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ORIGINAL PAPER

The 2-oxoacid dehydrogenase multienzyme complex of *Haloferax volcanii*

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Abstract Those aerobic archaea whose genomes have been sequenced possess four adjacent genes that, by sequence comparisons with bacteria and eukarya, appear to encode the component enzymes of a 2-oxoacid dehydrogenase multienzyme complex. However, no catalytic activity of any such complex has ever been detected in the archaea. In *Thermoplasma acidophilum*, evidence has been presented that the heterologously expressed recombinant enzyme possesses activity with the branched chain 2-oxoacids and, to a lesser extent, with pyruvate. In the current paper, we demonstrate that in *Haloferax volcanii* the four genes are transcribed as an operon in vivo. However, no functional complex or individual enzyme, except for the dihydrolipoamide dehydrogenase component, could be detected in this halophile grown on a variety of carbon sources. Dihydrolipoamide dehydrogenase is present at low catalytic activities, the level of which is increased three to fourfold when *Haloferax volcanii* is grown on the branched-chain amino acids valine, leucine and isoleucine.

Keywords *Haloferax* · Multienzyme complex · 2-Oxoacid dehydrogenase · RT-PCR · Archaea

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Abbreviations

BCOADHC	Branched-chain 2-oxoacid dehydrogenase complex
DCPIP	2,6-Dichlorophenolindophenol
E1	2-Oxoacid decarboxylase
E2	Dihydrolipoyl acyl-transferase
E3	Dihydrolipoamide dehydrogenase
FOR	Ferredoxin oxidoreductase
M_r	Relative molecular mass
OADHC	2-Oxoacid dehydrogenase complex
OGDHC	2-Oxoglutarate dehydrogenase complex
PDHC	Pyruvate dehydrogenase complex
TPP	Thiamine pyrophosphate

Introduction

In aerobic bacteria and eukaryotes, a family of 2-oxoacid dehydrogenase multienzyme complexes (OADHCs) are responsible for the oxidative decarboxylation of 2-oxoacids to their corresponding acyl-CoAs (Fig. 1) (Perham 1991, 2000; Perham et al. 2002). Members of this family include: the pyruvate dehydrogenase complex (PDHC), which catalyses the conversion of pyruvate to acetyl-CoA and so links glycolysis and the citric acid cycle; the 2-oxoglutarate dehydrogenase complex (OGDHC), which catalyses the conversion of 2-oxoglutarate to succinyl-CoA within the citric acid cycle; and the branched-chain 2-oxoacid dehydrogenase complex (BCOADHC), which oxidatively decarboxylates the branched-chain 2-oxoacids produced by the transamination of the branched-chain amino acids valine, leucine and isoleucine.

The complexes comprise multiple copies of three component enzymes: 2-oxoacid decarboxylase (E1),

