Three 2-oxoacid dehydrogenase operons in *Haloferax volcanii*: expression, deletion mutants and evolution

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Two unrelated protein families catalyse the oxidative decarboxylation of 2-oxoacids, i.e. the 2-oxoacid dehydrogenase complexes (OADHCs) and the 2-oxoacid ferredoxin oxidoreductases (OAFORs). In halophilic archaea, OAFORs were found to be responsible for decarboxylation of pyruvate and 2-oxoglutarate. Nevertheless, two gene clusters encoding OADHCs were found previously in *Haloferax volcanii*, but their biological function remained obscure. Here a third *oadhc* gene cluster of *H. volcanii* is presented. To characterize the function, the genes encoding the E1 subunit were inactivated in all three gene clusters by in-frame deletions. Under aerobic conditions none of the three mutants showed any phenotypic difference from the wild-type in various media. However, growth yields of two mutants were considerably lower than that of wild-type under nitrate-respirative conditions in complex medium. Northern blot analyses revealed (1) that polycistronic transcripts are formed and all three gene clusters are *bona fide* operons and (2) that transcription of all three operons is induced under anaerobic conditions compared to aerobic conditions. Taken together, the three *H. volcanii* enzymes do not fulfil one of the 'usual' aerobic functions of typical OADHCs, but decarboxylate an as-yet-unidentified novel substrate under anaerobic conditions. A survey of all 28 fully sequenced archaeal genomes revealed that nearly all archaea contain several OAFORs (three to four on average), suggesting that this protein family was already present in their last common ancestor. In contrast, only nine archaea encode one or two OADHCs, indicating that this protein family entered archaea by lateral transfer of the cognate genes from bacteria. This view is underscored by a phylogenetic tree of 33 archaeal and bacterial OADHCs.

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INTRODUCTION

Two different enzyme systems catalyse the oxidative decarboxylation of 2-oxoacids to yield acyl-CoA, i.e. 2-oxoacid dehydrogenase complexes (OADHCs) and 2-oxoacid ferredoxin oxidoreductases (OAFORs). In addition, some bacterial species contain pyruvate oxidase, which oxidatively decarboxylates pyruvate to yield acetyl phosphate. OADHCs and OAFORs are not homologous and they differ in subunit composition, coenzyme specificity and, last but not least, reaction mechanism. The OADHC mechanism involves one oxidation step and two electrons

are transferred, while the OAFOR mechanism includes two one-electron oxidation reactions and a radical intermediate. An overview of the different steps is given in Fig. 1.

OADHCs are widely distributed in eukaryotes and in aerobic bacteria and catalyse the following overall reaction:

They are composed of three different types of subunits called E1, E2 and E3, which catalyse different steps of the reaction (Fig. 1). E1 can either be a single polypeptide, or it comprises two subunits E1 α and E1 β . E2 is the core component onto which E1 and E3 are assembled. Most if not all OADHCs are very large complexes containing multiple copies of all subunits. Substrate specificity resides in the E1 and E2 component, while the same E3 (dihydrolipoamide dehydrogenase) can be a component of several different OADHCs. The OADHCs are multifunctional enzymes that couple several reactions. They are a paradigm for an extreme form of 'substrate channelling', i.e. the intermediates remain covalently bound to a

Abbreviations: ADHC, acetoin dehydrogenase complex; BCDHC, branched-chain 2-oxoacid dehydrogenase complex; BCFOR, branched-chain 2-oxoacid ferredoxin oxidoreductase; OADHC, 2-oxoacid dehydrogenase complex; OAFOR, 2-oxoacid ferredoxin oxidoreductase; OGDHC, 2-oxoglutarate dehydrogenase complex; OGFOR, 2-oxoglutarate ferredoxin oxidoreductase; PDHC, pyruvate dehydrogenase complex; PFOR, pyruvate ferredoxin oxidoreductase.

Two supplementary tables are available with the online version of this paper.

Fig. 1. The two different reaction mechanisms of oxidative decarboxylation of 2-oxoacids (Wanner & Soppa, 2002). (a) Mechanism of OADHCs and (b) mechanism of OAFORs. Abbreviations: CoA, coenzyme A; Fd, ferredoxin; FeS, iron-sulfur cluster; Lip, lipoamide; TPP, thiamine pyrophosphate.

‘swinging arm’ that transports them from one active site to the next (reviews e.g. Perham, 1991, 2000; Berg & de Kok, 1997). Thereby the local substrate concentration is increased, reactive intermediates are sequestered and their reaction with undesired cellular components is prevented, and the reaction can be precisely controlled.

