Genetic Examination and Mass Balance Analysis of Pyruvate/Amino Acid Oxidation Pathways in the Hyperthermophilic Archaeon *Thermococcus kodakarensis*

Kenta Nohara,^a Izumi Orita,^a Satoshi Nakamura,^a Tadayuki Imanaka,^b Toshiaki Fukui^a

Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama, Japan^a; Department of Biotechnology, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan^b

The present study investigated the simultaneous oxidation of pyruvate and amino acids during H₂-evolving growth of the hyperthermophilic archaeon *Thermococcus kodakarensis*. The comparison of mass balance between a cytosolic hydrogenase (HYH)-deficient strain (the Δ hyhBGS strain) and the parent strain indicated that NADPH generated via H₂ uptake by HYH was consumed by reductive amination of 2-oxoglutarate catalyzed by glutamate dehydrogenase. Further examinations were done to elucidate functions of three enzymes potentially involved in pyruvate oxidation: pyruvate formate-lyase (PFL), pyruvate:ferredoxin oxidoreductase (POR), and 2-oxoisovalerate:ferredoxin oxidoreductase (VOR) under the HYH-deficient background in *T. kodakarensis*. No significant change was observed by deletion of *pflDA*, suggesting that PFL had no critical role in pyruvate oxidation. The growth properties and mass balances of Δ porDAB and Δ vorDAB strains indicated that POR and VOR specifically functioned in oxidation of pyruvate and branched-chain amino acids, respectively, and the lack of POR or VOR was compensated for by promoting the oxidation of another substrate driven by the remaining oxidoreductase. The H₂ yields from the consumed pyruvate and amino acids were increased from 31% by the parent strain to 67% and 82% by the deletion of *hyhBGS* and double deletion of *hyhBGS* and *vorDAB*, respectively. Significant discrepancies in the mass balances were observed in excess formation of acetate and NH₃, suggesting the presence of unknown metabolisms in *T. kodakarensis* grown in the rich medium containing pyruvate.

The euryarchaeal order *Thermococcales*, composed of two major genera, *Thermococcus* and *Pyrococcus*, comprises hyperthermophiles capable of growing at temperatures around 100°C (1, 2). Many members of the *Thermococcales* are anaerobic sulfur-reducing heterotrophs preferring proteinaceous substrates and utilize elemental sulfur (S⁰) as a terminal electron acceptor evolving H₂S. In the absence of S⁰, some of these hyperthermophiles can grow on carbohydrates and related compounds, evolving hydrogen gas (H₂) by using protons as a terminal electron acceptor (3).

The energy-conserving metabolism of carbohydrates associated with H₂ evolution in the order *Thermococcales* has been well studied for *Thermococcus kodakarensis* and *Pyrococcus furiosus* (4). Pyruvate, added into the medium or generated from carbohydrates, is oxidized to acetyl coenzyme A (acetyl-CoA) by pyruvate:ferredoxin (Fd) oxidoreductase (POR) (5–8) and subsequently is converted to acetate concomitant with ATP synthesis by ADP-forming acetyl-CoA synthetases (9, 10). In addition to pyruvate oxidation, peptides and amino acids also are dissimilated simultaneously (11). Amino acids, which are directly incorporated into the cells or formed by degradation of peptides, are deaminated to the corresponding 2-oxoacids using 2-oxoglutarate as an amino acceptor by a number of aminotransferases with different substrate specificities (12–15). Glutamate formed by the transamination is regenerated to 2-oxoglutarate by alanine aminotransferase-mediated transfer of the amino group to pyruvate (15), resulting in the formation of alanine as one of the end products (15, 16). Although it is also possible that glutamate dehydrogenase (GDH) participates in the regeneration of 2-oxoglutarate through oxidative deamination of glutamate, GDH was suggested to act in the direction of glutamate formation when *T. kodakarensis* was grown with excess pyruvate (17). The amino acid-derived 2-oxoacids

then are oxidized to acyl-CoAs by a set of 2-oxoacid (2-ketoacid):Fd oxidoreductases (KORs), followed by conversion to the corresponding carboxylic acids concomitant with ATP synthesis. This is similar to the pyruvate oxidation pathway, and the final step is catalyzed by NDP-forming acyl-CoA synthetases, including ADP-forming acetyl-CoA synthetases, composed of possible 10 combinations of five α -subunits and two β -subunits (18–20).

In *Thermococcales* hyperthermophiles, two kinds of [NiFe] hydrogenase, membrane-bound hydrogenase (MBH) and cytosolic hydrogenase (HYH), are considered to participate in H₂ metabolism (21–28). MBH reduces protons to evolve H₂ using reduced ferredoxin (Fd_{red}), generated via catabolism of growth substrates, as an electron donor. It also exhibited proton-pumping activity during H₂ evolution, and the resulting proton-motive force was coupled with ATP synthesis driven by the membrane-bound ATP synthase directly or by being converted to sodium-motive force (21–24). It has been demonstrated genetically that MBH-lacking strains of *T. kodakarensis* and *P. furiosus* showed severe growth impairment under the H₂-evolving growth conditions (26–28). HYH in these hyperthermophiles acted in H₂ uptake rather than

Received 30 June 2014 Accepted 21 August 2014

Published ahead of print 25 August 2014

Address correspondence to Toshiaki Fukui, tfukui@bio.titech.ac.jp.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.02021-14>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.02021-14

H₂ evolution for the recovery of reducing equivalents from H₂ in the form of NADPH (25–28).

As mentioned above, 2-oxoacids, including pyruvate, are oxidatively decarboxylated to the corresponding CoA derivatives in an Fd-dependent manner by multiple KORs (29). Four kinds of KORs have been purified from *P. furiosus* and *Thermococcus litoralis*, and the following substrate specificities have been revealed: POR specifically catalyzes oxidation of pyruvate (5–8), and 2-oxoisovalerate:Fd oxidoreductase (VOR) shows activity with preference to 2-oxoacids derived from branched-chain amino acids (30, 31). Indolepyruvate:Fd oxidoreductase (IOR) recognizes 2-oxoacids from aromatic amino acids (32, 33), and 2-oxoglutarate:Fd oxidoreductase (OGOR) is specific to 2-oxoglutarate (34). POR is widely distributed in the three domains, whereas VOR, IOR, and OGOR have been found only in *Archaea* (29). Recently, it was demonstrated that OGOR from *T. kodakarensis* specifically was responsible for Glu/Gln degradation, as succinate production no longer was observed in the strain lacking OGOR (17). However, the details of functions and physiological roles of the set of KORs have been not thoroughly investigated to date.

