A 2-oxoacid dehydrogenase complex of *Haloferax volcanii* is essential for growth on isoleucine but not on other branched-chain amino acids

Marco Sisignano,\(^1\) Daniel Morbitzer,\(^1\) Jochem Gätgens,\(^2\) Marco Oldiges\(^2\) and Jörg Soppa\(^1\)

1 Goethe University, Biocentre, Institute for Molecular Biosciences, Max-von-Laue-Str. 9, D-60438 Frankfurt, Germany
2 Institute for Biotechnology 2, Forschungszentrum Jülich, D-52425 Jülich, Germany

The halophilic archaeon *Haloferax volcanii* contains three operons encoding 2-oxoacid dehydrogenase complexes (OADHCs) OADHC1–OADHC3. However, the biological role of these OADHCs is not known as previous studies have demonstrated that they cannot use any of the known OADHC substrates. Even the construction of single mutants in all three *oadhc* operons, reported recently, could not identify a substrate. Therefore, all three possible double mutants and a triple mutant were generated, and single, double and triple mutants were compared to the wild-type. The four mutants devoid of a functional OADHC1 had a reduced growth yield during nitrate-respirative growth on tryptone. A metabolome analysis of the medium after growth of the triple mutant in comparison to the wild-type revealed that the mutant was unable to degrade isoleucine and leucine, in contrast to the wild-type. It was shown that *oadhc1* mutants were unable to grow in synthetic medium on isoleucine, in contrast to the other mutants and the isogenic parent strain. However, all strains grew indistinguishably on valine and leucine. The transcript of the *oadhc1* operon was highly induced during growth on isoleucine. However, attempts to detect enzymic activity were unsuccessful, while the branched-chain OADHC (BCDHC) of *Pseudomonas putida* could be measured easily. Therefore, the growth capability of the triple mutant and the wild-type on the two first degradation intermediates of isoleucine was tested and provided further evidence that OADHC is involved in isoleucine degradation. Taken together, the results indicate that OADHC1 is a specialized BCDHC that uses only one (or maximally two) of the three branched-chain 2-oxoacids, in contrast to BCDHCs from other species.

INTRODUCTION

Two different enzyme systems catalyse the oxidative decarboxylation of 2-oxoacids to yield acyl-CoA, i.e. 2-oxoacid dehydrogenase complexes (OADHC) and 2-oxoacid ferredoxin oxidoreductases (OAFOR). In addition, some bacterial species contain pyruvate oxidase that oxidatively decarboxylates pyruvate to yield acetylphosphate. OADHCs and OAFORs are not homologous and 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.

OADHCs are widely distributed in eukaryotes and in aerobic bacteria, and catalyse the following overall reaction: 2-oxoacid + NAD\(^+\) + CoA → acyl-CoA + CO\(_2\) + NADH

They are composed of three different types of subunits called E1, E2 and E3 which catalyse different steps of the reaction. E1 can either be a single polypeptide, or it comprises two subunits E1\(\alpha\) and E1\(\beta\). 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 shared 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 ‘swinging arm’ that transports them from...
Seven different substrates for OADHCs are known, i.e. pyruvate, 2-oxoglutarate, 2-oxoisovalerate, 2-oxo-3-methylvalerate, 2-oxoisocaproate, 2-oxobutyrate and acetoin. 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, 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 OADHCs can cause human diseases, e.g. a PDHC deficiency can lead to mental retardation or, in more severe cases, death at birth, while branched-chain OADHC (BCDHC) deficiency causes ‘Maple syrup disease’ (Hengeveld & de Kok, 2002; Chuang 1998). A fourth OADHC catalyses decarboxylation of 2-oxobutyrate, an intermediate in methionine catabolism. The substrate of the fifth 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:

\[
2\text{-oxoacid} + 2\text{ferredoxin}_{\text{oxidized}} + \text{CoA} - \text{acyl-CoA} + \text{CO}_2 + 2\text{ferredoxin}_{\text{reduced}}
\]

