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4 **Temperature-Dependent Acetoin Production by *Pyrococcus furiosus* is Catalyzed by a**
5 **Biosynthetic Acetolactate Synthase and Its Deletion Improves Ethanol Production**
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Abstract

The hyperthermophilic archaeon, *Pyrococcus furiosus*, grows optimally near 100°C by fermenting sugars to acetate, carbon dioxide and molecular hydrogen as the major end products. The organism has recently been exploited to produce biofuels using a temperature-dependent metabolic switch using genes from microorganisms that grow near 70°C. However, little is known about its metabolism at the lower temperatures. We show here that *P. furiosus* produces acetoin (3-hydroxybutanone) as a major product at temperatures below 80°C. A novel type of acetolactate synthase (ALS), which is involved in branched-chain amino acid biosynthesis, is responsible and deletion of the *als* gene abolishes acetoin production. Accordingly, deletion of *als* in a strain of *P. furiosus* containing a novel pathway for ethanol production significantly improved the yield of ethanol. These results also demonstrate that *P. furiosus* is a potential platform for the biological production of acetoin at temperatures in the 70 to 80°C range.

Keywords: Archaea, Hyperthermophile, Genetic engineering, Acetolactate Synthase, Acetoin, Ethanol

1. Introduction

Acetoin, or 3-hydroxybutanone, is an important four-carbon compound that serves as a building block for valuable bio-based chemical compounds and is a common flavor additive and preservative in the food industry (1). Acetoin can be chemically synthesized from fossil feedstocks, but biologically produced acetoin is preferable for use in the food and cosmetics industries despite its higher cost (2). The most efficient acetoin producers are species from the bacterial genera *Bacillus*, *Paenibacillus*, *Serratia*, *Enterobacter* and *Klebsiella*. These microorganisms produce acetoin via a well-characterized pathway that involves two key enzymes, acetolactate synthase (ALS)¹ and acetolactate decarboxylase (ALDC), versions of which have been extensively characterized in *Lactococcus lactis* (3). Naturally produced acetoin has been in demand throughout the past decade, leading to the metabolic engineering of many *Bacillus* and *Clostridium* strains for producing acetoin and butanediol or co-producing butanol and acetoin (2, 4). To date, most reports on acetoin and butanediol production involve mesophiles, with optimal growth temperatures ranging from 30-40°C, making the processes more prone to contamination (5). To reduce the risks of contamination during fermentation, thermophilic organisms have been considered. For example, a recently isolated strain of *Geobacillus* that grows optimally at 45-55°C produced up to 90 mM acetoin (6).

Herein, we report that the hyperthermophilic archaeon *Pyrococcus furiosus* produces acetoin in a temperature-dependent manner. *P. furiosus* is an obligate heterotroph that grows optimally near 100°C by fermenting simple and complex sugars to acetate, carbon dioxide and molecular hydrogen (7). With the recent availability of a genetic system (8), *P. furiosus* has been

¹ Abbreviations used are as follows: ALS, α -acetolactate synthase; ALDC, α -acetolactate decarboxylase; AOR, aldehyde ferredoxin oxidoreductase; AHAS, α -acetohydroxy acid synthase; ILV, isoleucine, leucine and valine; POR, pyruvate ferredoxin oxidoreductase; ACSI, acetyl-CoA synthase I; DM, defined medium.

exploited and engineered to produce biofuels and desired products via a strategy based on a temperature-dependent metabolic switch (9-12). This approach involves the expression of genes and pathways from organisms with growth optima almost 30°C lower than that of *P. furiosus*. As such, cultivation at these lower temperatures drives product formation from a heterologous pathway, while minimizing host metabolism, but not to the extent that cell function is completely compromised.

Using metabolic engineering hosts at temperatures substantially below their normal growth temperatures represents a new strategy for producing bio-based chemicals and fuels (10, 12). Although *P. furiosus* is one of the best-studied hyperthermophiles, very little is known about its metabolic function at suboptimal growth temperatures. Genome-wide transcriptional analyses of cold-shocked and cold-adapted *P. furiosus* cells demonstrated that the expression of a surprising number of genes were either up-regulated or down-regulated upon a temperature shift from 98° to 72°C (13). However, no analyses of metabolites were performed during growth at the lower temperature. Herein, we demonstrate that *P. furiosus* produces acetoin as a major end product at growth temperatures below 85°C in a temperature-dependent manner, and that this is accomplished via a unique type of ALS whose gene expression and biochemical function are also temperature-dependent. Growth studies on deletion strains lacking ALS showed that it is necessary for branched-chain amino acid biosynthesis and, furthermore, that its absence can improve biofuel production in *P. furiosus* by redirecting the carbon flow toward ethanol.