Whole-genome analysis of *T. kodakarensis* has identified *pflD* (35), encoding a homolog of pyruvate formate-lyase (PFL) that catalyzes CoA-dependent cleavage of pyruvate to formate and acetyl-CoA (36). In addition, *pflA*, encoding a homolog of PFL-activating enzyme that probably activates PFL by forming the stable radical form (37), was identified as a neighbor of *pflD* on the *T. kodakarensis* chromosome. The presence of *pflD* is rather rare in *Archaea*, and it is found in a few *Thermococcus* species (*T. kodakarensis*, *T. litoralis*, and *T. sibiricus*) but not in *Pyrococcus* spp. among the order *Thermococcales*. In mesophilic facultative anaerobes, such as *Escherichia coli*, PFL has an important role in anaerobic metabolism, and formate generated by PFL is degraded to H₂ and CO₂ by the formate hydrogen-lyase (FHL) complex (38, 39). Although no obvious homolog of the FHL complex was identified in *T. kodakarensis*, the general function of PFL raises the possibility that PflDA in cooperation with an unknown formate oxidoreductase establishes a bypass pathway for pyruvate oxidation in this hyperthermophile.

In this study, we initially confirmed and assessed in detail the effects of gene deletion for HYH and then investigated the functions of POR, VOR, and PFL in the pyruvate/amino acid oxidation during H₂-evolving growth by *T. kodakarensis* on the basis of changes in mass balance caused by the deletion of the corresponding genes.

MATERIALS AND METHODS

Microorganisms and culture conditions. *E. coli* DH5α was used for general DNA manipulation, and *E. coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. *T. kodakarensis* strains and plasmids used in this study are listed in Table 1. *T. kodakarensis* strains were grown anaerobically at 85°C in a nutrient-rich medium (ASW-YT) composed of a 0.8-fold dilution of artificial seawater (40), 10 g/liter yeast extract, and 5.0 g/liter tryptone with either 5.0 g/liter S⁰ (ASW-YT-S⁰) or 5.0 g/liter sodium pyruvate (ASW-YT-Pyr). Minimal medium (ASW-AA-S⁰) containing the nutritionally required amino acids plus trace minerals, vitamins, and 5.0 g/liter elemental sulfur was prepared as described previously (40).

Target gene disruption in *T. kodakarensis*. DNA manipulation was carried out by standard procedures, and the sequences of oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. PCR was carried out with KOD-Plus-ver.2 DNA polymerase

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>T. kodakarensis</i> strains		
KOD1	Wild type	45
KUW1	Δ <i>pyrF</i> Δ <i>trpE</i> (uracil and tryptophan auxotroph)	42
KUW1Δ <i>hyh</i>	KUW1 derivative, Δ <i>hyhBGS</i> L	This study
KUW1ΔΔ <i>hyh</i> _pfl	KUW1 derivative, Δ <i>hyhBGS</i> L Δ <i>pflD</i> A	This study
KUW1ΔΔ <i>hyh</i> _por	KUW1 derivative, Δ <i>hyhBGS</i> L Δ <i>porDAB</i>	This study
KUW1ΔΔ <i>hyh</i> _vor	KUW1 derivative, Δ <i>hyhBGS</i> L Δ <i>vorDAB</i>	This study
Plasmids		
pUC118	Amp ^r , general cloning vector	TaKaRa Bio
pUD3	pUC118 derivative, <i>pyrF</i> marker cassette	41
pUD3-Δ <i>hyh</i>	pUD3 derivative, <i>hyhBGS</i> L homologous regions	This study
pUD3-Δ <i>pfl</i>	pUD3 derivative, <i>pflD</i> A homologous regions	This study
pUD3-Δ <i>por</i>	pUD3 derivative, <i>porDAB</i> homologous regions	This study
pUD3-Δ <i>vor</i>	pUD3 derivative, <i>vorDAB</i> homologous regions	This study

(Toyobo, Osaka, Japan). Plasmids for gene disruption of *hyhBGS*L (pUD3-Δ*hyh*), *pflD*A (pUD3-Δ*pfl*), *porDAB* (pUD3-Δ*por*), and *vorDAB* (pUD3-Δ*vor*) in *T. kodakarensis* were constructed based on pUD3 harboring a *pyrF* marker cassette (41). pUD3-Δ*hyh* and pUD3-Δ*vor* were constructed as follows. DNA fragments containing the respective target genes, *hyhBGS*L (*tk2072*-*tk2069*) and *vorDAB* (*tk1979*-*tk1981*), together with the flanking regions (approximately 800 bp), were amplified from *T. kodakarensis* KOD1 genomic DNA with primer sets *hyhBGS*L800–5′/*hyhBGS*L800–3′ and *vorD800*–5′/*vorB800*–3′, respectively. The amplified DNA fragments were inserted into pUD3 at the *Hinc*II site. Inverse PCRs with primers *hyhBGS*L-inv-5′/*hyhBGS*L-inv-3′ and *vor*-inv-5′/*vor*-inv-3′ then were carried out to amplify the respective homologous regions along with the plasmid backbone, thereby removing the coding regions. The amplified fragments were self-ligated after 5′-phosphorylation to obtain pUD3-Δ*hyh* and pUD3-Δ*vor*.

pUD3-Δ*por* was constructed similarly, with slight changes. A DNA fragment containing *porDAB* (*tk1982*-*tk1984*) together with the flanking regions was amplified with a primer set of *porD800*–5′/*porB800*–3′ and inserted into pUC118 at the *Hinc*II site. The plasmid was digested by *Cla*I and *Nde*I to remove the coding regions of *porDAB* and subsequently was self-ligated after blunting of the ends. The fragment of the connected homologous regions was excised from the vector by *Eco*RV and inserted into pUD3 at the *Hinc*II site.

pUD3-Δ*pfl* was constructed by the insertion of a fragment of upstream and downstream regions of *pflD*A (*tk0289*-*tk0290*) into pUD3 at the *Hinc*II site. The fragment was prepared by fusion PCR with the individual fragments of the upstream region amplified using *pflD800*–5′/*pflD800*-fusion-3′ primers and the downstream region amplified using *pflA800*-fusion-5′/*pflA800*–3′ primers.

Transformation of *T. kodakarensis* KUW1 (Δ*pyrF* Δ*trpE*) with the constructed vectors, and selection of transformants formed by pop-in/pop-out recombination based on uracil prototrophy of *pyrF*⁺ strains and resistance of *pyrF* mutant strains to 5-fluoro-orotic acid monohydrate (5-FOA), were performed according to the procedure described previously

FIG 1 Structures of *T. kodakarensis* *vor-por* and *pfl* gene clusters. The oligo(T) sequences that potentially acted as archaeal transcription terminators are indicated with a “V” (46).

