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Dihydrolipoamide Dehydrogenase from the Halophilic Archaeon *Haloferax volcanii*: Homologous Overexpression of the Cloned Gene

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The gene encoding dihydrolipoamide dehydrogenase from the halophilic archaeon, *Haloferax volcanii*, has been subcloned and overexpressed in the parent organism by using the halophilic archaeal rRNA promoter. The recombinant protein has been purified to homogeneity and characterized with respect to its kinetic, molecular, and salt-dependent properties. A dihydrolipoamide dehydrogenase-minus mutant of *H. volcanii* has been created by homologous recombination with the subcloned gene after insertion of the mevinolin resistance determinant into the protein-coding region. To explore the physiological function of the dihydrolipoamide dehydrogenase, the growth properties of the mutant halophile have been examined.

Dihydrolipoamide dehydrogenase catalyzes the oxidation of dihydrolipoamide: dihydrolipoamide + $NAD^+ \rightleftarrows$ lipoamide + NADH + H^+. In members of the domains *Bacteria* and *Eucarya*, the enzyme normally performs this function as an integral component of the pyruvate, 2-oxoglutarate, and branched-chain 2-oxoacid dehydrogenase multienzyme complexes (19, 20) or of the glycine cleavage system (16). In all of these systems, the lipoyl moiety is covalently bound to the enzyme complexes, where its reduction and reoxidation are part of the catalytic cycle.

Whereas the role in these complexes is the only established function of dihydrolipoamide dehydrogenase, the enzyme has now been found in a variety of *Bacteria*, *Eucarya*, and *Archaea* in the apparent absence of the multienzyme systems (10, 11, 23, 24, 26). Such observations are indicative of a new function for the dehydrogenase (8), although its precise nature is still to be established. Clearly the identification of a new role requires evidence from both molecular genetics and protein structure, and to this end we have chosen to study in detail the dihydrolipoamide dehydrogenase from the moderately halophilic archaeon, *Haloferax volcanii.*

The discovery of dihydrolipoamide dehydrogenase and its substrate lipoic acid in the halophilic *Archaea*, and of the apparent absence of the 2-oxoacid dehydrogenase complexes in these organisms, is reviewed in references 9 and 12. The gene encoding the enzyme has been cloned and sequenced from *H. volcanii* (29), and from sequence alignments it is clearly related to the complexed dihydrolipoamide dehydrogenases from *Bacteria* and *Eucarya*. In addition, shuttle vectors with antibiotic resistance markers for *H. volcanii* are now available (13–15) to enable the necessary molecular biological analysis of this halophilic gene and its enzyme product.

In this paper, we report the subcloning and expression of the *H. volcanii* gene, the purification and characterization of the recombinant dihydrolipoamide dehydrogenase, and the creation and growth analysis of a *H. volcanii* strain that lacks the functional enzyme.

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MATERIALS AND METHODS

Reagents and enzymes. Bovine serum albumin, DL-lipoamide, RNase A, low-melting-point agarose, novobiocin, and polyethylene glycol (PEG) 600 were purchased from Sigma. PEG 600 was purified by the method of Klebe et al. (17) as outlined by Cline et al. (7). Vent DNA polymerase was obtained from New England Biolabs. *Taq* DNA polymerase, T4 DNA ligase, and NAD^+ were from Boehringer Mannheim. Magic Minipreps were purchased from Promega. Geneclean II was from Stratech. Restriction endonucleases and T4 DNA polymerase were obtained from New England Biolabs or Gibco BRL. Hydroxylapatite was purchased from Bio-Rad. Mevinolin was a gift from Merck Sharp & Dohme Research Laboratories, Harlow, England.

DL-Dihydrolipoamide was prepared by the reduction of DL-lipoamide with $NaBH_4$ (22); the preparation was shown to be 98% pure, as judged by titration of the thiol groups with 5,5′-dithiobis-(2-nitrobenzoic acid).

Plasmid pNAT82, which contains the cloned *H. volcanii* gene for dihydrolipoamide dehydrogenase (29), was a kind gift from K. J. Stevenson (University of Calgary, Calgary, Alberta, Canada).

