503 and significant similarity with the highly thermostable ADH from the thermoacidophilic archaebacterium *Sulfolobus solfataricus*. The alignment of these sequences led to the identification of three amino acid replacements probably responsible for the higher thermostability of the novel bacillar ADH.

Adh-hT gene expression in *Escherichia coli*, a fast purification procedure and the characterization of the recombinant enzyme are also described.

Alcohol dehydrogenases (ADH) catalyze the reversible oxidation of various alcohol substrates with reduction of a pyridine nucleotide [1], their coenzyme and/or substrate specificity depending on the specific ADH [2, 3]. These enzymes are widely distributed in several organisms such as bacteria, yeasts, plants and higher eukaryotes [1].

Some thermostable ADH have been purified from thermophiles [4–7]; we are engaged in the purification and characterisation of an enzyme (ADH-hT) bearing mainly an ethanol-dehydrogenase activity [8], from the novel and more thermophilic LLD-R strain (NCIMB 12403) of *Bacillus stearothermophilus* [9].

Over the last few years, studies have increasingly focused on proteins isolated from thermophilic, eubacterial and/or archaebacterial sources. These proteins are generally very stable to common denaturing agents [10], and can be easily distinguished and isolated when the coding genes are cloned and expressed in mesophilic host cells. However, the biological systems resistant to extreme conditions can also serve as useful models to investigate the properties responsible for the stabilization of biomolecules.

This study describes the construction of a gene library of the *B. stearothermophilus* LLD-R chromosome in *Escherichia coli*, the screening for the gene encoding the thermostable ADH using colony-hybridization techniques as well as the isolation of a genomic fragment carrying the complete coding and regulatory sequences of the *adh-hT* gene. Moreover, the transfer of this gene into a competent *E. coli* strain, which grows at high cell density, led to the selection of a clone which expresses the protein in reasonable amounts. The recombinant protein was also purified from the crude extracts of this *E. coli* clone by merely applying a strategy based on heat precipitation and one single step of affinity chromatography.

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Abbreviations. ADH, alcohol dehydrogenase; ORF, open reading frame.

Note. The novel *adh-hT* nucleotide sequence data reported here has been submitted to the EMBL sequence data bank and is available under accession number Z27089.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes and chemicals

Cells of *B. stearothermophilus* (LLD-R) were kindly supplied by Prof. Mario De Rosa (Istituto di Biochimica delle Macromolecole, University of Naples). *E. coli* BO3310 and Rb791 [11] were used as host cells for library propagation and expression, respectively.

Plasmid vector pGEM7Zf(+) and deoxynucleotides were obtained from Promega Biotec. pUC18 plasmid vector, restriction and modification enzymes were purchased from Boehringer Mannheim and Promega Biotec. Sequenase version 2.0 dideoxynucleotide sequencing kit was purchased from United States Biochemicals.

Radioactive materials were obtained from Amersham International.

Construction of LLD-R genomic bank

Isolation of chromosomal high-molecular-mass DNA from *B. stearothermophilus* LLD-R was performed as described by Barker [12]. DNA was partially digested with *Sau*3AI endonuclease (1-h incubation with 0.15 U enzyme/μg DNA), and fragments in the molecular-mass range of 2–4 kb were isolated using polyacrylamide gel electrophoresis and electroelution of the specific DNA from the excised portion of the gel. Partial filling in with *E. coli* DNA polymerase

(Klenow fragment), dGTP and dATP of the selected DNA fragments allowed their insertion into the plasmid vector pGEM7Zf(+), the ends of which had been previously made compatible by linearization with Xho I endonuclease and partial filling in with the Klenow fragment, dCTP and dTTP. Propagation and amplification of the gene library was obtained by transforming of *E. coli* BO3310 competent cells with the ligation mixture (hexammine cobalt chloride procedure) and growing the transformants in selection medium (Luria-Bertani medium containing 100 μg/ml ampicillin) for 4 h, according to Sambrook et al. [13].

Isolation and sequence of *adh-hT* clones

The LLD-R genomic bank in pGEM7Zf(+) was screened with a ^{32}P-end-labeled mixture of 35-residue oligonucleotides (5×10^6 cpm/ml; 3000Ci/mmol) deduced from the N-terminal sequence of ADH-hT. The specificity of the probe and stringency conditions were previously determined using Southern blot analysis of the genomic DNA digested with different restriction enzymes.