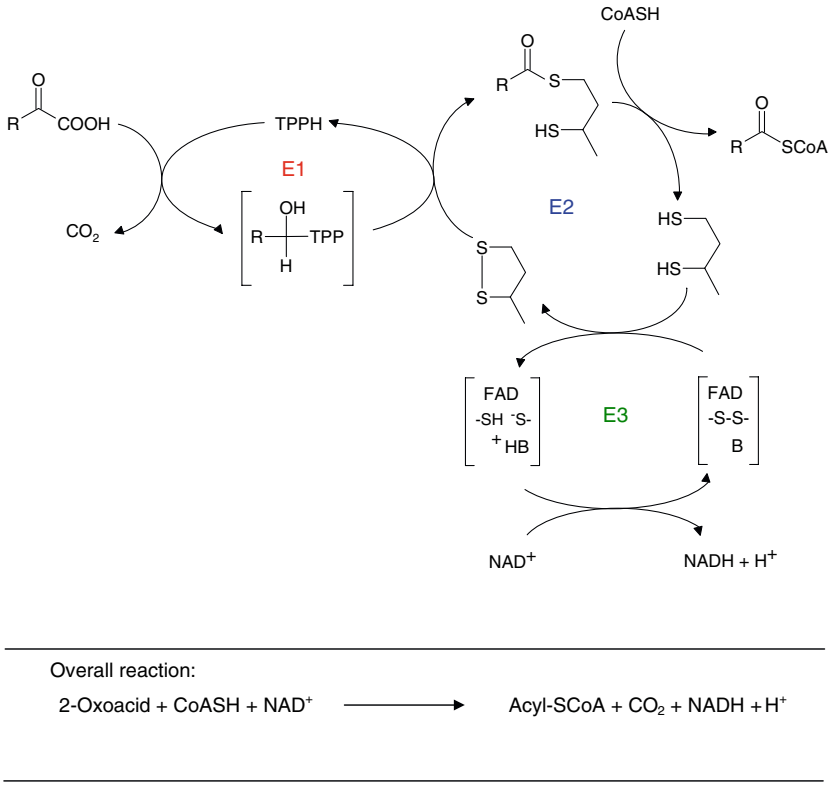

Fig. 1 Reaction mechanism of the 2-oxoacid dehydrogenase complexes: E1 2-oxoacid decarboxylase, E2 dihydrolipoyl acyl-transferase and E3 dihydrolipoamide dehydrogenase. B a histidine base on E3; Lip enzyme-bound lipoic acid; CoASH Coenzyme-A; TPP-H thiamine pyrophosphate

dihydrolipoyl acyl-transferase (E2) and dihydrolipoamide dehydrogenase (E3). E2 forms the structural core of the complex, with multiple polypeptide chains associating into octahedral (24-mer) or icosahedral (60-mer) configurations, depending on the particular complex and the source organism (Izard et al. 1999; Mattevi et al. 1992). E1 and E3 bind non-covalently to the E2 core. E1 may occur as a homodimer or as an $\alpha_2\beta_2$ hetero-tetramer, depending upon the source and the type of complex, although in all cases E3 is a dimer of identical subunits. E2 also forms the catalytic core of the complex, with a covalently attached lipoyl moiety serving to connect the active sites of each enzyme and channelling substrate through the complex (Fig. 1) (Perham 2000; Perham et al. 2001).

No OADHC activity has ever been detected in the archaea (Danson et al. 2007); instead, the oxidation of 2-oxoacids is catalysed by an unrelated and structurally simpler family of 2-oxoacid ferredoxin oxidoreductases (FORs). This family includes the pyruvate FOR, the 2-oxoglutarate FOR and the 2-oxoisovalerate FOR, which catalyse the oxidative decarboxylation of pyruvate, 2-oxoglutarate and the branched-chain 2-oxoacids, respectively (Kerscher and Oesterhelt 1981, 1982; Schut et al. 2001). The subunit composition of the FORs varies with the source organism; for example, the pyruvate FOR from *Halobacterium halobium* has an $\alpha_2\beta_2$ structure (Plaga et al. 1992), from *Sulfolobus* an $\alpha\beta$ structure (Zhang et al. 1996)

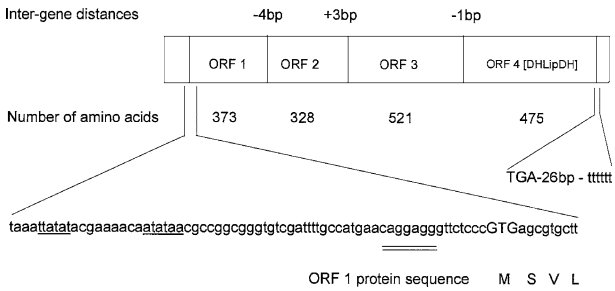

Fig. 2 The arrangement and inter-gene distances (bp) of the four open reading frames constituting the proposed *Hfx. volcanii* 2-oxoacid dehydrogenase complex operon. The corresponding number of amino acids of the protein products is given. The DNA sequence at the 5′ end of ORF 1, plus the upstream region, is shown to illustrate the proposed promoter sequences (*underlined*), the Shine-Dalgarno sequence (*doubly underlined*) and the GTG start codon. The proposed transcriptional stop signal is also shown

and from *Pyrococcus furiosus* an $\alpha_2\beta_2\gamma_2\delta_2$ octamer (Schut et al. 2001). The FOR's catalytic reaction does not involve a lipoic acid moiety or NAD^+; rather, the acyl-moiety formed on decarboxylation of the 2-oxoacid is handed on direct to coenzyme-A, and the reducing equivalents to ferredoxin via the enzyme's iron–sulphur centre.

