Supporting Information for

Single gene insertion drives bioalcohol production by a thermophilic archaeon

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Fig. S1 to S10

Tables S1 and S2

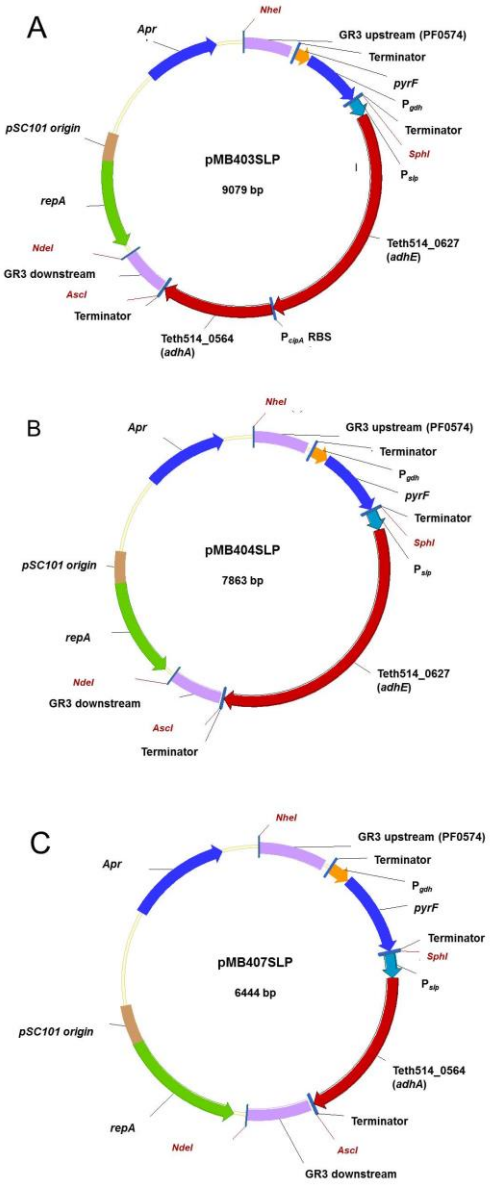

Fig. S1. Maps of plasmids used in this study. (*A*) pMB403SLP, (*B*) pMB404SLP and (*C*) pMB407SLP were used to transform *Pyrococcus furiosus* strain COM1, using *pyrF* as selective marker (1). They contained the gene(s) *adhE* and/or *adhA* from *Thermoanaerobacter* strain X514 under the control of the strong constitutive promoter $P_{slp.}$ They were inserted between the converging genes PF0574 and PF0575 (2). See Materials and Methods for details.

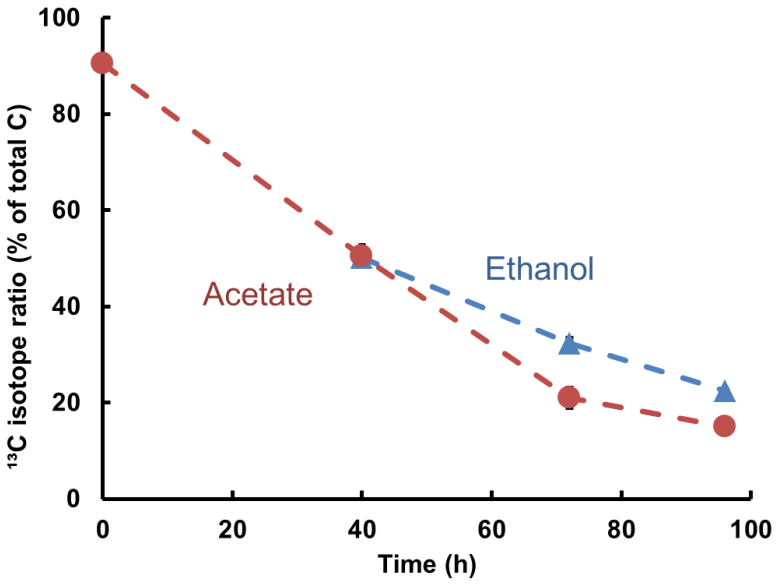

Fig. S2. Formation of ^{13}C-ethanol from ^{13}C-acetate by *P. furiosus* strain A. Percentage ratio of ^{13}C vs. total C (^{12}C+^{13}C) determined in ethanol (blue triangles) and acetate (red circles) in cultures incubated at 72°C on unlabeled maltose (5 g L^{-1}, 15 mM) supplied with 8 mM of double-labeled ^{13}C-acetate. Average of three independent cultures (n=3; ± SD).