2. Materials and methods

2.1 PCR product and vector construction

A linear PCR construct was designed to delete the *als* gene (PF0935) from the *P. furiosus* genome. The *pyrF* pop-out marker cassette containing 65 bp identical flanking regions for marker removal via homologous recombination (10) was sandwiched between ~0.5 kb homologous flanking regions of the *als* gene. Each PCR product was obtained separately, and the full linear ~2 kb PCR construct was assembled via splicing by overlap extension and PCR (14).

The pDN005 vector was designed to insert an ALS overexpression cassette into the *P. furiosus* genome. The *als* expression cassette, consisting of the 182 bp *slp* promoter (10), the *als* gene (PF0935) and a 19 bp terminator sequence of the *hpyAI* gene (5'-aatcttttagcacttt), was assembled with the backbone vector pGL009 containing the *pyrF* popout cassette and ~0.5 kb homologous flanking regions targeting the intergenic space between convergent genes PF1232 and PF1233 (termed genome region 5) (11). The pDN005 vector was assembled using Gibson Assembly (NEB) (15) and was sequence-verified.

2.2 Strain construction

All strains used and constructed are listed in **Table 1**. The linear *als* deletion construct was transformed into *P. furiosus* COM1, as previously described (8). Transformants were cultured and purified twice on liquid and solid defined cellobiose medium (8). Genomic DNA was isolated using the ZymoBeadTM Genomic DNA Kit (Zymo Research) and isolates were screened by PCR, using primers that target outside of the homologous flanking regions. A strain

containing the *als* deletion was sequence-verified and designated MW250 (herein referred to as Δ ALS).

Markerless versions of the ADHA strain (11) and the markerless Δ ALS strain were constructed by counter-selection for the loss of the *pyrF* marker cassette, as previously described (16, 17) to allow further genetic manipulation. The markerless strains were designated as MW610 and MW260, respectively. MW610 subsequently was transformed with the linear *als* deletion construct in the same manner described above to create an *als* deletion in the ADHA strain background. A sequence-verified isolate was saved, designated MW618, and is referred herein as ADHA- Δ ALS. MW260 was transformed with the linearized pDN005 plasmid in order to complement the *als* deletion with a highly expressed *als* gene. Transformants were screened and purified, as detailed above. This strain was designated MW268 and is referred herein as GR5:ALS.

2.3 Growth and cell protein quantification

Strains were cultured in artificial seawater medium containing the following components per liter: 1x base salt (18), 1x trace minerals (18), 0.26 μ M sodium tungstate, 0.25 μ g resazurin, 0.5 g cysteine, 1g sodium bicarbonate, 0.5 g sodium sulfide, 5 g maltose, and either 0.5 or 2 g yeast extract for minimal (YM) and rich (YMR) complex medium, respectively. For growth curves using medium lacking branched chain amino acids, yeast extract was replaced with 1x amino acids mix (either including or lacking the branched chain amino acids Val, Leu, and Ile) and 1x vitamin mix (8). After all components were added, media were adjusted to pH 6.8 prior to bottling and the bottle head space was replaced with argon, with cycling three times between

vacuum and argon to ensure no trace of oxygen was present. In this study, growth was performed in 100 mL serum bottles containing 50 mL of medium, unless otherwise indicated. Fresh cultures to be used as inocula were prepared a day in advance of experiments, and inocula were standardized by cell count using a Petroff-Hausser counting chamber. During growth, 1 mL samples were collected periodically. Cell pellets were obtained by centrifugation at 10,000 x g for 10 min and used for protein quantification. Spent media were saved for further metabolite identification and quantification (see below). Protein was quantified as follows: cells were lysed by adding 1 mL water with vortexing and a freeze-thaw cycle, insoluble cell debris was removed by 1 min centrifugation at 10,000 x g, and soluble protein was quantified with a BioRad protein assay kit.