The oligonucleotide mixture used as hybridization probe was directly purchased from Beckman. Its sequence was as follows:

5′GGTTCTTTGAATTGTTC$^{G}_{T}$AC$^{G}_{T}$AC$^{G}_{T}$GC$^{G}_{T}$GCTTTCAT-3′.

The hybridizations were carried out in NaCl/Cit (0.75 M NaCl, 75 mM trisodium citrate, pH7.0) at 37°C and the final washing at 50°C, in the same buffer. Clones shown to be positive in the first selection were purified after a second colony-hybridization screening.

Replica filters were used in all experiments as a control of signal specificity. Sequencing was carried out following Sanger's dideoxynucleotide termination method with Sequenase version 2.0 sequencing kit (USB), on double-stranded DNA using the universal M13 forward and reverse primers and [^{35}S]dAdoP[S]PP (1000Ci/mmol). Sequencing of suitable restriction fragments from the positive clones subcloned into pUC18 plasmid vector was the method followed. All sequences were determined in duplicate and on both strands, and stored, aligned and analyzed using MicroGenie DNA analysis computer program obtained from Beckman.

Search and analysis of sequence similarity

A computer search was made to find ADH from different sources which have similar sequences to the ADH-hT, using data from the Genetic Sequence Data Bank (GenBank) and the National Medical Research Foundation (NBRF) provided by Beckman MicroGenie. Gene/protein sequences of thermostable ADH from *B. stearothermophilus* NCA 1503 strain [14] and *Sulfolobus solfataricus* [5] were also recorded and analyzed with the appropriate MicroGenie programs. Prediction of the secondary structure and comparison of the sequences was carried out using MicroGenie protein analysis and homology programs.

Gene-expression analysis

pRC1$_7$ plasmid, carrying the complete coding sequence of the *adh-hT* gene, was transferred into *E. coli* Rb791 by transformation as described above. Enzyme assays were performed on crude extracts of one plate-selected clone at different growth times, using a non-transformed clone as reference for endogenous ADH-activity levels at the same growth times.

Enzyme and protein assay

ADH activity was assayed spectrophotometrically at 60°C. The oxidation of ethanol was monitored by an increase in absorbance at 340 nm due to the reduction of NAD^+, using a Varian DMS-100 recording spectrophotometer. The 1-ml reaction mixture contained 20 mM ethanol, 2 mM NAD^+, 20 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 8.0 and the extract or the purified protein to be assayed. 1 U enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol NAD^+ in 1 min at 60°C under the described conditions. ADH-hT thermophilicity was determined by assaying enzyme activity under the mentioned standard conditions but gradually varying the temperature from 25°C to 70°C (5°C at a time).

ADH-hT thermostability was investigated at 50°C, 60°C, 65°C and 70°C. The purified enzyme (24 μg) was incubated at the specific temperature in 30 mM sodium phosphate, pH 7.0, 7 mM 2-mercaptoethanol in a final volume of 0.6 ml. 50-μl aliquots were assayed at different times under standard conditions. Protein concentration was determined as described by Bradford [15] using bovine serum albumin as the standard.

Recombinant-enzyme purification

The following procedure refers to a 1-l cell culture. Cells of *E. coli* Rb791, transformed with the pRC1$_7$ plasmid, were grown aerobically for 16 h with rotary shaking and at 37°C in Terrific Broth medium [13]. After harvesting by centrifugation (3000×*g*, 10 min at 4°C) cells were washed in 30 ml ice-cold lysis buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.0), and resuspended in 24 ml of the same buffer containing 0.7 mM phenylmethylsulphonyl fluoride. Lysis was performed by the addition 340 μl lysozyme (10 mg/ml) and incubating for 20 min at room temperature. 320 μl sodium deoxycholate (100 mg/ml) was added to the suspension, which was then incubated at room temperature for 30 min with 30 μg DNase I and centrifuged (5000×*g*, 15 min, 4°C).

Addition of protamine sulphate to a final concentration of 1 mg/ml at 4°C, and a 15-min centrifugation at 5000×*g* at 4°C allowed the removal of the nucleic acid component.