There is growing evidence to suggest that some archaea may possess an OADHC (Danson et al. 2004, 2007). Whilst no whole complex activity has been found

in any archaeon, we first discovered in *Haloferax volcanii* four genes, whose predicted protein sequences show significant identity to the OADHC components E1α, E1β, E2 and E3 of bacteria and eukaryotes (Jolley et al. 2000). Analysis of the gene sequences and of the upstream region indicated that the genes may comprise an operon (Fig. 2). Interestingly, only E3 activity could be found in cell extracts of this halophile (Danson et al. 1984), although the essential cofactor of the complex, lipoic acid, was detected (Pratt et al. 1989). E3-knockout studies in *Hfx. volcanii* failed to elucidate any metabolic function (Jolley et al. 1996). The recent sequencing of a number of archaeal genomes has revealed the presence of similar putative OADHC-encoding operons in the aerobic archaea, including *H. halobium*, *T. acidophilum*, *Aeropyrum pernix*, *Pyrobaculum aerophilum* and *Sulfolobus solfataricus*. Evidence that they may encode functional proteins comes from our recent work with the genes from *T. acidophilum*; heterologous co-expression of the putative E1α and E1β genes in *Escherichia coli* resulted in an active $\alpha_2\beta_2$ decarboxylase component with specificity for the branched-chain 2-oxoacids and, to a lesser extent, pyruvate (Heath et al. 2004). Moreover, we have now expressed in *E. coli* the *T. acidophilum* genes encoding the putative E2 and E3 components, and have assembled them and the E1 enzyme to form a complex with overall 2-oxoacid dehydrogenase activity with the same substrate specificity as the recombinant E1$\alpha_2\beta_2$ enzyme (Heath 2006).

The presence of an active E3 component in *Hfx. volcanii* in the absence of whole-complex activity raises an interesting question concerning its transcription from a 4-gene operon. Homologous expression of the cloned E3 gene only gave active enzyme when a strong promoter from the rRNA operon of *Halobacterium cutirubrum* was added to the 5′ end of the gene, suggesting that its normal in vivo expression might be from a transcript of the whole operon (Jolley et al. 1996). This hypothesis has been tested in the current paper, along with growth studies on a variety of carbon sources to see if the whole complex activity can be induced under defined nutrient conditions.

Materials and methods

Organism and reagents

Haloferax volcanii (DSM 3757) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Oligonucleotide primers were obtained from MWG-Biotech AG, Germany. Random DNA primers were supplied by Promega, Southampton, UK. SuperScript™II Reverse Transcriptase, RNaseOut™ and RNase H were provided by Invitrogen, Paisley, UK. Turbo™ DNase and DNase Inactivation Reagent were from Ambion, Huntingdon, UK. Vent® DNA polymerase and ThermoPol reaction buffer were purchased from New England (UK) Biolabs, Hitchen, UK. Unless mentioned otherwise, all other chemicals were supplied from Sigma-Aldrich, Gillingham, UK.

Isolation of RNA

Hfx. volcanii was grown in 18% (w/v) saltwater modified growth medium (MGM) consisting of 14.4% (w/v) NaCl, 1.8% (w/v) $MgCl_2{\cdot}6H_2O$, 2.1% (w/v) $MgSO_4{\cdot}7H_2O$, 0.42% (w/v) KCl, 0.5% (w/v) peptone and 0.1% (w/v) yeast extract, pH 7.2, at 42°C with shaking at 200 rpm (Dyall-Smith 2006).

At the mid-exponential phase, a 1 ml sample was removed to a pre-chilled 1.5 ml microcentrifuge tube and the cells were pelleted by centrifugation (11,000*g*, 1 min, 4°C). RNA was then extracted according to the ‘‘manual method’’ described by Dyall-Smith (2006). Prior to RT-PCR, the isolated RNA was treated with Turbo™ DNase at 37°C for 30 min, after which the mixture was incubated with the manufacturer’s DNase Inactivation Reagent for 2 min at 25°C. Any precipitate was removed by centrifugation at 10,000*g* for 1.5 min.

