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Supplementary Information for
Removal of phosphoglycolate in hyperthermophilic archaea

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Supporting Information Text

Results

Gene disruption of TK0150 and its effect on growth

5,10-Methylenetetrahydrofolate is known as a C1 donor for multiple reactions (1-4). As serine hydroxymethyltransferase reactions are reversible (1, 5), growth of ΔTK0150 cells in the presence of serine is most likely due to the serine hydroxymethyltransferase reaction proceeding in the direction of 5,10-methylenetetrahydrofolate and glycine formation. In order to evaluate whether the serine auxotrophy observed in the ΔTK0150 strain was due to a shortage of 5,10-methylenetetrahydrofolate and not an impairment in the serine biosynthesis pathway per se, we examined the effects of adding an alternative C1 donor to the medium. Ketopantoate is a precursor of CoA biosynthesis in *T. kodakarensis*, and is generated from ketoisovalerate by ketopantoate hydroxymethyltransferase (KPHMT) (***SI Appendix*, Fig. S4*B***) (6, 7). The addition of ketopantoate should be able to provide 5,10-methylenetetrahydrofolate through the reverse reaction catalyzed by KPHMT. When ketopantoate was supplemented into ASW-AA-S^0 without serine, growth of ΔTK0150 was partially restored (***SI Appendix*, Fig. S5*B* and Fig. S7*A***), suggesting that 5,10-methylenetetrahydrofolate was generated from ketopantoate by the KPHMT reaction proceeding in the direction of ketoisovalerate formation. The lack of complete complementation is most likely due to insufficient levels of KPHMT activity to provide adequate levels of serine. To confirm that the KPHMT reaction was actually proceeding in the direction of ketoisovalerate generation, we examined valine auxotrophy. Wild-type *T. kodakarensis* does not harbor a pathway for valine biosynthesis, and displays valine auxotrophy. However, valine can be synthesized by an aminotransferase reaction with ketoisovalerate as an amino group acceptor. The TK0186 protein, described above, displays aminotransferase activity on ketoisovalerate. We can thus confirm the generation of ketoisovalerate through KPHMT activity by supplementing ketopantoate to the medium in the absence of valine. As shown in ***SI Appendix*, Fig. S7*B***, the addition of ketopantoate, although with a decrease in growth rate, complemented the valine auxotrophy of *T. kodakarensis* KU216, indicating that KPHMT can function to generate ketoisovalerate from ketopantoate (***SI Appendix*, Fig. S5*C***). Interestingly, KU216 cells displayed higher growth rates when serine was additionally removed from the medium (***SI Appendix*, Fig. S7*C***). This can be explained by the fact that serine is also a C1 donor leading to 5,10-methylenetetrahydrofolate generation via serine hydroxymethyltransferase. Removal of serine would result in a decrease in 5,10-methylenetetrahydrofolate, pulling the ketopantoate hydroxymethyltransferase reaction towards 5,10-methylenetetrahydrofolate and ketoisovalerate generation (***SI Appendix*, Fig. S5*D***). When the ΔTK0150 strain was grown under this condition, a decrease in cell yield was observed, similar to the results of the strain grown in medium without serine (***SI Appendix*, Fig. S7*A***). This is due to the fact that 5,10-methylenetetrahydrofolate can no longer be produced from glycine, and serine biosynthesis relies solely on the production of 5,10-methylenetetrahydrofolate from ketopantoate (***SI Appendix*, Fig. S5*D***).

Methods

Composition of growth medium. ASW-YT-S^0 and ASW-YT-Pyr medium were composed of 0.8×ASW (8), 5.0 g L^{-1} yeast extract, 5.0 g L^{-1} tryptone, and 0.8 mg L^{-1} resazurin with 2.0 g L^{-1} elemental sulfur or 5.0 g L^{-1} sodium pyruvate, respectively. ASW-AA-S^0 medium was composed of 0.8×ASW, a mixture of 20 amino acids, minerals, a mixture of vitamins (8), 0.8 mg L^{-1} resazurin, and 2.0 g L^{-1} elemental sulfur. Modified versions of these media, ASW-YT-m1 medium (***SI Appendix*, Table S3**) and ASW-AA-m1 medium (***SI Appendix*, Table S4**), were also used. Solid medium used to isolate transformants were based on ASW-AA-S^0 medium and supplemented with 10 g L^{-1} Gelrite, 7.5 g L^{-1} 5-fluoroorotic acid (5-FOA), 10 mg L^{-1} uracil and 4.5 mL of 1 M NaOH. Elemental sulfur was replaced with 0.2% (v/v) polysulfide solution (8).

Construction of gene expression plasmids. The TK0683 and TK1734 coding regions with extensions homologous to the plasmid ends were amplified by PCR using *T. kodakarensis* KOD1 genomic DNA as a template and inserted into pET21a(+) expression plasmid (Merck KGaA, Darmstadt, Germany) by infusion cloning. TK0186, TK0551, TK1094 and TK2301 coding regions were amplified so that additional restriction sites were incorporated. After digestion with NdeI and EcoRI for TK0186 or BamHI for TK0551, TK1094 and TK2301, DNA fragments were ligated with pET21a(+) digested with the same enzymes. All plasmids were

sequenced, and respectively named pET-TK0683, pET-TK1734, pET-TK0186, pET-TK0551, pET-TK1094 and pET-TK2301. Primers used for plasmid construction are listed in ***SI Appendix*, Table S5**.

Gene expression in *E. coli* and purification of the recombinant proteins. *E. coli* BL21-CodonPlus(DE3)-RIL was transformed with pET-TK0683, pET-TK1734, pET-TK0186, pET-TK0551, pET-TK1094 or pET-TK2301. Transformants were cultivated in LB medium until OD_{660} reached 0.4-0.8. Isopropyl-β-D-1-thiogalactopyranoside (0.1 mM) was added, and cultures were continued for another 4 h. As TK1094 protein was insoluble, cells harboring pET-TK1094 were cultivated at 37˚C for 18 h without the addition of isopropyl-β-D-1-thiogalactopyranoside. Cells were collected by centrifugation (4˚C, 5,000×*g*, 15 min), washed with 50 mM Tris-HCl (pH 8.0) containing 1% NaCl, and centrifuged again (4˚C, 5,000×*g*, 15 min). Cells were re-suspended with 50 mM Tris-HCl (pH 7.5) and sonicated with TOMY UD-201 (TOMY SEIKO, Tokyo, Japan) (OUTPUT:4, DUTY:50, 20-30 min). After centrifugation (4˚C, 20,400×*g*, 15 min), the supernatant was treated for 10 min at 90˚C (TK1734 and TK2301) or 85˚C (TK0186, TK0551, TK0683 and TK1094) and centrifuged (4˚C, 15,000×*g*, 15 min) to remove thermolabile proteins.

For TK1734 and TK2301 proteins, supernatants were applied to a ResourceQ column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with 50 mM Tris-HCl (pH 8.0 at 80˚C). Proteins were eluted with a linear gradient of 0 to 1.0 M NaCl. Fractions including TK1734 or TK2301 protein were collected and centrifuged with an Amicon Ultra centrifugal filter unit (MWCO 3,000) (EMD Millipore, Billerica, MA, USA). Samples were then mixed with approximately equal volumes of 3 M $(NH_4)_2SO_4$ and separated by hydrophobic interaction chromatography (Resource ISO, GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8.0 at 80˚C) containing 1.5 M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient of 1.5 M to 0 M $(NH_4)_2SO_4$. Fractions containing TK1734 or TK2301 protein were collected and centrifuged with an Amicon Ultra centrifugal filter unit (MWCO 3,000). Finally, proteins were applied to gel-filtration chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare) and separated with a mobile phase of 50 mM Tris-HCl (pH 8.0 at 80˚C) containing 0.15 M NaCl. TK0551 and TK0683 proteins were purified with the same procedures without the use of hydrophobic interaction chromatography. Different mobile phases were used for the anion exchange chromatography (50 mM Tris-HCl, pH 7.5 at room temperature) and gel-filtration chromatography (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5 at room temperature). TK0186 and TK1094 proteins were purified with anion exchange chromatography and gel-filtration chromatography with the procedures described for the TK1734 and TK2301 proteins. Protein concentrations were determined with the Protein Assay System (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA) as a standard.

Construction of gene disruption plasmids. Coding regions of TK0150, TK0186, TK0551, TK0683, TK1734, and TK2301 with approximately 1,000 bps each of 5'- and 3'-flanking regions were amplified by PCR using *T. kodakarensis* KU216 genomic DNA as a template. Each fragment was ligated with HincII-digested pUD3 plasmid, containing a *pyrF* marker cassette. Inverse PCR was carried out to remove the regions to be deleted, and the amplified fragments were self-ligated. Sequences of the resulting six disruption plasmids were confirmed. Primers used for construction of the disruption plasmids are listed in ***SI Appendix*, Table S6**.

Transformation of *T. kodakarensis*. For gene disruption, *T. kodakarensis* KU216 (Δ*pyrF*) was used as the host strain. KU216 cells grown in ASW-YT-S^0 medium for 12 h at 85˚C were harvested (4˚C, 15,000×*g*, 5 min) from 3 mL culture. Cells were resuspended in 200 μL 0.8×ASW and kept on ice for 30 min. Three micrograms of the disruption plasmid were added to the cells and the mixture was kept on ice for 1 h. The mixture was subjected to heat shock for 45 s at 85˚C and kept on ice for 10 min. The mixture was inoculated into uracil-free synthetic medium (ASW-AA-S^0 (ΔTK0150, ΔTK0551, ΔTK0683) or ASW-AA-m1-S^0 (ΔTK0186, ΔTK1734, ΔTK2301)), and incubated at 85˚C for 2 d. Cells were cultivated in the same medium for another 2 d to enrich transformants harboring the *pyrF* gene via single crossover insertion. Cells were spread onto solid medium (ASW-AA (ΔTK0150, ΔTK0551, ΔTK0683) or ASW-AA-m1 (ΔTK0186, ΔTK1734, ΔTK2301)) with 0.75% 5-FOA and 10 μg mL^{-1} uracil and incubated for 24 h to select cells whose *pyrF* gene was removed via a second recombination event. Genotypes of the obtained colonies were confirmed by PCR and DNA sequencing analysis. Primers used for analyses of deletion mutants are listed in ***SI Appendix*, Table S6**.

Tracer-based metabolomic analysis with ^{13}C-glycolate. *T. kodakarensis* KU216 was grown in 10 mL ASW-YT-m1-Pyr supplemented with 0.08 mM Na_2S and 0.01% (w/v) 1,2-^{13}C-labeled sodium glycolate. Cells were grown for 20 h, collected by centrifugation and washed with 5 mL 0.8×ASW. Preparation of protein-derived amino acids from the cells and the conditions of subsequent analysis using Microfluidic capillary electrophoresis-mass spectrometry (CE–MS) are described in Fukuyama et al. (9). Briefly, protein-derived amino acids were prepared by hydrolyzation with 12N HCl. The purified protein-derived amino acids were analyzed by using a ZipChip CE system (908devices, Boston, MA, USA) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). The obtained MS data was analyzed using Qual Browser in Xcalibur version 4.3.73.11.

Glycolate measurement in the culture medium using LC–MS/MS. *T. kodakarensis* KU216 was grown in 10 mL ASW-YT-m1-Pyr for 20 h under the microaerobic condition defined above. After cultivation, cells were removed by centrifugation (4˚C, 5000×*g*, 15 min) followed by filtration with a 0.2 μm filter (Steradisc S-2502S; Kurabo Industries, Osaka, Japan). Glycolate concentrations were measured after derivatization with 3-nitrophenylhydrazine referring to a previous report (10). LC–MS/MS measurement was performed on an ExionLC liquid-chromatograph coupled with a quadrupole time-of flight mass spectrometer X500R QTOF system (SCIEX, Toronto, Canada) with an electrospray ionization source in negative ion mode. The samples were separated with an ACQUITY UPLC BEH C18 column (2.1×100 mm, 1.7 μm; Waters, Milford, MA) kept at 40˚C using a mobile phase combination of water (A) and 0.01% formic acid in methanol (B). The gradient was started from 18% B, and the percentage of B was linearly raised to 90% in 15 min after injection at a flow rate of 0.2 mL min^{-1}. During the analysis, samples were kept at 4˚C in the auto sampler. The injection volume was 10 μL. MS/MS data of glycolate were collected in multiple reaction monitoring (MRM) mode as the mass transition pair of m/z 210.05→152.05. The capillary voltage and the collision energy voltage were set at 4000V and -30V, respectively. Quantitative analyses of glycolate based on MRM chromatograms were performed using SCIEX OS software ver 1.7.