Typically OAFORs operate in energy metabolism of anaerobic bacteria. Again, the best characterized family member is the one using pyruvate as a substrate (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 thus OADHCs are not needed (Kerscher & Oesterhelt, 1981). Therefore, it was a surprise that four genes encoding an OADHC were discovered (Jolley et al., 1996, 2000). While 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 (ε3, HVO_2961) indicated that none of the seven known OADHC substrates is used by this OADHC (Jolley et al., 1996; genes HVO_2958–HVO_2961 at www.halolex.mpig.de). Subsequently, analysis of nitrate respiration-deficient mutants led to the discovery of a second oadhc gene cluster encoding OADHC2, but it was also concluded that none of the seven known substrates is used (Wanner & Soppa, 2002; genes HVO_2595–HVO_2597). Analysis of transcriptome changes following a shift from Casamino acids to glucose under aerobic conditions led to the identification of genes of a third gene cluster, oadhc3 (Zaigler et al., 2003; genes HVO_0666–HVO_0669).

The construction and phenotypic characterization of single in-frame deletion mutants of the genes encoding the E1ζ subunits of the three OADHCs revealed that the growth yield of the oadhc1 mutant is considerably lower than that of the wild-type in complex medium under nitrate-respirative conditions (van Ooyen & Soppa, 2007). In line with this observation it was revealed that the transcript level of the oadhc1 operon is higher under anaerobic than under aerobic conditions (van Ooyen & Soppa, 2007). The transcript levels of the other two oadhc operons were also enhanced during nitrate-respirative growth, but no or only a slight decrease of the growth yields of the ∆oadh3 and ∆oadh2 mutants were detected under these conditions.

In an extension of the previous study, we present here the construction and characterization of the three double mutants and a triple mutant of the three oadhc operons of Haloferax volcanii. A metabolome analysis of culture supernatants, growth capability analyses and an expression analysis has led to the identification of the substrate specificity of OADHCl, more than 10 years after its discovery.

**METHODS**

**Materials.** Enzymes for molecular genetic approaches were obtained from MBI Fermentas, Qiagen and Promega. Products from Qiagen were used for the isolation of plasmids and PCR fragments. DIG-dUTP for the labelling of probes for Southern and Northern blot analyses was obtained 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 H. volcanii strain H26 was obtained from Thorsten Allers (University of Nottingham) and was grown as described by Allers et al. (2004). The Escherichia coli XL1-blue MRF+ was obtained from Stratagene and was grown in standard media (Sambrook et al., 1989). Pseudomonas putida DSM 6125 was obtained from DSMZ (www.dsmz.de) and was grown in a complex medium recommended by the supplier.

**Construction of in-frame deletion mutants.** The in-frame deletion mutants were generated using the so-called Pop-In/Pop-Out method which was developed by Bitan-Banin et al. (2003) and subsequently optimized (Allers et al., 2004; Hammelmann & Soppa, 2008). Construction of suicide vectors including versions of the target genes carrying internal in-frame deletions has been described previously (pJ01, pJ02 and pJ03; van Ooyen & Soppa, 2007). They were used to construct three single mutants with in-frame deletions in the genes encoding the E1ζ subunit of OADHC1–OADHC3, respectively. The three possible double mutants were constructed by transforming the single mutants with a vector targeting a second oadhc gene, followed by Pop-In/Pop-Out selection. The triple mutant was constructed by transforming a double mutant with a vector targeting the third oadhc gene, followed by selection. In all cases, strain H26, the parent strain of the respective deletion experiment, the Pop-In variant and the Pop-Out variant were characterized by Southern blotting using all necessary probes to verify the two to three deletion events (data not shown), as exemplified for single mutants in a previous publication (see Fig. 2 in van Ooyen & Soppa, 2007). All strains used in this study are summarized in Table 1.