Two-step RT-PCR

For 5 min, 1 µl (50 ng/µl) random primers, 10 µl Turbo DNase treated RNA and 1 µl 10 mM dNTP were heated to 65°C and then rapidly chilled on ice. To this were added 4 µl Invitrogen’s 5 × first-strand buffer, 2 µl 0.1 M dithiothreitol and 1 µl of the RNase inhibitor, RNaseOUT™ (40 units/µl). The tube contents were gently mixed and incubated at 25°C for 2 min. Subsequently, 1 µl (200 units) of SuperScript™II Reverse Transcriptase was added, mixed gently and then incubated at 25°C for 10 min. The RT step was carried out by incubating the tube at 42°C for 50 min, after which the enzyme was inactivated by heating at 70°C for 15 min. Finally, the RNA was removed by the addition of 1 µl (2 units) RNase H and incubating at 37°C for 20 min.

The cDNA from the RT reaction was then PCR-amplified using gene-specific primers. Approximately 100 ng target DNA was mixed with 100 pmol of each primer, 2 mM dNTP, 5 µl 10 × ThermoPol reaction buffer, 4 µl DMSO, 1 µl Vent® DNA polymerase and 50 µl sterile Milli-Q water. The tubes were preheated for 5 min to 96°C in an Eppendorf Mastercycler and the following PCR protocol was then used: denaturation at 96°C for 1.5 min, annealing at 56°C for 1.25 min and extension at 72°C for

3 min. The final extension was carried out at 72°C for 10 min.

Control reactions for the RT step were carried out by omitting the reverse transcriptase, as were the normal controls (no DNA and single primers) for the PCR step.

Enzyme assays

Assays of whole complex activity were performed at 37°C in a final volume of 1 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 0.2 mM TPP, 2.5 mM NAD^+, 1 mM $MgCl_2$, 2 M KCl, 2.6 mM cysteine-HCl, 0.13 mM Coenzyme-A and 2 mM of the 2-oxoacid substrate. The assay was started by the addition of the 2-oxoacid substrate and its progress monitored by the increase in $A_{340\ nm}$ (Domingo et al. 1999).

Enzymic activity of the E1 2-oxoacid decarboxylase component was assayed spectrophotometrically at 595 nm by following the 2-oxoacid dependent reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) (molar extinction coefficient = 22,000 $M^{-1}\ cm^{-1}$) (Lessard and Perham 1994). Assays were carried out in 20 mM potassium phosphate buffer (pH 7.0), 2 mM $MgCl_2$, 0.2 mM TPP and 2 M KCl in a final volume of 1 ml. Buffer and cell extract were pre-incubated for 10 min at 37°C, after which 50 μM DCPIP was added and the assay then started with the 2-oxoacid substrate.

Assays of dihydrolipoamide dehydrogenase (E3 component) were carried out at 37°C in 50 mM Tris–HCl (pH 8.0) buffer, containing 2 M KCl, 1 mM EDTA, 1 mM NAD^+ and 0.4 mM dihydrolipoamide, in a final volume of 1 ml (Jolley et al. 1996). Progress was monitored by the increase in A_{340}.

In all assays, 1 unit of enzyme activity is defined as the amount required to produce 1 μmol of product per minute.

Aerobic growth on single carbon sources

For growth on single carbon sources, *Hfx. volcanii* was grown in a minimal medium comprising 0.3% (w/v) carbon source, 14.4% (w/v) NaCl, 1.8% (w/v) $MgCl_2{\cdot}6H_2O$, 2.1% (w/v) $MgSO_4{\cdot}7H_2O$, 0.42% (w/v) KCl, 5 mM NH_4Cl, 1 mM K_2HPO_4 buffer, 20 mM Tris–HCl (pH 7.5), and 0.1% (v/v) trace elements solution, at 42°C with shaking at 200 rpm. The stock trace elements solution consisted of 0.36 mg/ml $MnCl_2{\cdot}4H_2O$, 0.44 mg/ml $ZnSO_4{\cdot}7H_2O$, 2.3 mg/ml $FeSO_4{\cdot}7H_2O$ and 0.05 mg/ml $CuSO_4{\cdot}5H_2O$. Growth was routinely monitored by measuring the optical density at 600 nm. Where required, the medium was solidified by the addition of 1.5% (w/v) agar.