The best-characterized OADHC is the pyruvate dehydrogenase complex (PDHC), which generates acetyl-CoA, a central intermediate in energy and anabolic metabolism (e.g. Milne *et al.*, 2006; Frank *et al.*, 2005). Another OADHC, the 2-oxoglutarate dehydrogenase complex (OGDHC), is part of the citric acid cycle. A third OADHC is involved in degradation of the three branched-chain amino acids valine, leucine and isoleucine and decarboxylates the three 2-oxoacids that are generated by their transamination. Malfunction of any of these three OADHCs can cause human diseases, including neurological impairment and the ‘maple syrup disease’ (Hengeveld & de Kok, 2002; Chuang, 1998). A fourth OADHC catalyses the decarboxylation of 2-oxobutyrate, an intermediate in methionine catabolism. The last known substrate of an OADHC is acetoin, which can be used as an electron source under anaerobic conditions by a variety of bacteria (Krüger *et al.*, 1994, and references therein).

The second enzyme family, the OAFORs, catalyse the following overall reaction:

Typically, OAFORs operate in energy metabolism and catabolism of anaerobic bacteria. Again, the best-characterized family member is the one using pyruvate as a substrate (reviews: Ragsdale, 2003; Charon *et al.*, 1999). More than 25 years ago it was discovered that in halophilic archaea the oxidative decarboxylation of pyruvate and 2-oxoglutarate under aerobic conditions is catalysed by ‘anaerobic’ OAFORs instead of ‘aerobic’ OADHCs, and therefore OADHCs are not needed (Kerscher & Oesterhelt, 1981). Therefore it was a surprise that, in addition, the genes encoding an *oadhc* operon were discovered (Jolley *et al.*, 1996, 2000). While a Northern blot analysis showed that the genes are expressed, biochemical analyses as well as physiological characterization of a deletion mutant of the dihydrolipoamide dehydrogenase gene (*e3*) indicated that none of the seven known OADHC substrates is used by this OADH (Jolley *et al.*, 1996). Subsequently, a second *oadhc* gene cluster was found to play a role during nitrate respiration, but it was also concluded that none of the seven known substrates is used (Wanner & Soppa, 2002). An analysis of transcriptome changes following a shift from Casamino acids to glucose under aerobic conditions led to the identification of genes of a third *oadhc* gene cluster of *Haloferax volcanii* (Zaigler *et al.*, 2003). Here we present the entire third gene cluster. To enable the identification of the biological roles of all three gene clusters, in-frame deletion of the respective genes for the E1 subunit were constructed and the growth phenotypes were characterized in a variety of media. In addition, transcription of the gene cluster under aerobic and anaerobic conditions has been studied. The distribution of OADHCs and OAFORs in archaea with fully sequenced genomes was investigated, and a phylogenetic OADHC tree was constructed.

METHODS

Materials. The plasmid pTA131 was obtained from Thorsten Allers (Nottingham University, UK). Enzymes for molecular genetic techniques were obtained from MBI Fermentas, Qiagen and Promega. For the isolation of DNA fragments and plasmids, products of Qiagen were used. DIG-dUTP for the generation of probes was from Roche, an anti-DIG antibody and CDP-Star for visualization of probe hybridization were from Sigma-Aldrich. For sequence determination, the ‘Big-Dye-Terminator kit’ from Applied Biosystems was used.

Micro-organisms and culture conditions. The *Haloferax volcanii* strain H26 was obtained from Thorsten Allers (Nottingham University, UK) and was grown as described by Allers *et al.* (2004). The *Escherichia coli* strain X1blue-MRF' was obtained from Stratagene and grown in standard media (Sambrook *et al.*, 1989).

Construction of in-frame deletion mutants. The in-frame deletion mutants were constructed using the so-called pop-in-pop-out method developed by Bitan-Banin *et al.* (2003) and optimized by Allers *et al.* (2004). In short, a version of the target gene carrying an internal deletion is constructed by cloning two PCR fragments, representing the 5'-region and the 3'-region of the gene, respectively, into the plasmid pTA131. The oligonucleotides used for PCR fragment generation, together with all other oligonucleotides used in this study, are listed in Supplementary Table S1 (available with the online

version of this paper). The plasmids pJO1, pJO2 and pJO3 were generated, which contain deleted versions of the E1 subunit of OADHC1, OADHC2 and OADHC3, respectively. They were used to transform *H. volcanii* strain H26, which has a defect in the *pyrE2* gene. Due to the presence of an intact *pyrE2* on pTA131, transformants can be selected that have integrated the plasmid via homologous recombination at the respective *oadhc* locus (growth on uracil-free medium). Clones that have experienced a second homologous recombination event (pop-out) can be selected by their ability to grow in the presence of 5-fluoroorotic acid (5-FOA), which is toxic for cells with an intact *pyrE2* gene. If the deletion mutant has no growth defect under the applied conditions, about half of the clones should have the deletion version of the gene, and the other half should have regained the wild-type chromosomal arrangement. Deletion mutants were identified first by PCR with small culture aliquots as templates, and then verified by Southern blot hybridization using standard procedures. Genomic DNA was digested with *Bst*NI for the analysis of *oadhc1* and with *Mbo*I for the analysis of *oadhc2* and *oadhc3*. The sequences of the oligonucleotides used for probe construction are included in Table S1.