**Characterization of growth phenotypes.** The H. volcanii parent strain and mutants were grown in complex and synthetic media in 100 ml Klett flasks as described previously (van Ooyen & Soppa, 2007).
2-Oxoacid dehydrogenase complexes of *H. volcanii*

**Table 1.** *H. volcanii* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Relevant features</th>
<th>Reference</th>
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<tr>
<td>H26</td>
<td>Wild-type</td>
<td><em>ApyrE</em> for Pop-In/Pop-Out</td>
<td>Allers et al. (2004)</td>
</tr>
<tr>
<td>Δoadh1</td>
<td>H26</td>
<td>In-frame deletion in <em>oadh1</em></td>
<td>van Ooyen &amp; Soppa (2007)</td>
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<td>H26</td>
<td>In-frame deletion in <em>oadh2</em></td>
<td>van Ooyen &amp; Soppa (2007)</td>
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<td>Δoadh3</td>
<td>H26</td>
<td>In-frame deletion in <em>oadh3</em></td>
<td>van Ooyen &amp; Soppa (2007)</td>
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<td>Δoadh1</td>
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<td>This study</td>
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<td>This study</td>
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<td>Δoadh1,2,3</td>
<td>Δoadh1,2</td>
<td>In-frame deletion in <em>oadh1</em>+2+3</td>
<td>This study</td>
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2007). Cultures were inoculated with less than 0.01 vols of exponentially growing precultures grown in the same medium. The optical density was monitored using a Klett Colorimeter. For anaerobic growth the flasks were gassed with nitrogen and sealed with rubber septa. Branched-chain amino acids were added to a final concentration of 0.3 % (w/v), and the sodium nitrate concentration (anaerobic growth) was 50 mM. In all cases, three biological replicates were performed, and mean values ± 50 were calculated.

**Metabolome analysis.** For preparation of the samples, 13 ml culture supernatant was lyophilized. The dried residue was redissolved and derivatized in 50 µl 20 mg methoxyamine hydrochloride ml−1 in pyridine for 90 min at 35 °C followed by a 60 min treatment at 35 °C with 80 µl N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). Standard substances for peak identification of isoleucine, leucine and valine were dissolved in water at 10 mg ml−1. A 5 µl volume of standard solution was lyophilized and derivatized as described above. The GC/MS system consisted of an Agilent 6890N system and a GCT Premier TOF-MS (Waters). The mass spectrometer was tuned and calibrated according to the manufacturer’s recommendations using heptacosa (perfluorotri-n-butylamine).

For GC/MS analysis, sample volumes of 1 µl were injected with a split ratio of 1:2 with injector temperature of 280 °C. Gas chromatography was performed on a 30 m Factor4 VF-5ms capillary column with 0.25 mm i.d. and 0.25 µm film thickness (Varian). The carrier gas used was helium at a constant flow rate of 1 ml min−1. The temperature programme after injection was 3 min isothermal heating at 80 °C, followed by a 14 °C min−1 oven temperature ramp to 300 °C, followed by 5 min isothermal heating, followed by a 10 °C min−1 oven temperature ramp to 320 °C and a final 5 min isothermal heating at 320 °C. The GC system was then temperature-equilibrated for 1 min at 80 °C prior to injection of the next sample.

The MS interface was set to 300 °C and the ion source temperature was adjusted to 250 °C. Ions were generated by positive EI (70 eV) and mass spectra were recorded at cycle times of 0.15 s with a scanning range of m/z 50–600. The chromatograms and mass spectra were evaluated using the MassLynx software package (Waters). For peak identification and relative comparison of peak areas, compound specific mass fragments and retention times of valine (m/z=145) and leucine/isoleucine (m/z=159) were used based on measurements of reference standards. For 2-oxo-3-methylvalerate, no reference standard was available. Based on retention time behaviour of amino acids and their corresponding ketoacids, an earlier retention time window of 2-oxo-3-methylvalerate compared to isoleucine was deduced. Following standard EI fragmentation patterns of derivatized ketoacids specific fragment ions of m/z=216, 200 and 114 were calculated for 2-oxo-3-methylvalerate and used for identification in the GC/MS chromatograms.

**Northern blot analysis.** Isolation of RNA, probe preparation and Northern blot analysis were performed as described previously (van Ooyen & Soppa, 2007). Digoxigenin-labelled probes were generated using the same oligonucleotides as in a previous study (van Ooyen & Soppa, 2007).