Anaerobic growth

Anaerobic cultures of *Hfx. volcanii* were grown using a medium consisting of 18.8% (w/v) NaCl, 4.3% (w/v) $MgSO_4{\cdot}7H_2O$, 0.25% (w/v) KCl, 0.07% (w/v) $CaCl_2{\cdot}2H_2O$, 50 mM Tris–HCl (pH 7.2), 0.5 % (w/v) bacto-tryptone, and 0.3 % (w/v) bacto-yeast extract. The medium was distributed into 250 ml bottles in 50 ml aliquots; some were supplemented with 0.7% arginine, others with 50 mM sodium nitrate, some with both, and the remainder left with no supplementation (Wanner and Soppa 2002). The bottles were sealed with rubber septa, autoclaved, and then inoculated with a starting culture of aerobically grown *Hfx. volcanii*. The cultures were flushed with nitrogen for 15–20 min and then incubated at 42°C with shaking at 50 rpm. Growth was monitored by withdrawing a sample with a syringe while flushing with nitrogen.

Results

RT-PCR

Total RNA was isolated and purified from mid-log phase cells of aerobically grown *Hfx. volcanii*, using the method of Dyall-Smith (2006). After treatment with Turbo™ DNase, the preparation showed two major bands on agarose gel electrophoresis, corresponding to 23S and 16S rRNA, plus a smear of mRNA species. PCR amplification of the glucose dehydrogenase gene from this RNA preparation, using gene-specific primers, gave no detectable products, indicating the absence of genomic DNA. The use of glucose dehydrogenase, both here as a DNA probe and later as a control in the RT-PCR reaction, is based on activity assays in cell extracts, which showed the enzyme to be produced and catalytically active.

A two-step RT-PCR reaction was carried out on the purified RNA, using random primers for the RT step, and primers specific for the E1α, E1β, E2 and E3 genes for the PCR amplification of the cDNA. Using primers designed to give short-length products (in the range 200–500 bp) from the individual genes, DNA bands of the expected size were obtained in each reaction (Fig. 3, Table 1). Moreover, RT-PCR with primers to the glucose dehydrogenase gene gave a band of the expected size. When the reverse transcriptase was omitted from the RT-PCR reaction, no products were seen with any combination of the primers, indicating that the products seen in the full RT-PCR were generated from mRNA and not from any contaminating DNA. An equally successful set of data was obtained using a different set of gene-specific PCR primers designed to generate DNA of lengths 950–1,600 bp (data not shown).

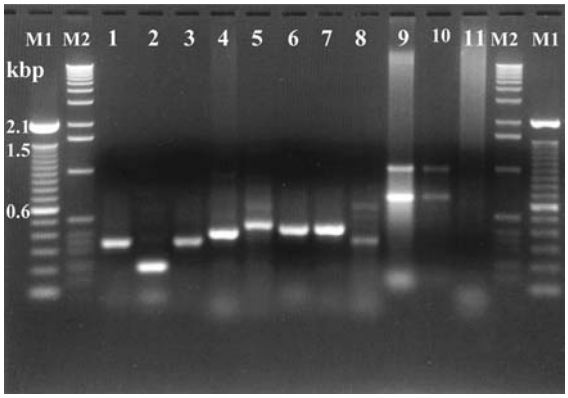

Fig. 3 Agarose gel electrophoresis of RT-PCR generated fragments of the individual genes encoding the components of the putative 2-oxoacid dehydrogenase complex from *Hfx. volcanii.* Marker lanes M1 and M2 contain 100 bp ladder and 1 kb ladder. RT-PCR reaction: *lanes 1*, *2* and *3*: E1α; *lane 4*: E1β; *lane 5*: E2; *lanes 6* and *7*: E3; *lane 8*: glucose dehydrogenase; *lanes 9* and *10*: untreated RNA and Turbo^{TM} DNase-treated RNA; *lane 11*: PCR control. See Table 1 for the predicted and determined sizes of the RT-PCR generated fragments