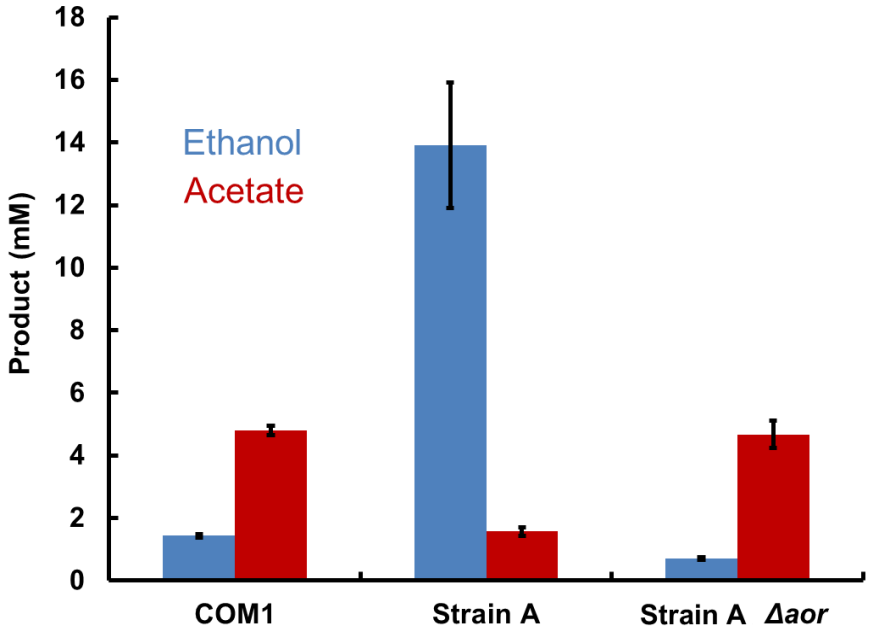

Fig. S3. Effect of *aor* deletion on ethanol formation from maltose. Formation of ethanol (blue bars) and acetate (red bars) in Strains COM1, A (harboring *adhA*) and AΔ*aor* (strain A with *aor* gene deleted) when incubated with maltose (5 g L^{-1}, 15 mM) at 72°C for 3 days (n=3; ± SD).