The cleared supernatant was heated at 65°C for 10 min and again centrifuged, as above. This step was repeated after a fivefold concentration by ultrafiltration through a YM10 membrane (cut-off 10 kDa) in an Amicon cell. The enzyme was purified by affinity chromatography on Matrex Gel Blue A (Amicon); the supernatant was mixed with the affinity gel (2.5 ml) in a shaking batch at 4°C overnight; the gel was then packed in a glass column (1-cm diameter), washed with 25 ml 50 mM sodium phosphate, pH 7.0, 0.6 M NaCl and with 25 ml of the elution buffer containing only 0.2 mM NAD^+. The protein was finally eluted with 10 ml 50 mM sodium phosphate, pH 7.0, 20 mM ethanol and 2 mM NAD^+. The purified enzyme was concentrated, dialyzed against 50 mM sodium phosphate, pH 6.0, 100 mM NaCl and 5 mM 2-mercaptoethanoland stored at −20°C in the same buffer containing 50% glycerol.

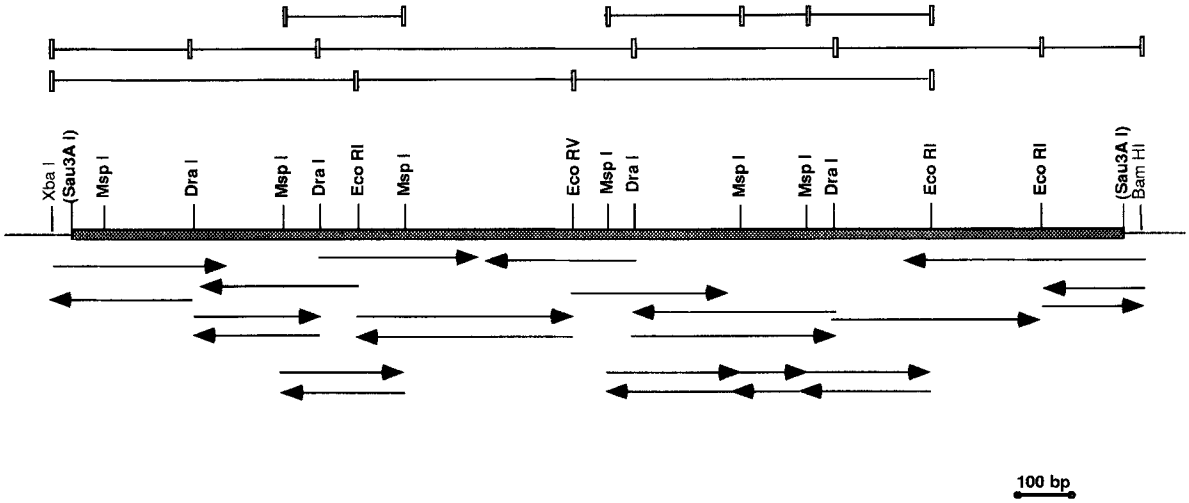

Fig. 1. Restriction endonuclease map of the genomic fragment in pRC1$_7$ plasmid carrying *adh-hT* gene. Restriction fragments subcloned for sequencing are indicated in the interrupted lines at the top. The arrows indicate the sequencing strategy used.

Electrophoretic analysis and native-molecular-mass determination

ADH enzymes were detected using isoelectric focusing or electrophoresis on a native 7% polyacrylamide slab gel stained for ADH activity. The gel was incubated at 60°C (the time depending on the amount of loaded protein) and stained with a solution containing the enzyme-assay components and 1 mg/ml of phenazine methosulphate and an equal amount of nitrobluetetrazolium. All purification steps were analyzed using SDS/PAGE as described by Laemli [16]; protein bands were stained either by Coomassie Brilliant Blue R250 (Bio-Rad) or silver stain (Sigma).

Purified recombinant ADH-hT was analyzed using gel-filtration chromatography. The protein was extensively dialysed against 50 mM sodium phosphate, pH 7.0, containing 0.2 M NaCl and loaded onto a Hi-Load Superdex 200 column (2.6 cm×60 cm, Pharmacia) connected to a FPLC system (Pharmacia) equilibrated in the same buffer.

Chromatography was performed at a flow rate of 2 ml/min with the same buffer after a calibration run under the same conditions, using apoferritin (44.3 kDa), yeast ADH (150 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa) as molecular-mass standards.

RESULTS

Cloning and sequencing

The strategy used to establish a representative gene library in pGEM7Zf(+) plasmid vector was highly efficient for the insertion of a single DNA fragment from *B. stearothermophilus* (LLD-R) genome into each vector molecule. The probe was designed from a reverse translation of the ADH-hT N-terminal sequence. Eight clones out of 5×10^4 were found to hybridize with the probe. Using restriction enzyme and Southern blot analysis, one clone, pRC1$_7$, was found to comprise a genomic region of 2.3 kb encompassing the entire coding sequence (Fig. 1).