Glycolate detection in the culture medium using LC–high resolution MS (HRMS). Glycolate in the culture medium was prepared as described previously with some modifications (11). Briefly, the culture medium was filtered with a 0.2-μm filter and then acidified with 12 N HCl to a final concentration of 0.1 M. To concentrate glycolate, 2 mL of ethyl acetate was added to 12 mL of the sample. After shaking and phase separation, the organic solvent fraction was transferred to a 17-mL glass test tube, with repeated extraction of glycolate from the remaining aqueous fraction using another 2 mL of ethyl acetate. Glycolate was concentrated by evaporation under a N_2 stream from the organic solvent fractions at room temperature. Measurement of exact mass of glycolate using LC–HRMS was performed with an UltiMate 3000RS liquid chromatography (Thermo Fisher Scientific) coupled to electrospray ionization high resolution mass spectrometry analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Sample separations were performed with an Intrada Organic Acid column 3 μm 2.0 × 150 mm, (Imtakt, Kyoto, Japan) with a linear gradient of 5%-95% solvent B (90% 100 mM ammonium formate, 10% acetonitrile, 0.1% formic acid) over 20 min with a constant flow of 200 μL/min; solvent A (10% acetonitrile, 0.1% formic acid). The column oven was set at 40˚C. Full scan MS spectra were acquired in the Orbitrap mass analyzer (polarity: Negative, *m/z* range: 70–210, resolution:120,000 full width at half maximum (FWHM)) using internal calibration (EASY-IC). MS/MS spectra were acquired in the Orbitrap mass analyzer (mass range: Normal, scan range mode: Auto, resolution:15,000 FWHM) and Stepped Higher-energy collisional dissociation MS/MS fragmentation with normalized collision energy of 15%, 35% and 45%

Table S1. Presence or absence of gene homologs of Rubisco and 2-PG phosphatase on various archaeal genomes.

Phylum	Genus	Organism	Rubisco	PGP
Euryarchaeota	Methanocaldococcus	*Methanocaldococcus jannaschii*	■	
		Methanocaldococcus fervens	■	
		Methanocaldococcus vulcanius	■	
		Methanocaldococcus sp. FS406-22	■	
		Methanocaldococcus infernus		
		Methanocaldococcus bathoardescens	■	
		Methanocaldococcus lauensis SG7	■	
		Methanocaldococcus lauensis SG1	■	
	Methanotorris	*Methanotorris igneus*	■	
	Methanococcus	*Methanococcus maripaludis* S2		
		Methanococcus maripaludis C5		
		Methanococcus maripaludis C6		
		Methanococcus maripaludis C7		
		Methanococcus maripaludis X1		
		Methanococcus maripaludis KA1		
		Methanococcus maripaludis OS7		
		Methanococcus maripaludis DSM 2067		
		Methanococcus aeolicus		
		Methanococcus vannielii		
		Methanococcus voltae		
	Methanothermococcus	*Methanothermococcus okinawensis*	■	
	Methanofervidicoccus	*Methanofervidicoccus* sp. A16		
	Methanothermobacter	*Methanothermobacter thermautotrophicus*		
		Methanothermobacter marburgensis		
		Methanothermobacter wolfeii		
		Methanothermobacter tenebrarum		
		Methanothermobacter sp. CaT2		
		Methanothermobacter sp. EMTCatA1		
		Methanothermobacter sp. MT-2		
		Methanothermobacter sp. KEPCO-1		
		Methanothermobacter sp. THM-1		
		Methanothermobacter sp. THM-2		
	Methanosphaera	*Methanosphaera stadtmanae*		
		Methanosphaera sp. BMS		
		Methanosphaera sp. ISO3-F5		
	Methanobrevibacter	*Methanobrevibacter ruminantium*		
		Methanobrevibacter smithii		
		Methanobrevibacter sp. AbM4		
		Methanobrevibacter millerae		
		Methanobrevibacter sp. YE315		
		Methanobrevibacter olleyae		
		Methanobrevibacter arboriphilus		
		Methanobrevibacter sp. TLL-48-HuF1		
	Methanobacterium	*Methanobacterium lacus*		
		Methanobacterium paludis		
		Methanobacterium sp. MB1		
		Methanobacterium formicicum BRM9		
		Methanobacterium formicicum DSM1535		
		Methanobacterium congolense		
		Methanobacterium subterraneum		
		Methanobacterium sp. MZ-A1		
		Methanobacterium sp. BRmetb2		
		Methanobacterium sp. BAmetb5		
		Methanobacterium alkalithermotolerans		
		Methanobacterium ferruginis		
	Methanothermus	*Methanothermus fervidus*		
	Methanopyrus	*Methanopyrus kandleri*		
	Archaeoglobus	*Archaeoglobus fulgidus* DSM 4304	■	■
		Archaeoglobus fulgidus DSM 8774	■	■
		Archaeoglobus profundus	■	
		Archaeoglobus veneficus	■	■
		Archaeoglobus sulfaticallidus	■	■
	Ferroglobus	*Ferroglobus placidus*	■	■
	Geoglobus	*Geoglobus acetivorans*	■	■
		Geoglobus ahangari	■	■
	Pyrococcus	*Pyrococcus furiosus* DSM 3638	■	■
		Pyrococcus furiosus COM1	■	■
		Pyrococcus horikoshii	■	■
		Pyrococcus abyssi	■	■
		Pyrococcus sp. NA2	■	■
		Pyrococcus yayanosii	■	■
		Pyrococcus sp. ST04	■	■
		Pyrococcus kukulkanii	■	■
		Pyrococcus chitonophagus	■	■
	Thermococcus	*Thermococcus kodakarensis*	■	■
		Thermococcus onnurineus	■	■
		Thermococcus gammatolerans	■	■
		Thermococcus sibiricus	■	■
		Thermococcus barophilus	■	■
		Thermococcus sp. 4557	■	■
		Thermococcus sp. AM4	■	■
		Thermococcus cleftensis	■	■
		Thermococcus litoralis	■	■
		Thermococcus paralvinellae	■	■
		Thermococcus nautili	■	■
		Thermococcus eurythermalis	■	■
		Thermococcus guaymasensis	■	■
		Thermococcus sp. 2319x1	■	■
		Thermococcus peptonophilus	■	■
		Thermococcus piezophilus		
		Thermococcus gorgonarius	■	■
		Thermococcus celer	■	■
		Thermococcus barossii	■	■
		Thermococcus sp. 5-4	■	■
		Thermococcus siculi	■	■
		Thermococcus thioreducens	■	■
		Thermococcus profundus	■	■
		Thermococcus radiotolerans	■	■
		Thermococcus pacificus	■	■
		Thermococcus sp. P6	■	■
		Thermococcus indicus	■	■
		Thermococcus camini	■	■
		Thermococcus aciditolerans	■	■
		Thermococcus argininiproducens	■	■
	Palaeococcus	*Palaeococcus pacificus*	■	■

Phylum	Genus	Organism	Rubisco	PGP
Euryarchaeota	Methanosarcina	*Methanosarcina barkeri* Fusaro	■	
		Methanosarcina barkeri MS	■	
		Methanosarcina barkeri Wiesmoor	■	
		Methanosarcina barkeri 227	■	
		Methanosarcina barkeri 3	■	
		Methanosarcina acetivorans	■	
		Methanosarcina mazei Go1	■	
		Methanosarcina mazei Tuc01	■	
		Methanosarcina mazei S-6	■	
		Methanosarcina mazei C16	■	
		Methanosarcina vacuolata	■	
		Methanosarcina sp. Kolksee	■	
		Methanosarcina lacustris	■	
		Methanosarcina sp. MTP4	■	
		Methanosarcina sp. WH1	■	
		Methanosarcina sp. WWM596	■	
		Methanosarcina siciliae C2J	■	
		Methanosarcina siciliae HI350	■	
		Methanosarcina siciliae T4/M	■	
		Methanosarcina thermophila TM-1	■	
		Methanosarcina thermophila CHTI-55	■	
		Methanosarcina horonobensis	■	
		Methanosarcina flavescens	■	
	Methanococcoides	*Methanococcoides burtonii*	■	
		Methanococcoides methylutens	■	
		Methanococcoides orientis	■	
	Methanohalophilus	*Methanohalophilus mahii*	■	
		Methanohalophilus halophilus	■	
	Methanohalobium	*Methanohalobium evestigatum*	■	
	Methanosalsum	*Methanosalsum zhilinae*	■	
	Methanolobus	*Methanolobus psychrophilus*	■	
		Methanolobus zinderi	■	
		Methanolobus mangrovi	■	
		Methanolobus sediminis	■	
	Methanomethylovorans	*Methanomethylovorans hollandica*	■	
	Methanimicrococcus	*Methanimicrococcus* sp. Hf6		
		Methanimicrococcus sp. Es2		
	Methanothrix	*Methanothrix thermoacetophila*	■	■
		Methanothrix soehngenii	■	
		Methanothrix harundinacea	■	■
	Methanospirillum	*Methanospirillum hungatei*	■	
		Methanospirillum sp. J.3.6.1-F.2.7.3	■	
	Methanocorpusculum	*Methanocorpusculum labreanum*	■	
	Methanoculleus	*Methanoculleus marisnigri*	■	■
		Methanoculleus bourgensis MS2	■	
		Methanoculleus bourgensis MAB1	■	
		Methanoculleus chikugoensis	■	■
		Methanoculleus submarinus	■	■
		Methanoculleus receptaculi	■	■
	Methanolacinia	*Methanolacinia petrolearia*	■	
	Methanoplanus	*Methanoplanus endosymbiosus*	■	
		Methanoplanus sp. FWC-SCC4	■	
	Methanofollis	*Methanofollis aquaemaris*	■	■
	Methanogenium	*Methanogenium organophilum*	■	■
		Methanogenium sp. S4BF	■	■
	Methanomicrobium	*Methanomicrobium antiquum*	■	■
	Methanoregula	*Methanoregula boonei*	■	■
		Methanoregula formicica	■	■
	Methanosphaerula	*Methanosphaerula palustris*	■	■
	Methanocella	*Methanocella paludicola*	■	
		Methanocella conradii	■	
		Methanocella arvoryzae	■	
	Halobacterium	*Halobacterium salinarum* NRC-1		■
		Halobacterium salinarum R1		■
		Halobacterium salinarum NRC-34001		■
		Halobacterium sp. DL1		
		Halobacterium hubeiense		■
		Halobacterium sp. GSL-19		■
		Halobacterium sp. BOL4-2		■
		Halobacterium noricense		■
		Halobacterium litoreum	■	■
	Halodesulfurarchaeum	*Halodesulfurarchaeum formicicum* HTSR1	■	
		Halodesulfurarchaeum formicicum HSR6	■	
	Halanaeroarchaeum	*Halanaeroarchaeum sulfurireducens* HSR2	■	
		Halanaeroarchaeum sulfurireducens M27-SA2	■	
		Halanaeroarchaeum sp. HSR-CO	■	
	Salarchaeum	*Salarchaeum* sp. JOR-1	■	■
	Halarchaeum	*Halarchaeum* sp. CBA1220		■
	Halalkalicoccus	*Halalkalicoccus jeotgali*		■
	Halococcus	*Halococcus dombrowskii*		■
	Halorussus	*Halorussus limi*		■
		Halorussus gelatinilyticus		■
		Halorussus salilacus		■
		Halorussus vallis		■
	Salinarchaeum	*Salinarchaeum* sp. Harcht-Bsk1		
		Salinarchaeum sp. IM2453		■
	Natranaeroarchaeum	*Natranaeroarchaeum sulfidigenes*	■	■
	Haloarcula	*Haloarcula marismortui*		■
		Haloarcula hispanica ATCC 33960		■
		Haloarcula hispanica N601		■
		Haloarcula sp. CBA1115		■
		Haloarcula taiwanensis		■
		Haloarcula sp. JP-L23	■	■
		Haloarcula sinaiiensis		■
	Natronomonas	*Natronomonas pharaonis*	■	■
		Natronomonas moolapensis		■
		Natronomonas halophila		■
		Natronomonas salina		■
	Halorhabdus	*Halorhabdus utahensis*		
		Halorhabdus tiamatea		
		Halorhabdus sp. CBA1104		■
		Halorhabdus sp. SVX81		
		Halorhabdus sp. BNX81		