2.4 Cell Suspension Experiments

P. furiosus ADHA and ADHA-ΔALS strains were grown at 72 °C for 46 h to reach late log phase ($<1 \times 10^8$ cells per milliliter), pelleted by centrifugation ($6,000 \times g$) for 10 min, and then resuspended in 1x base salt (18) and 100 mM MOPS buffer pH 7.5 (1/10 of the original culture volume to achieve a 10× concentration). Maltose and/or pyruvate were added to achieve 10 mM and 100 mM concentration, respectively. Then the headspace was replaced with argon and the assay tubes were incubated at 72 °C, 50 rpm.

2.5 Metabolites quantification

Ethanol, acetate, and acetoin in spent media were measured and quantified using an Agilent 7890A Gas-Chromatography instrument, equipped with a Carbowax/20m column and an FID detector. The spent media was acidified with formic acid to 100 mM concentration prior to injecting.

2.6 Cytoplasmic extract preparation

To obtain whole cell extract for ALS activity assays, strains were cultured in YMR medium to stationary phase. Cells were collected and suspended in 50 mM MOPS pH 7.5 under strict anaerobic conditions and then lysed by three rounds of 45 sec sonication (Qsonica Q55, amplitude 30) and 15 sec rest. To remove the membrane fraction, cell lysates were centrifuged at 100,000 x g for 30 min. The cytoplasmic extract was stored in stoppered glass vials at -20 °C until used.

2.7 ALS assay

The ALS activity assay was modified from a previously published method which allows the indirect quantitation of α -acetolactate by measuring acetoin formation (19). The assay was conducted anaerobically in sealed vials containing 10 μ M of flavin adenine dinucleotide (FAD), 1 mM of thiamine pyrophosphate (TPP) and 10 mM of pyruvate in 50 mM EPPS buffer pH 8.4, supplemented with 2 mM $MgCl_2$. Whole cell extracts were added, and the assay vials were incubated at temperature ranging from 60 to 95°C to activate ALS, thereby catalyzing the condensation and decarboxylation of two pyruvates to one α -acetolactate . Aliquots (100 μ L)

were taken from the assay vials at intervals of 0, 5, 10, 30 and 60 min. The assay samples were acidified with 1% (vol/vol) H₂SO₄ at 60°C to chemically convert α-acetolactate to acetoin. Acetoin was detected by adding equal volumes of creatine (1.5%, wt/vol) and α-naphthol (5%, wt/vol) in 5 N of NaOH to the assay samples. The amount of acetoin produced was quantitated by the absorbance at 525 nm (19).

2.8 ALDC assay

The α-acetolactate decarboxylase activity assay was modified from a previously published method (20). Ethyl-2-acetoxy-2-methylacetoacetate (Sigma-Aldrich) was treated with 0.5 M NaOH for approximately 60 min at room temperature to produce α-acetolactate, the direct substrate of ALDC. The assay vials contained the substrate solution (approximately 5 mM final concentration) in 50 mM EPPS buffer, pH 8.4, supplemented with 2 mM MgCl₂. Whole cell extract was added and vials were incubated at 4, 25, 60, 70, 80 and 95 °C for 30 minutes. A control vial lacking cell extract was also included. Acetoin was detected as described in section 2.7.

3. Results and discussion

3.1 Acetoin is produced by wild-type *P. furiosus* during growth at sub-optimal temperatures

A strain of *P. furiosus*, termed ADHA, was previously engineered to heterologously express an alcohol dehydrogenase gene (*adhA*) from *Thermoanaerobacter* sp. X514 that grows optimally near 70°C. The ADHA strain was shown to produce ethanol optimally between 70 and 80°C by way of the heterologous AdhA functioning in concert with the *P. furiosus* enzyme aldehyde ferredoxin oxidoreductase (AOR), a pathway termed the AOR/AdhA pathway (11). However, metabolite analyses showed that strain ADHA generated acetoin in addition to ethanol. To determine if this was a property inherent to the recombinant strain, acetoin production was compared in strain ADHA, the wild type (DSM3638) and in the genetic background control strain COM1c (17). COM1c is a uracil prototrophic version of the COM1 uracil auxotrophic genetic background strain and is used as a control in place of COM1 since there is a slight phenotype associated with uracil auxotrophy even when uracil is supplied in the growth medium.