**Enzymic tests.** The enzymic activities of the *P. putida* BCDHC with the three substrates 2-oxovalerate, 2-oxo-3-methylvalerate and 2-oxoisocaproate were determined in cytoplasmic extracts according to Lessard & Perham (1994). The assay contained 20 mM potassium phosphate buffer, pH 7, 2 mM MgCl2, 20 mM 2-oxoacid, 50 µM DCPIP (2,6-dichlorophenolindophenol) and varying concentrations of cytoplasmic extract. The assays were performed at 30 °C. Reduction of the blue oxidized form of DCPIP to the colourless reduced form was followed at 600 nm. For attempts to measure BCDHC activity in cytoplasmic extracts of *H. volcanii*, the same assay was used, except that potassium chloride was added to final concentrations between 0.8 and 2 M, substrate concentrations were varied and incubation times were prolonged up to overnight.

**RESULTS**

**Construction of in-frame deletion mutants**

In a previous study, single in-frame deletion mutants of the genes encoding the E1z subunits of all three OADHCs (see Fig. 1) were constructed (van Ooyen & Soppa, 2007). The growth yield of the Δoadhc1 mutant was about 40 % lower than that of the parent strain under nitrate-respirative conditions in complex medium, while the growth yields of the Δoadhc2 and Δoadhc3 mutants were only 20 % lower than or identical to that of the parent strain, respectively. To test whether the different OADHCs might have overlapping substrate specificities and thus single gene mutants might not develop the null-phenotype, three double mutants were constructed representing all possible combinations of the three OADHCs. Furthermore, a triple mutant was constructed which was totally devoid of a functional gene for an E1z subunit. An optimized procedure of the so-called Pop-In/Pop-Out method was used for mutant construction (Hammelmann & Soppa, 2008). In all cases the genomic organization of the respective oadhc gene clusters was analysed by Southern blot analysis, and the successful construction of the four mutants was verified (data not shown). All eight strains used in this study are summarized in Table 1.
Phenotypic characterization

At first the three double mutants, the triple mutant and the isogenic parent strain H26 were grown in synthetic medium with pyruvate or 2-oxoglutarate, respectively, as sole carbon source. All five strains grew identically with both carbon sources, corroborating that pyruvate and 2-oxoglutarate are not substrates for any of the three OADHCs (Fig. 2). Next, nitrate-respirative growth of double and triple mutants was compared to the parent strain. The complex medium which had been used in the previous study (van Ooyen & Soppa, 2007) contained both tryptone and yeast extract. This time the two C-sources were tested in separate experiments. As shown in Fig. 3(a), the parent strain H26 and all four mutants grew indistinguishably on yeast extract/nitrate, indicating that the substance which had been differentially oxidized in the parent strain and the Δoadhc1 mutant in complex medium (van Ooyen & Soppa, 2007) was not present in yeast extract. In contrast, on tryptone/nitrate, three of the mutants exhibited a growth yield reduction of about 40% (Fig. 3b), similar to the observation with the Δoadhc1 mutant in complex medium (van Ooyen & Soppa, 2007). Therefore, the double and triple mutants did not show a synthetic phenotype, indicating that they did not have overlapping substrate specificities with OADHC1. The oadhc2/oadhc3 double mutant, which contained a single functional OADHC, OADHC1, grew at least as well as the parent strain. This indicates that OADHC1 is essential for utilization of a substance present in tryptone (and absent from yeast extract) and that it catalyses the oxidative decarboxylation of an intermediate of the degradation pathway. Therefore, a global comparison of the media after growth of either the parent strain or the triple mutant appeared to be a promising approach to identify a substance which could not be used by the mutant.