Phylum	Genus	Organism	Rubisco	PGP
Euryarchaeota	Halomicrobium	*Halomicrobium mukohataei*	■	■
		Halomicrobium mukohataei JP60	■	■
		Halomicrobium sp. LC1Hm	■	■
		Halomicrobium sp. ZPS1	■	■
	Halorientalis	*Halorientalis* sp. IM1011	■	■
	Halapricum	*Halapricum salinum*	■	
		Halapricum desulfuricans	■	■
	Halosimplex	*Halosimplex rubrum*		■
		Halosimplex pelagicum		■
		Halosimplex litoreum		■
	Halococcoides	*Halococcoides cellulosivorans*		
	Salinirubellus	*Salinirubellus salinus*	■	■
	Halocatena	*Halocatena salina*		
	Haloquadratum	*Haloquadratum walsbyi* DSM 16790		■
		Haloquadratum walsbyi C23		■
	Haloferax	*Haloferax volcanii*	■	■
		Haloferax mediterranei	■	■
		Haloferax gibbonsii	■	■
		Haloferax alexandrinus	■	■
		Haloferax larsenii	■	■
		Haloferax lucentense	■	■
	Halogeometricum	*Halogeometricum borinquense*	■	■
	Haloplanus	*Haloplanus rubicundus* CBA1112		■
		Haloplanus rubicundus CBA1113		■
		Haloplanus aerogenes	■	■
		Haloplanus rallus	■	■
	Halobellus	*Halobellus limi*	■	■
	Haloprofundus	*Haloprofundus* sp. MHR1		■
	Halorubrum	*Halorubrum lacusprofundi*		■
		Halorubrum sp. PV6		■
		Halorubrum sp. BOL3-1		■
		Halorubrum ezzemoulense		■
		Halorubrum sp. CBA1229		■
		Halorubrum sodomense		■
		Halorubrum salinarum		■
		Halorubrum sp. 2020YC2		■
	Salinigranum	*Salinigranum rubrum*	■	■
	Halohasta	*Halohasta litchfieldiae*		■
	Halolamina	*Halolamina* sp. CBA1230		■
	Halobaculum	*Halobaculum halophilum*	■	■
		Halobaculum salinum		■
		Halobaculum magnesiiphilum		
		Halobaculum roseum		
		Halobaculum rubrum		■
		Halobaculum sp. CBA1158		■
	Halalkaliarchaeum	*Halalkaliarchaeum desulfuricum*	■	■
		Halalkaliarchaeum sp. AArc-CO		■
	unclassified Haloferacales	Halophilic archaeon DL31		
	Haloterrigena	*Haloterrigena turkmenica*		■
		Haloterrigena salifodinae	■	■
		Haloterrigena alkaliphila		■
	Natrialba	*Natrialba magadii*	■	■
	Halopiger	*Halopiger xanaduensis*		■
	Natrinema	*Natrinema* sp. J7-2	■	■
		Natrinema pellirubrum		■
		Natrinema versiforme		■
		Natrinema pallidum	■	■
		Natrinema zhouii		■
		Natrinema halophilum	■	■
		Natrinema thermotolerans		■
		Natrinema longum		■
	Natronobacterium	*Natronobacterium gregoryi*		■
	Halovivax	*Halovivax ruber*		
	Natronosalvus	*Natronosalvus rutilus*	■	■
		Natronosalvus halobius		■
	Natronococcus	*Natronococcus occultus*		■
	Halostagnicola	*Halostagnicola larsenii*		■
	Halobiforma	*Halobiforma lacisalsi*		■
	Natrarchaeobaculum	*Natrarchaeobaculum aegyptiacum*	■	■
		Natrarchaeobaculum sulfurireducens AArc-Mg	■	■
		Natrarchaeobaculum sulfurireducens AArc1	■	■
		Natrialbaceae archaeon AArc-T1-2	■	■
	Natronorubrum	*Natronorubrum bangense*	■	■
		Natronorubrum aibiense	■	■
		Natronorubrum daqingense		■
	unclassified Nanohaloarchaea	Nanohaloarchaea archaeon SG9		■
	Methanoliparum	Candidatus Methanoliparum sp. LAM-1		
	Methanonatronarchaeum	*Methanonatronarchaeum* sp. AMET6-2	■	
		Methanonatronarchaeum sp. AMET-SI		
Thermoplasmatota	Thermoplasma	*Thermoplasma acidophilum*		
		Thermoplasma volcanium		
	Picrophilus	*Picrophilus oshimae*		
	Ferroplasma	*Ferroplasma acidarmanus*		
		Ferroplasma acidiphilum		
	Cuniculiplasma	*Cuniculiplasma divulgatum*		
	unclassified Thermoplasmatales	Thermoplasmatales archaeon BRNA1	■	■
	Methanomethylophilus	*Methanomethylophilus alvi*	■	■
	Methanomassiliicoccus	Candidatus Methanomassiliicoccus intestinalis Issoire-Mx1	■	
	Methanoplasma	Candidatus Methanoplasma termitum	■	■
	Methanogranum	Candidatus Methanogranum sp. U3.2.1	■	■
	unclassified Thermoplasmata	Methanogenic archaeon ISO4-H5	■	■
	Aciduliprofundum	*Aciduliprofundum boonei*	■	■
		Aciduliprofundum sp. MAR08-339	■	■

Phylum	Genus	Organism	Rubisco	PGP
Thermoproteota	Aeropyrum	*Aeropyrum pernix*		■
		Aeropyrum camini		■
	Staphylothermus	*Staphylothermus marinus*	■	■
		Staphylothermus hellenicus	■	■
	Ignicoccus	*Ignicoccus hospitalis*		■
		Ignicoccus islandicus		■
		Ignicoccus pacificus		■
	Desulfurococcus	*Desulfurococcus amylolyticus* 1221n	■	
		Desulfurococcus amylolyticus DSM 16532	■	
		Desulfurococcus mucosus	■	
	Thermosphaera	*Thermosphaera aggregans*	■	
	Ignisphaera	*Ignisphaera aggregans*	■	
	Thermogladius	*Thermogladius calderae*	■	
	Hyperthermus	*Hyperthermus butylicus*	■	
	Pyrolobus	*Pyrolobus fumarii*		
	Pyrodictium	*Pyrodictium delaneyi*	■	■
		Pyrodictium abyssi	■	■
	Pyrofollis	*Pyrofollis japonicus*	■	■
	Sulfurisphaera	*Sulfurisphaera tokodaii*		■
		Sulfurisphaera ohwakuensis		■
	Saccharolobus	*Saccharolobus solfataricus* P2		■
		Saccharolobus solfataricus 98/2		■
		Saccharolobus solfataricus SULA		■
		Saccharolobus solfataricus SARC-B		■
		Saccharolobus solfataricus SARC-C		■
		Saccharolobus shibatae		■
		Saccharolobus caldissimus		■
	Sulfolobus	*Sulfolobus acidocaldarius* DSM 639		■
		Sulfolobus acidocaldarius N8		■
		Sulfolobus acidocaldarius Ron12/I		■
		Sulfolobus acidocaldarius SUSAZ		■
		Sulfolobus islandicus L.S.2.15		■
		Sulfolobus islandicus M.14.25		■
		Sulfolobus islandicus M.16.27		■
		Sulfolobus islandicus M.16.4		■
		Sulfolobus islandicus Y.G.57.14		■
		Sulfolobus islandicus Y.N.15.51		■
		Sulfolobus islandicus L.D.8.5		■
		Sulfolobus islandicus HVE10/4		■
		Sulfolobus islandicus REY15A		■
		Sulfolobus islandicus LAL14/1		■
		Sulfolobus sp. A20		■
		Sulfolobus sp. E5-1-F		■
		Sulfolobus sp. E11-6		■
		Sulfolobus sp. S-194		■
	Metallosphaera	*Metallosphaera sedula* DSM 5348		■
		Metallosphaera sedula MJ1HA		■
		Metallosphaera cuprina		■
		Metallosphaera hakonensis JCM 8857 = DSM 7519		■
		Metallosphaera prunae		■
		Metallosphaera tengchongensis		■
		Metallosphaera javensis		■
	Acidianus	*Acidianus hospitalis*		■
		Acidianus manzaensis		■
		Acidianus brierleyi		■
		Acidianus sulfidivorans		■
		Acidianus ambivalens		■
		Acidianus sp. HS-5		■
	Sulfodiicoccus	*Sulfodiicoccus acidiphilus*		■
	Stygiolobus	*Stygiolobus azoricus*		■
		Stygiolobus caldivivus		■
	Sulfuracidifex	*Sulfuracidifex tepidarius*		■
		Sulfuracidifex metallicus		■
	unclassified Sulfolobales	Sulfolobales archaeon HS-7		■
	Pyrobaculum	*Pyrobaculum aerophilum*		
		Pyrobaculum islandicum		
		Pyrobaculum calidifontis		
		Pyrobaculum arsenaticum		■
		Pyrobaculum ferrireducens		■
		Pyrobaculum oguniense		■
		Pyrobaculum neutrophilum		
		Pyrobaculum sp. WP30		
	Caldivirga	*Caldivirga maquilingensis*		
	Thermoproteus	*Thermoproteus tenax*		
		Thermoproteus uzoniensis		
	Vulcanisaeta	*Vulcanisaeta distributa*		■
		Vulcanisaeta moutnovskia		■
		Vulcanisaeta souniana		
	Thermofilum	*Thermofilum pendens*	■	
		Thermofilum adornatum 1910b	■	
		Thermofilum adornatum 1505	■	
	Infirmifilum	*Infirmifilum uzonense*	■	
		Infirmifilum lucidum	■	
	Acidilobus	*Acidilobus saccharovorans*		
		Acidilobus sp. 7A		
	Caldisphaera	*Caldisphaera lagunensis*		
	Fervidicoccus	*Fervidicoccus fontis*		

Phylum	Genus	Organism	Rubisco	PGP
Nitrososphaerota	Nitrosopumilus	*Nitrosopumilus maritimus*		
		Candidatus Nitrosopumilus sediminis		
		Candidatus Nitrosopumilus koreensis		
		Nitrosopumilus piranensis		
		Nitrosopumilus adriaticus		
		Candidatus Nitrosopumilus sp. SW		
		Nitrosopumilus cobalaminigenes		
		Nitrosopumilus oxyclinae		
		Nitrosopumilus ureiphilus		
		Nitrosopumilus sp. K4		
		Nitrosopumilus zosterae		
	Nitrosomarinus	Candidatus Nitrosomarinus catalina		
	Nitrosarchaeum	*Nitrosarchaeum* sp. AC2		
	Nitrososphaera	Candidatus Nitrososphaera gargensis		
		Nitrososphaera viennensis		
		Candidatus Nitrososphaera evergladensis		
	Nitrosocosmicus	Candidatus Nitrosocosmicus oleophilus		
		Candidatus Nitrosocosmicus franklandus		
	Nitrosocaldus	Candidatus Nitrosocaldus cavascurensis		
	Cenarchaeum	*Cenarchaeum symbiosum*		
	Caldarchaeum	Candidatus Caldarchaeum subterraneum		
	Nitrosopelagicus	Candidatus Nitrosopelagicus brevis		
	Nitrosotenuis	Candidatus Nitrosotenuis cloacae		
		Candidatus Nitrosotenuis sp. DW1		
		Candidatus Nitrosotenuis chungbukensis		
	Nitrosotalea	Candidatus Nitrosotalea devanaterra		
	Conexivisphaera	*Conexivisphaera calida*		
Korarchaeota	Korarchaeum	Candidatus Korarchaeum cryptofilum		
Bathyarchaeota	unclassified Bathyarchaeota	Candidatus Bathyarchaeota archaeon BA1		
		Candidatus Bathyarchaeota archaeon BA2		
Nanoarchaeota	Nanoarchaeum	*Nanoarchaeum equitans*		
	Nanopusillus	Candidatus Nanopusillus acidilobi		
	Nanobdella	*Nanobdella aerobiophila*		
Micrarchaeota	Mancarchaeum	Candidatus Mancarchaeum acidiphilum Mia14		
	Fermentimicrarchaeum	Candidatus Fermentimicrarchaeum limneticum		
	Micrarchaeum	Candidatus Micrarchaeum sp. A_DKE		
Nanohaloarchaeota	Nanohalobium	Candidatus Nanohalobium constans		
Lokiarchaeota	Lokiarchaeum	Candidatus Lokiarchaeum sp. GC14_75		
		Candidatus Lokiarchaeum sp. B-35		
	Prometheoarchaeum	Candidatus Prometheoarchaeum syntrophicum		
unclassified Archaea		Archaeon GW2011_AR10		
		Archaeon GW2011_AR20		

The presence of a gene homolog is indicated in black.