The hybridization of the probe to bacterial chromosomal DNA produced results consistent with the restriction maps of the isolated clone (Fig. 1), thus confirming that the recombinant clone suffered no rearrangements during the cloning procedures.

A 1020-bp open reading frame (ORF) starting at ATG (position 359) and ending at TAA (position 1376) was found. The number of amino acid residues and the molecular mass of the polypeptide (36.32 kDa) predicted from this ORF (Fig. 2) agreed with that determined by amino acid analysis and with the apparent molecular mass of the wild-type protein subunit [8].

Similarity comparison analysis

The ADH-hT sequence was compared to those stored in Microgenie GenBank and NBRF under standard conditions of stringency, and a low degree of similarity with other mesophilic ADH was found. The results of this analysis essentially agreed with those reported for the ADH-T sequence (ADH enzyme from *B. stearothermophilus* strain, NCA 1503) by Sakoda and Imanaka [14].

Alignment of ADH-hT, ADH-T and SsADH (ADH from the thermophilic archaebacterium *S. solfataricus*)[5] was also performed (Fig. 3). A high degree of similarity (91%) was found between the enzymes from the *B. stearothermophilus* strains while a score of 36% was observed when the ADH-hT sequence was compared with that from *S. solfataricus*, both values referring only to identical residues.

Computer prediction of secondary structure performed on ADH-T, ADH-hT and horse liver ADH produced results in agreement with the known three-dimensional structure of the latter [17], the N-terminal regions showing the most significant differences. Residues 1−30 of horse liver ADH, organized in two β-strands interrupted by a non-structured linker region [17, 18] are substituted by amino acids (1−20) with high helix-forming potential in the bacillus ADH. Moreover, this helix-forming tendency is more pronounced and uninterrupted in the ADH-hT N-terminus (Fig. 4).

Gene expression and protein purification

The presence of putative, canonically eubacterial, Pribnow box and Shine-Dalgarno sequences in the 5′ flanking region of the *adh-hT* gene (Fig. 2) led to the transfer of the pRC1$_7$ plasmid into a high-cell-density-growing strain of *E. coli* in order to detect thermostable and thermophilic ADH activity in cell homogenates. Assays on crude extracts re-