Metabolome analysis of media after growth of wild-type or mutant

The triple mutant and the parent strain H26 were grown anaerobically in medium with tryptone and nitrate. At late exponential phase and 2 days after reaching stationary phase, aliquots were taken and the cells were removed by centrifugation. The compositions of the supernatants were analysed by GC/MS analysis, an established method used to study the metabolome and functional genomics (Fiehn...
et al., 2000; Oldiges et al., 2007). When the results for the branched-chain amino acids valine, leucine and isoleucine were normalized to equity for all compounds, a specific metabolic pattern for the three compounds was observed. The samples from the medium before cultivation showed all three amino acids to be present (Fig. 4a, d). The culture supernatant sample of the parent strain H26 showed an almost constant peak area for valine (Fig. 4e), but peak areas decreased by a factor of 13.8 and 15.5 for leucine and isoleucine, respectively (Fig. 4b). This gives rise to the conclusion that leucine and isoleucine are degraded, but not valine. In contrast, the triple mutant appeared to be unable to degrade valine, leucine and isoleucine (Fig. 4c, f). While the peak area of leucine only slightly increased (+7%), the isoleucine and valine peak area increased by 288% compared to the initial medium concentration. In all samples, no peak for 2-oxo-3-methylvalerate, the corresponding ketoacid of isoleucine, was identified.

Growth of wild-type and mutants on branched-chain amino acids

The indication that OADHC1 is involved in the degradation of leucine and isoleucine under nitrate-respirative conditions was surprising because it had been shown that

\( H. volcanii \) (strain WR340) is unable to grow anaerobically with branched-chain amino acids and nitrate (Wanner, 2000). Nevertheless, we tested whether the parent strain H26 could grow anaerobically with leucine/nitrate or isoleucine/nitrate, and confirmed that it was unable to grow (data not shown). Therefore, we tested whether \( H. volcanii \) can use branched-chain amino acids under aerobic conditions. Fig. 5(a) shows that neither the wild-type nor any of the mutants could grow with valine as sole carbon source. Fig. 5(b) shows growth of the parent strain, the three double mutants and the triple mutant with leucine as sole carbon source. All five strains grew rather poorly but indistinguishably, indicating that under aerobic conditions none of the three OADHCs is involved in the decarboxylation of the transamination product of leucine (2-oxoisocaproate). Fig. 5(c) shows growth of the parent strain and all seven single, double and triple mutants with isoleucine as sole carbon source. All mutants that carry a deletion in the \( oadhc1 \) gene cluster, i.e. four single, double and triple mutants, were totally unable to grow on isoleucine (curves designated by \( \Delta \)), indicating that OADHC1 is essential for the decarboxylation of the transamination product of isoleucine, 2-oxo-3-methylvalerate. The \( \Delta oadhc2 \) single mutant exhibited a very reproducible slight growth difference compared to the parent strain (curve designated with \( \# \)), but it can be excluded that OADHC2 is involved in isoleucine degradation because the \( \Delta oadhc2 \Delta oadhc3 \) double mutant grew indistinguishably from the parent strain. To gain a further indication that OADHC1 is involved in isoleucine degradation, the (differential) expression of the \( oadhc1 \) operon was analysed.

Expression analysis

The \( H. volcanii \) wild-type was grown in synthetic medium with isoleucine and with pyruvate, respectively, as sole carbon and energy source. The cultures were grown to exponential phase for more than 15 generations to fully adapt them to the two substrates. Exponentially growing cells were harvested at a cell density of about \( 5 \times 10^8 \) cells ml\(^{-1}\) and RNA was isolated. The expression of the \( oadhc1 \) operon was analysed by Northern blot analysis. Fig. 6 shows that the operon was highly expressed during growth on isoleucine, while no transcript could be detected when the cells were grown on pyruvate. As has been reported earlier (Jolley et al., 2000; van Ooyen & Soppa, 2007), the \( oadhc1 \) genes were transcribed into a single polycistronic transcript of about 5100 nt (data not shown), the size of which fits well with the combined size of the four genes (Fig. 1).