Table S2. List of strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relAI*	Stratagene (La Jolla, CA)
BL21-CodonPlus(DE3)-RIL	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) dcm^+ Tet^r *gal* λ(DE3) *endA* Hte (*argU ileY leuW* Cam^r)	Stratagene
Thermococcus kodakarensis		
KOD1	Wild-type	(12)
KU216*	KOD1 Δ*pyrF*	(13)
ΔTK0150-3*	KU216 ΔTK0150	This study
ΔTK0150-26	KU216 ΔTK0150	This study
ΔTK0186-17*	KU216 ΔTK0186	This study
ΔTK0186-25	KU216 ΔTK0186	This study
ΔTK0186-28	KU216 ΔTK0186	This study
ΔTK0186-33	KU216 ΔTK0186	This study
ΔTK0548*	KU216 ΔTK0548	(14)
ΔTK0551-1*	KU216 ΔTK0551	This study
ΔTK0551-2	KU216 ΔTK0551	This study
ΔTK0683-13*	KU216 ΔTK0683	This study
ΔTK0683-15	KU216 ΔTK0683	This study
ΔTK1094*	KU216 ΔTK1094	(15)
ΔTK1734-2*	KU216 ΔTK1734	This study
ΔTK1734-4	KU216 ΔTK1734	This study
ΔTK1734-5	KU216 ΔTK1734	This study
ΔTK1734-6	KU216 ΔTK1734	This study
ΔTK2268*	KU216 ΔTK2268	(14)
ΔTK2301-1*	KU216 ΔTK2301	This study
ΔTK2301-2	KU216 ΔTK2301	This study
ΔTK2301-3	KU216 ΔTK2301	This study
ΔTK2301-4	KU216 ΔTK2301	This study
Plasmids		
pUC118	Amp^r general cloning vector	Takara (Kyoto, Japan)
pUD3	pUC118 derivative; *pyrF* marker cassette (P_{pyrF}::*pyrF*)	(16)
pUDTK0150	pUD3 derivative; TK0150 disruption vector	This study
pUDTK0186	pUD3 derivative; TK0186 disruption vector	This study
pUDTK0551	pUD3 derivative; TK0551 disruption vector	(17)
pUDTK0683	pUD3 derivative; TK0683 disruption vector	(17)
pUDTK1734	pUD3 derivative; TK1734 disruption vector	This study
pUDTK2301	pUD3 derivative; TK2301 disruption vector	This study
pET-21a(+)	Amp^r general expression vector	Novagen (Madison, Wis)
pET-TK0186	pET-21a(+) derivative; TK0186	This study
pET-TK0551	pET-21a(+) derivative; TK0551	This study
pET-TK0683	pET-21a(+) derivative; TK0683	This study
pET-TK1734	pET-21a(+) derivative; TK1734	This study
pET-TK2301	pET-21a(+) derivative; TK2301	This study

T. kodakarensis mutant strains with asterisk were used for activity measurement of cell extracts or growth experiments.

Table S3. Composition of ASW-YT-m1 medium.

Component	Concentration (mg L^{-1})
NaCl	16000
$MgCl_2 \cdot 6H_2O$	2400
$MgSO_4 \cdot 7H_2O$	4800
$(NH_4)_2SO_4$	800
$NaHCO_3$	160
$CaCl_2 \cdot 2H_2O$	240
KCl	400
KH_2PO_4	336
NaBr	40
$SrCl_2 \cdot 6H_2O$	16
$Fe(NH_4)$citrate	8
KI	3.32
H_3BO_3	1.236
$NiCl_2 \cdot 6H_2O$	2.38
Yeast extract	5000
Tryptone	5000
Resazurin	0.8

Table S4. Composition of ASW-AA-m1 medium.

Component	Concentration (mg L^{-1})	Component	Concentration (μg L^{-1})
NaCl	16000	$MnSO_4 \cdot 5H_2O$	2500
$MgCl_2 \cdot 6H_2O$	2400	$CoCl_2$	500
$MgSO_4 \cdot 7H_2O$	4800	$ZnSO_4$	500
$(NH_4)_2SO_4$	800	$CuSO_4 \cdot 5H_2O$	50
$NaHCO_3$	160	$AlK(SO_4)_2$	50
$CaCl_2 \cdot 2H_2O$	240	H_3BO_3	50
KCl	400	$Na_2MoO_4 \cdot 2H_2O$	50
KH_2PO_4	336	Nicotinic acid	400
NaBr	40	Biotin	160
$SrCl_2 \cdot 6H_2O$	16	Calcium pantothenate	400
$Fe(NH_4)$citrate	8	Lipoic acid	400
KI	3.32	Folic acid	160
H_3BO_3	1.236	*p*-Aminobenzoic acid	400
$NiCl_2 \cdot 6H_2O$	2.38	Thiamine	400
Resazurin	0.8	Riboflavin	400
L-Alanine	75	Pyridoxine	400
L-Cysteine·HCl·H_2O	250	Cobalamin	400
L-Aspartic acid	50		
L-Glutamic acid	200		
L-Phenylalanine	75		
Glycine	200		
L-Histidine·HCl·H_2O	100		
L-Isoleucine	100		
L-Lysine·HCl	100		
L-Leucine	100		
L-Methionine	75		
L-Asparagine·H_2O	100		
L-Proline	125		
L-Glutamine	50		
L-Arginine·HCl	250		
L-Serine	75		
L-Threonine	100		
L-Valine	200		
L-Tryptophan	75		
L-Tyrosine	100		

Table S5. List of primers used for expression vector construction in this study.

Use	Gene	Type	Primer name	5'-sequence-3'
For expression vector construction	TK0186	A	TK0186-f	TTTTGGATCCCATATGCAGGAAAAGCTTGAGAACAA
			TK0186-r	TTTGAATTCTCACTTGAGCTTCTTTATCTCCTCCTC
	TK0551	A	ldhA1-F	GGGCATATGAGGCCGAGAGTTCTTGTGACATTTAAG
			ldhA1-R	GGGGGATCCTCACAGCATCTTAACTTCTTCCGGCGGGCG
	TK0683	A	ldhA2-infusion-F	GAAGGAGATATACATATGAGGCCTAAGGTTTTCATAACCCGTGCC
			ldhA2-infusion-R	GCTCGAATTCGGATCTCAGAAGCCGGGTTTTCTAACCTTCACGAC
	TK1734	A	TK1734-infusion-f	AAGGAGATATACATATGACGAGAAAAATCGGCATTATCTTCG
			TK1734-infusion-r	GCTCGAATTCGGATCCCTAAAGGGCAGCCTCCAGATACCTT
	TK2301	A	TK2301-f	GGGCATATGACAATAAAGGCAATATCTGTCG
			TK2301-r	GGGGGATCCTCAGGTCTTTTCTTCGGGGAGATAT
For DNA sequencing of target gene	TK0186	B	TK0186seqF1	TACGGAATCCCAATGAGCAAG
			TK0186seqF2	ATGAGCCTCGAGAGGAGAAAGC
			TK0186seqF3	GAAGACGGCCACCTCGACAG
	TK0551	B	TK0551seqF1	AAGTACGCCGACGTTGATTT
			TK0551seqF2	AAGCTCATCAGGAGGGGTGA
			TK0551seqF3	GAAAGAATTAAGCTCCTTGA
			TK0551seqR1	TTCCCTGTTCACGAGGTCTT
			TK0551seqR2	CTCGTTGATGATATGGTAGG
			TK0551seqR3	GTCGGTGTAAACGAACTTCC
	TK0683	B	TK0683seqF1	GAGCACTTTGAGGTTGAGGT
			TK0683seqF2	GAGGCCGACCACTTCACCCG
			TK0683seqF3	ATGATAAACGAGGAAAGGCT
			TK0683seqR1	GAGGGTCGGAGGTACTTCAC
			TK0683seqR2	GTACTGGGTTTCCTTAGTCA
			TK0683seqR3	TATCAGCCTCCTGGCCGTTG
	TK1734	B	TK1734seqF	CGAGAAGCTCAAGTACGGAACGCTCG
			TK1734seqR	CGAGCGTTCCGTACTTGAGCTTCTCGTAGGTTAAC
	TK2301	B	TK2301seqF	AGCTGAAAAAGAGGTATCCCGAGGCTC
			TK2301seqR	GAGTATCCACTCCTCGTCCATGTTGGTCAGGTAGA
	universal	C	T7Pmodified	CGCGAAATTAATACGACTCACTATAGG
			T7Tmodified	CCAAGGGGTTATGCTAGTTATTGCT

A: Primers for amplifying target gene to be overexpressed in *E. coli* with appropriate restriction sites. Underlined sequences indicate the restriction sites.
B: Primers annealing within the target genes.
C: Primers annealing outside of multi cloning region including NdeI, EcoRI and BamHI sites and used for sequencing target genes.

Table S6. List of primers used for genetic analysis in this study.

Use	Gene	Type	Primer name	5'-sequence-3'
For disruption vector construction	TK0150	D	pTK0150F	ATACACTTTGGTGAGAAAAGAAAAAGTTCA
			pTK0150R	ACCAGCTCCGCAGTCCTCACGGCCTCAT
		E	D1fTK0150	TGGGCTTTTTCTTCTTCATTATTTCCTTAT
			U1rTK0150	GAACATCACCGTCCATGGTTGCAACTT
	TK0186	D	dTK0186F1	GACCTCCGTTGGATCGCGCATC
			dTK0186R1	GCTACTACTACCTCCCAAAGG
		E	dTK0186F2	AGTTCTACAAGCCGAGGAGG
			dTK0186R2	CGACTATCATTACCTCGACCTTGC
	TK1734	D	dTK1734F1	CCAGCGCCGTTATCCCGAAAC
			dTK1734R1	AGCGAGAACACGGAGTGCGTTG
		E	dTK1734F2	ATGGACGCAGGAGAGCTCATAAAGAAG
			dTK1734R2	GAGGGAAAAGAGGAAGAGGG
	TK2301	D	dTK2301F1	AGGGTTGAGCAGGTGAGTATC
			dTK2301R1	CCACAACTGGCTGATAGCG
		E	dTK2301F2	AGGGTGGTTGTCATGTTCAAG
			dTK2301R2	TTATCTCACCCGGAAAATGAGGG
For DNA sequencing of 5'- and 3'-flanking regions of target gene	TK0150	F	TK0150sqf1	CCTATAAAAGCTCTCTTTG
			TK0150sqf2	ATCCAGAATCGTCGCCCTTG
			TK0150sqr1	AATAACAGGAGGAGAGTATA
			TK0150sqr2	AGGACAACTGCAAGGAGCGT
	TK0186	F	dTK0186seqF1	CGGAGGAGAAGATTAGGGAAG
			dTK0186seqF2	GTGCTATTCAGCCCTGAGGGAGATC
			dTK0186seqF3	GAAATTCCGAAAGCCATAAAAC
			dTK0186seqF4	GCATTGAGTCCACCGACGAG
			dTK0186seqF5	GATGAAGGCTTCTGAGGTTAGGG
			dTK0186seqR1	CCCTAACCTCAGAAGCCTTCATC
			dTK0186seqR2	TTCCAAGCGTTAATGTACAAAAAG
			dTK0186seqR3	CGAGTTCTGGAAAGAGATG
			dTK0186seqR4	CGACGTCATAATGACTCAC
	TK0551	F	SEQDldhA1-F1	CGAAGTTCTGGCGGTAGTTG
			SEQDldhA1-F2	AACCCTGATGAGCGGCCTCG
			SEQldhA1-F3	AGGGCCGGGAAGAGAACCTC
			SEQldhA1-F4	TTTCCCGGTTTCTCCAAAAG
			SEQldhA1-R1	GGTTTGTGTTCTCGTGTTCA
			SEQldhA1-R2	ATCGTCAATTCCAATGTGGA
			SEQldhA1-R3	CCCCCTTGCCAGCTTCAGCA
			SEQldhA1-R4	AAGACCGTAGGGTTCGAGGA
	TK0683	F	SEQldhA2-F1	CCCTAATGAGCTTCGGTGCG
			SEQldhA2-F2	CCTCTCGGGCCTTTGGAGCA
			SEQldhA2-F3	GGAAACCTGATACCGCTCCT
			SEQldhA2-F4	AACACAGAAAGTGAGCGCTT
			SEQldhA2-R1	ATTTCCCCTTCAATTTCACG
			SEQldhA2-R2	CCTCGCCGGTCAGCTTCCCT
			SEQldhA2-R3	GTCTCGGGTGAATCGTATGA
			SEQldhA2-R4	ATCAGTCCTTATAAAACTGA
	TK1734	F	dTK1734seqF1	AAACATGCGTACGAACTCTACAC
			dTK1734seqF2	GTTTATCAATTATGACAGGGGTG
			dTK1734seqF3	CGCTTCCAGTTGCCAACTCT
			dTK1734seqR1	GTTTCAAGATTTACTCAAAAAC
			dTK1734seqR2	AGAGTTGGCAACTGGAAGCG
	TK2301	F	dTK2301seqF1	CTGTAACGGTTCTCGCCATC
			dTK2301seqF2	CCCTCTGGCCACGGGTATC
			dTK2301seqF3	GTTTCAGGTCCGCTCTTTGGGTTCG
			dTK2301seqR1	GCGTTCTTTCATACCTCAG
			dTK2301seqR2	CGAACCCAAAGAGCGGACCTGAAAC
	universal	G	M4S	CTGGCGAAAGGGGGATGTGC
			RVS	ACACTTTATGCTTCCGGCTC
For PCR analysis of gene disruptants	TK0150	H	dTK0150outF	AAAGTGGAGTGAAGTACCACCTGAGCTT
			dTK0150outR	CCTTCGACGCTATCACCGCCCGTTT
		I	dTK0150inF	ATGATCGAAGTCGGGGAATACAAGGTCA
			dTK0150inR	TCAAAGGCTCCTCAGGTATTCGGCGTAGG
	TK0186	H	dTK0186outF	TACTCCGGTGGAGTAACGTCAAAGGC
			dTK0186outR	TCCACCCTCAAGCTCAGGCCCGACTC
		I	dTK0186inF	CAGGAAGCCAGCAGGCGCT
			dTK0186inR	CGATGACCCTTGGAATGTGC
	TK0551	H	CHDldhA1-F	TCGGCTATCTCACGAGCATAGGCGAAAACG
			CHDldhA1-R	ACTCCCGCTCCCGGTAGGCCAGAAGCTCAT
		I	CHldhA1-F	GAGTTCTTGTGACATTTAAGATGAAGAGCA
			CHldhA1-R	TTCCGGCGGGCGAACCTTCACGACTTCCCT
	TK0683	H	CHDldhA2-F	ATACTCGTTGGAACGCAGATAGGGGCTGGA
			CHDldhA2-R	TTTGAAAGCTCCTCTCTGAGCTTTGGAAGG
		I	CHldhA2-F	AGGTTTTCATAACCCGTGCCATTCCCGAGA
			CHldhA2-R	GGTTTTCTAACCTTCACGACTTCCTTATTC
	TK1734	H	dTK1734outF	ATCCCAATCGTAGTTCCTGGCATAC
			dTK1734outR	AACTTTCCGTCGTACTCATCG
		I	dTK1734inF	ATGACGAGAAAAATCGGCATTATCTTC
			dTK1734inR	CTAAAGGGCAGCCTCCAGATAC
	TK2301	H	dTK2301outF	AAGAGCCTATCCCTTCAATCTTTG
			dTK2301outR	CATAGGCTACTACCGCCCGC
		I	dTK2301inF	ATGACAATAAAGGCAATATCTGTCG
			dTK2301inR	TCAGGTCTTTTCTTCGGGG