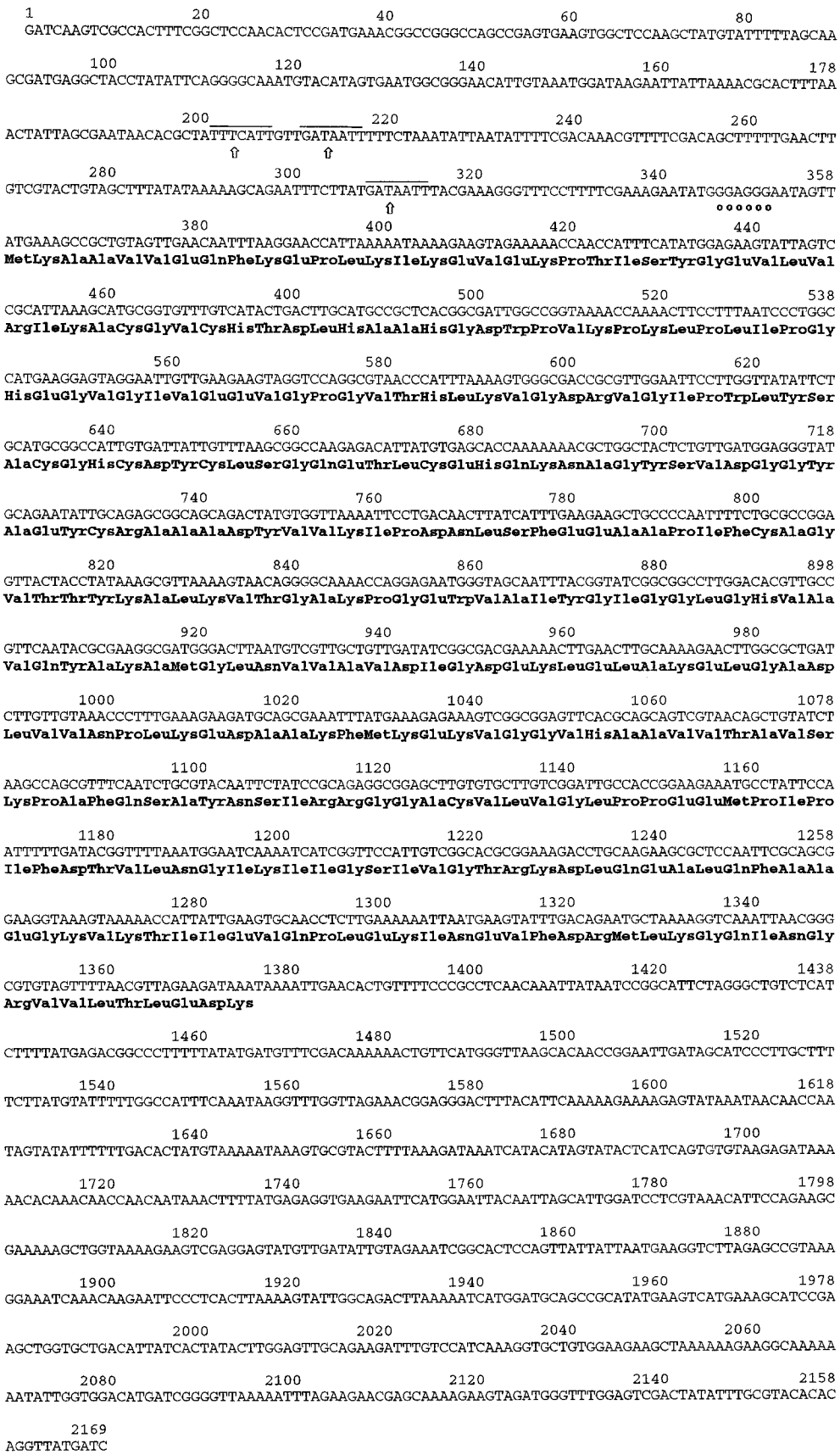

Fig. 2. Nucleotide sequence of the *adh-hT* gene. The open reading frame is indicated by the deduced protein sequence shown above the nucleotide sequence. The putative Shine-Dalgarno core sequence is underlined by circles and the possible locations for the Pribnow box consensus are indicated by arrows and overlined.