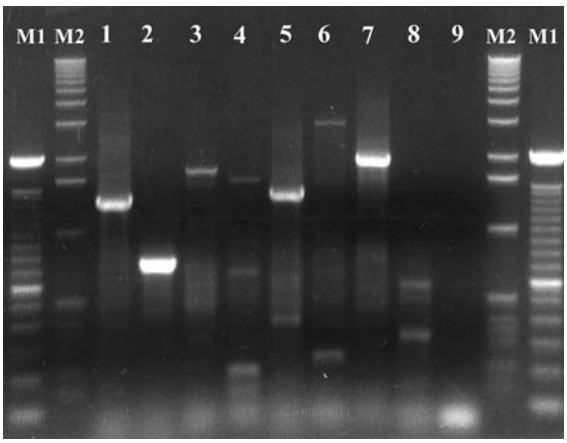

Fig. 4 Agarose gel electrophoresis of RT-PCR generated fragments using inter-gene primers to investigate the transcription of the components of the putative 2-oxoacid dehydrogenase complex from *Hfx. volcanii.* Marker lanes M1 and M2 contain 100 bp ladder and 1 kb ladder, respectively. RT-PCR reaction: *lanes 1* and *2*: E1α-E1β; *lane 3*: E1α-E2; *lane 4*: E1α-E3; *lane 5*: E1β-E2; *lane 6*: E1β-E3; *lane 7*: E2-E3; *lane 8*: glucose dehydrogenase; *lane 9*: PCR control. See Table 1 for the predicted and determined sizes of the RT-PCR generated fragments

Table 1 Size of the fragments generated by RT-PCR from isolated RNA of *Hfx. volcanii*

RT-PCR fragment	Gel band	Predicted size (bp)	Experimentally determined size (bp)
E1α	Fig. 3, lane 1	343	331
E1α	Fig. 3, lane 2	203	216
E1α	Fig. 3, lane 3	345	351
E1β	Fig. 3, lane 4	392	386
E2	Fig. 3, lane 5	447	449
E3	Fig 3, lanes 6 and 7	416	429
E1α–E1β	Fig. 4, lane 1	1355	1288
E1α–E1β	Fig. 4, lane 2	743	705
E1α–E2	Fig. 4, lane 3	1794	1758
E1α–E3	Fig. 4, lane 4	3325	No RT-PCR product
E1β–E2	Fig. 4, lane 5	1437	1396
E1β–E3	Fig. 4, lane 6	2968	3126
E2–E3	Fig. 4, lane 7	1987	1928

The predicted sizes were calculated from the known gene sequences and the positions to which the gene-specific primers were designed to bind. The determined sizes were calculated from the gels shown in Figs. 3 and 4 from a plot of log (bp of markers) versus distance migrated

These data provide strong evidence that all four genes of the putative 2-oxoacid dehydrogenase operon are transcribed under these growth conditions. Furthermore, RT-PCR using the same primers, but in inter-gene combinations, indicated that the four genes can be transcribed as an operon (Fig. 4, Table 1). That is, products of the expected size were generated with the following pairs of primers: E1α–E1β, E1α–E2, E1β–E2, E1β–E3 and E2–E3. No product could be observed using primers E1α-E3, but this may be due to a combination of a large transcript (expected to be 3325 bp) and the known instability of mRNA. Again, omission of the reverse transcriptase resulted in no PCR products, showing that we are indeed detecting mRNA.

Growth studies and enzyme levels

As previously reported, catalytic activity of dihydrolipoamide dehydrogenase (DHLipDH; E3) could be detected in *Hfx. volcanii* grown on a complex (MGM) medium (Table 2) (Danson et al. 1984). However, also as found before, neither whole complex activity nor activity of the E1 decarboxylase component could be found. For the whole complex and E1 assays, the 2-oxoacid substrates used were pyruvate, 2-oxoglutarate, 4-methyl-2-oxopentanoate, 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate, these being the known respective substrates for the pyruvate, 2-oxoxglutarate and branched-chain 2-oxoacid dehydrogenase complexes of bacteria and eukaryotes.