Strains and culture conditions. *H. volcanii* WFD11 and strains derived from it were grown at 37°C in 18% (wt/vol) salt-water modified growth medium, consisting of 14.4% NaCl, 1.8% $MgCl_2 \cdot 6H_2O$, 2.1% $MgSO_4 \cdot 7H_2O$, 0.42% KCl, 0.5% (wt/vol) peptone, and 0.1% yeast extract (pH 7.2). Minimal medium was used for growth experiments and consisted of the same salts as minimal growth medium with 0.5% (vol/vol) glycerol, 0.05% (wt/vol) succinate, 10 mM NH_4Cl, 1 mM K_2HPO_4 buffer, 20 mM Tris-HCl (pH 7.5), and 0.1% (vol/vol) trace elements solution. Trace elements solution consisted of 0.36 mg of $MnCl_2 \cdot 4H_2O$ per ml, 0.44 mg of $ZnSO_4 \cdot 7H_2O$ per ml, 3.3 mg of $FeSO_4 \cdot 7H_2O$ per ml, and 0.05% $CuSO_4 \cdot 5H_2O$.

Plasmid purification. Plasmids isolated from *H. volcanii* were prepared by using Magic Minipreps or the alkaline lysis method (25). The only modification of the procedures required was the addition of 1 M NaCl to the initial resuspension buffer, to prevent the cells lysing at this stage. Medium-scale plasmid stocks were prepared by a method based on that described by Ausubel et al. (1). One hundred milliliters of cells from an overnight culture was harvested by centrifugation at 1,300 × *g* in a benchtop centrifuge for 10 min. The cells were resuspended in 1 M NaCl–50 mM glucose–25 mM Tris-HCl (pH 8.0)–10 mM EDTA and lysed by the addition of 8 ml of 0.2 M NaOH–1% sodium dodecyl sulfate (SDS). After the mixture was left on ice for 10 min, 6 ml of 3 M potassium acetate (pH 4.8) was added quickly, and the mixture was left on ice for 15 to 30 min, after which the precipitate was removed by centrifugation. Nucleic acids were precipitated from the supernatant by addition of 18 ml of isopropanol and incubation on ice for 30 min. The precipitate was removed by centrifugation and resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. One milliliter of 6 M LiCl was added, and the mixture was left on ice for 15 min. The precipitate was removed by centrifugation, and DNA was isolated from the supernatant by ethanol precipitation. The DNA was resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and incubated with 0.1 mg of RNase A per ml at 37°C for 30 min. RNase was inactivated by adding 0.5% SDS and heating to 75°C for 10 min. An equal volume of 6 M LiCl was added, and the mixture was left for 15 min at room temperature. The precipitate was removed, and the DNA was extracted from the supernatant by ethanol precipitation followed by extraction with phenol and chloroform.

PCR amplification of the dihydrolipoamide dehydrogenase gene. Two primers were designed to bind to the respective ends of the dihydrolipoamide dehydro-

genase gene and introduce suitable restriction sites for cloning into expression vectors (their positions relative to the published sequence [29] are indicated in parentheses after each primer name): KAJ-1 (nucleotides [nt] 11 to 39, 5′ agggcgtcggatcccgaaccgcgtgaagg 3′ [29-mer]) and KAJ-2 (nt 1574 to 1544, 5′ tctcttaagcttggaactcgatgccgacctg 3′ [31-mer]).

Reaction mixtures contained approximately 100 ng of target DNA (pNAT82), 0.1 μM each primer, and 50 μM each deoxynucleoside triphosphate. For reactions with Vent DNA polymerase, the reaction mixture also contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100. For reactions with *Taq* DNA polymerase, the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. Reaction mixtures were incubated at 96°C for 5 min before addition of 1 to 2.5 U of Vent DNA polymerase or *Taq* DNA polymerase, and 30 cycles were performed of the following program: 96°C for 75 s, 60°C for 60 s, and 72°C for 90 s.

Construction of plasmids. Plasmid DNA was digested with restriction endonucleases, and fragments were isolated by electrophoresis. DNA was recovered from low-melting-point agarose by using Geneclean II (BresaTech). Specific fragments were ligated together by using T4 DNA ligase.