Enzymic assays

Next, we determined the enzymic activity and substrate specificity of OADHC1. Several earlier attempts to measure OADHC activity with any of the seven known OADHC substrates had been unsuccessful; therefore, the enzyme assay was first established with cell extracts of \( P. putida \), a

![Fig. 3. Anaerobic growth of the parent strain, the three double mutants and the triple mutant with yeast extract/nitrate (a) and with tryptone/nitrate (b). ■, Parent strain; ▼, \( \Delta oadhc2 \Delta oadhc3 \) double mutant.](http://mic.sgmjournals.org)
species known to contain a BCDHC. Specific activity was measured with all three BCDHC substrates [average values of three independent cultures ± SD are given in pkat (mg protein)$^{-1}$]: 2-oxoisocaproate, 12.2 ± 1.3; 2-oxo-3-methylvalerate, 25.0 ± 9.0; 2-oxoisovalerate, 17.0 ± 8.0.

Subsequently, extensive attempts were performed to adapt the enzymic assay for the measurement of BCDHC activity in cell extracts of *H. volcanii*. However, no activity could be detected with any of the three potential substrates. Therefore, another approach was chosen to corroborate that the transamination product of isoleucine, 2-oxo-3-methylvalerate, is a substrate for OADHC1.

**Growth on intermediates of isoleucine catabolism**

The first two steps of isoleucine degradation are shown in Fig. 7(a), i.e. the transamination to 2-oxo-3-methylvalerate and the oxidative decarboxylation to 2-methylbutyryl-CoA. If OADHC1 catalyses the second step, as indicated by the results presented above, a Δoadhc1 mutant should be unable to grow on the first intermediate, 2-oxo-3-methylvalerate, but should be able to grow on the second and all subsequent intermediates of the degradation pathway, if suitable transporters are present in *H. volcanii*. Therefore the wild-type, the oadhc1 single mutant and the triple mutant were grown in synthetic medium with 2-oxo-3-methylvalerate and with 2-methylbutyrate as sole carbon sources. The results are shown in Fig. 7(b, c). As predicted, both mutants were unable to grow on 2-oxo-3-methylvalerate, in contrast to the wild-type, indicating that 2-oxo-3-methylvalerate is the substrate of OADHC1. Unfortunately, none of the strains grew on 2-methylbutyrate, possibly because *H. volcanii* is unable to import this substance.

**DISCUSSION**

Several previous publications came to the conclusion that OADHC1 and, moreover, none of the three OADHCs can decarboxylate branched-chain 2-oxoacids or can use any of the other known OADHC substrates (e.g. Jolley et al., 1996, 2000; Wanner & Soppa, 2002; van Ooyen & Soppa, 2007; Al-Mailem et al., 2008). The conclusions were partly based...
on the inability to detect any enzymic activity, despite a variety of trials. Furthermore, *H. volcanii* was reported to be unable to grow on any of the three branched-chain amino acids under aerobic conditions (Jolley et al., 1996) and under nitrate-respiratory conditions (Wanner, 2000). Recently, it was reported that *H. volcanii* can in fact grow aerobically on all three branched-chain amino acids with growth yields in the order isoleucine > leucine > valine, but a renewed attempt to detect an enzymic activity failed, like all previous attempts (Al-Mailem et al., 2008). Therefore, it was very surprising to find that the substances in tryptone, which can be degraded by the wild-type but not by the triple *oadhc* mutant, were the branched-chain amino acids leucine and isoleucine. This result, together with the following results, provide compelling evidence that the transamination product of isoleucine, 2-oxo-3-methylvalerate, is a substrate of OADHC1: (1) all four mutants with a deletion in the *oadhc1* operon are unable to grow on isoleucine, in contrast to the isogenic parent strain H26; (2) expression of the *oadhc1* operon is highly induced during growth on isoleucine, and the transcript is undetectable during growth on pyruvate; (3) the mutant cannot grow on 2-oxo-3-methylvalerate, in contrast to the wild-type; and (4) the enzymic activity of the E3 subunit is enhanced during growth on isoleucine (Al-Mailem et al., 2008). Whether or not the transamination product of leucine, 2-oxoisocaproate, is also a substrate of OADHC1 could not be clarified. Metabolome analysis indicated that leucine is differentially degraded by the parent strain and the triple mutant; on the other hand, the parent strain and all mutants grew indistinguishably with leucine as sole carbon source under aerobic conditions. However, there are no indications that the transamination product of valine, 2-oxoisovalerate, is a substrate of OADHC1.