(42). In the case of *vorDAB* deletion, ASW-YT-Pyr plate medium containing 0.75 g/liter 5-FOA and 5.0 g/liter sodium pyruvate was used for isolation of the pop-out strain. The successful recombination was verified by PCR using appropriate primer sets (see Fig. S1 in the supplemental material).

Determination of growth properties and measurement of H₂ and CO₂ in gas phase. *T. kodakarensis* strains were precultured in 100 ml of ASW-YT-Pyr at 85°C for 14 h, and the cells were inoculated into 120 ml of the same medium (1%, vol/vol) in a 100-ml bottle (40 ml headspace) with an Omnifit GL45 cap having two valve ports (Diva Industries, Danbury, CT). The bottle was incubated in an oil bath at 85°C, a portion of the broth was taken through one valve port every 2 h, and the optical density at 600 nm (OD₆₀₀) was measured by using an S1200 diode array spectrophotometer (Biochrom, Berlin, Germany). Another valve port was directly connected to a 0.5-liter-volume aluminum bag having one syringe port (GL Sciences, Tokyo, Japan) to collect gas evolved during the cultivation. A portion of the collected gas was taken through the syringe port by using a 500-μl gas-tight syringe (Hamilton, Reno, NV), and the concentrations of H₂ and CO₂ were determined by a gas chromatograph (GC-2014; Shimadzu, Kyoto, Japan) equipped with a stainless packed column (3.0 mm by 4 m; Shincarbon ST; Shinwa Chemical Industries, Kyoto, Japan) (carrier gas, Ar) and a thermal conductivity detector (current value, 50 mA). After that, the collected gas phase in the aluminum bag was transferred to a 1,011-ml vacuum bottle (GL Science), and the gas volume was calculated from the pressure change in the bottle measured by using a digital pressure meter.

The S⁰-dependent growth properties of *T. kodakarensis* strains were determined as follows. The cells were precultured in 100 ml of ASW-YT-S⁰ at 85°C for 14 h, except for the KUW1Δ*hyh_vor* strain, which was precultured in ASW-YT-Pyr due to the growth defect in S⁰-dependent condition, and then the cells were inoculated into 8 ml of ASW-YT-S⁰ (5%, vol/vol) in a tightly capped test tube. The optical density at 600 nm of the culture was directly measured every 2 h with the S1200 spectrophotometer.

Analyses of substrates and products in culture medium. After 12 to 14 h of cultivation of *T. kodakarensis* strains in ASW-YT-Pyr, the culture broth was centrifuged (5,000 × g, 20 min, 4°C), and the supernatant was ultrafiltrated with a Vivaspin 500 (3,000-molecular-weight cutoff; Sartorius, Göttingen, Germany). The concentration of each carboxylic acid in the supernatant was determined by ion chromatography (ICS-2100; Dionex, CA) equipped with a series of two ion exchange columns (IonPac AS11-HC and IonPac AS-19; both 4.0 mm by 250 mm; both from Dionex) and a conductivity detector (current value, 140 mA). The mobile phase was a gradient of KOH from 0.5 mM to 60 mM. The concentration of each amino acid in the supernatant was determined by high-performance liquid chromatography (HPLC) after acid hydrolysis and derivatization. The acid hydrolysis of the supernatant was done in the presence of 12 N HCl (Fe-free) (Nacalai Tesque, Kyoto, Japan) under reduced pressure in a sealed glass tube at 110°C for 24 h. The hydrolysate was reacted with phenylisothiocyanate according to the procedure described previously (43), and the derivatives were analyzed using a

Prominence HPLC apparatus (Shimadzu) equipped with a reverse-phase column (3.0 mm by 75 mm; Shim-pack XR-ODS; Shimadzu). The mobile phase was a linear gradient of acetonitrile in the range of 5% to 36% in 5 mM potassium phosphate buffer (pH 6.9), and the absorbance at 254 nm was monitored. The concentration of acetate was enzymatically determined by using F-kit acetate (Roche Diagnostics, Basel, Switzerland), and that of NH₃ was measured by using an ammonia test (Wako Pure Chemicals, Osaka, Japan). The amount of biomass was calculated based on the estimation that the organic fraction of the cell is C₅H₇NO₂, and this comprises 90% of cell dry weight (11).

RESULTS

Construction of *T. kodakarensis* mutants lacking genes for POR, VOR, or PFL potentially involved in pyruvate oxidation. It has been reported that POR and VOR from *P. furiosus* were heterotetramers, wherein the γ-subunit was shared with the two enzymes, and the genes were clustered as *porG* (*vorG*)-*vorDAB*-*porDAB* (6). *T. kodakarensis* also possesses a gene cluster with the same organization (*tk1978*-*tk1984*) as that shown in Fig. 1A, of which deduced amino acid sequences were highly homologous to the counterparts from *P. furiosus* and *T. litoralis* (>80% identities). PFL and its activating enzyme, encoded by *pflDA* (*tk0289*-*tk0290*) (Fig. 1B), shared 33% and 30% identities with the corresponding homologs from *E. coli*, respectively. The genes of POR, VOR, or PFL were individually deleted in *T. kodakarensis* by homologous recombination to evaluate the contribution of the enzymes to pyruvate/amino acid oxidation in this hyperthermophile. We used an *Hyh*-deficient strain, the KUW1Δ*hyh* mutant, which was constructed by deletion of *hyhBGS*L (*tk2072*-*tk2069*) in *T. kodakarensis* KUW1 (Δ*pyrF* Δ*trpE*) as a host strain in order to simplify the H₂-evolving metabolisms by blocking the uptake of the evolved H₂ (26, 27). Considering the presence of a common γ-subunit for POR and VOR, the knockout mutants of POR and VOR were constructed by deletion of the genes encoding δ, α, and β subunits of POR (*porDAB*) and those of VOR (*vorDAB*), respectively. The strain lacking PFL was constructed by deletion of almost the entire region of *pflDA* except an 8-bp region of the 3' end of *pflA* overlapped with the downstream gene *tk0291*, encoding a hypothetical protein. The genotype of the resulting KUW1Δ*hyh*, KUW1ΔΔ*hyh_por*, KUW1ΔΔ*hyh_vor*, and KUW1ΔΔ*hyh_pfl* strains were confirmed by PCR analyses as shown in Fig. S1 in the supplemental material.