Therefore, in an attempt to induce the synthesis of all the components of the putative complex, *Hfx. volcanii* was grown on a variety of carbon sources and the enzyme activities were measured (Table 2). DHLipDH activity was found in cells grown on all the nutrients tested, with significantly increased levels (over those grown on MGM) when the branched-chain amino acids (valine, leucine and isoleucine) were the sole carbon sources. However, in none

Table 2 Growth of *Hfx. volcanii* on a variety of carbon sources

Carbon source	Growth density	DHLipDH activity
MGM	+++	0.22
MGM (+lip)	+++	0.20
Pyruvate	+++	0.20
Pyruvate (+lip)	+++	0.35
Glucose	+++	0.25
Glucose (+lip)	+++	0.30
Glycerol	+++	0.05
Acetate	++	0.40
Glutamate	+	0.03
Citrate	+	0.02
Alanine	++	0.03
Lactate	+	0.10
Isoleucine	+++	0.93
Isoleucine (+lip)	+++	0.85
Leucine	++	0.90
Leucine (+lip)	++	0.80
Valine	+	0.70
Valine (+lip)	+	0.60

Growth density: (+) $OD_{600\ nm} < 0.5$, (++) $OD_{600\ nm}$ 0.5–1.0, (+++)$OD_{600\ nm} > 1.0$; DHLipDH enzymic activity is expressed in U/mg protein; (*+lip*) lipoic acid added to the medium

of the conditions could whole complex or E1 activity be found. Furthermore, growth of *Hfx. volcanii* anaerobically gave similar data as those of the aerobically-grown cells, with DHLipDH activity levels approximately the same as those found in the aerobic MGM grown cells.

Lipoic acid is an essential cofactor of the 2-oxoacid dehydrogenase complexes and in bacteria can be synthesised de novo or taken in from the environment (Morris et al. 1995); in both routes it is ligated to the side-chain amino group of a lysine residue of the E2 component. The addition of lipoic acid to the media had no significant effect on whether or not the complex or its components could be detected in cell extracts (Table 2).

Gel filtration

Analytical gel filtration of cell extracts of *Hfx. volcanii* was performed on an Amersham Biosciences Äkta FPLC system, using a Superdex 200 10/300 GL column equilibrated with 50 mM Tris–HCl (pH 8.0) containing 2 M KCl and 1 mM EDTA at a flow rate of 0.5 ml/min. This was to determine whether the DHLipDH present in cell extracts is associated with a multienzyme complex or is in an unassociated form. With cells grown aerobically on isoleucine and on acetate, and anaerobically on MGM with nitrate as electron acceptor, DHLipDH eluted as a single peak with an estimated M_r of 120,000; that is, none was associated with high M_r material characteristic of a multienzyme complex. As a control, a cell extract of *E. coli* was also gel filtered in the same buffer, but with a reduced salt concentration of 0.1 M KCl. DHLipDH activity eluted in two main peaks, one associated with pyruvate dehydrogenase whole complex activity in the excluded volume of the column and the other at an M_r value of approximately 100,000, corresponding to the uncomplexed DHLipDH dimer.

Discussion

The question addressed in this paper concerns the in vivo transcription of the four genes proposed to encode the components of a 2-oxoacid dehydrogenase complex in *Hfx. volcanii*. That is, in the absence of any detectable whole complex activity in cell extracts, but in the presence of an active E3 enzyme (encoded by the last gene of the cluster), are all four genes transcribed and, if so, are they transcribed as a single message?