Preparation of *H. volcanii* genomic DNA. Two hundred microliters of stationary-phase culture was harvested and resuspended immediately in the same volume of distilled water to lyse the cells. An equal volume of buffer-saturated phenol (pH 8.0) was added, the mixture was incubated at 65°C for 10 min, and the aqueous layer was removed. The DNA was ethanol precipitated and then washed and resuspended in 200 μl of distilled water. DNA was further purified by using Geneclean II.

Transformation of *H. volcanii*. Transformations were performed by the PEG method described by Charlebois et al. (6) and Cline et al. (7). Freshly inoculated cultures of *H. volcanii* were grown until late log phase (A_{500} of 0.8 to 1.0) and then harvested by centrifugation at 3,300 × *g* for 15 min at room temperature. The cells were then washed in a 1/10 volume of buffered spheroplasting solution (1 M NaCl, 27 mM KCl, 50 mM Tris-HCl [pH 8.2], 15% [wt/vol] sucrose) and resuspended in buffered spheroplasting solution containing 15% (wt/vol) glycerol. Spheroplasts were formed by addition of 45 mM EDTA (pH 8.0) for 10 min at room temperature; they were then incubated with DNA for 5 min, after which an equal volume of 60% PEG 600 was added. The cells were incubated for 20 to 30 min at room temperature, after which they were allowed to recover in 18% salt-water modified growth medium for a few hours. Selection of transformants was achieved by plating the cells onto 18% salt-water minimal growth medium agar containing 0.3 μg of novobiocin or 4 μg of mevinolin per ml and incubating them at 37°C for 5-10 days.

Preparation of cell extracts. *H. volcanii* cells were harvested by centrifugation at 10,800 × *g* for 25 min at 4°C. The cells were then resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–2 M KCl and lysed by sonication. Cell debris was removed by centrifugation at 15,600 × *g* for 30 min at 4°C. Protein was estimated by the method of Bradford (3), using bovine serum albumin as a standard.

Assay of dihydrolipoamide dehydrogenase. Enzyme activity was assayed at 37°C in 10 mM Tris-HCl (pH 8.0) buffer containing 2 M KCl, 1 mM EDTA, 1 mM NAD^+, and 0.4 mM dihydrolipoamide. The reaction, in a final volume of 1 ml, was started with enzyme, and its progress was monitored by the increase in A_{340}.

Purification of recombinant dihydrolipoamide dehydrogenase. Cell extracts of *H. volcanii* transformed with pMDS24 were heated in a PEG bath at 95°C for 6 min. Precipitated protein was removed by centrifugation and loaded onto a hydroxylapatite column (2.2 by 20 cm) equilibrated with 50 mM potassium phosphate (pH 7.0) containing 1 M NaCl. The column was washed with equilibration buffer and eluted with a 50 to 500 mM potassium phosphate gradient containing 1 M NaCl. Fractions containing dihydrolipoamide dehydrogenase activity were pooled, concentrated in Centricon-30 tubes, and further purified by molecular exclusion chromatography on a Pharmacia FPLC Superdex-200 column equilibrated with 50 mM potassium phosphate–2 M KCl (pH 7.0). Fractions with dihydrolipoamide dehydrogenase activity were pooled.

RESULTS

Subcloning and homologous expression of the *H. volcanii* dihydrolipoamide dehydrogenase gene. The *H. volcanii* dihydrolipoamide dehydrogenase gene was PCR amplified from pNAT82 by using oligonucleotides KAJ-1 and KAJ-2, which introduced a *Hin*dIII restriction site at the 3′ end of the gene, and a *Bam*HI site at the 5′ end prior to the putative box A promoter region. These oligonucleotides produced a major PCR product of about 1,500 bp, which was subsequently cloned into the shuttle vector pMDS20 (15), to create pMDS21, and transformed into *H. volcanii* WFD11 cells. No expression of dihydrolipoamide dehydrogenase was detected in transformed cells, either in assays of the cell extract or upon SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the soluble and insoluble fractions.