Characterization of growth phenotypes. For the characterization of growth phenotypes, complex medium as well as synthetic media with diverse carbon sources and electron donors were used (Wanner & Soppa, 2002). Nitrate (50 mM) was added as electron acceptor during anaerobic growth. All growth experiments were performed at 42 °C. For aerobic growth, small-scale experiments were performed in 96-well plates using 150 µl of the respective medium and 5 µl inoculum. Thereby the wild-type and the three mutants could be tested simultaneously in many different media in quadruplicate measurements. The OD₆₀₀ values were recorded using a microtitre plate reader (Molecular Devices). Attempts to use microtitre plates sealed with a transparent foil for anaerobic growth were not successful, because optical density measurements were not very reproducible. Therefore initial experiments were performed in 5 ml volumes using test tubes that fit into a Klett colorimeter (Klett Manufacturing Co.). After inoculation, the tubes were gassed with nitrogen and sealed with rubber septa. All final experiments were performed in 30 ml medium using 100 ml Klett flasks that allow the use of a Klett colorimeter for optical density determination. For nitrate-respirative growth, the flasks were sealed with rubber septa after inoculation and replacing the air with nitrogen. In all cases three biological replicates were performed, and means and standard deviations were calculated.

Northern blot analysis. Isolation of total RNA, probe preparation, and Northern blot analysis were performed as described previously (Herrmann & Soppa, 2002). Three micrograms of total RNA was used for the detection of the *oadh1* and *oadh3* transcripts, 5 µg of total RNA was used for the analysis of the *oadh2* transcript. The primers used for probe construction are included in Table S1. For quantification the films were scanned and the pictures were analysed with the software 'ImageJ' (<http://rsb.info.nih.gov/ij/index.html>). The background was determined locally for each band and subtracted from the signal.

Bioinformatic analyses. The program 'Clone Manager Professional Suite' (Sci Ed Central, www.scienced.com) was used for *in silico* vector construction and sequence analysis.

The analysis of the distribution of the OADHCs and the OAFORs in all fully sequenced archaeal genomes was performed in two steps. First the COG numbers of all subunits were determined (<http://www.ncbi.nlm.nih.gov/COG>). Then, members of the COGs in all archaeal genomes were identified with the 'gene search' tool of the 'Comprehensive Microbial Resource' of TIGR (<http://www.tigr.org>) and the number of genes in each genome was tabulated.

Construction of phylogenetic trees was done as described previously (Wanner & Soppa, 2002). The programs ProtPars, ProtDist, Fitch and CONSENSE of the PHYLIP program package (Felsenstein, 1996) were used at the Server of the Institut Pasteur (<http://bioweb.pasteur.fr/seqanal/phylogeny/phytip-uk.html>). One hundred bootstrap replications were performed, and consensus trees were calculated. Phylogenetic trees were visualized with the program TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

RESULTS

Three gene clusters encoding 2-oxoacid dehydrogenases

Two different *oadhc* gene clusters have been described for *H. volcanii*, i.e. the *oadhc1* gene cluster containing four genes for a complete OADHC (Jolley *et al.*, 2000), and the *oadhc2* gene cluster containing three genes for the E1 α and E1 β subunits and an isolated lipoyl domain (Wanner & Soppa, 2002). The characterization of transcriptome changes after a shift of *H. volcanii* cultures from Casamino acid medium to glucose medium led to the discovery that *H. volcanii* contains differentially regulated genes that belong to a third *oadhc* gene cluster (Zaigler *et al.*, 2003). A shotgun DNA microarray with a onefold coverage of the genome was used and the genome sequence was not available at that time, therefore the genomic localization of the newly identified genes could not be analysed. Recently, the nearly complete genome sequence of *H. volcanii* has become available (<http://www.tigr.org>), and it was used to identify the genes of the third *oadhc* gene cluster. It comprises genes encoding E1 α , E1 β and E2; a gene encoding E3 is missing. However, directly downstream of the gene cluster, but in the opposite direction, a gene encoding another E2 subunit was identified. Fig. 2 gives an overview of all three gene clusters. The previous studies have failed to find a biological function for OADHC1 (Jolley *et al.*, 2000) and identified a function of OADHC2 during nitrate-respirative growth, but used a randomly generated mutant with an uncharacterized mutation (Wanner & Soppa, 2002). Therefore we decided to construct in-frame deletion mutants of the genes encoding the E1 subunits of all three gene clusters and to use these defined mutants in the search for the biological roles of the three OADHCs of *H. volcanii*.