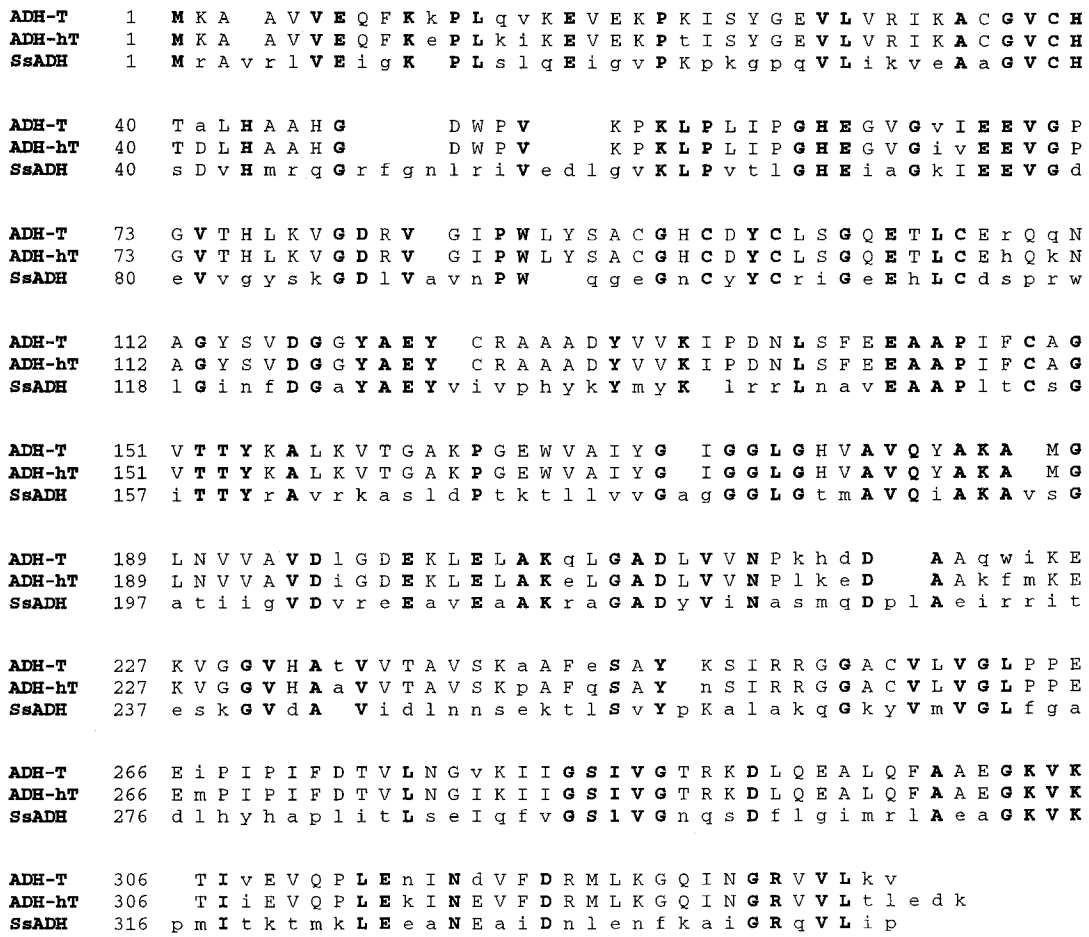

Fig. 3. Alignment of three thermostable ADH from thermophilic sources: *B. stearothermophilus* NCA1503 ADH-T; *B. stearothermophilus* LLD-R ADH-hT (this work); *S. solfataricus* SsADH. The sequences are listed from top to bottom following the increasing thermostabilities of the corresponding proteins. Relative amino acid positions are indicated for each sequence on the left and gaps are introduced to optimize amino acid matching. Capital letters indicate positions occupied by the same residue in two protein sequences. Bold capital letters are used to show maximum local similarities.

vealed a 20-fold higher ADH activity in Rb791 cells transformed with the $pRC1_7$ plasmid: this activity was completely unaltered after a 15-min incubation of the crude extract at 65 °C. Endogenous *E. coli* enzymes (assayed in protein extracts of non-transformed Rb791 cells) were fully inactivated by the same thermal treatment. Moreover, the presence of the recombinant protein in the cell extract of the $pRC1_7$ transformed clone was confirmed by isoelectric focusing and zymography, using the whole extract of an *E. coli* non-transformed clone as a negative control and the purified wild-type protein as a positive control. The thermophilic (recombinant and wild type) and mesophilic (*E. coli*) ADH are perfectly distinguishable on the basis of their different electrophoretic mobilities (Fig. 5 A and B) and pI values (data not shown).

Recombinant ADH-hT was purified to apparent homogeneity using a heating/affinity-chromatography procedure, as described in Materials and Methods. Fig. 6 shows the SDS/PAGE analysis of the active sample after different purification steps. After the first and second heat-precipitation steps of the host proteins, purification was about twofold and fivefold, respectively. Affinity chromatography on Matrex Gel Blue A represented the most effective purification step, yielding an increase of over 200-fold in the specific activity. The material recovered after affinity chromatography was homogeneous. Table 1 shows the results of a standard purification procedure.

The structural and functional properties of the purified protein allowed the definite identification of the recombinant protein as ADH-hT. For example, the enzyme was expressed with a specific activity very close to that of the wild-type protein, as calculated either for purified proteins (about 420 U/mg) or in crude extract of both *Bacillus* LLD-R and ADH-hT expressing *E. coli* cells (about 2 U/mg). The recombinant enzyme also showed electrophoretic mobilities, pI (4.9) and native molecular mass (homotetramer, 150 kDa) identical to those determined for the wild-type protein. Thermostability (half-life of 2 h at 70 °C) and thermophilicity (65 °C optimum temperature) were also virtually identical to the wild-type enzyme.

DISCUSSION

The novel strain of *B. stearothermophilus* LLD-R was chosen by the authors on the basis of its ability to grow, up to 75 °C (optimum 70 °C), on a wide range of carbohydrates and to produce high amounts of ethanol as a result of microbial fermentation. The higher thermoresistance and ethanol production, when compared to the type strain NCA 1503 (op-