All strains were cultured at 78°C for 36 h as well as at 98°C for 12 h (to late stationary phase) in minimal maltose medium (YM), and the spent medium was analyzed for metabolite identification and quantification. As shown in **Figure 1A**, acetate is produced at 78 and 98°C in all three strains but, surprisingly, acetoin is produced at significant concentrations only at 78°C, and the ADHA has the highest acetoin formation at 78°C. To determine the temperature range of acetoin production, the ADHA strain was cultured in YM medium at temperatures ranging from 70°C to 93°C for six days, and acetoin production was analyzed at the end point of growth (**Figure 1B**). The highest amount of acetoin (6 mM) was produced between 70°C to 78°C. In

contrast, acetoin accumulation dropped rapidly above 80°C, and only 0.3 mM acetoin was formed at 98°C. Hence, *P. furiosus* produces acetoin as one of its major metabolites in a temperature-dependent manner.

Figure 1. *P. furiosus* produces acetoin at sub-optimal growth temperatures. (A) Acetate (left) and acetoin (right) accumulation after 36 h of growth at 78 °C (open bars) or 12 h of growth at 98 °C (closed bars) for the indicated strains (see Table 1). (B) Temperature dependence of acetoin formation in the ADHA strain. Samples were collected from cultures grown at temperatures ranging from 70°C to 93°C. Ethanol was produced by the ADHA strain at concentrations up to 4 mM. Each strain was grown in biological triplicate.

3.2 Acetoin is produced via spontaneous breakdown of α -acetolactate

P. furiosus contains no homolog for ALDC, the enzyme that catalyzes the decarboxylation of α -acetolactate to acetoin as a mechanism bacteria use to produce acetoin (3). Hence, the formation of acetoin in *P. furiosus* is likely due to the spontaneous breakdown of α -acetolactate during growth at high temperatures (21) (**Figure 2A**). To validate this hypothesis, approximately 5 mM α -acetolactate substrate solution was incubated at different temperatures with and without *P. furiosus*, ADHA strain, whole cell extract (**Figure S2**). When the assay solutions were incubated at 4 °C and 25 °C, there was virtually no acetoin formation, while at 60 °C and above, α -acetolactate breakdown to acetoin was observed equally in samples with or without added cell extract. At 70 °C and above, about 80% of the α -acetolactate was converted to acetoin after 30 min (**Figure S2**). This result leads us to believe that *P. furiosus* does not contain an ALDC or a non-specific decarboxylase that could catalyze the conversion of α -acetolactate to acetoin, and that acetoin formation is merely due to the spontaneous breakdown of α -acetolactate at temperatures above 70 °C.

Figure 2. Acetoin formation via ALS and its genomic context. (A) Schematic of proposed pathway for acetoin formation. (B) Diagram of ALS-containing gene cluster including ILV biosynthetic genes. (*als*: α -acetolactate synthase, PF0935; *ilvC*: ketol-acid reductoisomerase, PF0936; *leuA1*: 2-isopropylmalate synthase, PF0937; *leuC*: 3-isopropylmalate dehydratase large subunit, PF0938; *leuD*: 3-isopropylmalate dehydratase small subunit, PF0939; *leuB*: 3-isopropylmalate dehydrogenase, PF0940; *leuA2*: α -isopropylmalate transferase, PF0941; *ilvD*: dihydroxy-acid dehydratase; PF0942). (C) Schematic of carbon flow from glucose to pyruvate, acetoin and ethanol in the recombinant ADHA strain (POR: pyruvate ferredoxin oxidoreductase, ACS: acetyl-CoA synthase; AOR: aldehyde oxidoreductase; AdhA: alcohol dehydrogenase A; ALS: α -acetolactate synthase). Glucose fermentation branches to acetoin production pathway.

3.3 ALS activity is temperature-dependent

There are two enzymes in nature that are known to produce α -acetolactate from pyruvate, a catabolic ALS encoded by the *alsSD* operon, which is involved in the production of acetoin and butanediol, and an anabolic α -acetohydroxy acid synthase (AHAS) that participates in biosynthesis of the branched chain amino acids isoleucine, leucine and valine (ILV) (21). Some species contain both enzymes and/or multiple copies of AHAS (**Figure S1**). The flux of α -acetolactate toward amino acid biosynthesis is regulated via allosteric activation of ALDC by either valine or leucine (21). *P. furiosus* contains one ALS enzyme, encoded by PF0935, and it is presumed to be involved in branched chain amino acid biosynthesis, since its gene is clustered with those involved in ILV biosynthesis (PF0935-PF0942; **Figure 2B**).