Construction of in-frame deletion mutants

The construction of the three in-frame deletion mutants was performed with the so-called 'pop-in-pop-out' method as described by Allers *et al.* (2004), using the plasmid pTA131 and the strain H26 constructed by Thorsten Allers (Nottingham University, UK). Three plasmids were generated, pJO1 to pJO3, which harbour deletion variants of the E1 gene of the three gene clusters, and were used to transform H26. After selection of the 'pop-in' clones, which have the plasmid integrated into their chromosome and have both the wild-type gene and the deletion variant,

Fig. 2. Three *oadh* operons and construction of in-frame deletion mutants. The genomic organization of the *oadh1*, *oadh2* and *oadh3* genes is shown schematically on the left (a, c, e). In each case, the situations in the wild-type, the pop-in mutant and the pop-out mutant are shown from top to bottom. The integrated plasmid with the deletion variant of the *e1* gene is indicated above the genome of the pop-in mutant. The probe used for Southern blot analysis is indicated by an arrow below the genome representation. Relevant restriction sites are indicated by vertical arrows, and the sizes of hybridizing fragments are included. The Southern blots are shown on the right (b, d, f). The lanes from left to right represent the wild-type, the pop-in mutant and the in-frame deletion mutant. Relevant fragments are indicated by their sizes and horizontal arrows.

the 'pop-out' clones that had lost the wild-type gene were selected. Fig. 2(a, c, e) schematically shows the genomic organization of the three gene clusters in the wild-type, in

the pop-in mutant, and in the pop-out mutant that exclusively carries the *in vitro*-constructed in-frame deletion variant. Relevant restriction sites, the probes used

for Southern blot analysis, and the sizes of the genomic fragments that should hybridize with the probes are indicated. The results of Southern blots that were used to analyse the genomic arrangement of the gene clusters in wild-type and mutants are also included in Fig. 2(b, d, f). The *oadhc2* probe gave the clearest result without any cross-hybridization. Although in the remaining two cases unspecific cross-hybridization to a few additional bands appeared in all strains, the differences between the wild-type and the mutants are clearly visible (relevant fragments are indicated by arrows). Thus the in-frame deletion of all three genes was successful. It showed that none of the genes is essential under the conditions used for selection (aerobic growth in complex medium).

Phenotypic comparison of the three *oadhc* mutants and the wild-type

Aerobic growth of the three mutants and the wild-type was tested in synthetic media with nine different carbon sources/electron donors and in complex medium. In all cases, four biological replicates were used. The cultures were grown in 96-well microtitre plates, and growth was monitored as OD₆₀₀. No phenotypic differences could be detected, either in growth rates or in growth yields. The results are summarized in Table 1. Growth curves of 30 ml cultures grown under three selected conditions are shown in Fig. 3(a, c, e). Also under these conditions (greater volume, a better aeration than in microtitre plates) the growth curves of the three mutants and the wild-type were identical when cultures were grown on glucose, on Casamino acids and in complex medium.

Similarly, wild-type and mutants grew indistinguishably under nitrate-respirative conditions with glucose, pyruvate or Casamino acids as electron donors. In contrast, two of

the mutants showed a clear phenotypic difference from the wild-type when grown under nitrate-respirative conditions in complex medium or a mixture of complex and glucose medium. The growth yields are summarized in Table 2. Interestingly, the growth defect of the *oadhc1* mutant is more severe in complex medium, while that of the *oadhc2* mutant is higher in a mixture of complex and glucose medium. Growth curves of 30 ml cultures grown under three selected conditions are shown in Fig. 3(b, d, f), illustrating that the two mutants have a longer lag phase, a lower growth rate and a lower growth yield than the wild-type only in complex medium (Fig. 3f), but not with glucose or Casamino acids as electron donors (Fig. 3b, d). The data show that OADH1 and 2 play an important role under specific conditions, but that they are not essential. No phenotype of the *oadhc3* mutants could be detected.

Regulation of expression of the *oadhc* operons

The genes of the *oadhc1* gene cluster were reported to be transcribed into a polycistronic mRNA under aerobic conditions (Jolley *et al.*, 2000). The *oadh2* genes were postulated to be transcribed together, since they overlap, but this has not been experimentally proven (Wanner & Soppa, 2002). The *oadh3* genes have not been investigated yet. Therefore we decided to determine the expression level of the wild-type genes and the deletion variants by Northern blot analysis. Because two of the three deletion mutants had a phenotype under anaerobic conditions, aerobic expression during exponential phase was compared to anaerobic expression during exponential as well as stationary phase. The results are summarized in Fig. 4. First, the sizes of all transcripts were determined. In all cases, polycistronic transcripts were found with sizes in excellent agreement with those predicted from the genomic organization. Therefore all three *oadhc* gene clusters are

Table 1. Comparison of aerobic growth of wild-type and the three *oadhc* mutants

Mean values of four biological replicates are tabulated; standard deviations are shown in parentheses.