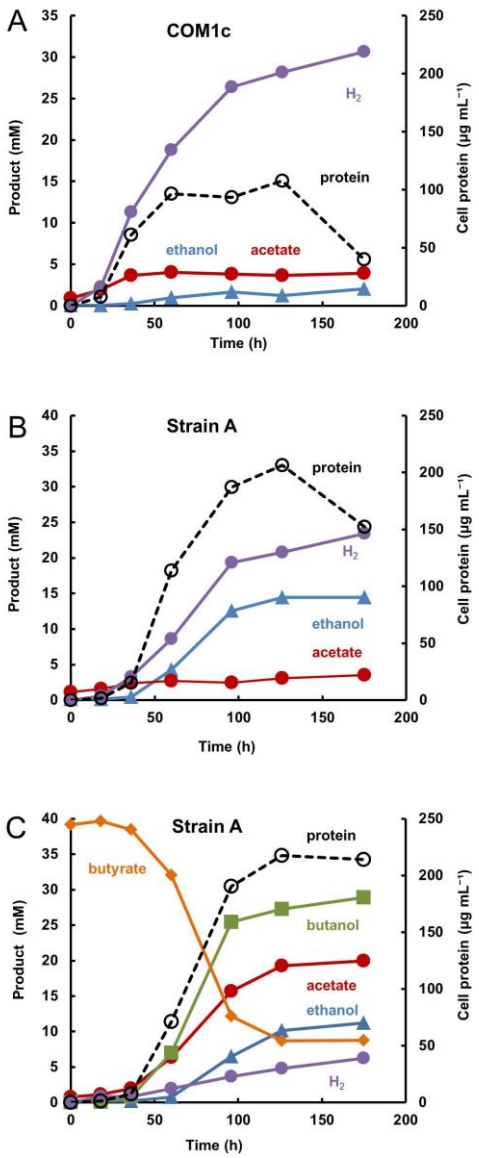

Fig. S4. Conversion of sugars to ethanol and butyrate to butanol by *P. furiosus.* (*A*) Product formation from maltose in strain COM1c. (*B*) Formation of ethanol from maltose by strain A. (*C*) Formation of butanol from butyrate by strain A. Concentration of metabolites are represented as follows: red circles, acetate; blue triangles, ethanol; purple circles, H_2 (represented as mmol per L medium); open black circles, total cell protein (µg cell protein per mL medium); orange diamonds, butyrate; green squares, butanol.

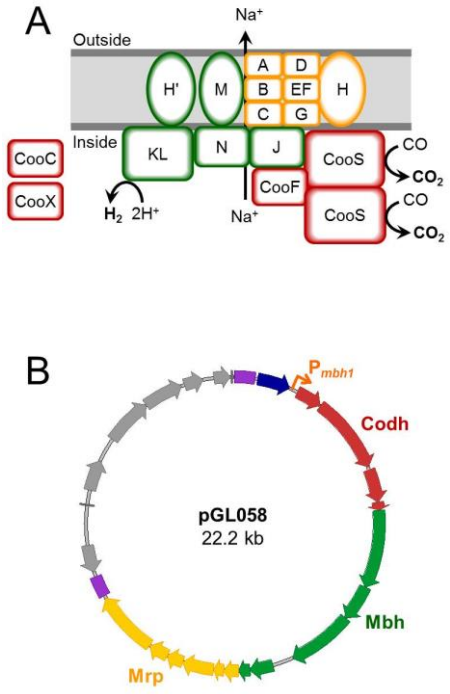

Fig. S5. The *T. onnurineus* Codh complex and vector for insertion of the Codh expression construct into the *P. furiosus* chromosome. (*A*) A schematic representation of the Ton CODH complex encoded by TON_1017-1031. This shows subunits of carbon monoxide dehydrogenase (*Codh*) in red, membrane-bound hydrogenase (*Mbh*) in green, and the Na+/H+ transporter (*Mrp*) in yellow. *CooC* (TON_1019) encodes the maturation protein for *CooS*, and the function of *CooX* encoded by TON_1020 is not known. (*B*) The BAC-based vector pGL058 containing the *Codh* operon TON_1017-1031 gene cluster (*Codh*, TON_1017-20, red; *Mbh*, TON_1021-25, green; Mrp, TON_1025-31, yellow) under transcriptional control of the promoter for *P. furiosus* membrane-bound hydrogenase P_{mbh1}; the *pyrF* marker cassette (indigo) and 5' and 3' homologous recombination regions (purple) used for targeted insertion into the *P. furiosus* chromosome. The BAC vector backbone features are shown in grey (from left to right: cat marker, *oriS, repE, sopA, sopB, sopC* and *cos*).