The inability to measure an enzymic activity of either E1 or the whole complex is still puzzling. Possible reasons include (1) that the haloarchael E1 cannot use the artificial electron acceptor DCPIP, in contrast to BCDHCs from other species, and (2) that the E3 subunit dissociates from the rest of the complex during the generation of a cytoplasmic extract. Another puzzling result was the fact that the wild-type degrades isoleucine during nitrate-respirative growth in complex medium, but is unable to

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**Fig. 5.** Aerobic growth of the parent strain, the three double mutants and the triple mutant in synthetic medium with valine (a), leucine (b) or isoleucine (c) as sole carbon source. (a–c). Parent strain; ◆, *∆oadhc2 ∆oadhc3* double mutant. (c) Growth curves of the three single mutants are also included. 1, All mutants containing a *∆oadhc1* deletion (one single, two double and the triple mutant); *, *∆oadhc2* single mutant; #, *∆oadhc3* single mutant, *∆oadhc2 ∆oadhc3* double mutant.

**Fig. 6.** Differential expression of *oadhc1* in cells grown in synthetic medium with either pyruvate (Pyr) or isoleucine (Ile) as sole carbon source. Top: Northern blot analysis with an *oadhc1*-specific probe. Bottom: ethidium-bromide-stained 16S rRNA.
grow anaerobically with isoleucine and nitrate. A possible explanation is that the synthetic medium lacks a precursor for a prosthetic group of an enzyme that is only involved in the anaerobic, but not in the aerobic isoleucine degradation pathway.

In contrast to typical BCDHCs of many species, which use all three 2-oxoacids as substrates, OADHC1 seems to be specialized to the oxidative decarboxylation of 2-oxo-3-methylvalerate (and perhaps 2-oxoisocaproate). To our knowledge this is the first report of a specialized 'BCDH' which uses only one of the three branched-chain 2-oxoacids. This specialty might be explained by the phylogenetic history of the oadh1 operon in haloarchaea. The genes most probably entered a haloarchaeal ancestor via lateral transfer of a bcdhc operon from a bacterium (event '1' in Fig. 5; van Ooyen & Soppa, 2007), because all sequenced haloarchaeal genomes contain at least one oadh1 operon, in contrast to the genomes of most other Archaea. If the genome of this ancient haloarchaeon encoded only enzymes for the rest of the transport and degradation pathway of isoleucine, but not the other two branched-chain amino acids, this might have led to the specialization of the OADHC during evolution. Alternatively, the bacterial donor might already have encoded a specialized BCDHC and specialized bacterial BCDHCs are still to be discovered. The Thermoplasma group probably also obtained the oadh genes via lateral transfer of bcdhc genes from a bacterium (event '3' in Fig. 5 in van Ooyen & Soppa, 2007). In this case it was shown that the recent archaeal genes still encode a bona fide BCDHC that uses all three 2-oxoacids as substrates (Heath et al., 2004, 2007). In addition, it also decarboxylates pyruvate, albeit with reduced efficiency.

The growth capabilities of the double and triple mutants revealed that OADH2 and OADH3 do not use pyruvate, 2-oxoglutarate or any of the three branched-chain 2-oxoacids as substrates. Unfortunately, no indication could be found which substrate they might use. Both operons were most probably obtained by lateral transfer of genes encoding acetoin dehydrogenase complexes from a bacterium into the genome of H. volcanii (event ‘4’ in Fig. 5 in van Ooyen & Soppa, 2007). However, it has been shown previously that H. volcanii cannot grow on acetoin (Wanner, 2000). Therefore, the double and triple mutants will be used to test further 2-oxoacids as possible substrates.

Taken together, the combination of molecular genetics, metabolomics and physiology enabled us to identify the substrate of one of the three OADHCs of H. volcanii, OADHC1, which has been a mystery for more than a decade. It is the first BCDHC that uses only one (or maximally two) of the three branched-chain 2-oxoacids that are generated by transamination of the three branched-chain amino acids, and might therefore be designated a 2-oxo-3-methylvalerate dehydrogenase complex.

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REFERENCES


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