Growth properties of the *T. kodakarensis* mutants. The growth properties of the constructed strains were evaluated in nutrient-rich media supplemented with S⁰ (ASW-YT-S⁰; H₂S-evolving conditions) or sodium pyruvate (ASW-YT-Pyr; H₂-

FIG 2 Growth property of *T. kodakarensis* strains in ASW-YT medium containing either S⁰ or sodium pyruvate. The cells were cultivated at 85°C, and optical densities at 600 nm were measured.

evolving conditions). Compared to those of the parent strain K UW1, the K UW1Δhyh strain showed a similar growth rate and slightly higher final cell density in both media (Fig. 2). Among the modified strains derived from the K UW1Δhyh strain, the most drastic change was observed for the K UW1ΔΔhyh_vor strain; growth was severely impaired in ASW-YT-S⁰. The growth rate and final cell density of the K UW1ΔΔhyh_vor strain in ASW-YT-Pyr also were decreased, although the change was less significant than that in ASW-YT-S⁰. The K UW1ΔΔhyh_por strain showed slightly lower growth ability than the K UW1Δhyh control strain. Almost no change in growth properties was observed for the K UW1ΔΔhyh_pfl strain.

Mass balance of pyruvate/amino acid oxidation in *T. kodakarensis* and the effects of *hyh* deletion. Although previous reports described an increase of H₂ evolution by gene disruption of Hyh acting on H₂ uptake in *T. kodakarensis* (26, 27), the alterations in pyruvate/amino acid oxidation have not been investigated in detail. Hence, the K UW1 and K UW1Δhyh strains were cultivated in ASW-YT-Pyr, and the changes in concentrations of substrates and end products in the culture broth and those of H₂ and CO₂ in the evolved gas phase were compared (Table 2). Among

TABLE 2 Consumption of substrates and production of fermentation products during the batch cultivation in ASW-YT-Pyr

Strain	Consumption (mmol/liter of culture)					Production (mmol/liter of culture)						
	OD ₆₀₀	Pyruvate	Amino acids ^a	NH ₃	Acetate	Alanine	Acids ^b	Formate	CO ₂	H ₂	NH ₃	Biomass ^c
K UW1	1.12 ± 0.08	37.0 ± 0.3	10.2 ± 1.0	4.50 ± 0.07	24.8 ± 1.1	20.4 ± 0.4	7.5 ± 0.2	0.97 ± 0.13	21.5 ± 1.8	14.7 ± 1.0	8.01 ± 0.40	3.14 ± 0.22
K UW1Δhyh	1.51 ± 0.09	32.9 ± 0.01	17.0 ± 0.1		31.7 ± 1.8	14.4 ± 1.6	11.5 ± 0.3	0.18 ± 0.02	33.4 ± 1.9	33.6 ± 1.9	8.24 ± 0.68	4.24 ± 0.26
K UW1ΔΔhyh_pfl	1.54 ± 0.05	32.8 ± 0.4	16.0 ± 1.9		32.5 ± 1.8	15.4 ± 0.4	12.7 ± 0.01	0.22 ± 0.03	34.1 ± 4.0	33.9 ± 2.7	5.84 ± 0.29	4.31 ± 0.13
K UW1ΔΔhyh_por	0.73 ± 0.02	22.9 ± 0.2	21.4 ± 0.6		8.3 ± 0.1	23.4 ± 0.7	16.7 ± 0.6	0.01 ± 0.00	17.8 ± 1.4	18.3 ± 0.6	7.69 ± 0.04	2.05 ± 0.07
K UW1ΔΔhyh_vor	1.19 ± 0.08	32.3 ± 0.1	9.4 ± 0.3		36.6 ± 0.2	8.9 ± 0.1	7.2 ± 0.1	0.15 ± 0.02	33.9 ± 2.2	34.0 ± 2.5	3.34 ± 0.23	

^a Tnp consumption is estimated from indoleacetate (derived from Tnp) production. Ala is not included in the sum.
^b The amount includes carboxylic acids corresponding to amino acids, except for formate and acetate. Thioglycolate (derived from Cys), 5-aminovaleate (Lys), 4-guanidinobutanoate (Arg), and imidazoleacetate (His) production was estimated from the corresponding amino acid consumption.
^c Based on the assumption that the organic fraction of cells is C₅H₇NO₂, and this comprises 90% of dry cell weight (11).

(A) Amino acid consumption

(B) Acid production

FIG 3 Consumption of amino acids (A) and production of the corresponding acids (B) during the cultivation of *T. kodakarensis* strains in an ASW-YT-Pyr medium. Each strain was cultivated for 12 h, except the KUW1ΔΔhyh_por strain, which was cultivated for 14 h. The letters in parentheses in panel B indicate the corresponding amino acids. Pro was estimated to be degraded after the conversion to Glu and formed succinate as the corresponding acid.

the amino acids and the corresponding carboxylic acids to be analyzed, the concentration of Trp could not be determined due to degradation during the acid hydrolysis of proteins/peptides, and thioglycolate (derived from Cys), 5-aminovalerate (Lys), 4-guanidinobutanoate (Arg), and imidazoleacetate (His) could not be detected by ion chromatography. Therefore, the consumption of Trp and production of the four acids were estimated from the production of indoleacetate (derived from Trp) and consumption of the corresponding amino acids, respectively. In Table 2 and the data below, the terms “amino acids” and “acids” indicated the sum of 19 amino acids besides alanine and that of 15 carboxylic acids except for acetate and formate, respectively. The consumption of each amino acid and the production of each acid are shown in Fig. 3.

The strain KUW1, expressing *hyhBGS*L, evolved 14.7 mmol/liter of culture of H₂ on pyruvate, which was much lower than the 21.5 mmol/liter of culture of CO₂ (Table 2). The amount of the produced acids corresponded to 74% of the consumed amino acids, and the tendency of less formation of acids always was ob-

served for *T. kodakarensis* strains in this study. The alanine formation was about two times greater than amino acid consumption, and consumption of NH₃ and production of formate during the cultivation were detected. Val, Leu, Ile, Phe, Ser, Asp/Asn, and Glu/Gln were preferred amino acids for KUW1 (Fig. 3), which agreed with the results in a previous continuous cultivation of *T. kodakarensis* KOD1 (11). The results also were consistent with substrate specificities of KORs and acyl-CoA synthetases, except for Asp/Asn (18–20, 30–34). The consumption of Val, Ile, and Phe coincided well with the formation of the corresponding acids, whereas Leu, Ser, Asp/Asn, and Glu/Gln were consumed more than the formation of the corresponding acids. This discrepancy was especially significant for Leu, as about only half of isovalerate was produced compared to the consumption of Leu.