Medium	H26 (wt) final OD ₆₀₀	Percentage of wt		
		$\Delta oadh1E1\alpha$	$\Delta oadh2E1\alpha$	$\Delta oadh3E1\alpha$
Synthetic medium with the following C source:				
Glucose	0.203 (0.009)	102 (4.3)	101 (5.8)	97 (7.7)
Casamino acids	0.374 (0.028)	95 (2.0)	96 (2.9)	99 (4.2)
Pyruvate	0.406 (0.032)	96 (2.3)	92 (6.0)	94 (8.3)
Succinate	0.189 (0.018)	101 (8.2)	104 (6.6)	100 (8.8)
Glycerol	0.233 (0.020)	101 (1.6)	98 (5.0)	105 (8.1)
Sucrose	0.399 (0.032)	102 (8.3)	98 (5.8)	94 (5.8)
Xylose	0.266 (0.013)	93 (5.6)	92 (3.5)	99 (6.1)
Fructose	0.262 (0.022)	95 (6.3)	96 (1.6)	100 (7.4)
Galactose	0.115 (0.016)	114 (12.8)	83 (9.1)	119 (16.0)
Complex medium (CM)	0.498 (0.024)	98 (6.8)	96 (4.5)	98 (5.6)
CM + glucose synthetic medium (1:1)	0.573 (0.042)	94 (2.1)	98 (2.8)	103 (3.3)

Fig. 3. Characterization of growth phenotypes. Growth of the wild-type (crosses), the *oadh1* deletion mutant (squares), the *oadh2* deletion mutant (triangles), and the *oadh3* deletion mutant (diamonds) were compared in different media and conditions. Aerobic growth is shown on the left (a, c, e), and nitrate-respirative growth is shown on the right (b, d, f). From top to bottom, three different electron donors/carbon sources were used, i.e. synthetic medium with glucose (a, b), synthetic medium with Casamino acids (c, d) and complex medium (e, f). Growth was monitored with a Klett colorimeter. In all cases, mean values of three independent biological replicates are shown. For clarity, the standard deviations are shown only in (f), because they would hide the data points in (a–e). The reproducibility was the same under all conditions.

bona fide operons. The polycistronic transcripts of the deletion mutants had the expected size difference from the wild-type transcripts, providing additional proof that the three deletions had been successfully constructed.

The relative levels of the *oadh1* and *oadh3* transcripts were determined densitometrically and are summarized in Table 3. Interestingly, transcript levels were higher in the mutants than in the wild-type (Fig. 4a, c). This could be explained either by an unexpected difference in transcript stability or by a feedback loop present only in the wild-type, which

leads to downregulation of transcription when enough active OADHC is present in the cell. Comparison of transcript levels in aerobic and anaerobic cultures revealed that these two *oadhc* operons are induced three- to fivefold under nitrate-respirative conditions. The *oadh3* transcript level is even higher in stationary than in exponential phase, indicating that OADHC3 might be involved not only in anaerobic respiration during growth, but also in maintenance metabolism, enhancing the survival of resting cells. The *oadh2* transcript was much more difficult to detect than the other two transcripts. Despite considerable efforts

Table 2. Comparison of nitrate-respirative growth of wild-type and the three *oadhc* mutants

Mean values of four biological replicates are tabulated; standard deviations are shown in parentheses.

	H26 (wt) final OD ₆₀₀	Percentage of wt		
		$\Delta oadh1E1\alpha$	$\Delta oadh2E1\alpha$	$\Delta oadh3E1\alpha$
Glucose	70 (2.0)	97 (3.7)	101 (2.7)	99 (5.5)
Casamino acids	120 (4.8)	101 (5.1)	107 (4.0)	102 (6.9)
Pyruvate	69 (4.9)	92 (7.0)	96 (6.5)	101 (4.5)
Complex medium (CM)	171 (3.8)	57 (2.1)	81 (2.6)	98 (3.8)
CM + glucose synthetic medium (1:1)	219 (6.1)	77 (3.7)	64 (6.2)	99 (2.6)

(e.g. using three different probes) it was not possible to see the transcripts in Northern blot analyses with total RNA isolated from exponentially growing cultures. The transcript of the wild-type and of the mutant strain could only be detected in anaerobic cultures in stationary phase (Fig. 4b). The higher transcript level under anaerobic conditions is in congruence with the phenotype of the mutant, which was observed solely during nitrate-respirative growth. In addition, the accumulation in stationary phase indicates that OADHC2 might have a specific function for survival in the resting state.

The data show that all three OADHCs of *H. volcanii* fulfil their major or their only function under anaerobic conditions, in contrast to the usual role of OADHCs during aerobic metabolism of bacteria or eukaryotes.

Evolution of archaeal 2-OADHCs

Two alternative views of the evolution of archaeal OADHCs have been proposed: (1) some archaeal genomes acquired *oadhc* genes by lateral gene transfer from bacteria (Wanner & Soppa, 2002) and (2) the common ancestor of all archaea and bacteria possessed one (or several) *oadhc* genes, and they were retained in aerobic species, but got lost from anaerobic species (Heath *et al.*, 2004). A first approach to clarify the evolution of archaeal OADHCs was to tabulate the *oadhc* genes present in the 28 completely sequenced archaeal genomes. Supplementary Table S2 (available with the online version of this paper) contains the results as well as the distribution of *oafor* genes in archaea. Only 9 out of 28 species contain genes for 12 OADHCs, while 23 archaeal species contain genes for 71 OAFORs. Four additional archaeal genomes contain genes either for α -subunits or for β -subunits of OAFORs, so that only the very reduced genome of *Nanoarchaeum equitans* is devoid of any *oafor* gene. This extremely different distribution indicates that the last common ancestor of all archaea already contained several OAFORs involved in decarboxylation of pyruvate, 2-oxoglutarate and branched-chain amino acids, while – in contrast – it was devoid of OADHCs.