It may be that the putative box A is not a functional promoter, and therefore a strong promoter from the rRNA operon of *Halobacterium cutirubrum* (4) was added to the 5′ end of the gene (Fig. 1). The P2 promoter of the rRNA operon was synthesized as follows. Two overlapping oligonucleotides (WGW-1 (5′ atcgatgcccttaagtacaacagggtacttcgg 3′ [33-mer]) and WGW-2 (5′ gcggatccgttcgcattccaccgaagtaccctgtt 3′ [35-mer]) were designed to bind to the consensus region of the promoter as well as to all of the region downstream as far as two bases past the known transcription starting point. The forward oligonucleotide (WGW-1) has half of an *Eco*RV site, and the reverse (WGW-2) contains a *Bam*HI site. The oligonucleotides were annealed and extended with T4 DNA polymerase, and the product was run on a 10% polyacrylamide gel. The band was excised and purified, cut with *Bam*HI, and initially cloned into the *Eco*RV and *Bam*HI sites of pUK21 (30). This construct was designated pMDS65. The promoter was cut out of pMDS65 with *Eco*RI and *Bam*HI and annealed to the *Bam*HI end of the dihydrolipoamide dehydrogenase PCR product. This fragment was ligated into pWL102 (18) at the *Kpn*I and *Xba*I sites, to create pMDS24. *H. volcanii* WFD11 cells transformed with pMDS24 had a level of dihydrolipoamide dehydrogenase activity 15-fold higher than that of wild-type cells (0.45 and 0.03 U/mg of protein, respectively).

Purification of the recombinant dihydrolipoamide dehydrogenase. The recombinant dihydrolipoamide dehydrogenase was purified to electrophoretic homogeneity from pMDS24-transformed *H. volcanii* WFD11 as described in Materials and Methods (Table 1). The properties of the purified recombinant enzyme were found to be as follows: specific activity = 24 U/mg of protein (37°C); K_m (NAD) = 80 μM; K_m (dihydrolipoamide) = 18 μM; subunit relative molecular weight = 66,500 (determined by SDS-PAGE); and maximal enzymatic activity at 2 to 3 M KCl and pH 9. The spectrum of the protein shows the presence of a flavin cofactor. These characteristics are comparable with those of dihydrolipoamide dehydrogenase purified from cells of *H. volcanii* (28); specific activity = 11 to 16 U/mg of protein (25°C); K_m (NAD) = 80 μM; and subunit relative molecular weight = 64,500 (SDS-PAGE).

Creation of a dihydrolipoamide dehydrogenase-minus strain of *H. volcanii*. The dihydrolipoamide dehydrogenase gene of *H. volcanii* was inactivated by insertion of a mevinolin resistance gene into the *Xho*I site of the coding region (Fig. 2). The correct construction was confirmed by examining the size of the *Xho*I region of the gene by PCR. PCR primers KAJ-8 (nt 381 to 399), 5′ tcgagaagctctgtaaggc 3′ [19-mer]) and KAJ-9 (nt 786 to 768), 5′ atgtcgatgccgagctctt 3′ [19-mer]) were designed so as to bind to the dihydrolipoamide dehydrogenase gene approximately 200 bp either side of the insertion point.

Results of PCRs performed with chromosomal DNA from wild-type and mutant strain BAS-5005 were compared. The reaction mixture was the same as for the amplification of the dihydrolipoamide dehydrogenase gene, and the following program was used for 30 cycles: 96°C for 75 s, 37°C for 90 s, and 72°C for 5 min. The reaction performed with wild-type chromosomal DNA as the template yielded a product of 400 bp, while that containing chromosomal DNA from strain BAS-5005 produced a product of approximately 2.5 kbp. Both products are consistent with the insertion of the mevinolin resistance marker, as expected.