D: Primers for amplifying target genes and their 5'- and 3'-flanking regions.
E: Primers for inverse PCR amplifying 5'- and 3'-flanking regions and entire plasmid to exclude target genes.
F: Primers for sequencing 5'- or 3'-flanking region of target genes.
G: Primers annealing outside of multi cloning site of pUD3.

H: Primers annealing outside of homologous regions for homologous recombination.
I: Primers annealing within the target genes.

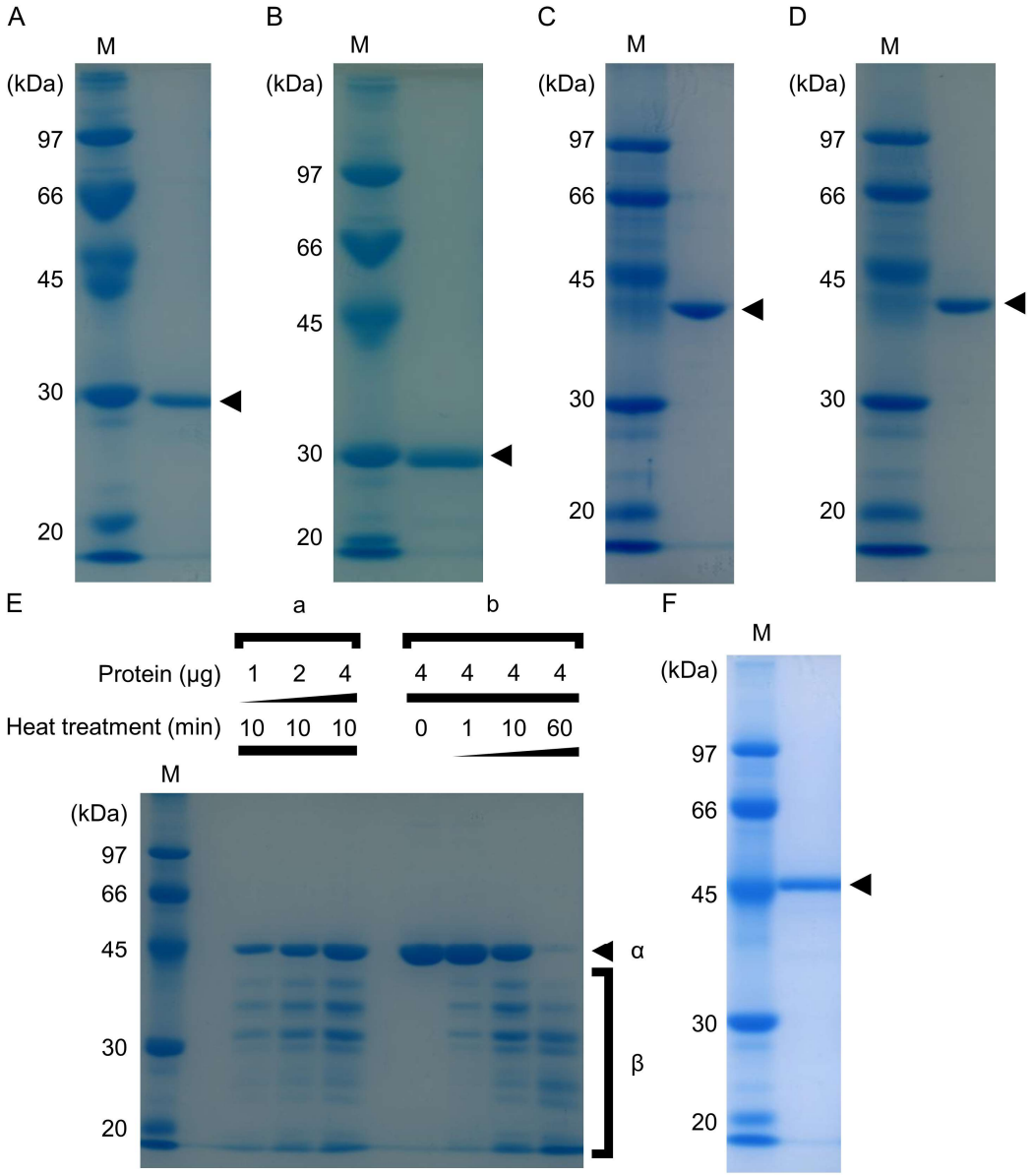


Fig. S1. SDS-PAGE analysis of the purified recombinant proteins. (*A* to *D* and *F*) The genes TK1734 (*A*), TK2301 (*B*), TK0551 (*C*), TK0683 (*D*) and TK1094 (*F*) were expressed in *E. coli* and the recombinant proteins were purified with procedures described in the Methods section. The homogeneity of each sample was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. M represents molecular weight marker. (*E*) The recombinant protein of TK0186 expressed in *E. coli* was purified as described in the Methods section and analyzed by SDS-PAGE/Coomassie Brilliant Blue staining. M represents molecular weight marker. (a) Different amounts of protein were incubated at 98˚C for 10 min in SDS-PAGE buffer. (b) Protein samples mixed with SDS-PAGE buffer were incubated at 98˚C for various periods of time. (α) Main protein band corresponding to intact TK0186 protein, (β) low molecular weight proteins corresponding to fragments of the TK0186 protein.

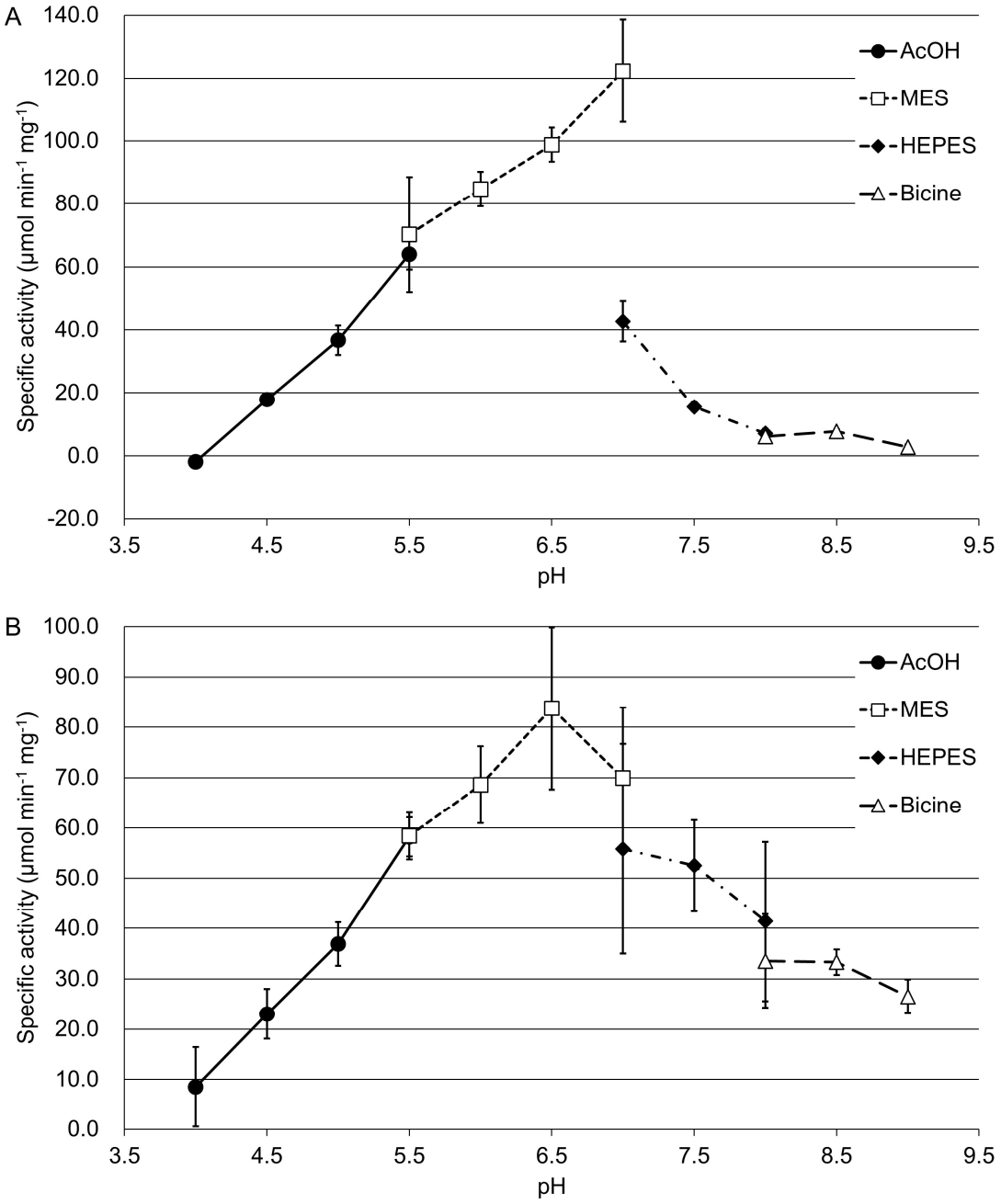


Fig. S2. Effect of pH on the 2-PG phosphatase activity of the TK1734 and TK2301 proteins. The phosphatase activities of the TK1734 (*A*) and TK2301 (*B*) recombinant proteins were measured in mixtures based on various buffers with concentrations of 50 mM. Symbols: closed circles, acetate-NaOH (pH 4.0-5.5); open squares, MES-NaOH (pH 5.5-7.0); closed diamonds, HEPES-NaOH (pH 7.0-8.0); open triangles, Bicine-NaOH (pH 8.0-9.0). The data represent the average of three independent experiments and are shown with the SD values.