The deletion of *hyhBGS*L increased evolution of both H₂ and CO₂, and the increase of H₂ was larger than that of CO₂, leading to equal production of H₂ and CO₂ (~33 mmol/liter of culture) by the KUW1Δhyh strain (Table 2). This strain showed less consumption of pyruvate and less formation of alanine and for-

mate than the parent strain KUW1. Notably, unlike KUW1, the KUW1 Δ hyh strain produced NH₃ during S⁰-independent growth on pyruvate. The equimolar formation of H₂ and CO₂ and production of NH₃ also were seen for all of the Hyh-deficient strains examined in this study. As shown in Fig. 3, the KUW1 Δ hyh strain showed tendencies for the amino acid consumption and acid formation that were similar to those of KUW1, except it showed significantly more consumption of Thr.

Based on the two major fates of pyruvate, conversion to alanine and acetate, it can be expected that the sum of the produced acetate and alanine corresponded to the consumption of pyruvate. Nevertheless, in the determined mass balances for KUW1 and the KUW1 Δ hyh mutant, the actual sums of acetate and alanine (45.2 and 46.1 mmol/liter of culture, respectively) were remarkably larger than those of pyruvate consumption (37.0 and 32.9 mmol/liter of culture, respectively).

Effects of PFL or POR knockout on metabolism in *T. kodakarensis*. Almost no change in mass balances was observed between the *pflDA* deletion strain KUW1 Δ hyh_pfl and the KUW1 Δ hyh parent strain (Table 2). Although formate formation was detected for both strains, the levels were low (0.18 to 0.22 mmol/liter of culture) and not altered by the disruption of *pflDA*. The results strongly suggested that the small amount of formate was not produced by Pfl-mediated cleavage of pyruvate.

In contrast, quite drastic changes were observed in the stoichiometry of pyruvate/amino acid oxidation by the *porDAB* deletion strain KUW1 Δ hyh_por; more consumption of amino acids and less consumption of pyruvate were observed compared to those by the KUW1 Δ hyh strain (Table 2), and alanine production and pyruvate consumption almost matched each other (~23 mmol/liter of culture). As shown in Fig. 3, this strain showed more consumption for most of the detectable amino acids than the KUW1 Δ hyh strain, particularly for Gly, Val, Leu, Ile, Met, Phe, and Glu/Gln. The consumption of Leu and Thr was significantly higher than the formation of the corresponding acids, as seen for the KUW1 Δ hyh strain.

Effects of VOR knockout on metabolism in *T. kodakarensis*. Deletion of *vorDAB* resulted in a drastic increase and decrease in the formation of acetate and alanine, respectively, leading to a much larger ratio of acetate to alanine (4.1) than that by the KUW1 Δ hyh control strain (2.2) (Table 2). The amino acid consumption by the KUW1 Δ hyh_vor strain remarkably decreased compared to that by the KUW1 Δ hyh strain, while it was well balanced to alanine formation, as seen for the other Hyh-deficient strains. Despite the great impact of the deletion of *vorDAB* on catabolism, H₂ and CO₂ were evolved in equal amounts to levels as high as those by the KUW1 Δ hyh strain.

The KUW1 Δ hyh_vor strain was scarcely able to consume Val, Leu, and Ile and completely lost the ability to produce the corresponding acids (Fig. 3), whereas the consumption of Phe and Tyr and the formation of the corresponding acids were rather increased compared to the levels for the KUW1 Δ hyh strain.

DISCUSSION

The present study determined the mass balance of the simultaneous oxidation of pyruvate and amino acids concomitant with H₂ evolution by *T. kodakarensis* grown in the absence of S⁰, and the cellular consumption of substrates and production of end products in the estimated catabolic pathways are presented in Fig. 4. Pyruvate played two roles as an acceptor for the amino acid-derived amino group via glutamate, forming alanine, and as an en-

ergy source to be oxidized to acetate. As shown in Fig. 4A, the determined mass balance for the *hyhBGS*L deletion strain KUW1 Δ hyh rather agreed with the estimated metabolisms except for a few points. The H₂/CO₂ ratio was 1.0, and the sum of the cellular production of acids/formate and that of acetyl-CoA estimated by subtraction of alanine formation from pyruvate consumption (20 mmol/liter of culture · OD₆₀₀) was comparable to the formation of H₂ and CO₂ (22 mmol/liter of culture · OD₆₀₀). The alanine formation also was comparable to the amino acid consumption, suggesting most amino groups in the consumed amino acids were transferred to pyruvate. The production of acids was slightly lower than the consumption of amino acids, which was probably due to the anabolic metabolism of amino acids. One severe discrepancy in the stoichiometry, not only for the KUW1 Δ hyh strain but also for other strains examined in this study, was excess production of acetate, which is discussed below.

Although the actual amount of CO₂ evolved by the strain KUW1 was less than that by the KUW1 Δ hyh strain, this was due mainly to the lower cell growth of KUW1 (Table 2), as the cellular formation of CO₂ by KUW1 (Fig. 4B) was similar to that of the KUW1 Δ hyh strain (Fig. 4A). On the other hand, the H₂/CO₂ ratio for KUW1 was 0.68, which indicated the uptake of H₂ by HYH as reported previously (26, 27). It was further clarified that KUW1 showed the formation of less H₂ and more alanine than the KUW1 Δ hyh strain, and NH₃ was consumed during the cultivation, in contrast to the significant production of NH₃ by the KUW1 Δ hyh strain. These facts strongly suggested that NADPH formed by the Hyh-driven reoxidation of H₂ was used in glutamate formation through GDH-catalyzed reductive amination of 2-oxoglutarate, and the amino group in the resulting glutamate consequently was transferred to pyruvate to form alanine (Fig. 4B). Indeed, the increase of alanine formation attributed to the function of Hyh (8.7 mmol/liter of culture · OD₆₀₀) nearly corresponded to the change of NH₃ concentration (−9.3 mmol/liter of culture · OD₆₀₀) observed for KUW1. This suggests that the reducing equivalent recovered from H₂ was consumed mostly by GDH; thus, it hardly contributed to anabolic metabolisms in *T. kodakarensis*, at least under the cultivation conditions examined. The results also demonstrated an advantage of the deletion of *hyhBGS*L in the improvement of the yield of H₂ to the consumed substrates (amino acids and pyruvate) from 31% to 67%.