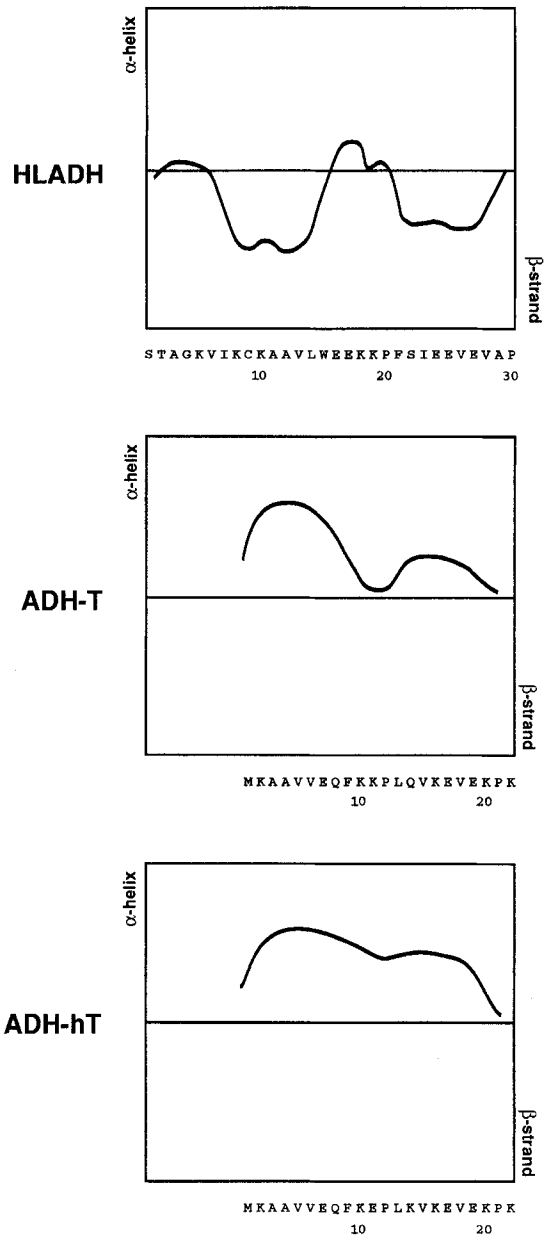

Fig. 4. Prediction of the secondary structure of the N-terminal regions of horse liver ADH E subunit (HLADH), ADH from *B. stearothermophilus* NCA1503 (ADH-T) and ADH from *B. stearothermophilus* LLD-R (ADH-hT, present work). The plot indicates the α-helix-forming (upper area in the diagrams) or β-strand-forming potential (lower area) of the amino acid stretches examined. Amino acid sequences are positioned to give the maximal similarity overlap, and numbered.

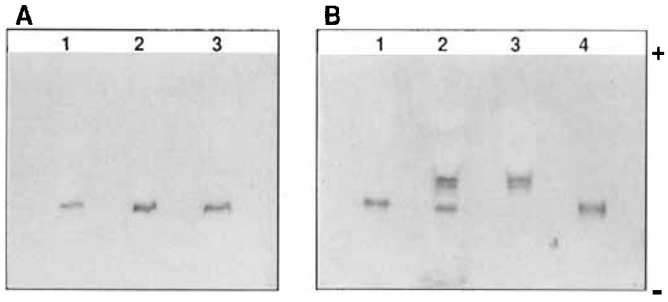

Fig. 5. Non-denaturing PAGE of ADH. The cell extracts of *E. coli* Rb791 transformed with the plasmid carrying the complete *adh-hT* gene were electrophoresized on a polyacrylamide slab gel at pH 8 and the bands detected using the tetrazolium-linked coupling system (ADH zymography). Total proteins from non-transformed *E. coli* cells and purified wild-type ADH from *B. stearothermophilus* LLD-R were used as negative and positive controls, respectively, for the identification of the recombinant ADH-hT. In some experiments, the cell extracts of recombinant *E. coli* were heated for 10 min at 65 °C, centrifuged and loaded. Lanes 1A and 1B, recombinant ADH-hT from prior heated *E. coli* extracts. Lane 2A, recombinant and wild-type ADH-hTs loaded into the same slot. Lanes 3A and 4B, wild-type ADH-hT. Lane 2B, extracts of non-transformed *E. coli* (endogenous ADH) and recombinant ADH-hT loaded in the same slot. Lane 3B, extracts of non-transformed *E. coli*.

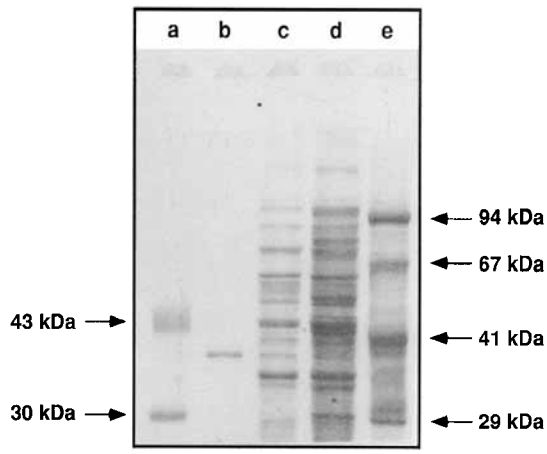

Fig. 6. SDS/PAGE analysis of recombinant ADH-hT after each step of purification. Lane a and e, molecular-mass protein standards. Lane b, sample after Matrex Blue A affinity chromatography. Lane c, extract after two thermal steps (heated for 10 min at 65 °C and centrifuged). Lane d, crude extract of recombinant *E. coli* Rb791.