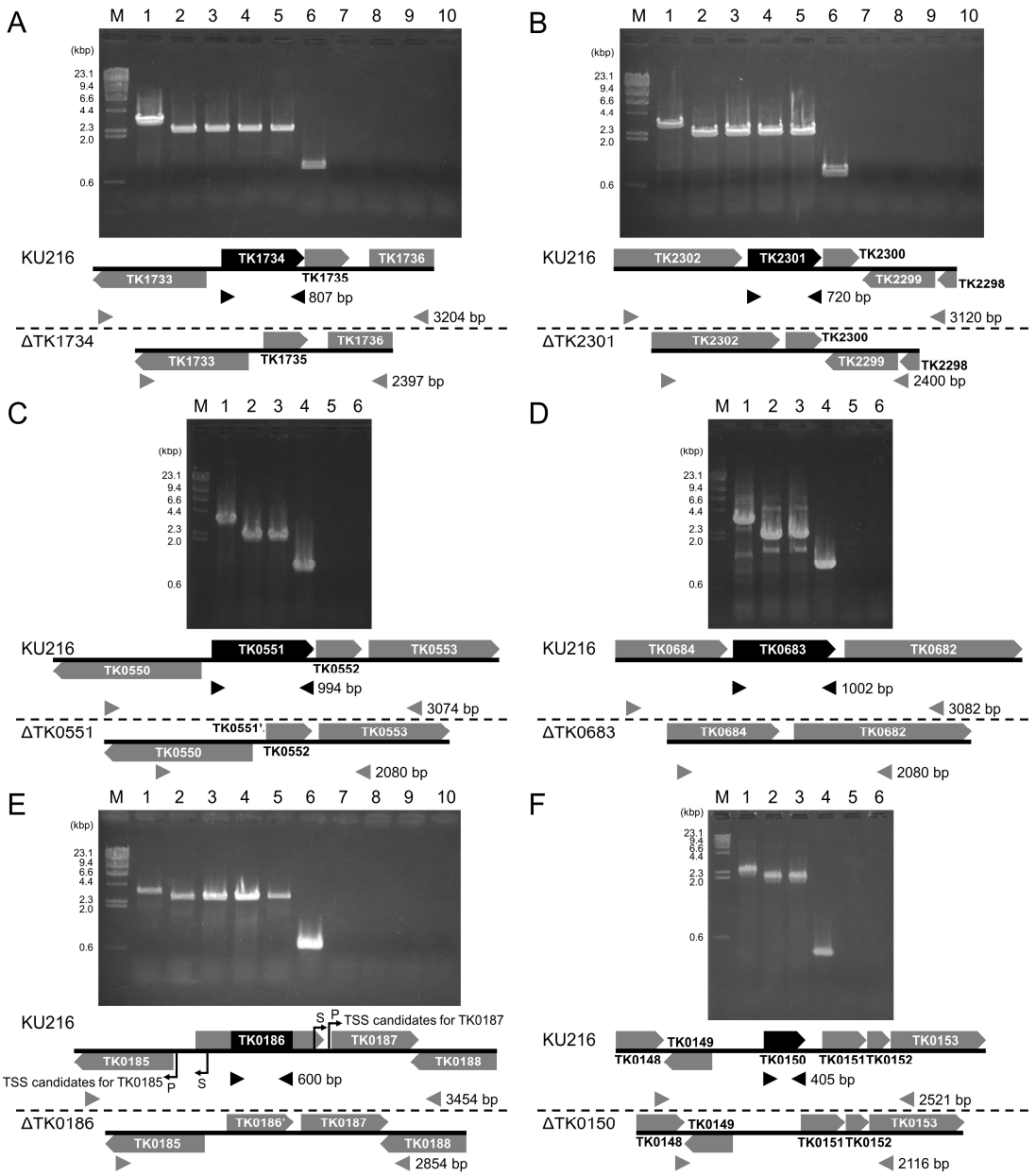


Fig. S3. PCR analysis of the gene disruption strains. The genotypes of individual gene disruption strains were analyzed by PCR using primer sets annealing outside of the 5'- and 3'-homologous regions for homologous recombination (outside pair, indicated by gray arrowheads) and the 5'- and 3'-terminal regions of the coding region of the target genes (inside pair, indicated by black arrowheads). (*A*) Lanes 1-5: outside, lanes 6-10: inside. Lanes 1, 6: *T. kodakarensis* KU216, lanes 2, 7: ΔTK1734-2, lanes 3, 8: ΔTK1734-4, lanes 4, 9: ΔTK1734-5, lanes 5, 10: ΔTK1734-6. (*B*) Lanes 1-5: outside, lanes 6-10: inside. Lanes 1, 6: *T. kodakarensis* KU216, lanes 2, 7: ΔTK2301-1, lanes 3, 8: ΔTK2301-2, lanes 4, 9: ΔTK2301-3, lanes 5, 10: ΔTK2301-4. (*C*) Lanes 1-3: outside, lanes 4-6: inside. Lanes 1, 4: *T. kodakarensis* KU216, lanes 2, 5: ΔTK0551-1, lanes 3, 6: ΔTK0551-2. (*D*) Lanes 1-3: outside, lanes 4-6: inside. Lanes 1, 4: *T. kodakarensis* KU216, lanes 2, 5: ΔTK0683-13, lanes 3, 6: ΔTK0683-15. (*E*) Lanes 1-5: outside, lanes 6-10: inside. Lanes 1, 6: *T. kodakarensis* KU216, lanes 2, 7: ΔTK0186-17, lanes 3, 8: ΔTK0186-25, lanes 4, 9: ΔTK0186-28, lanes 5, 10: ΔTK0186-33. Arrows P and S represent primary and secondary transcription initiation sites, respectively (18). (*F*) Lanes 1-3: outside, lanes 4-6: inside. Lanes 1, 4: *T. kodakarensis* KU216, lanes 2, 5: ΔTK0150-3, lanes 3, 6: ΔTK0150-26. λDNA digested with HindIII was used as a DNA marker (M).

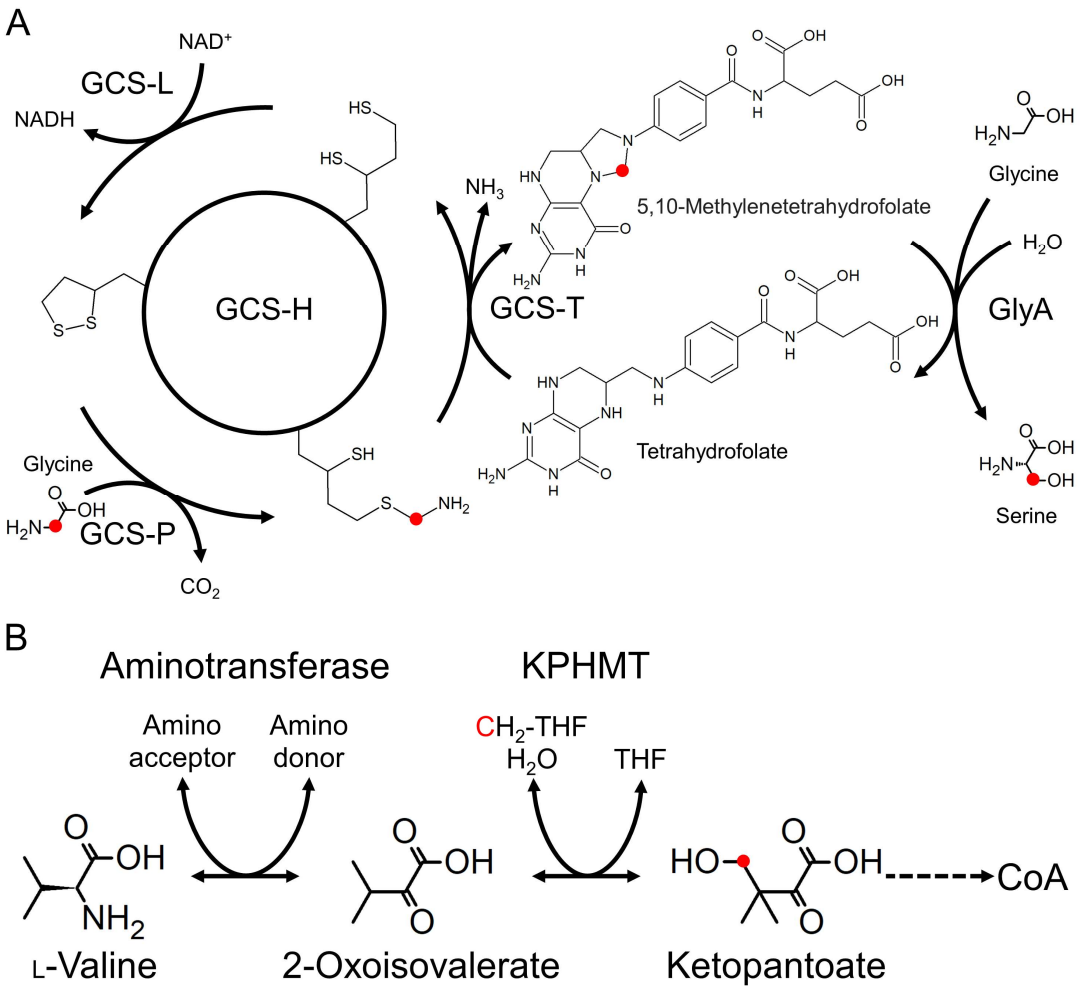


Fig. S4. C1 transfer reactions in *T. kodakarensis*. (*A*) The glycine cleavage system and C1 transfer. The glycine cleavage system is composed of four components: T-protein (aminomethyltransferase), P-protein (glycine decarboxylase), L-protein (dihydrolipoyl dehydrogenase) and H-protein, a protein modified with lipoic acid that interacts with all other components. In the system, P-protein first transfers an aminomethyl group from glycine onto the lipoic acid, which is attached to the H-protein linked with decarboxylation. Next, T-protein transfers the methylene carbon onto a tetrahydrofolate (THF) molecule, releasing ammonia and generating 5,10-methylenetetrahydrofolate (CH_2-THF). The reduced lipoic acid through the reactions of P- and T-protein is re-oxidized by L-protein utilizing NAD^+. The produced CH_2-THF is used as a C1 carrier for various C1 transfer reactions such as the serine hydroxymethyl transferase (GlyA) reaction. (*B*) Metabolism linking valine and coenzyme A biosynthesis. *T. kodakarensis* does not harbor a *de novo* synthesis pathway of 2-oxoisovalerate. Therefore, coenzyme A (CoA) biosynthesis in *T. kodakarensis* is dependent on 2-oxoisovalerate generation from valine. Valine is converted to 2-oxoisovalerate via transamination catalyzed by aminotransferases such as the TK0186 protein which was confirmed to display activity in this study. The produced 2-oxoisovalerate is next converted to ketopantoate by ketopantoate hydroxymethyltransferase (KPHMT). The reaction is known to utilize CH_2-THF as a C1 donor. The growth measurements of ΔTK0150 suggested that the KPHMT reaction shares the same C1 carrier with the GlyA reaction in *T. kodakarensis*.