Enzyme assays for dihydrolipoamide dehydrogenase confirmed the successful construction of cells lacking the enzyme; that is, catalytic activity was undetectable in extracts of the mutant strain, the limit of detectability being <0.01% of the

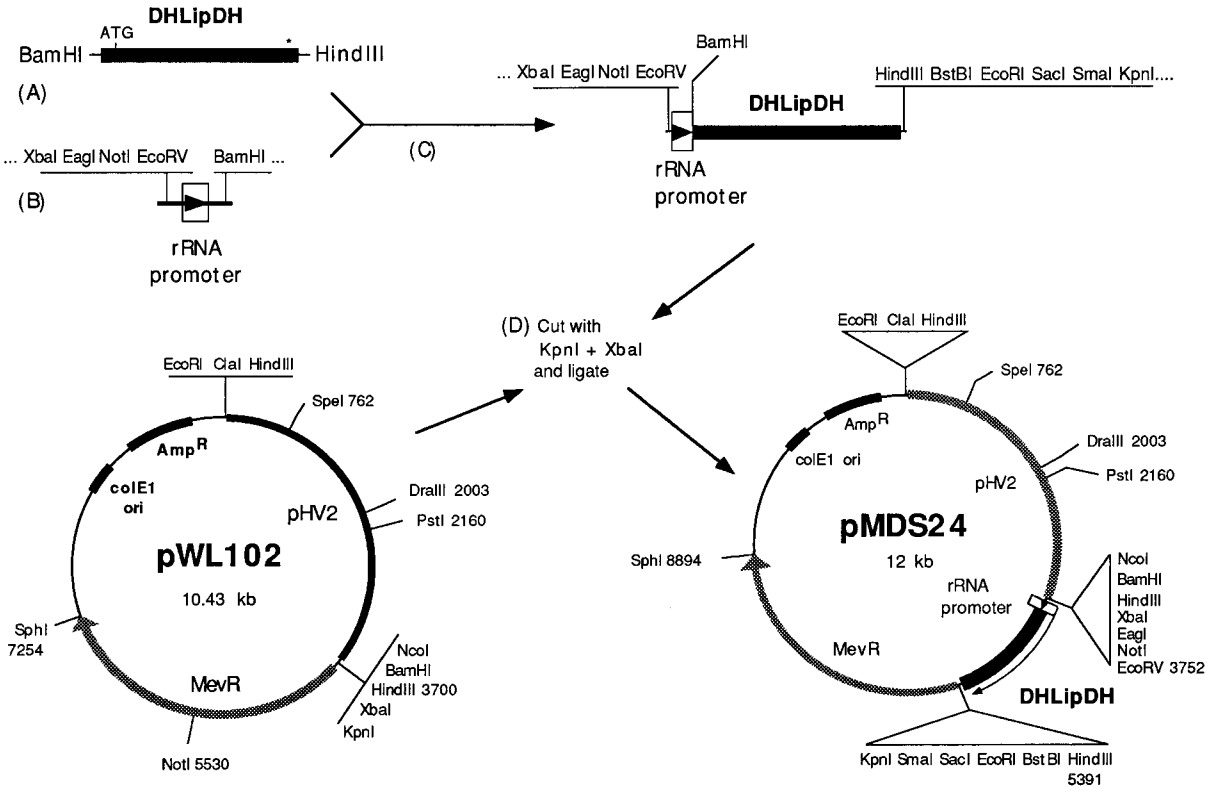

FIG. 1. Construction of a plasmid vector for the expression of dihydrolipoamide dehydrogenase in *H. volcanii*. (A) The *H. volcanii* dihydrolipoamide dehydrogenase (DHLipDH) gene was PCR amplified by using primers with terminal *Bam*HI and *Hin*dIII sites (see text) and cloned into plasmid pMDS20. (B) The P2 promoter of the *H. cutirubrum* rRNA operon was synthesized by using DNA oligonucleotides with terminal *Eco*RV and *Bam*HI sites (see text) and cloned into plasmid pUK21. (C) The cloned rRNA promoter was fused to the 5′ end of the dihydrolipoamide dehydrogenase gene by using their common *Bam*HI sites. (D) The rRNA promoter-dihydrolipoamide dehydrogenase gene fusion was cut with *Xba*I and *Kpn*I and ligated to the shuttle plasmid pWL102 (also cut with *Xba*I and *Kpn*I) to form plasmid pMDS24.

activity assayable in the wild-type organism. (As a control, the activities of the central metabolic enzyme, citrate synthase, were measured and found to be 0.14 and 0.11 U/mg of protein in the wild-type and mutant cells, respectively.)