To clarify the evolution of archaeal OADHCs further, phylogenetic trees were constructed with the E1 α - and

E1 β -subunits, which are responsible for substrate specificity. It was shown earlier that the bacterial and eukaryotic OGDHCs form a distinct subfamily only distantly related to any archaeal E1 (Wanner & Soppa, 2002). This was verified in initial trees, and therefore the OGDHC subfamily was omitted from further analysis. For final tree construction, the *oadhc* genes found in the 28 completely sequenced archaeal genomes, the three *oadhc* genes of *H. volcanii*, and 16 bacterial and eukaryotic *oadhc* genes encoding enzymes of experimentally proven functions were used. E1 α and E1 β trees were constructed using the parsimony as well as the distance matrix program of the PHYLIP program package of Felsenstein (1996). One hundred bootstrap replications were performed, and consensus trees were generated. As one example, the E1 α distance matrix-based tree is shown in Fig. 5. While the four trees differed in some details, they all had the following results in common: (1) the acetoin dehydrogenase complexes (ADHCs) form one monophyletic group, (2) the pyruvate dehydrogenase complexes (PDHCs) and branched-chain 2-oxoacid dehydrogenase complexes (BCDHCs) do not form monophyletic groups, (3) eight haloarchaeal OADHCs form one monophyletic group (compare the branch numbered 1 in Fig. 5), (4) their nearest neighbours are a BCDHC and a PDHC from Gram-positive bacteria (compare branch no. 2), (5) the OADHCs from three *Thermoplasma* and *Picrophilus* species form one group (branch no. 3), (6) OADHCs 2 and 3 from *H. volcanii* and the two OADHCs from *S. solfataricus* are included in the monophyletic ADHC group (branch no. 4), and (7) the OADHCs of Gram-negative bacteria form one group, including three different substrate specificities (branch no. 5). The relevance of these results for the evolution of archaeal OADHCs is discussed below.

DISCUSSION

H. volcanii was shown to harbour a third *oadhc* gene cluster in addition to the two gene clusters described previously (Jolley *et al.*, 2000; Wanner & Soppa, 2002). The substrate specificities of all three OADHCs remain unresolved. The group of Michael Danson and we failed to detect an enzymic activity in cytoplasmic extracts with any of the

Fig. 4. Regulation of expression of the three operons *oadhc1* (a), *oadhc2* (b) and *oadhc3* (c). Cultures of the wild-type and the three in-frame deletion mutants were grown to exponential or stationary phase, as indicated, and the transcripts of the gene clusters were detected by Northern blot analysis. For the detection of the *oadhc1* and *oadhc3* transcripts, 3 µg total RNA was loaded per lane, while 5 µg was loaded for the detection of *oadhc2*. Equal loading was verified using the bands of rRNAs stained with ethidium bromide (shown above the hybridization signals). In each case, a single polycistronic transcript was found, proving that all three clusters are *bona fide* operons. The positions of relevant size markers are indicated at the left side. Transcript sizes were determined using standard curves with additional marker bands. The lengths predicted from the genomic localization (Fig. 2) are shown below the Northern blots.

Table 3. Transcript levels of the three *oadhc* operons during aerobic and nitrate-respirative growth

After scanning of the Northern blots, the signals were quantified with ImageJ, yielding arbitrary units. All *oadh1* and all *oadh3* values were normalized to the signal of the respective wild-type transcript during exponential growth.

Condition	Growth phase	<i>oadh1</i>		<i>oadh3</i>	
		wt	Mutant	wt	Mutant
Aerobic respiration	Exponential	1	1.2	1	1.2
Nitrate respiration	Exponential	2.9	3.4	3.3	8
Nitrate respiration	Stationary	2.2	2.9	5.1	5.2

seven currently known OADHC substrates, indicating that all three haloarchaeal OADHCs have novel substrates. The assumption that the haloarchaeal OADHCs do not fulfil the roles known from bacterial and eukaryotic enzymes is corroborated by the phenotypic analyses of the three in-frame deletion mutants. Under aerobic conditions, no phenotypic difference from the wild-type could be detected in a variety of different media, indicating that the OADHCs are not involved in the aerobic degradation of sugars, branched-chain amino acids or methionine. Under nitrate-respirative conditions, phenotypic differences only occur in complex medium, not in glucose medium or Casamino acid medium, excluding that the OADHCs are involved in the degradation of glucose or amino acids. Future experiments will aim to identify the constituents present in yeast extract or/and tryptone that can be used by the wild-type but cannot be degraded by the *oadhc1* and *oadhc2* mutant. To date, no phenotype could be detected for the *oadhc3* mutant and thus determination of its substrate specificity is even more challenging. In addition to the mutant phenotypes, also the expression analysis showed that the OADHCs fulfil their main (or only) function under anaerobic conditions, in contrast to the well-characterized bacterial and eukaryotic enzymes. The transcript levels of all three *oadhc* operons is higher in stationary phase than in exponential phase, suggesting that the enzymes might be involved in maintenance metabolism. Taken together, the results indicate that the three haloarchaeal OADHCs decarboxylate novel substrates and play different biological roles compared to their bacterial and eukaryotic homologues.