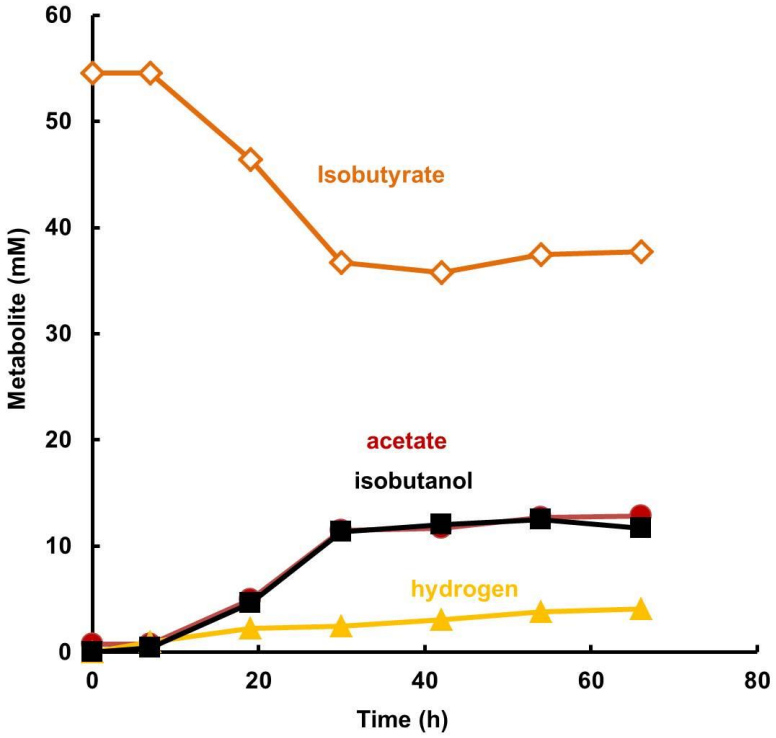

Fig. S6. Reduction of organic acids to alcohols by *Pyrococcus furiosus* strain A/Codh in the absence of CO. Strain A/Codh was incubated at 75 °C under argon and the production and utilization of alcohols and organic acids were determined. Less than 1 mM ethanol was produced under these conditions.

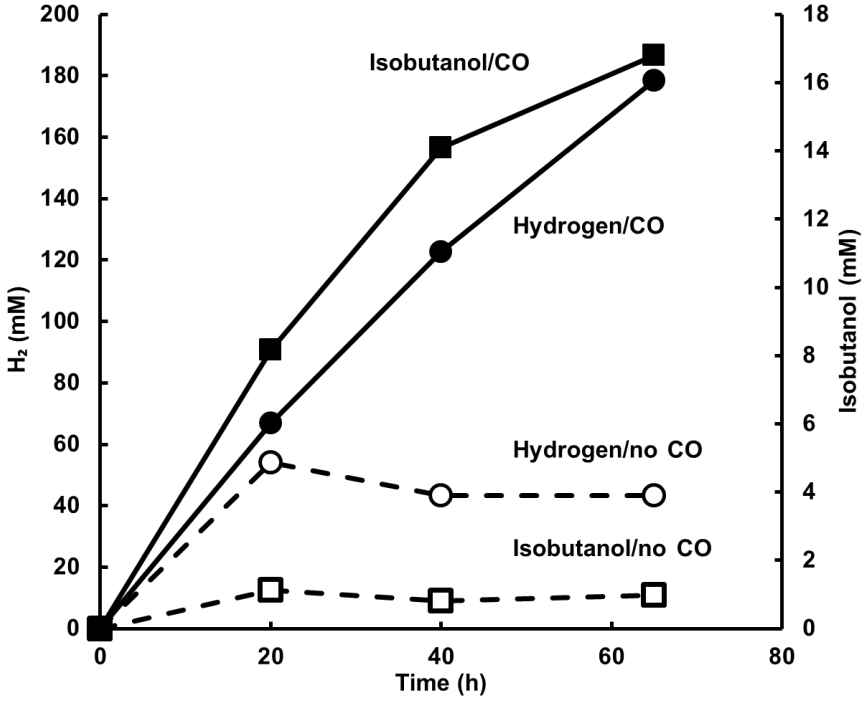

Fig. S7. Reduction of organic acids to alcohols by *Pyrococcus furiosus* strain A/Codh cell suspensions. Formation of hydrogen (circles) and of isobutanol (squares) from isobutyrate in the presence (filled symbols) or absence (open symbols, dotted lines) of CO as the only electron donor by a 10-fold concentrated cell suspension (~$3x10^9$ cells/ml).