We have used a two-step RT-PCR method, as did Wang et al (2004), to detect transcription. The data reported provide convincing evidence that the four genes are transcribed in vivo when *Hfx. volcanii* is grown on a peptone-yeast extract medium (MGM), and that they can be transcribed as a single message, consistent with the genes constituting an operon. Separate transcription of the individual genes cannot be ruled out, although homologous expression studies of the sub-cloned gene encoding the E3 component suggested that this is not the case, at least for that gene. The genome sequence of *Hfx. volcanii* is complete [http://archaea.ucsc.edu /cgi-bin/hgGateway?db = haloVolc1], and the E3 gene is represented only once; moreover, when this gene is knocked out by insertional mutagenesis, all E3 enzymic activity is lost (Jolley et al. 1996). Therefore, the use of gene-specific primers involving the E3 gene in the intergene PCR step of the RT-PCR ensures that transcription of the four putative OADHC genes are specifically detected.

Our finding that the putative OADHC genes are transcribed in vivo raises the question of why no whole complex activity could be detected, even though catalytic activity of E3 is present. One obvious possibility is that there may be mutations in the genes rendering one or more of the other components inactive. Genome analyses argue strongly against the presence of non-functional ORFs in prokaryotes (Alimi et al. 2000), and the presence of these four tightly spaced genes in all aerobic archaeal genomes adds further support to the fact that they encode functional enzymes. Indeed, all four protein products are active and assemble into a functional complex when the corresponding genes from *T. acidophilum* are heterologously expressed (Heath 2006).

Secondly, the mRNA detected in *Hfx. volcanii* may be the result of leaky transcription, giving such low levels of

protein components that complex formation does not take place. Analysis of cell extracts by gel filtration shows that the E3 enzyme is not part of a protein complex, and the failure to detect E1 activity suggests that the E1 components are not synthesised. Control of complex formation may therefore be at the level of translation rather than transcription.

A third possibility is that a complex is formed, but the substrate specificity is for a different 2-oxoacid than the ones tried, even though the substrates tested are those that are active with the *T. acidophilum* recombinant complex (Heath et al. 2004; Heath 2006) and with all known bacterial and eukaryal OADHCs (Perham 2000). Furthermore, the gel filtration results argue against any form of complex being present.

In an attempt to induce the production of active complex, *Hfx. volcanii* was grown on a range of single carbon nutrients, whose metabolism might involve or be enhanced by the presence of an active OADHC. However, no whole complex activity could be induced, although the level of the E3 component, dihydrolipoamide dehydrogenase, was increased three to fourfold when cells were grown on the branched-chain amino acids, valine, leucine and isoleucine. The significance of this stems from our observations that the metabolic transamination of these will produce the branched-chain 2-oxoacids that are the substrates of the recombinant *Thermoplasma* OADHC complex, and might indicate that the halophilic complex has the same or similar substrate specificity.

An obvious route to answer some of these questions raised would be to express the *Hfx. volcanii* genes recombinantly. Heterologous expression in *E. coli* has been attempted with the E1 and E3 genes. In both cases, inclusion bodies formed; resolubilisation and reactivation with high salt concentrations successfully produced an active E3 component (Connaris et al. 1999), but all attempts with the E1α and E1β proteins failed (Al-Mailem, Hough and Danson, unpublished observations). Similarly, homologous expression in *Hfx. volcanii* of the sub-cloned E3 gene yielded active enzyme (Jolley et al. 1996), but expression of an E1$\alpha\beta$ construct failed to yield protein that could be observed by SDS-PAGE or through E1 activity assays.

Finally, unlike the other aerobic archaea, Wanner and Soppa (2002) found that the *Hfx. volcanii* genome contains a partial OADHC gene cluster in addition to the one that is the subject of this paper. The cluster contains three genes that code for proteins with significant sequence identities to an E1α, an E1β and an unattached lipoyl domain; it is devoid of the genes for E2 and E3. The genes were isolated by complementation of a nitrate-respiration deficient mutant of *Hfx. volcanii*, suggesting an in vivo function, but physiological and biochemical analyses failed to identify any metabolic substrate.

Clearly, the presence of OADHC genes in the archaea is an intriguing situation, the characterisation of which, in a range of genera, will provide valuable information on the evolution of metabolism and a system with which to study multienzyme complex assembly and function in extreme environmental conditions. The findings reported in this paper are one further step in achieving this functional characterisation.

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