Although PFL-mediated cleavage of pyruvate was one possible pathway for formate formation in *T. kodakarensis*, the concentration of formate was not changed by the deletion of *pflDA* (Table 2). Moreover, the KUW1 Δ hyh_pfl strain showed almost no differences in growth property and mass balance compared to the KUW1 Δ hyh parent strain, indicating that the PFL homolog did not participate in pyruvate/amino acid oxidation in *T. kodakarensis*. Interestingly, a higher level of formation of formate was observed for KUW1 than for the KUW1 Δ hyh strain (Table 2), suggesting some of the reducing equivalent recovered from H₂ by HYH was used to reduce CO₂ to formate by an unknown formate oxidoreductase. We assumed that the unknown formate dehydrogenase was related to a gene, *tk2076*, showing 40% identity to the formate dehydrogenase α -subunit FdhF from *E. coli*, and this was supported by the observation that the deletion of *tk2076* from KUW1 decreased formate production to a level similar to that by the KUW1 Δ hyh strain (data not shown).

This study also elucidated that POR and VOR were specifically involved in the oxidation of pyruvate and branched-chain amino

FIG 4 Mass balances of pyruvate/amino acid oxidation in *T. kodakarensis* KUW1Δhyh (A), KUW1 (B), KUW1Δhyh_por (C), and KUW1Δhyh_vor (D) strains. The cellular consumption and production were calculated as mmol/liter of culture · OD₆₀₀. The formation of acetyl-CoA shown in parentheses was estimated by subtraction of alanine formation from pyruvate consumption. An unidentified source for the excess formation of acetate or acetyl-CoA is shown by an "X."

acids *in vivo*, respectively. In particular, the pyruvate consumption by the KUW1Δhyh_por strain was well matched with alanine formation, and the evolution of CO₂ and H₂ was comparable to that of acid production. These results most likely were because the pyruvate oxidation was nearly completely blocked by the deletion of *porDAB*; thus, the energy conservation was shifted from the oxidation of pyruvate/amino acids to the oxidation of amino acids only (Fig. 4C). VOR appeared not to participate in the pyruvate oxidation, although it has been reported that VOR from *T. litoralis* showed weak activity to pyruvate (30). The deletion of *vorDAB* diminished the oxidation of branched-chain amino acids (Fig. 3 and 4D), which was consistent with the substrate specificity (30), as well as suggesting that VOR has a unique role in the oxidation of branched-chain amino acids in *T. kodakarensis*. The remaining oxidation ability for amino acids in the KUW1Δhyh_vor strain was supposed to be mediated by OGOR oxidizing 2-oxoglutarate derived from Glu/Gln and IOR preferring 2-oxoacids derived from aromatic amino acids. The related metabolic changes by the *vorDAB* deletion were the decrease in alanine formation and increase in pyruvate consumption. The KUW1Δhyh_vor strain showed a much lower growth rate and final cell density than the KUW1Δhyh strain in ASW-YT-S⁰, whereas such growth impairment was not significant in ASW-YT-Pyr (Fig. 2). It was estimated that the VOR-mediated oxidation of

branched-chain amino acids was an important energy-conserving pathway in *T. kodakarensis* grown in the presence of S⁰, and the weakened amino acid oxidation in the KUW1Δhyh_vor strain was compensated for by the enhancement of pyruvate oxidation when pyruvate was available. From the viewpoint of microbial H₂ production, the deletion of *vorDAB* was a beneficial modification, because the H₂ yield from the consumed substrates was increased up to 82% from 67% by the KUW1Δhyh parent strain. We also attempted the double deletion of *vorDAB* and *porDAB* in *T. kodakarensis* KUW1 and KUW1Δhyh strains, but this was unsuccessful even after several trials using selection medium supplemented with S⁰, starch, and an increased concentration of aromatic amino acids. This result suggested that the action of VOR or POR is essential for the growth of *T. kodakarensis*.

The most severe discrepancy in the mass balances determined in this study was the excess formation of acetate or alanine toward pyruvate consumption. Considering that alanine formation was well balanced with amino acid consumption, as described above, this discrepancy probably was attributed to the excess formation of acetate in *T. kodakarensis*. The levels of excess acetate (or acetyl-CoA) reached 7.3 to 12.1 mmol/liter · OD₆₀₀ (Fig. 4), and a similar discrepancy in acetate formation by *T. kodakarensis* has been reported by Yokooji et al. (17). Although we initially considered that the excess acetate was formed through the hydrolysis of acetylated

compounds in rich medium during cell growth, acetate was not detected after acid hydrolysis of the fresh rich medium (data not shown). Another possible source of the excess acetate was assumed to be some sugars in the rich medium. However, this was unlikely, because the degradation of sugars to acetate led to the formation of more H_2 than CO_2 (2:1 with hexoses), which did not agree with the equimolar formation of H_2 and CO_2 for the *hyh-BGSL* deletion strains examined. These results suggested the presence of an acetate-generating pathway from an unidentified source (indicated with an "X" in Fig. 4) in *T. kodakarensis*. Interestingly, such an excess formation of acetate was not observed in continuous cultivation of *T. kodakarensis* with a rich medium and batch cultivation of *P. furiosus* with a semisynthetic medium (11, 16). We also observed the formation of NH_3 in the *Hyh*-deficient strains of *T. kodakarensis*. As the present results exhibited almost stoichiometric transfer of an amino group in amino acids to pyruvate, such release of free NH_3 during the oxidation of amino acids was unexpected. Another disagreement was seen between consumption of some amino acids and formation of the corresponding acids, particularly for Leu and Thr, as shown in Fig. 3. It was supposed that some Leu residues were consumed by anabolic reactions in *T. kodakarensis* lacking the Leu biosynthesis pathway (35). The higher level of consumption of Thr may be caused by threonine dehydrogenase activity in *T. kodakarensis* (17, 44). Given the formation of acetyl-CoA by the threonine dehydrogenase-initiated degradation of Thr, this side pathway may contribute to the excess formation of acetate, although this would not be sufficient to explain the large amount of excess acetate. The results of mass balance analyses in this study suggested the presence of unknown metabolisms of some compounds in rich medium during batch cultivation of *T. kodakarensis*. Further analysis will be needed to elucidate these phenomena in the hyperthermophilic archaeon.

ACKNOWLEDGMENT

This work was supported by a research grant from the Institute of Fermentations, Osaka, Japan.