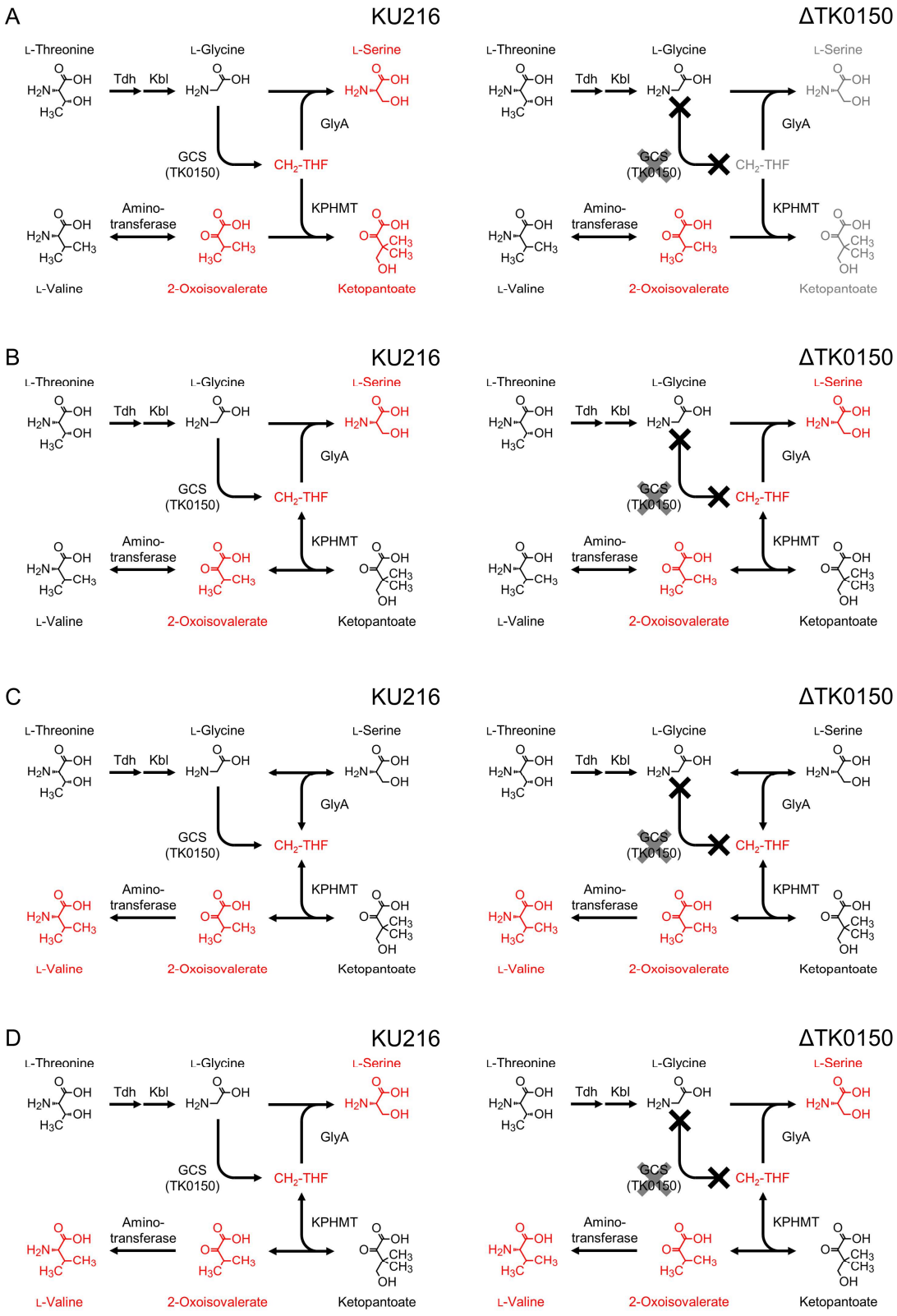


Fig. S5. Metabolic links for 5,10-methylenetetrahydrofolate generation and utilization. Compounds colored in black indicate the components present in the medium. Compounds colored in red (or gray) indicate those expected to be (or not to be) synthesized in the corresponding cells (KU216/ΔTK0150). (*A*) ASW-AA-S^0-Ura^+-Ser^- medium. (*B*) ASW-AA-S^0-

Ura^{+}-Ser^{-} medium with ketopantoate. (*C*) ASW-AA-S^{0}-Ura^{+}-Val^{-} medium with ketopantoate. (*D*) ASW-AA-S^{0}-Ura^{+}-Ser^{-}-Val^{-} medium with ketopantoate.

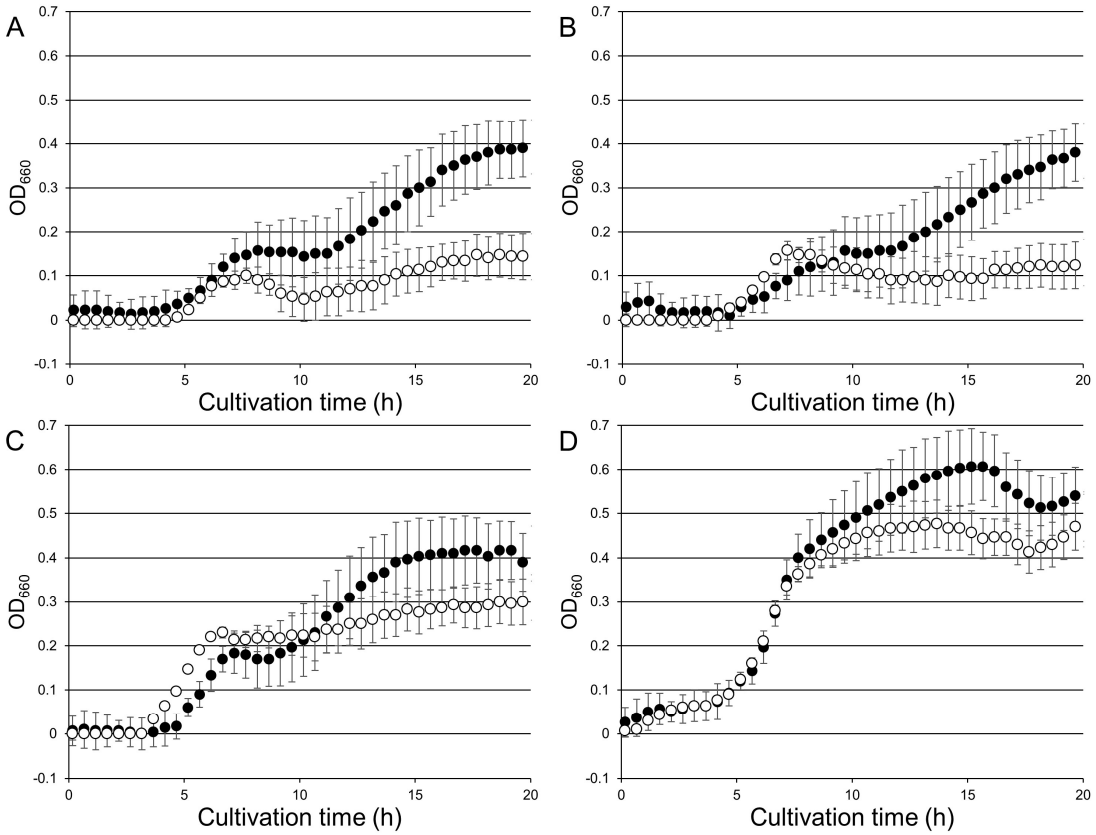


Fig. S6. Growth properties of *T. kodakarensis* KU216 and ΔTK1734 strains under microaerobic conditions. KU216 (closed circles) and ΔTK1734 (open circles) strains were cultivated in 10 mL ASW-YT-m1-Pyr medium in 20-mL vials supplemented with 25 g L^{-1} uridine. All media were left in an anaerobic box for 24 h prior to inoculation to allow dissolved gases to equilibrate with the atmosphere in the anaerobic box (oxygen concentration: ~0.1%). Media for *A* were prepared without Na_2S (microaerobic conditions), and the concentration of Na_2S in *B*, *C* and *D* were 0.08 mM, 0.16 mM and 0.32 mM, respectively. Error bars indicate the SD values of three independent culture experiments.

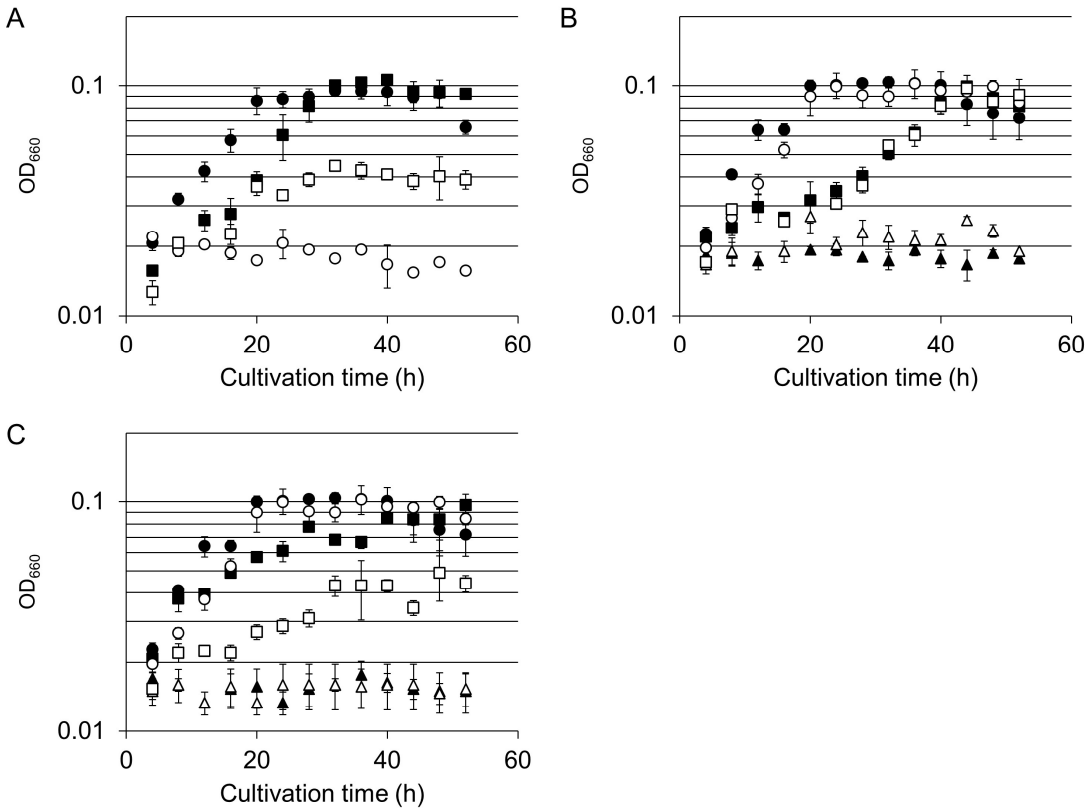


Fig. S7. Growth properties of *T. kodakarensis* KU216 and ΔTK0150 strains. (*A*) Effects of ketopantoate on serine auxotrophy were examined by cultivating cells in ASW-AA-S^0-Ura^+-Ser^- medium with or without 2 mM ketopantoate. Symbols: KU216 with (closed squares) or without ketopantoate (closed circles), ΔTK0150 with (open squares) or without ketopantoate (open circles). (*B*) Effects of ketopantoate on valine auxotrophy was examined by cultivating cells in ASW-AA-S^0-Ura^+-Val^- medium with or without 2 mM ketopantoate. Symbols: KU216 with valine (closed circles), without valine and with (closed squares) or without (closed triangles) ketopantoate, ΔTK0150 with valine (open circles), without valine and with (open squares) or without (open triangles) ketopantoate. (*C*) Effects of ketopantoate in ASW-AA-S^0-Ura^+-Ser^--Val^- medium with or without 2 mM ketopantoate. Symbols: KU216 with serine and valine (closed circles), without serine and valine, and with (closed squares) or without (closed triangles) ketopantoate, ΔTK0150 with serine and valine (open circles), without serine and valine, and with (open squares) or without (open triangles) ketopantoate. Error bars indicate the SD values of three independent culture experiments.