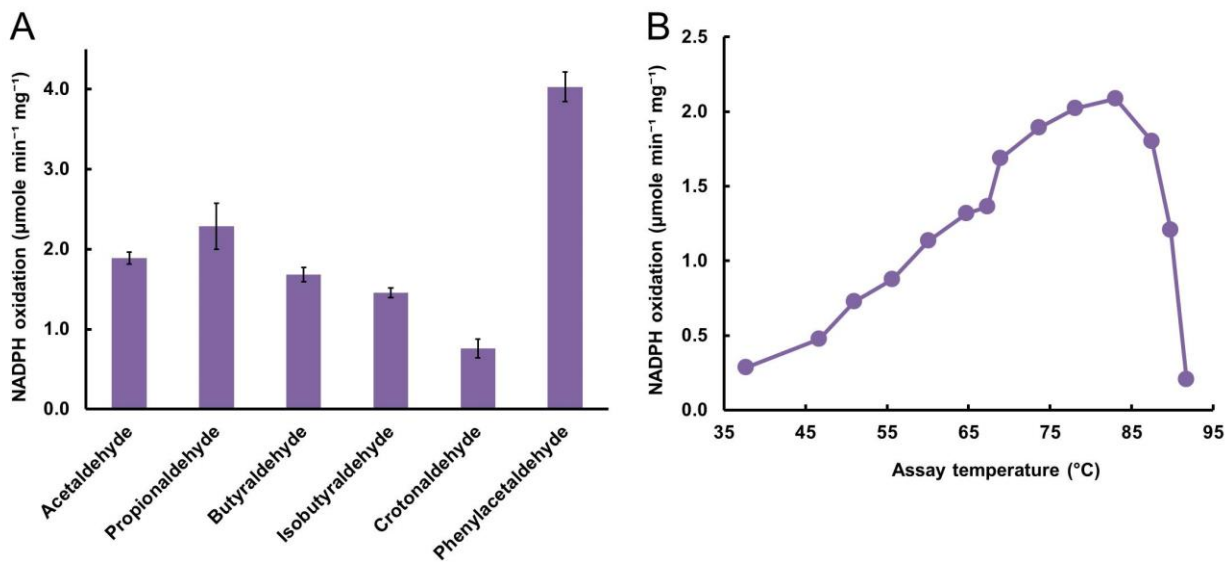

Fig. S8. Substrate specificity and temperature optimum of *T.* X514 AdhA. (*A*) Relative specific activity of AdhA in the cell extracts of strain A with various aldehydes. (*B*) Specific activity of AdhA at different temperatures (n=3; ± SD).