REFERENCES

- Huber R, Stetter KO. 2001. Discovery of hyperthermophilic microorganisms. *Methods Enzymol.* 330:11–24. [http://dx.doi.org/10.1016/S0076-6879\(01\)30367-1](http://dx.doi.org/10.1016/S0076-6879(01)30367-1).
- Itoh T. 2003. Taxonomy of nonmethanogenic hyperthermophilic and related thermophilic archaea. *J. Biosci. Bioeng.* 96:203–212. [http://dx.doi.org/10.1016/S1389-1723\(03\)80183-4](http://dx.doi.org/10.1016/S1389-1723(03)80183-4).
- Amend JP, Shock EL. 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiol. Rev.* 25:175–243. <http://dx.doi.org/10.1111/j.1574-6976.2001.tb00576.x>.
- Bräsen C, Esser D, Rauch B, Siebers B. 2014. Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. *Microbiol. Mol. Biol. Rev.* 78:89–175. <http://dx.doi.org/10.1128/MMBR.00041-13>.
- Blamey JM, Adams MW. 1993. Purification and characterization of pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochim. Biophys. Acta* 1161:19–27. [http://dx.doi.org/10.1016/0167-4838\(93\)90190-3](http://dx.doi.org/10.1016/0167-4838(93)90190-3).
- Kletzin A, Adams MW. 1996. Molecular and phylogenetic characterization of pyruvate and 2-ketoisovalerate ferredoxin oxidoreductases from *Pyrococcus furiosus* and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima*. *J. Bacteriol.* 178:248–257.
- Ma K, Hutchins A, Sung SJ, Adams MW. 1997. Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Pyrococcus furiosus*, functions as a CoA-dependent pyruvate decarboxylase. *Proc. Natl. Acad. Sci. U. S. A.* 94:9608–9613. <http://dx.doi.org/10.1073/pnas.94.18.9608>.
- Menon AL, Hendrix H, Hutchins A, Verhagen MF, Adams MW. 1998. The δ -subunit of pyruvate ferredoxin oxidoreductase from *Pyrococcus furiosus* is a redox-active, iron-sulfur protein: evidence for an ancestral relationship with 8Fe-type ferredoxins. *Biochemistry* 37:12838–12846. <http://dx.doi.org/10.1021/bi980979p>.
- Mai X, Adams MW. 1996. Purification and characterization of two reversible and ADP-dependent acetyl coenzyme A synthetases from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* 178:5897–5903.
- Glasemacher J, Bock AK, Schmid R, Schönheit P. 1997. Purification and properties of acetyl-CoA synthetase (ADP-forming), an archaeal enzyme of acetate formation and ATP synthesis, from the hyperthermophile *Pyrococcus furiosus*. *Eur. J. Biochem.* 244:561–567. <http://dx.doi.org/10.1111/j.1432-1033.1997.00561.x>.
- Kanai T, Imanaka H, Nakajima A, Uwamori K, Omori Y, Fukui T, Atomi H, Imanaka T. 2005. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakarensis* KOD1. *J. Biotechnol.* 116:271–282. <http://dx.doi.org/10.1016/j.jbiotec.2004.11.002>.
- Ward DE, de Vos WM, van der Oost J. 2002. Molecular analysis of the role of two aromatic aminotransferases and a broad-specificity aspartate aminotransferase in the aromatic amino acid metabolism of *Pyrococcus furiosus*. *Archaea* 1:133–141. <http://dx.doi.org/10.1155/2002/959031>.
- Andreotti G, Cubellis MV, Nitti G, Sannia G, Mai X, Marino G, Adams MW. 1994. Characterization of aromatic aminotransferases from the hyperthermophilic archaeon *Thermococcus litoralis*. *Eur. J. Biochem.* 220:543–549. <http://dx.doi.org/10.1111/j.1432-1033.1994.tb18654.x>.
- Matsui I, Matsui E, Sakai Y, Kikuchi H, Kawarabayashi Y, Ura H, Kawaguchi S, Kuramitsu S, Harata K. 2000. The molecular structure of hyperthermostable aromatic aminotransferase with novel substrate specificity from *Pyrococcus horikoshii*. *J. Biol. Chem.* 275:4871–4879. <http://dx.doi.org/10.1074/jbc.275.7.4871>.
- Ward DE, Kengen SWM, van der Oost J, de Vos WM. 2000. Purification and characterization of the alanine aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its role in alanine production. *J. Bacteriol.* 182:2559–2566. <http://dx.doi.org/10.1128/JB.182.9.2559-2566.2000>.
- Kengen SWM, Stams AJM. 1994. Formation of l-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. *Arch. Microbiol.* 161:168–175. <http://dx.doi.org/10.1007/BF00276479>.
- Yokooji Y, Sato T, Fujiwara S, Imanaka T, Atomi H. 2013. Genetic examination of initial amino acid oxidation and glutamate catabolism in the hyperthermophilic archaeon *Thermococcus kodakarensis*. *J. Bacteriol.* 195:1940–1948. <http://dx.doi.org/10.1128/JB.01979-12>.
- Shikata K, Fukui T, Atomi H, Imanaka T. 2007. A novel ADP-forming succinyl-CoA synthetase in *Thermococcus kodakarensis* structurally related to the archaeal NDP-forming acetyl-CoA synthetases. *J. Biol. Chem.* 282:26963–26970. <http://dx.doi.org/10.1074/jbc.M702694200>.
- Awano T, Wilming A, Tomita H, Fukui T, Imanaka T, Atomi H. 2014. Characterization of two members among the five ADP-forming acyl coenzyme A (acyl-CoA) synthetases reveals the presence of a 2-(imidazole-4-yl) acetyl-CoA synthetase in *Thermococcus kodakarensis*. *J. Bacteriol.* 196:140–147. <http://dx.doi.org/10.1128/JB.00877-13>.
- Scott JW, Poole FL, Adams MW. 2014. Characterization of ten heterotetrameric NDP-dependent acyl-CoA synthetases of the hyperthermophilic archaeon *Pyrococcus furiosus*. *Archaea* <http://dx.doi.org/10.1155/2014/176863>.
- Silva PJ, van den Ban ECD, Wassink H, Haaker H, de Castro B, Robb FT, Hagen WR. 2000. Enzymes of hydrogen metabolism in *Pyrococcus furiosus*. *Eur. J. Biochem.* 267:6541–6551. <http://dx.doi.org/10.1046/j.1432-1327.2000.01745.x>.
- Sapra R, Bagramyan K, Adams MW. 2003. A simple energy-conserving system: proton reduction coupled to proton translocation. *Proc. Natl. Acad. Sci. U. S. A.* 100:7545–7550. <http://dx.doi.org/10.1073/pnas.1331436100>.
- Schut GJ, Boyd ES, Peters JW, Adams MW. 2013. The modular respiratory complexes involved in hydrogen and sulfur metabolism by heterotrophic hyperthermophilic archaea and their evolutionary implications. *FEMS Microbiol. Rev.* 37:182–203. <http://dx.doi.org/10.1111/j.1574-6976.2012.00346.x>.
- Pisa KY, Huber H, Thomm M, Müller V. 2007. A sodium ion-dependent