To determine if *P. furiosus* contained ALS activity, we assayed for the conversion of pyruvate to α -acetolactate in the ADHA strain which also contains a pathway for ethanol production (see diagram in **Figure 2C**). The ADHA strain was grown at 77.5°C, within the optimal temperature range for acetoin production (see Figure 1B), and the cytoplasmic extract was assayed from 60 to 95°C. As shown in **Figure 3A**, ALS specific activity peaked between 80 and 85°C, indicating that the enzyme is optimally active at 15 to 20°C below the optimal growth temperature of *P. furiosus*. To determine how growth temperature affects ALS activity, ALS specific activity was assayed at 80°C in cytoplasmic extracts prepared from cells grown at temperatures ranging from 70 to 93°C. ALS activity, normalized to total protein detected in whole cell extract, was maximal between 75 and 77.5°C, and decreased as the temperature increased, with very low activity measured in extract from cells grown above 85°C (**Figure 3B**). These results suggest that the enzyme responsible for ALS activity in *P. furiosus* is maximally expressed between 72 and 80°C. The observed temperature-dependent activity profile of ALS correlates with the increased expression of this gene at the lower growth temperatures, since

previous transcriptomic analyses showed that expression of *als* increased by ~10 fold when *P. furiosus* was grown at 72 versus 98°C (13). All of these data correlate well with the temperature-dependent formation of acetoin *in vivo* (see **Figure 1B**) and suggest that ALS is the enzyme responsible.

Figure 3. ALS activity is temperature-dependent. (A) Temperature profile for ALS activity in the cytoplasmic fraction of strain ADHA grown at 77.5°C. (B) ALS activity profile in cytoplasmic extracts of strain ADHA grown at various temperatures. Assays were performed at 80°C. The assay substrate, pyruvate, becomes unstable at temperatures above 85°C. Cultures were grown in triplicate and assays were performed in triplicate.

3.4 ALS is responsible for acetoin formation in *P. furiosus*

To determine whether the ALS enzyme encoded by the gene PF0935 is solely responsible for acetoin production in *P. furiosus*, deletions of *als* were constructed in the *P. furiosus* COM1 strain as well as in the ADHA strain (**Table 1**). These strains will be referred to herein as Δ ALS and ADHA- Δ ALS, respectively. To analyze growth and metabolite formation (ethanol, acetate and acetoin), the Δ ALS and ADHA- Δ ALS strains were grown at 75°C, along with their parental COM1c and ADHA strains using production of soluble cell protein as a measure of growth. While the growth of ADHA- Δ ALS was similar to COM1c (**Figure 4A**), the ADHA strain grew slightly better than COM1c while the Δ ALS strain exhibited a lag phase (**Figure 4A**).

Figure 4. ALS deletion abolishes acetoin formation and improves ethanol production in the ADHA strain. The strains are designated as follows: COM1c (blue, diamond), Δ ALS (red,

square), ADHA (green, triangle) and ADHA- Δ ALS (purple, circle). Growth curves based on soluble protein content of samples from cultures grown in YM medium at 75°C (A). Specific metabolite accumulation at 57 h of growth was measured (B), and the concentrations of acetoin (C), ethanol (D) and acetate (E) were determined. All growths and analyses were performed in biological triplicate.

The Δ ALS and ADHA- Δ ALS strains produced no acetoin, even after 57 h of growth, while specific acetoin production by the parental COM1c and ADHA strains was comparable (105.1 ± 24.6 and 102.0 ± 27.3 $\mu\text{mol mg}^{-1}$ protein, respectively; **Figure 4B**). These results confirm that ALS is responsible for acetoin production in *P. furiosus*. Furthermore, they suggest that acetoin production is beneficial to the cell, since both strains lacking ALS did not grow as well as their respective parental strains. The ADHA strain produced the highest amount of acetoin (6.1 ± 1.0 mM) (**Figure 4C**). These data also suggest that *P. furiosus* is unable to take up acetoin, since the concentration of acetoin continues to rise throughout the growth phase for the COM1c and ADHA strains. Only one archaeon, *Sulfolobus solfataricus*, has been reported to utilize acetoin as an energy source and, although it contains an *als* homolog similar to *P. furiosus als*, it is not known to produce acetoin (22).

During growth on carbohydrates, *P. furiosus* metabolizes glucose via a modified Embden-Meyerhof pathway (23, 24), and the resulting pyruvate is converted to acetyl-CoA via pyruvate ferredoxin oxidoreductase (POR; (25)). Acetyl-CoA is then converted to acetate and CO₂ via acetyl-CoA synthase I (ACSI) in an energy-conserving manner (26, 27). We, therefore, hypothesized that deletion of *als* would increase the flux of pyruvate through ACSI (**Figure 2C**).