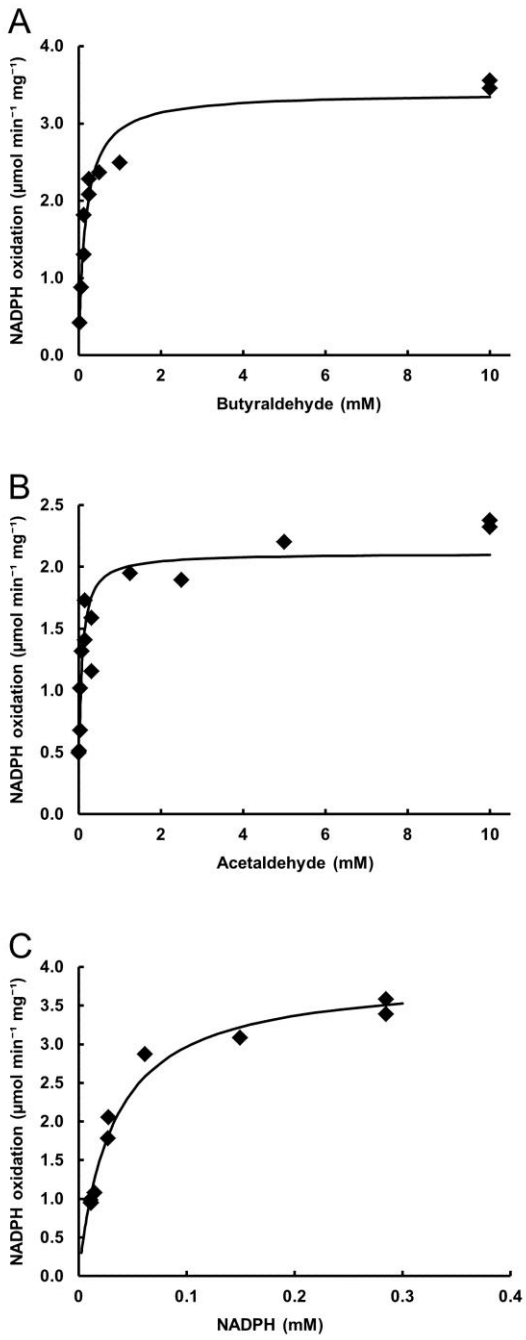

Fig. S9. Michaelis-Menten kinetics of *T*. X514 AdhA. Specific activity of AdhA in the cell extracts of *P. furiosus* strain A at different substrate concentrations of (*A*) butyraldehyde, (*B*) acetaldehyde and (*C*) NADPH.

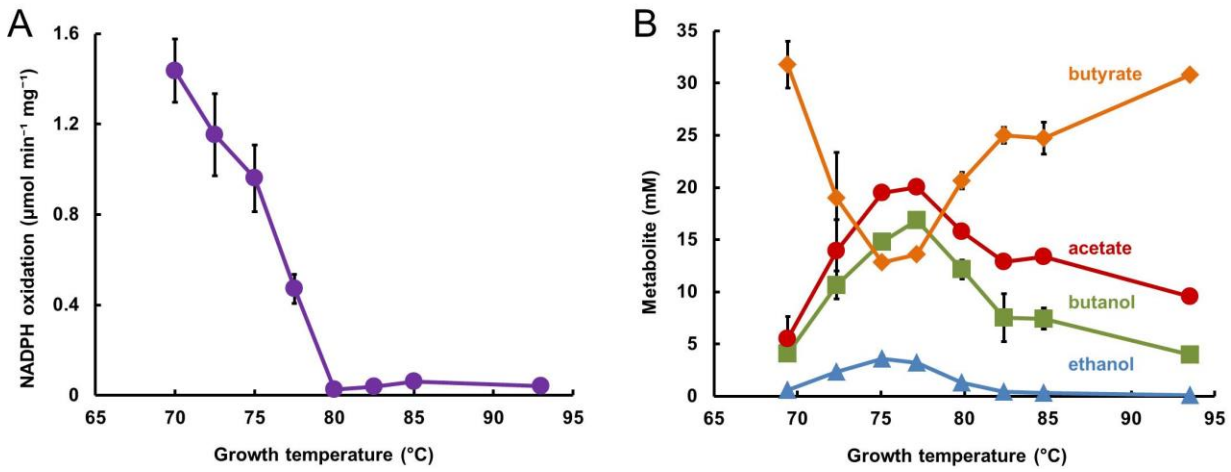

Fig. S10. Temperature dependence of the AOR/AdhA synthetic pathway.

(*A*) Activity of AdhA in cell extracts of strain A grown at different temperatures. (*B*) Conversion of butyrate (40 mM; orange diamonds) to butanol (green squares) and acetate (red circles) by strain A incubated at different temperatures in the presence of maltose (15 mM). Only minor amounts of ethanol (blue triangles) were formed. All experimental data represent the average of three independent cultures (n=3; ± SD).

Table S1.

Strains used in this study.

Strain Name	Strain ID	Relevant genotype	Parent	Source
DSM 3638	MW001	Wild Type	n/a	(3)
COM1	MW002	Δ*pyrF*	DSM 3638	(1)
COM1c	MW004	Δ*pyrF* P_{gdh}*pyrF*	COM1	(4)
EA	MW606	Δ*pyrF* P_{gdh}*pyrF* P_{slp}(*Teth*514_0627;*Teth*514_0564)	COM1	This study
E	MW607	Δ*pyrF* P_{gdh}*pyrF* P_{slp}*Teth*514_0627	COM1	This study
A	MW608	Δ*pyrF* P_{gdh}*pyrF* P_{slp}*Teth*514_0564	COM1	This study
AΔ*pyrF*	MW610	Δ*pyrF* P_{slp}*Teth*514_0564	MW608	This study
AΔ*aor*	MW611	Δ*aor* Δ*pyrF* P_{gdh}*pyrF* P_{slp}*Teth*514_0564	MW610	This study
A/Codh	MW258	Δ*pyrF* P_{gdh}*pyrF* P_{slp}*Teth*514_0564;P_{mbh1} TON_1017-1031	MW610	This study

Table S2.