To test whether a plasmid-borne copy of the dihydrolipoamide dehydrogenase gene would transform the mutant strain to dihydrolipoamide dehydrogenase-plus, the mevinolin resistance marker of pMDS24 was replaced with the novobiocin resistance gene, and the resulting plasmid was transformed into the dihydrolipoamide dehydrogenase-minus (MevR) strain of *H. volcanii*. Enzyme activity was found to be 30-fold higher than that of the wild type. Similar experiments with pMDS21 (containing the enzyme-coding region but without the rRNA promoter) produced transformants showing no enzyme activity, confirming that the dihydrolipoamide dehydrogenase gene is not expressed from the putative box A promoter.

TABLE 1. Purification of *H. volcanii* dihydrolipoamide dehydrogenase[a]

Step	Total activity (U)	Protein (mg/ml)	Sp act (U/mg)	Recovery (%)
Cell extract	2.4	6.1	0.4	
Heat step	1.9	2.0	1.0	79
Hydroxylapatite	1.2	0.34	3.5	50
Gel filtration	1.2	0.05	24.0	50

[a] The recombinant dihydrolipoamide dehydrogenase was purified from pMDS24-transformed *H. volcanii* WFD11 as described in Materials and Methods.

Growth studies on the dihydrolipoamide dehydrogenase-minus strain of *H. volcanii*. To probe the function of dihydrolipoamide dehydrogenase in halophilic *Archaea*, growth experiments were conducted on the dihydrolipoamide dehydrogenase-minus strain BAS-5005 and on the parent cells. Cells were grown on minimal medium agar, containing a range of sugars and amino acids, at concentrations of between 0.2 and 0.5% (wt/vol), as sole carbon sources. The results are summarized in Table 2. Surprisingly, no difference in growth characteristics could be detected between the mutant and wild-type *H. volcanii*. Growth in minimal growth medium of cells picked from the agar plates with pyruvate as the sole carbon source, followed by enzyme assays of cell extracts, demonstrated that the mutant strain had not reverted to the parental phenotype during the growth studies; that is, it contained no detectable levels of dihydrolipoamide dehydrogenase catalytic activity.

DISCUSSION

Using *H. volcanii* as a host system, we have succeeded in expressing the *H. volcanii* gene encoding the enzyme dihydrolipoamide dehydrogenase, to produce fully active recombinant protein. Using a variety of vector systems, we have attempted to express the active enzyme in *Escherichia coli* but without success, although this has been achieved for the enzymes malate dehydrogenase from *Haloarcula marismortui* (5) and