The amount of acetate produced in the spent media was analyzed and, as expected, acetate formation in Δ ALS and ADHA- Δ ALS was higher than in the parental strains COM1c and ADHA, respectively (**Figure 4D**). Specific acetate formation was ~30% higher in Δ ALS compared to COM1c and ~56% higher in ADHA- Δ ALS compared to ADHA (**Figure 4B**).

3.5 Deletion of ALS improves ethanol production

While *P. furiosus* is not known to natively produce ethanol in significant amounts, the engineered ADHA strain can convert acetate to ethanol through the AOR/AdhA pathway, while conserving energy via the recycling of ferredoxin (11). Therefore, we hypothesized that ethanol production would increase in the ADHA strain when ALS was deleted. While insignificant amount of ethanol were produced by COM1c and Δ ALS, ethanol production in ADHA- Δ ALS reached 4.0 ± 0.2 mM after 72 h of growth, which is about 25% higher than that produced in the ADHA strain (**Figure 4E**). In addition, specific ethanol production in ADHA- Δ ALS was about 50% higher than that in ADHA (107.5 ± 15.2 vs. 68.1 ± 10.0 $\mu\text{mol mg}^{-1}$ protein, respectively; **Figure 4B**).

To further investigate the impact of ALS deletion on the ADHA strain, a 10x concentrated whole cell suspension assay was employed to examine the distribution of major metabolites in the ADHA and ADHA- Δ ALS strains using an assay temperature of 72 °C, with the addition 10 mM maltose as the carbon source. Both strains produced ethanol linearly for 18 h. The ethanol production rate of ADHA- Δ ALS is at 1.9 mM per hour, 73% faster than that of the ADHA strain, 1.1 mM per hour. The AOR/AdhA synthetic pathway theoretically converts 0.5 mol glucose to 1 mol ethanol as shown in the equation below:

Therefore, the maximal theoretical yield of ethanol from 10 mM maltose is 40 mM. After 18 h, The ADHA-ΔALS strain produced 35.0 mM ± 2.0 mM ethanol, which is near 90% of the maximal yield, higher than the amount of ethanol produced by the ADHA strain, at 20.2 ± 2.8 mM or about 50% of the maximal yield (**Figure 5**). Since the flux from pyruvate to acetate is increased in the absence of ALS (**Figure 2C**), more acetate can be converted to ethanol in the ADHA-ΔALS strain via the AOR/AdhA pathway. Taken together, these results demonstrate that ALS is responsible for acetoin production in *P. furiosus*, and that deletion of the corresponding gene can be used to effectively increase product formation, depending on the growth condition, to either acetate or ethanol, at temperatures in the range of 72 to 80°C. It should be noted that the concentration of ethanol produced by the ADHA strain shown in **Figure 4E** is lower than that previously reported (11). This is because in the present study, all cultures were incubated with shaking, causing an increase in hydrogen production and a corresponding decrease in ethanol production.

Figure 5: ALS deletion improves ethanol yield in the ADHA strain. Formation of ethanol over time by the ADHA (opened bars) and the ADHA-ΔALS (closed bars) strains in the 10x-concentrated cell suspension assays. Both strains were supplemented with 10 mM maltose and 5

g L⁻¹ yeast extract in 100 mM MOPS buffer, pH 7.5, incubated at 72 °C and stirred at 50 rpm. All assays and analyses were performed in triplicate.

3.6 High level expression of ALS uncouples temperature-dependence of acetoin formation

To determine if addition of *als* would restore acetoin production in the Δ ALS strain, an *als* expression cassette utilizing the promoter (P_{slp}) of the highly expressed gene encoding the S-layer protein was inserted at genome region 5, a region that has been determined to have little to no transcriptional activity according to tiling array data (28). This strain will be referred to as GR5:ALS. To compare growth and metabolite production at 78°C versus 98°C, the GR5:ALS, Δ ALS and COM1c strains were grown in YM medium at both 78 and 98°C. At both temperatures, Δ ALS and COM1c strains have similar growth characteristics. However, at 78°C, GR5-ALS has a slower growth rate than the control, but not at 98°C (**Figure 6A**). Acetoin formation was absent in the Δ ALS strain, as expected, at both 78 and 98°C. However, GR5:ALS produced acetoin during growth at 78°C, and surpassed COM1c in acetoin production in late stationary phase (4.7 ± 0.7 vs. 3.4 ± 0.2 mM, respectively; **Figure 6B**). The GR5:ALS strain also produced acetoin even at 98°C, up to 1.6 ± 0.1 mM after 16 h of growth, which is three times higher than the acetoin formation of COM1c (0.50 ± 0.05 mM), although this concentration is still far lower than that produced at 78°C. Interestingly, even when the *als* gene is highly expressed at 98°C, not as much acetoin is produced compared to growth at 78°C. This could be due either to the lower specific activity of the enzyme ALS at the higher temperatures (**Figure 3A**) or to an increase in activity of ketol-acid reductoisomerase (IlvC), the ILV pathway enzyme