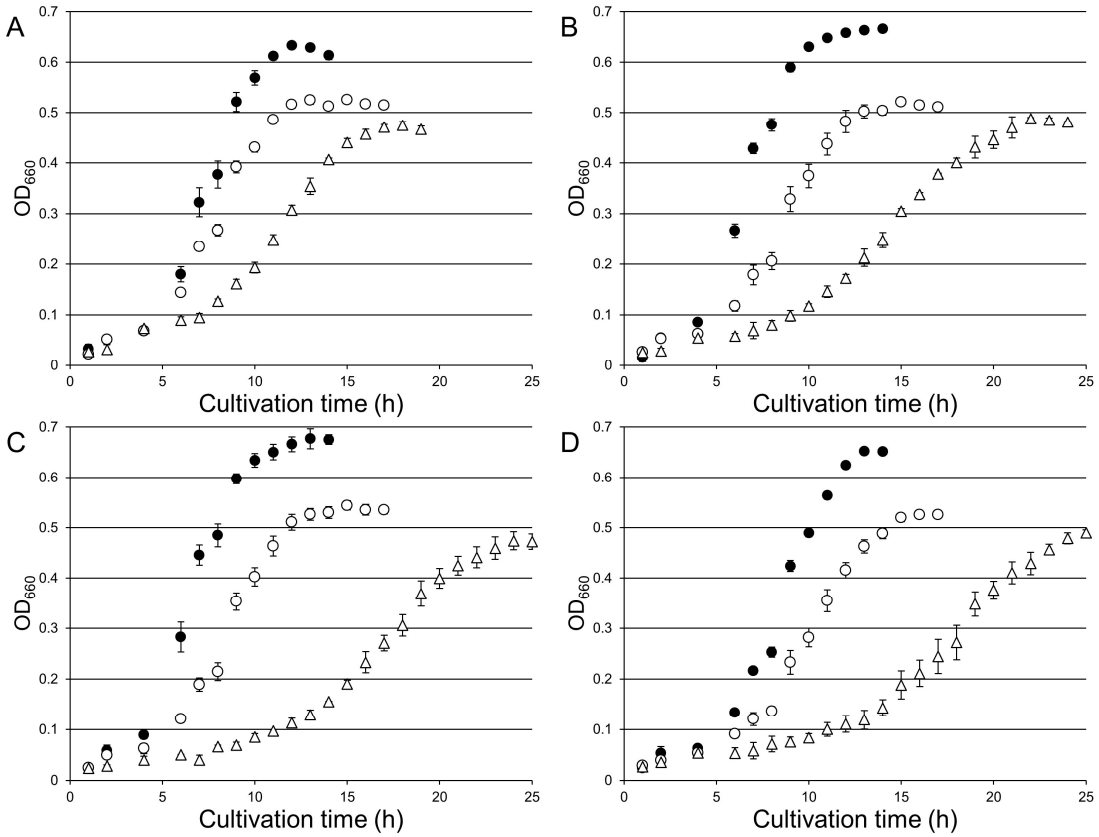
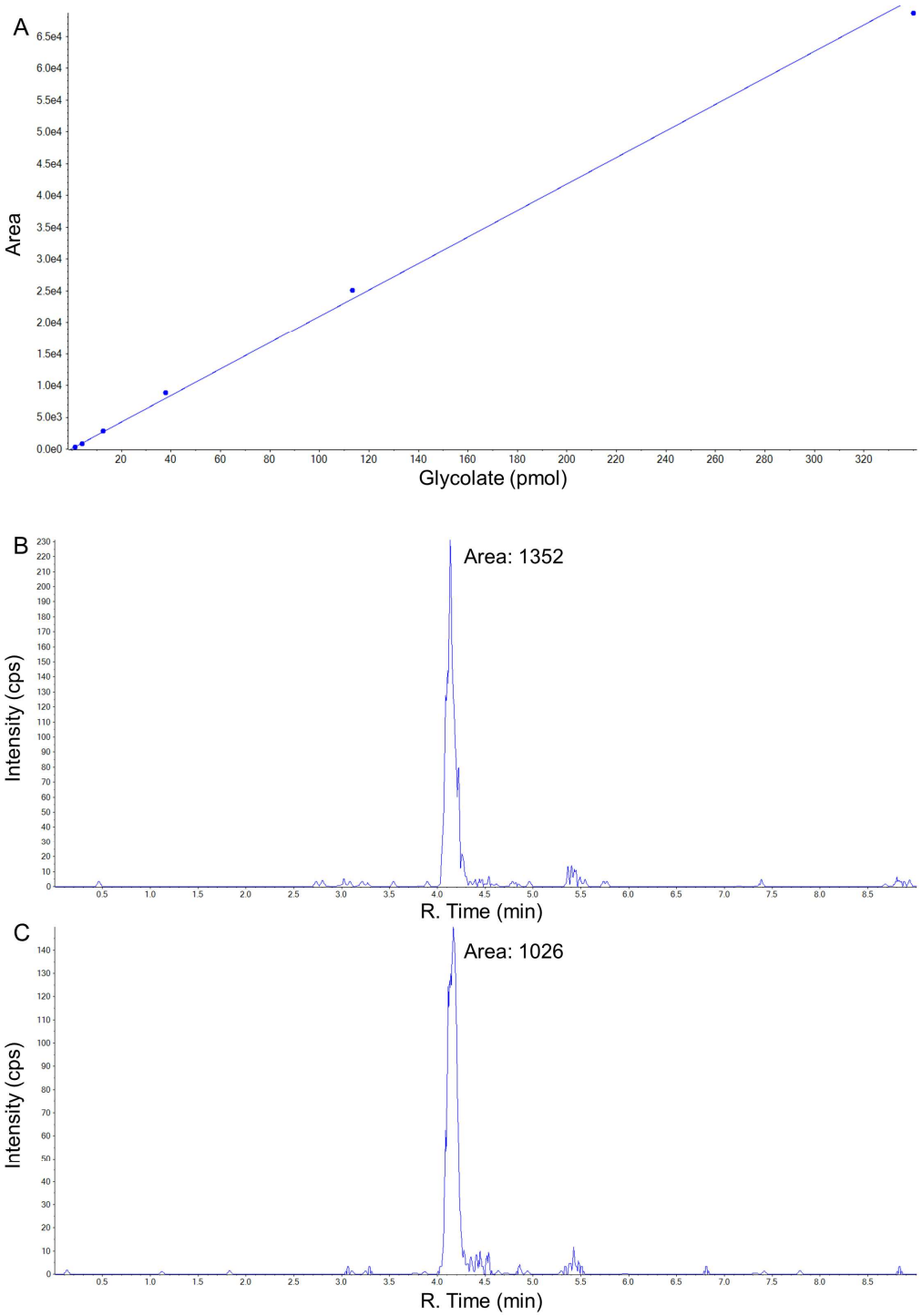
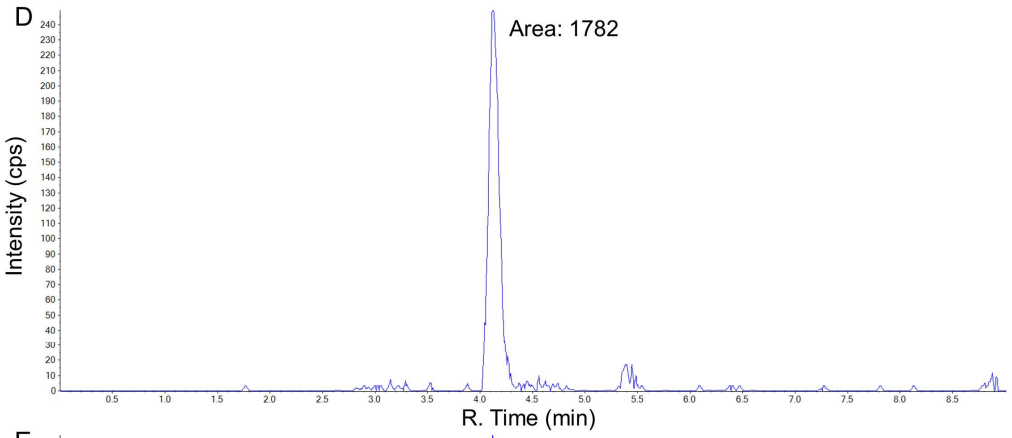


Fig. S8. Growth properties of *T. kodakarensis* KU216, ΔTK0683 and ΔTK0150 strains under microaerobic conditions. KU216 (closed circles), ΔTK0683 (open circles) and ΔTK0150 (open triangles) strains were cultivated in 10 mL ASW-YT-m1-Pyr medium in 30-mL test tubes. All media were left in an anaerobic box for 24 h prior to inoculation to allow dissolved gases to equilibrate with the atmosphere in the anaerobic box (oxygen concentration: ~0.1%). Media for *A* was prepared without Na_2S (microaerobic conditions), and the concentration of Na_2S in *B*, *C* and *D* were 0.08 mM, 0.16 mM and 0.32 mM, respectively. Error bars indicate the SD values of three independent culture experiments.


A
Area
Glycolate (pmol)
B
Area: 1352
Intensity (cps)
R. Time (min)
C
Area: 1026
Intensity (cps)
R. Time (min)


D
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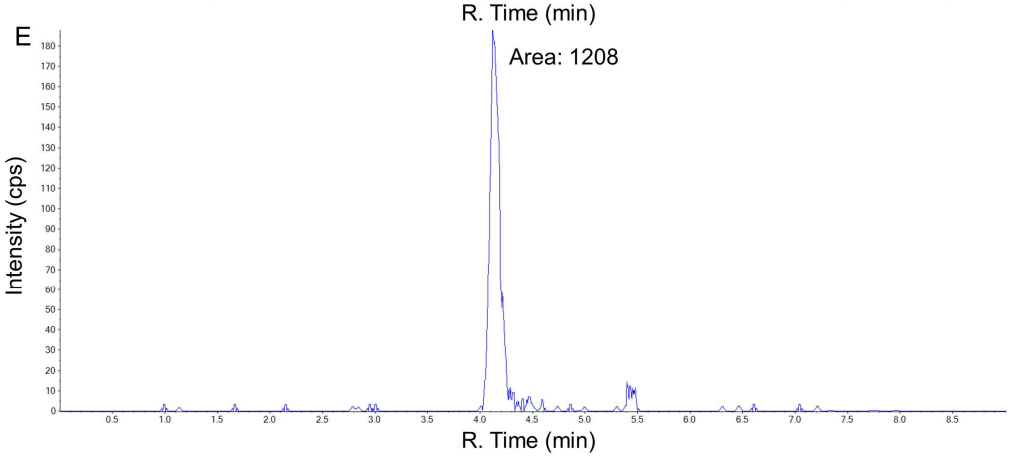
Intensity (cps)
R. Time (min)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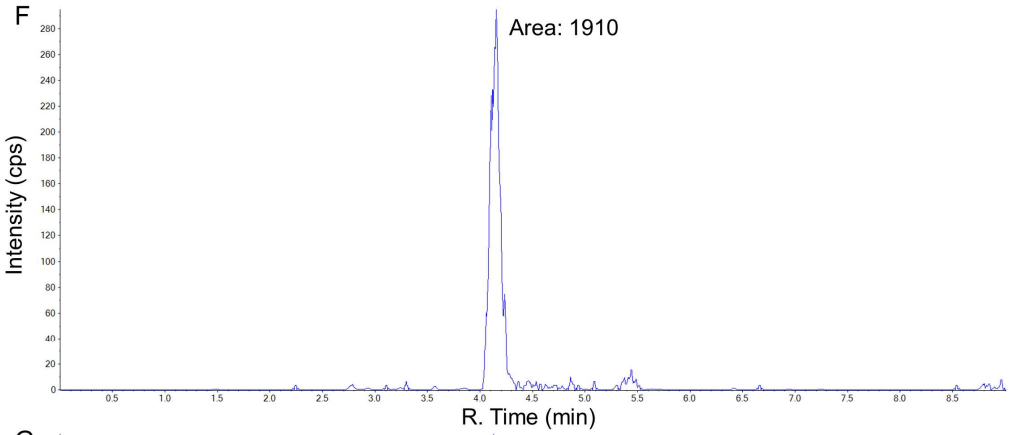
R. Time (min)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R. Time (min)

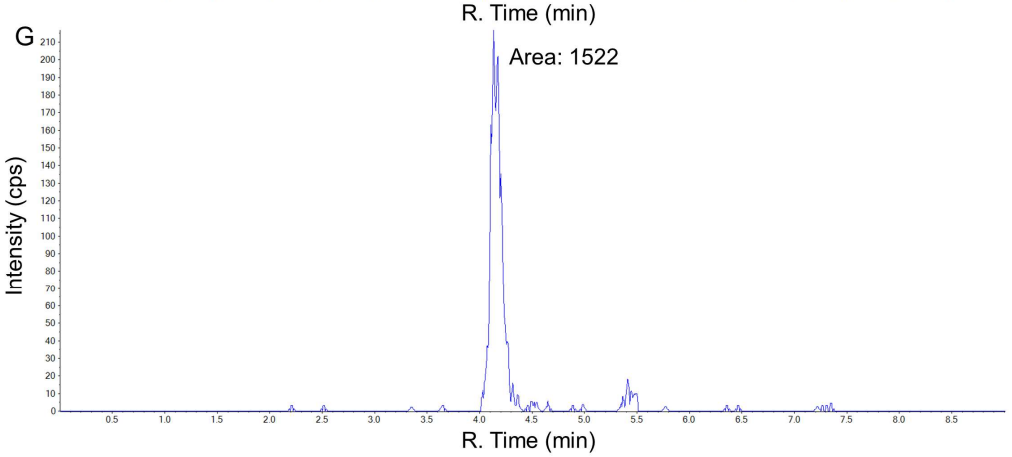
G
Area: 1522
Intensity (cps)
R. Time (min)

Fig. S9. Detection of glycolate in the culture supernatant after growth of *T. kodakarensis* KU216 under microaerobic conditions. The concentration of glycolate in the medium was examined by LC–MS/MS as described in the Methods section included in *SI Appendix*. *A* Calibration curve of amount of glycolate with peak area. Three sets of experiments were performed (*B*/*C*), (*D*/*E*) and (*F*/*G*). Each set represents data from medium with (*B*, *D*, *F*) or without (*C*, *E*, *G*) cell inoculation. Media in each set, including that used for pre-culture, were prepared together. Based on peak area, concentrations of glycolate deriving from *T. kodakarensis* were calculated as follows: B (24.414 μM) – C (18.163 μM) = 6.251 μM, D (32.652 μM) – E (21.656 μM) = 10.996 μM, F (35.112 μM) – G (27.677 μM) = 7.435 μM.

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