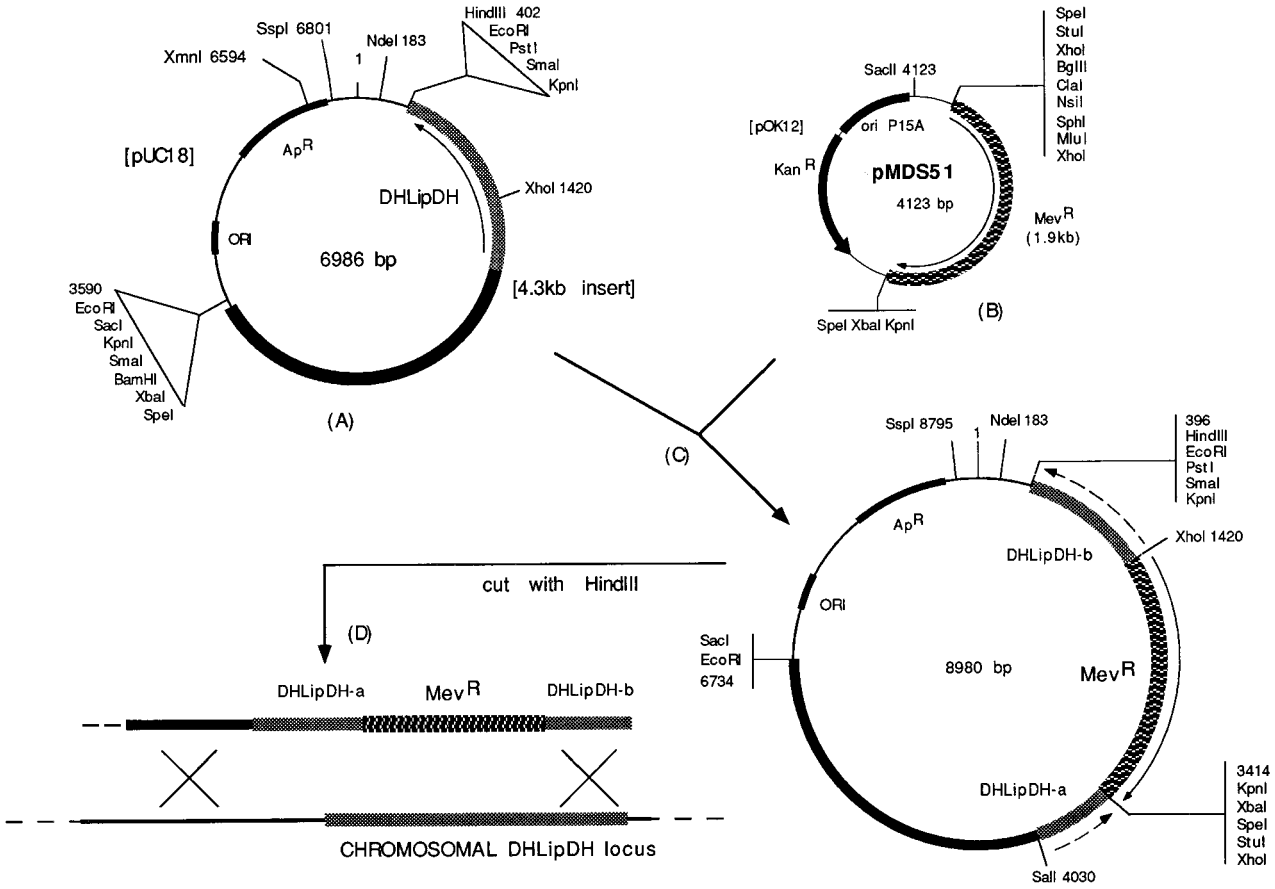

FIG. 2. Insertional inactivation of the dihydrolipoamide dehydrogenase gene of *H. volcanii*. To create a strain with an inactive dihydrolipoamide dehydrogenase (DHLipDH) gene, we constructed a plasmid which carried a mevinolin resistance (MevR) marker inserted into the *Xho*I site of the dihydrolipoamide dehydrogenase gene. This was introduced into *H. volcanii* cells and allowed to recombine at the dihydrolipoamide dehydrogenase locus. The plasmid was constructed as follows. (A) The 4.3-kb insert from plasmid pNAT82 (which includes the dihydrolipoamide dehydrogenase gene at one end) was excised with *Hin*dIII and *Xba*I and ligated to *Hin*dIII-*Xba*I-cut pUC18. (B) Plasmid pMDS51, containing a mevinolin resistance marker, was cut with *Xho*I and ligated to the *Xho*I-cut pUC18 clone depicted in panel A, thus inserting pMDS51 into the open reading frame of the dihydrolipoamide dehydrogenase gene. (C) The *E. coli* plasmid component of the inserted pMDS51 was removed by *Spe*I digestion, thus leaving only the mevinolin resistance marker. (D) The resulting plasmid was linearized at the *Hin*dIII site and introduced into *H. volcanii* cells, and mevinolin-resistant transformants were selected. Double recombination events between the introduced and chromosomal dihydrolipoamide dehydrogenase gene sequences (E) were expected to give rise to transformants with insertions at the *Xho*I site of the dihydrolipoamide dehydrogenase gene.