utilizing α -acetolactate, either of which would decrease the pool of α -acetolactate that can be spontaneously converted to acetoin. We hypothesized that *als* overexpression would effectively reduce pyruvate flux toward acetate. However, the GR5:ALS strain produced similar concentrations of acetate as the COM1c strain, while Δ ALS, as expected, produced the highest amount of acetate at 78°C (**Figure 6C**). Presumably, even though *als* is overexpressed, sufficient amounts of pyruvate must be oxidized and acetate produced to maintain the redox balance (via reduced ferredoxin production by POR) and ATP supply (via ACSI).

Figure 6: Expression of ALS restores acetoin production in the Δ ALS strain. Growth comparison between strains COM1c (blue, diamond), Δ ALS (red, square) and GR5:ALS (black,

circle) at 78 °C versus 98 °C in YM medium, showing growth (A), acetoin production (B), and acetate production (C). Growths were performed in biological triplicate.

In addition, ALS activity was measured in cytoplasmic extracts of strain GR5:ALS along with those of COM1c and Δ ALS, grown at 78°C and 98°C. The Δ ALS extract did not have any detectable ALS activity, confirming a clean genetic background for this strain. The extract of COM1c cells grown 98°C exhibited very low activity, and very low activity was detected in COM1c cells grown at 78°C growth ($0.020 \pm 0.001 \mu\text{mol min}^{-1} \text{mg}^{-1}$). Indeed, the activity of ALS in cells grown at 98°C is below the detection limit, even though a low concentration of acetoin is produced by COM1c at 98°C. In contrast, the specific activity of GR5:ALS cells grown at 78°C was approximately 20-fold higher ($0.34 \pm 0.03 \mu\text{mol min}^{-1} \text{mg}^{-1}$) than that of COM1c grown at 78°C. The dramatic increase in ALS activity in the GR5-ALS strain does not correspond with the marginal increase in acetoin production *in vivo* compared to the control strain COM1c.

To rule out the possibility that the availability of pyruvate is a major factor that limits acetoin production, acetoin formation in the ADHA strain was monitored in a 10x concentrated cell suspension assay with or without addition of 100 mM pyruvate. In medium with excess pyruvate (100 mM), only $8.5 \pm 0.8 \text{ mM}$ acetoin was detected after 18 h. However, it is still about five-fold higher than that detected in medium without additional pyruvate ($1.6 \pm 0.3 \text{ mM}$) (**Figure 7**). This result suggests that the availability of pyruvate is not what limits ALS activity. It is possible that the flux of pyruvate to ALS may be controlled by the redox balance (via POR

activity) and/or energy charge of the cell (via ACSI activity), although the mechanisms involved are not known.

Figure 7. Acetoin formation is increased in the presence of excess pyruvate. Acetoin production over time by the ADHA strain in a 10x concentrated cell suspension assay without (opened bars) and with 100 mM pyruvate (closed bars). In addition, 10 mM maltose and 5 g L⁻¹ yeast extract were also added. All assays were performed at 72 °C and 50 rpm, in triplicate.

It is unclear why *P. furiosus* produces acetoin rather than acetate (or ethanol with the ADHA strain) at sub-optimal growth temperatures, as there appears to be no energetic advantage. It is possible that cold-adapted growth causes a decrease in POR activity, relative to the glycolytic flux. The expression of the genes encoding POR do not change significantly between 72 and 98°C (13); however, its specific activity is ~20 fold lower at 70°C compared to 98°C (29). Expression of *als* may, therefore, be up-regulated at the lower temperature in response to pyruvate accumulation, either directly or indirectly. In addition, ALS activity could be regulated via branched-chain amino acids via feed-back inhibition as it is noted that as the amount of yeast extract used in media increases, the amount of acetoin produced decreases (data

not shown). The third possibility is that the second enzyme in the ILV biosynthesis pathway, ketol-acid reductoisomerase (encoded by PF0936), may be optimally active at 98°C and have decreased activity at lower temperatures where ALS is optimally active, thereby causing α -acetolactate to accumulate and be non-enzymatically converted to acetoin. In bacteria, acetoin formation is also driven by the production of acidic metabolites, such as acetate, that cause culture pH to drop. However, this is not the case for *P. furiosus*, since the medium pH during growth of the Δals strains (ΔALS and ADHA- ΔALS) and their parental strains (COM1c and ADHA) were similar (data not shown).