The unusual functional role of the haloarchaeal OADHCs might be explained by their evolutionary history. Several lines of arguments strongly suggest that OADHCs were not present in the last common ancestor of archaea, but that they entered archaeal genomes via horizontal gene transfer from bacteria. (1) Genes for OADHCs are present only in a

Fig. 5. Phylogeny of archaeal OADHCs. Phylogenetic trees of the E1 α and E1 β subunits were constructed using a parsimony and a distance matrix method. One hundred bootstrap replications were performed. The figure shows the distance matrix tree of the E1 α subunits. The tree includes 16 bacterial and eukaryotic OADHCs with an experimentally characterized function (PDHCs are boxed, BCDHCs are italic, ADHCs are surrounded by ovals, and the 2-oxobutyrate DHC of *P. putida* is indicated). The tree also includes all archaeal OADHCs found in fully sequenced genomes and the three OADHCs of *H. volcanii* (archaeal OADHCs are shown in bold). The following species abbreviations are used: A. pernix, *Aeropyrum pernix*; B. subtilis, *Bacillus subtilis*; C. magnum, *Clostridium magnum*; E. faecalis, *Enterococcus faecalis*; E. coli, *Escherichia coli*; H. marismortui, *Haloarcula marismortui*; H. salinarum, *Halobacterium salinarum*; H. volcanii, *Haloferax volcanii*; H. sapiens, *Homo sapiens*; K. pneumoniae, *Klebsiella pneumoniae*; M. xanthus, *Myxococcus xanthus*; N. pharaonis, *Natronosomonas pharaonis*; P. carbinolyticus, *Paracoccus carbinolyticus*; P. torridus, *Picrophilus torridus*; P. putida, *Pseudomonas putida*; P. aerophilum, *Pyrobaculum aerophilum*; R. eutropha, *Ralstonia eutropha*; S. avermiliis, *Streptomyces avermiliis*; S. solfataricus, *Sulfolobus solfataricus*; T. acidophilum, *Thermoplasma acidophilum*; T. volcanium, *Thermoplasma volcanium*; Z. mobilis, *Zymomonas mobilis*.

minority of archaea. (2) Their functions were not needed in the ancestor because the nearly universal distribution of OAFORs indicates that one or probably several members of this family was already present. (3) Even within one genus, the OADHC occurrence can vary, i.e. only one out of three *Sulfolobus* species contains OADHCs. (4) The archaeal OADHCs do not form a monophyletic group in the phylogenetic tree, which rules out that the archaeal ancestor had a single OADHC. If it had two or three OADHCs, two or three archaeal subclusters would be expected, that are remotely related to the bacterial subclusters of the respective substrate specificity. This is also not observed. (5) The most parsimonious explanation of the phylogenetic tree is that archaea acquired *oadhc* genes by very few gene transfer events, i.e. one transfer from a Gram-positive bacterium to an ancestor of halophiles (branch no. 1), one transfer to the *Thermoplasma* group (branch no. 3), one transfer to the *Aeropyrum*/*Pyrobaculum* group, and two to four transfers of *adhC* genes to *S. solfataricus* (and no other *Sulfolobus* species) and *H. volcanii* (and no other haloarchaeal species). In none of the trees do OADHCs 2 and 3 of *Haloferax* group together, and also the two *Sulfolobus* OADHCs do not cluster, which would argue in favour of four separate gene transfer events. But it might also be that only two transfer events occurred and the products of gene duplications in *H. volcanii* and *S. solfataricus* deviated due to the adaptation to new substrate specificities. All in all, five to seven gene transfer events can explain the distribution of OADHCs in archaea. In contrast, if an archaeal ancestor with at least two OADHCs is assumed, the number of gene loss events that would explain the current distribution is considerably higher and therefore much less parsimonious.

If the OADHCs entered archaeal species in which their normal functions were already fulfilled by OAFORs, three different subsequent scenarios seem possible. (1) Non-homologous gene replacement could have occurred, and the *oaf* genes could have been lost. This is not likely, because all OADHC-containing species still contain at least two different OAFORs. For *Thermococcus litoralis* it has been shown experimentally that it contains four different OAFORs, which have substrate specificities for pyruvate, 2-oxoglutarate, branched-chain 2-oxoacids and aromatic 2-oxoacids (Mai & Adams, 1996). (2) The enzymic activity is redundantly encoded by members of two very different protein families. It is possible that this happened in *Thermoplasma*, because the characterization of an E1 after heterologous production in *E. coli* revealed that it is specific for branched-chain 2-oxoacids (Heath *et al.*, 2004). (3) The OADHCs could have adapted to new substrates and to new cellular functions. As discussed above, it is not unlikely that this happened in *H. volcanii*. It also seems very possible that the as-yet-unstudied archaeal OADHCs present in additional species (Fig. 5) have adapted to new functions, and thus this protein family offers an ideal opportunity to study protein evolution.