Primers used in this study.

Primer	Sequence (5’ – 3’)	Source
P*slp*-SacII-F	gaatccccgcggaaatagatattatcggcaaacac	This study
P*slp*-AdhE-R	cttgtaataaggtaggcatttttctccacctcccaataatc	This study
AdhE-P*slp*-F	gattattgggaggtggagaaaaatgcctaccttattacaag	This study
AdhE-R2	gtttcccacactgcatatcacctgccattattctccataggc	This study
AdhE-SphI-R	gcatgcggtaccagcctcctattattctccataggcttttcta	This study
AdhA-F2	ctatggagaataatggcaggtgatatgcagtgtgggaaacaaaaataaatc	This study
AdhA-SphI-R	tacatgcatgcggtaccagcctcctattagaaagattcttcataaatc	This study
P*slp*-SphI-F	tacatgcatgcaaatagatattatcggcaaacac	This study
AdhA-AscI-R	aggcgcgcctaaaaaagattttagaaagattcttcataaatcttg	This study
AdhE-AscI-R	aggcgcgcctaaaaaagattttattctccataggcttttc	This study
åAdhA-P*slp*-F	gattattgggaggtggagaaaagtgtgggaaacaaaaataaatcc	This study
SP2.055	ttttctccacctcccaataatc	This study
AOR1	gatagctagcgaaacttctctgcatcgtcaaga	This study
AOR2	actcttcttttcaattaac	This study
AOR3	agaggtcaccaacatatttattg	This study
AOR4	tctacatatgatcgatctagaactttcagtattctcg	This study
AOR5	ggaaataaaaagttaattgaaaagaagagtcccgggaagccgctaag	This study
AOR6	caataaatatgttggtgacctctgcggccgcgtttaaacggc	This study
SP2.037	gcctttcagcattgtatatgg	This study
SP2.088	cttgaaaatgtttgaggaacacc	This study
SP.237	ctgagggagatatggttaatatg	This study
SP.238	ggaattactcacaaatgttccaacggccgcgtttaaacggc	This study
SP.239	ttggaacatttgtgagtaattcc	This study
SP.243	gaaccggaaaaagctggcatcgccaaacctccttaacatttg	This study
SP.244	atgccagctttttccggttc	This study
SP.245	catattaaccatatctccctcagacaacccattgatagtcatgtgc	This study

References for Supporting Information

1. Lipscomb GL, Stirrett K, Schut GJ, Yang F, Jenney FE, Scott RA, Adams MWW, & Westpheling J (2011) Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: construction of markerless deletions of genes encoding the two cytoplasmic hydrogenases. *Appl Environ Microbiol* 77**:** 2232-2238.
2. Keller MW, Schut GJ, Lipscomb GL, Menon AL, Iwuchukwu IJ, Leuko TT, Thorgersen MP, Nixon WJ, Hawkins AS, Kelly RM, *et al.* (2013) Exploiting microbial hyperthermophilicity to produce an industrial chemical, using hydrogen and carbon dioxide. *Proc Natl Acad Sci USA* 110**:** 5840-5845.
3. Fiala G & Stetter KO (1986) *Pyrococcus furiosus* sp.nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. *Arch Microbiol* 145**:** 56-61.
4. Thorgersen MP, Lipscomb GL, Schut GJ, Kelly RM, & Adams MWW (2014) Deletion of acetyl-CoA synthetases I and II increases production of 3-hydroxypropionate by the metabolically-engineered hyperthermophile *Pyrococcus furiosus*. *Metab Eng* 22**:** 83-88.