dihydrofolate reductase from *H. volcanii* (2). However, in both of these cases, the enzymes were not produced in an active form in *E. coli*, and refolding and/or salt activation treatments were necessary. Therefore, we envisage the expression of a halophilic enzyme in *H. volcanii* to be a potentially useful procedure for other enzymes, and our approach provides a vector expression system using the halophilic rRNA promoter that should be applicable to any cloned halophilic gene. In addition, expression in the halophile ensures that correct post-translational modifications are carried out, should they occur in the normal situation. Finally, the level of overexpression achieved for the dihydrolipoamide dehydrogenase will provide sufficient enzyme for future structural studies that will explore both the nature of protein halophilicity and the relationship of the enzyme to those dihydrolipoamide dehydrogenases that function in multienzyme complexes in *Bacteria* and *Eucarya*.

In addition, we have created a mutant strain of *H. volcanii* in which the gene for the dihydrolipoamide dehydrogenase has been interrupted so that the active enzyme is not produced. This has permitted us to explore the in vivo function of the enzyme in the halophilic archaeon by searching for nutrients that can no longer be metabolized. Unfortunately, we have not yet found a difference between the wild-type and mutant strains (Table 2).

In *Bacteria* and *Eucarya*, dihydrolipoamide dehydrogenase is an integral component of the pyruvate, 2-oxoglutarate, and branched chain 2-oxoacid dehydrogenase complexes; these enzyme activities have never been detected in the *Archaea*, and alternative enzyme systems for the first two are present in the form of pyruvate and 2-oxoglutarate ferredoxin oxidoreductases (see reference 21 and references therein). Therefore, it is not surprising that the mutant *H. volcanii* grew as well as the wild-type organism on nutrients metabolized through these enzymes, e.g., pyruvate, citrate, and glutamate. Growth of neither wild type nor mutant was supported by branched-chain amino acids.

In *E. coli*, dihydrolipoamide dehydrogenase has been implicated in the binding-protein-dependent transport of galactose and maltose (23, 24). However, the mutant *H. volcanii* showed no growth differences on glucose, galactose, fructose, and sucrose, indicating that the dihydrolipoamide dehydrogenase does not function in their transport systems. Finally, the weak growth of *H. volcanii* on glycine was not impaired by the ab-

TABLE 2. Growth properties of wild-type and dihydrolipoamide dehydrogenase-minus strains of *H. volcanii*

Carbon source[a]	Growth[b]	
	Wild type[c]	BAS-5005[d]
Acetate	+	+
Citrate	+	+
Fructose	+++	+++
Galactose	w	w
Glucose	++	++
Glutamate	++	++
Glycerol	+++	+++
Glycine	w	w
Isoleucine	−	−
Leucine	−	−
Pyruvate	+++	+++
Sorbitol	−	−
Succinate	++	++
Sucrose	++	++
Valine	−	−

[a] Cells were grown on minimal medium agar containing a range of compounds as sole carbon sources at concentrations of 0.2 to 0.5% (wt/vol).
[b] −, no growth; w, weak growth; +, ++, or +++, positive growth.
[c] *H. volcanii* WFD11.
[d] The mutant strain of *H. volcanii* that lacks dihydrolipoamide dehydrogenase activity.

sence of the enzyme, arguing against a role in a glycine cleavage enzyme complex.

Clearly, there are many more potential nutrients to test, and our search is continuing. However, the possibility remains that another enzyme takes over the normal function of the dihydrolipoamide dehydrogenase when it is absent, although no such precedent is known. Alternatively, dihydrolipoic acid is known to possess antioxidant properties (27), and a role for this cofactor in the repair of membrane thiol groups in mitochondria has been suggested (31). Therefore, the role of lipoic acid and dihydrolipoamide dehydrogenase in the halophilic *Archaea* may be in protection and repair, and this function may not be evident until the cells are stressed in some oxidative manner.

While the dihydrolipoamide dehydrogenase-minus mutant has not yet enabled us to identify the normal in vivo function of the enzyme, it has provided a host into which we can now introduce site-directed mutants of the protein. This is essential for our protein structure-function studies of the halophilic enzyme. In addition, the methodology that we have developed to create this mutant is directly applicable to other halophilic enzymes for which the appropriate gene has been cloned and sequenced.

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