- A₁A₀ ATP synthase from the hyperthermophilic archaeon *Pyrococcus furiosus*. FEBS J. 274:3928–3938. <http://dx.doi.org/10.1111/j.1742-4658.2007.05925.x>.
25. Kanai T, Ito S, Imanaka T. 2003. Characterization of a cytosolic NiFe-hydrogenase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. J. Bacteriol. 185:1705–1711. <http://dx.doi.org/10.1128/JB.185.5.1705-1711.2003>.
 26. Kanai T, Matsuoka R, Beppu H, Nakajima A, Okada Y, Atomi H, Imanaka T. 2011. Distinct physiological roles of the three [NiFe]-hydrogenase orthologs in the hyperthermophilic archaeon *Thermococcus kodakarensis*. J. Bacteriol. 193:3109–3016. <http://dx.doi.org/10.1128/JB.01072-10>.
 27. Santangelo TJ, Cuboňová L, Reeve JN. 2011. Deletion of alternative pathways for reductant recycling in *Thermococcus kodakarensis* increases hydrogen production. Mol. Microbiol. 81:897–911. <http://dx.doi.org/10.1111/j.1365-2958.2011.07734.x>.
 28. Schut GJ, Nixon WJ, Lipscomb GL, Scott RA, Adams MW. 2012. Mutational analyses of the enzymes involved in the metabolism of hydrogen by the hyperthermophilic archaeon *Pyrococcus furiosus*. Front. Microbiol. 3:163–168. <http://dx.doi.org/10.3389/fmicb.2012.00163>.
 29. Schut GJ, Menon AL, Adams MW. 2001. 2-Keto acid oxidoreductases from *Pyrococcus furiosus* and *Thermococcus litoralis*. Methods Enzymol. 331:144–158. [http://dx.doi.org/10.1016/S0076-6879\(01\)31053-4](http://dx.doi.org/10.1016/S0076-6879(01)31053-4).
 30. Heider J, Mai X, Adams MW. 1996. Characterization of 2-ketoisovalerate ferredoxin oxidoreductase, a new and reversible coenzyme A-dependent enzyme involved in peptide fermentation by hyperthermophilic archaea. J. Bacteriol. 178:780–787.
 31. Ozawa Y, Nakamura T, Kamata N, Yasujima D, Urushiyama A, Yamakura F, Ohmori D, Imai T. 2005. *Thermococcus profundus* 2-ketoisovalerate ferredoxin oxidoreductase, a key enzyme in the archaeal energy-producing amino acid metabolic pathway. J. Biochem. 137:101–107. <http://dx.doi.org/10.1093/jb/mvi012>.
 32. Mai X, Adams MW. 1994. Indolepyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. A new enzyme involved in peptide fermentation. J. Biol. Chem. 269:16726–16732.
 33. Ozawa Y, Siddiqui MA, Takahashi Y, Urushiyama A, Ohmori D, Yamakura F, Arisaka F, Imai T. 2012. Indolepyruvate ferredoxin oxidoreductase: an oxygen-sensitive iron-sulfur enzyme from the hyperthermophilic archaeon *Thermococcus profundus*. J. Biosci. Bioeng. 114:23–27. <http://dx.doi.org/10.1016/j.jbiosc.2012.02.014>.
 34. Mai X, Adams MW. 1996. Characterization of a fourth type of 2-keto acid-oxidizing enzyme from a hyperthermophilic archaeon: 2-ketoglutarate ferredoxin oxidoreductase from *Thermococcus litoralis*. J. Bacteriol. 178:5890–5896.
 35. Fukui T, Atomi H, Kanai T, Matsumi R, Fujiwara S, Imanaka T. 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. Genome Res. 15:352–363. <http://dx.doi.org/10.1101/gr.3003105>.
 36. Knappe J, Blaschkowski HP, Grobner P, Schmitt T. 1974. Pyruvate formate-lyase of *Escherichia coli*: the acetyl-enzyme intermediate. Eur. J. Biochem. 263:253–263.
 37. Buis JM, Broderick JB. 2005. Pyruvate formate-lyase activating enzyme: elucidation of a novel mechanism for glycyl radical formation. Arch. Biochem. Biophys. 433:288–296. <http://dx.doi.org/10.1016/j.abb.2004.09.028>.
 38. Bagramyan K, Trchounian A. 2003. Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from *Escherichia coli*. Biochemistry 68:1159–1170.
 39. Leonhartsberger S, Korsa I, Böck A. 2002. The molecular biology of formate metabolism in Enterobacteria. J. Mol. Microbiol. Biotechnol. 4:269–276.
 40. Sato T, Fukui T, Atomi H, Imanaka T. 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. J. Bacteriol. 185:210–220. <http://dx.doi.org/10.1128/JB.185.1.210-220.2003>.
 41. Kobori H, Ogino M, Orita I, Nakamura S, Imanaka T, Fukui T. 2010. Characterization of NADH oxidase/NADPH polysulfide oxidoreductase and its unexpected participation in oxygen sensitivity in an anaerobic hyperthermophilic archaeon. J. Bacteriol. 192:5192–5202. <http://dx.doi.org/10.1128/JB.00235-10>.
 42. Sato T, Fukui T, Atomi H, Imanaka T. 2005. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. Appl. Environ. Microbiol. 71:3889–3899. <http://dx.doi.org/10.1128/AEM.71.7.3889-3899.2005>.
 43. Koop DR, Morgan ET, Tarr GE, Coon MJ. 1982. Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. J. Biol. Chem. 257:8472–8480.
 44. Bashir Q, Rashid N, Jamil F, Imanaka T, Akhtar M. 2009. Highly thermostable L-threonine dehydrogenase from the hyperthermophilic archaeon *Thermococcus kodakaraensis*. J. Biochem. 146:95–120. <http://dx.doi.org/10.1093/jb/mvp051>.
 45. Atomi H, Fukui T, Kanai T, Morikawa M, Imanaka T. 2004. Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. Archaea 1:263–267. <http://dx.doi.org/10.1155/2004/204953>.
 46. Santangelo TJ, Cuboňová L, Skinner KM, Reeve JN. 2009. Archaeal intrinsic transcription termination in vivo. J. Bacteriol. 191:7102–7108. <http://dx.doi.org/10.1128/JB.00982-09>.