3.7 ALS is required for branched-chain amino acid biosynthesis in *P. furiosus*

ALS is involved in the biosynthesis of the branched-chain amino acids via the ILV biosynthesis pathway. In *P. furiosus*, this pathway is encoded by a gene cluster that contains ALS (PF0935-PF0942). ALS catalyzes the condensation and decarboxylation of two molecules of pyruvate to α -acetolactate (**Figure 2A**). α -acetolactate is the substrate for ketol-acid reductoisomerase, which is encoded by a gene (PF0936 or *ilvC*) located immediately downstream of *als* (**Figure 2B**). To determine whether ALS is required for ILV biosynthesis, ΔALS , COM1c and GR5:ALS strains were cultivated at 78°C in a defined medium using maltose as the carbon source and that either contained (DM) or lacked isoleucine, leucine and valine (DM-ILV) (**Figure 8**). The GR5:ALS strain displayed a long lag phase in both types of media. In the DM medium, both COM1c and ΔALS strains grew similarly (T_D of 3.3 and 3.2 h, respectively), while the doubling time for GR5:ALS was twice as long (5.8 h). The slower growth of GR5:ALS is possibly due to the burden of overexpressing *als* in addition to the

unfavorable growth temperature. However, in the absence of branched-chain amino acids (DM-ILV medium), the Δ ALS strain was unable to grow. In addition, the length of the lag phases of COM1c and GR5:ALS increased by 24 h in the DM-ILV medium. These data show that ALS is necessary for ILV biosynthesis, and that insertion of *als* in to the Δ ALS background successfully rescues the growth phenotype on a medium lacking ILV.

Figure 8. ALS is required for branched chain amino acid biosynthesis. Growth comparison between COM1c (blue, diamond), Δ ALS (red, square) and GR5:ALS (black, circle) in media with (DM) and without (DM-ILV) the branched-chain amino acids (leucine, isoleucine and valine). Cultures were grown at 78 °C in biological triplicate.

4. Conclusion

We show herein that the archaeon *P. furiosus* contains a functional ALS. This enzyme is responsible for the first step in branched chain amino acid biosynthesis from pyruvate to generate α -acetolactate . It also leads to acetoin production via spontaneous breakdown of α -acetolactate . These findings present several options to terms of engineering *P. furiosus* strains to produce desired products. First, current temperature-dependent biofuel producing strains can be improved by deleting *als* from their genomes, thereby directing carbon flow away from acetoin and towards the desired end product. Hence, as was shown here, deleting *als* leads to improved ethanol production in the ADHA strain, and the same should be true with a butanol-producing strain of *P. furiosus* (12). Second, *P. furiosus* is a potential platform for the biological production of acetoin at temperatures in the 70 to 80°C range. Studies are underway to exploit both of these options.

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Table 1. Strains used and constructed in this study.

Strain designation	Alias	Parent	Genotype/Description	Reference
DSM 3638	-	-	wild type	-
MW002	COM1	DSM 3638	$\Delta pyrF$	(8)
MW004	COM1c	COM1	$\Delta pyrF::pyrF$	(17)
MW250	ΔALS	COM1	$\Delta pyrF \Delta als::P_{gdh}pyrF$	this study
MW260	-	MW250	$\Delta pyrF \Delta als$	this study
MW268	GR5:ALS	MW260	$\Delta pyrF \Delta als$ GR5: $P_{slp}als$ $P_{gdh}pyrF$	this study
MW608	ADHA	COM1	$\Delta pyrF$ $P_{slp}adhA$ $P_{gdh}pyrF$	(11)
MW610	-	MW608	$\Delta pyrF$ $P_{slp}adhA$	this study
MW618	ADHA- ΔALS	MW610	$\Delta pyrF$ $P_{slp}adhA$ $\Delta als::P_{gdh}pyrF$	this study

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