timum 55 °C) and other thermophilic eubacteria, are interesting requisites for investigation of both biotechnological applicability and enzymology of its alcohol dehydrogenase. It was demonstrated that the *adh-hT* gene can be easily expressed and its regulatory region recognized in a heterologous and mesophilic expression system. The conserved thermophilicity and thermostability of the recombinant protein clearly indicate that the main physico-chemical parameters depend exclusively on the primary structure of the polypeptide chain and allow a fast and relatively easy purification strategy, with full recovery of the active enzyme. Computer analysis ascertained the structural homology and functional similarity of ADH-hT with the proteins of the ADH enzyme family. In fact, the suitable alignment with other mesophilic and thermophilic ADH, not restricted to the catalytic site and the structural zinc-binding domain [19], led to the identification and classification of ADH-hT as a medium-chain, NAD^+-dependent alcohol dehydrogenase [20].

It has been demonstrated [8] that ADH-hT shows similar thermophilicity but higher stability to temperature, organic solvents and denaturing agents than the protein isolated from the more characterized NCA 1503 strain (half-life at 70 °C of 2 h and 30 min, respectively); alignment of the two sequences showed that these differences might be due to amino acid substitutions [21], the most relevant of which seem to be Glu11→Lys, Lys14→Gln and Pro242→Ala. Computer prediction of the secondary structure and comparison with

Table 1. Purification of recombinant ADH from *B. stearothermophilus* LLD-R. Thermal steps consisted in the incubation of the protein extract from the *E. coli* clone expressing the *adh-hT* gene for 15 min at 65°C followed by centrifugation. After the first step a fivefold volume reduction was performed by ultrafiltration of the cleared supernatant from the first step.

Purification step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	U	U/mg	%	-fold
Crude extract	292	525	1.8	100	–
1st thermal step	146	533[a]	3.6	101	2.0
2nd thermal step	57	501	8.8	95	4.9
Matrex Gel Blue A	1.1	462	420	88	233

[a] Apparent value.

ADH from horse liver, for which crystal-structure standards are known [17], indicated that those two substitutions are located in regions not directly involved in the catalytic function. Lys11 is a highly conserved residue in the compared maize (ADH1, Lys20), horse liver (ADH E subunit, Lys19), human (ADH β1 subunit, Lys20) as well as other bacillar ADH [22]. Thus, the charge substitution due to a glutamic acid replacing a lysine and the introduction of a lysine in position 14 might contribute to the higher thermostability of ADH-hT through the generation of an additional surface salt bridge [21], which increases the helix-forming potential of the N-terminal region, as previously demonstrated both for some amino acid stretches of proteins [23] and for synthetic small peptides in solution [24]. Likewise, proline imposes rigidity to polypeptide chains particularly when it substitutes for small amino acid residues such as glycine and alanine in loops between elements of secondary structure [25, 26]. Therefore Pro242 can stabilize the native form of ADH-hT just by exploiting a change in the peptide backbone which contributes to an overall decrease in the entropy of the unfolded state. In fact, protein stability can be increased by substitutions that decrease the configurational entropy of unfolding [27]. The proline-stabilization theory makes even more sense when comparing SsADH to ADH-hT and ADH-T; this comparison reveals many of these Xaa-Pro substitutions especially in the N-terminal sequence of SsADH, the most thermostable among the NAD^+-dependent ADH investigated. Moreover, this ADH shows a relatively high degree of similarity (distributed along the entire polypeptides and not restricted to functional domains) to those from the *Bacillus* strains; this observation is surprising when results of similarity analysis of other functionally related proteins from Archaea and Eubacteria are considered. This suggests that thermostability is a selected phenotype common even to organisms that have recently been classified in very distant taxonomic domains [28].

The comparison of these and other available thermophilic gene/protein sequences could represent a good approach to investigate the structural properties responsible for stability to extreme conditions and, particularly, thermal stability. It also seems to be a more effective method than direct comparison with mesophilic counterparts because of the mentioned higher degree of similarity. In fact, these comparative studies can provide a more theoretical and targeted alternative to the current random-mutagenesis strategies aimed at generating new and specifically tailored proteins.

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