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REFERENCES

- Allers, T., Ngo, H. P., Mevarech, M. & Lloyd, R. G. (2004). Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. *Appl Environ Microbiol* **70**, 943–953.
- Berg, A. & de Kok, A. (1997). 2-Oxo acid dehydrogenase multienzyme complexes. The central role of the lipoyl domain. *Biol Chem* **378**, 617–634.
- Bitan-Banin, G., Ortenberg, R. & Mevarech, M. (2003). Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene. *J Bacteriol* **185**, 772–778.
- Charon, M. H., Volbeda, A., Chabriere, E., Pieulle, L. & Fontecilla-Camps, J. C. (1999). Structure and electron transfer mechanism of pyruvate:ferredoxin oxidoreductase. *Curr Opin Struct Biol* **9**, 663–669.
- Chuang, D. T. (1998). Maple syrup urine disease: it has come a long way. *J Pediatr* **132**, S17–S23.
- Felsenstein, J. (1996). Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. *Methods Enzymol* **266**, 418–427.
- Frank, R. A., Pratap, J. V., Pei, X. Y., Perham, R. N. & Luisi, B. F. (2005). The molecular origins of specificity in the assembly of a multienzyme complex. *Structure* **13**, 1119–1130.
- Heath, C., Jeffries, A. C., Hough, D. W. & Danson, M. J. (2004). Discovery of the catalytic function of a putative 2-oxoacid dehydrogenase multienzyme complex in the thermophilic archaeon *Thermoplasma acidophilum*. *FEBS Lett* **577**, 523–527.
- Hengeveld, A. F. & de Kok, A. (2002). Structural basis of the dysfunctioning of human 2-oxo acid dehydrogenase complexes. *Curr Med Chem* **9**, 499–520.
- Herrmann, U. & Soppa, J. (2002). Cell cycle-dependent expression of an essential SMC-like protein and dynamic chromosome localization in the archaeon *Halobacterium salinarum*. *Mol Microbiol* **46**, 395–409.
- Jolley, K. A., Rapaport, E., Hough, D. W., Danson, M. J., Woods, W. G. & Dyall-Smith, M. L. (1996). Dihydrolipoamide dehydrogenase from the halophilic archaeon *Haloferax volcanii*: homologous overexpression of the cloned gene. *J Bacteriol* **178**, 3044–3048.
- Jolley, K. A., Maddocks, D. G., Gyles, S. L., Mullan, Z., Tang, S. L., Dyall-Smith, M. L., Hough, D. W. & Danson, M. J. (2000). 2-Oxoacid dehydrogenase multienzyme complexes in the halophilic Archaea? Gene sequences and protein structural predictions. *Microbiology* **146**, 1061–1069.
- Kerscher, L. & Oesterhelt, D. (1981). Purification and properties of 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. *Eur J Biochem* **116**, 587–594.
- Krüger, N., Oppermann, F. B., Lorenz, H. & Steinbüchel, A. (1994). Biochemical and molecular characterization of the *Clostridium magnum* acetoin dehydrogenase enzyme system. *J Bacteriol* **176**, 3614–3630.
- Mai, X. & Adams, M. W. (1996). Characterization of a fourth type of 2-keto acid-oxidizing enzyme from a hyperthermophilic archaeon:

2-ketoglutarate ferredoxin oxidoreductase from *Thermococcus litoralis*. *J Bacteriol* **178**, 5890–5896.

Milne, J. L., Wu, X., Borgnia, M. J., Lengyel, J. S., Brooks, B. R., Shi, D., Perham, R. N. & Subramaniam, S. (2006). Molecular structure of a 9-MDa icosahedral pyruvate dehydrogenase subcomplex containing the E2 and E3 enzymes using cryoelectron microscopy. *J Biol Chem* **281**, 4364–4370.

Perham, R. N. (1991). Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein. *Biochemistry* **30**, 8501–8512.

Perham, R. N. (2000). Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. *Annu Rev Biochem* **69**, 961–1004.

Ragsdale, S. W. (2003). Pyruvate ferredoxin oxidoreductase and its radical intermediate. *Chem Rev* **103**, 2333–2346.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Wanner, C. & Soppa, J. (2002). Functional role for a 2-oxo acid dehydrogenase in the halophilic archaeon *Haloferax volcanii*. *J Bacteriol* **184**, 3114–3121.

Zaigler, A., Schuster, S. C. & Soppa, J. (2003). Construction and usage of a onefold-coverage shotgun DNA microarray to characterize the metabolism of the archaeon *Haloferax volcanii*. *Mol Microbiol* **48**, 1089–1105.

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