AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

GENETICS AND MOLECULAR BIOLOGY

A Genetic System for the Thermophilic Acetogenic Bacterium *Thermoanaerobacter kivui*

Mirko Basen,[a] Irina Geiger,[a] Laura Henke,[a] Volker Müller[a]

[a]Department of Molecular Microbiology & Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe University, Frankfurt am Main, Germany

ABSTRACT *Thermoanaerobacter kivui* is one of the very few thermophilic acetogenic microorganisms. It grows optimally at 66°C on sugars but also lithotrophically with H_2 + CO_2 or with CO, producing acetate as the major product. While a genome-derived model of acetogenesis has been developed, only a few physiological or biochemical experiments regarding the function of important enzymes in carbon and energy metabolism have been carried out. To address this issue, we developed a method for targeted markerless gene deletions and for integration of genes into the genome of *T. kivui*. The strain naturally took up plasmid DNA in the exponential growth phase, with a transformation frequency of up to 3.9×10^{-6}. A nonreplicating plasmid and selection with 5-fluoroorotate was used to delete the gene encoding the orotate phosphoribosyltransferase (*pyrE*), resulting in a Δ*pyrE* uracil-auxotrophic strain, TKV002. Reintroduction of *pyrE* on a plasmid or insertion of *pyrE* into different loci within the genome restored growth without uracil. We subsequently studied fructose metabolism in *T. kivui*. The gene *fruK* (TKV_c23150) encoding 1-phosphofructosekinase (1-PFK) was deleted, using *pyrE* as a selective marker via two single homologous recombination events. The resulting Δ*fruK* strain, TKV003, did not grow on fructose; however, growth on glucose (or on mannose) was unaffected. The combination of *pyrE* as a selective marker and the natural competence of the strain for DNA uptake will be the basis for future studies on CO_2 reduction and energy conservation and their regulation in this thermophilic acetogenic bacterium.

IMPORTANCE Acetogenic bacteria are currently the focus of research toward biotechnological applications due to their potential for *de novo* synthesis of carbon compounds such as acetate, butyrate, or ethanol from H_2 + CO_2 or from synthesis gas. Based on available genome sequences and on biochemical experiments, acetogens differ in their energy metabolism. Thus, there is an urgent need to understand the carbon and electron flows through the Wood–Ljungdahl pathway and their links to energy conservation, which requires genetic manipulations such as deletion or overexpression of genes encoding putative key enzymes. Unfortunately, genetic systems have been reported for only a few acetogenic bacteria. Here, we demonstrate proof of concept for the genetic modification of the thermophilic acetogenic species *Thermoanaerobacter kivui*. The genetic system will be used to study genes involved in biosynthesis and energy metabolism, and may further be applied to metabolically engineer *T. kivui* to produce fuels and chemicals.

KEYWORDS DNA uptake, *Thermoanaerobacter kivui*, acetogenesis, fructose metabolism, genetic system

Acetogenic microorganisms are a widespread ecophysiological guild of microorganisms that fulfill important functions in anoxic environments (1). To date, all isolated acetogens belong to the domain *Bacteria*, and within that domain many species, including the model organisms *Acetobacterium woodii*, *Moorella thermoacetica*, and

Received 5 October 2017 **Accepted** 11 November 2017

Accepted manuscript posted online 17 November 2017

Citation Basen M, Geiger I, Henke L, Müller V. 2018. A genetic system for the thermophilic acetogenic bacterium *Thermoanaerobacter kivui*. Appl Environ Microbiol 84:e02210-17. https://doi.org/10.1128/AEM.02210-17.

Editor Marie A. Elliot, McMaster University

Address correspondence to Mirko Basen, basen@bio.uni-frankfurt.de.

Clostridium ljungdahlii, are associated with the phylum *Firmicutes* (2). Recently, a metagenome study from a deep biosphere environment revealed the potential for acetogenesis in an uncharacterized archaeon (3). Many acetogens are metabolically versatile, able to degrade sugars, products of primary fermentations such as alcohols or C_1 compounds (methanol, formate, and CO), and methylated nitrogen compounds such as glycine betaine (4). An important and eponymous characteristic of acetogenic bacteria is that they thrive on the production of acetic acid from $H_2 + CO_2$ ($\Delta G^{o\prime} = -95$ kJ · mol^{-1}). Thus, they represent an important link in the anoxic food chain between primary fermenters that produce H_2 and CO_2 as some of their main products and acetoclastic methanogens (5). Acetate is formed from $H_2 + CO_2$ through the Wood-Ljungdahl pathway (6, 7). Notably, this pathway is likely the oldest CO_2-fixing pathway in nature (8). Moreover, it is also the only one that does not require a net investment of ATP. While the biochemistry of the Wood-Ljungdahl pathway (WLP) has been studied for decades, the mechanism of how an acetogen, the mesophilic bacterium *A. woodii*, conserves energy during growth on $H_2 + CO_2$ has only recently been found. Interestingly, in *A. woodii*, reduction of CO_2 is catalyzed by soluble enzymes, including the hydrogen-dependent carbon dioxide reductase (9). Energy is conserved by a membrane-bound ferredoxin:NAD$^+$ oxidoreductase, the Rnf complex (10). Genomes of other acetogens lack *rnf* genes but contain genes encoding all subunits of energy-converting hydrogenases (11), which led to a novel classification into Ech- or Rnf-containing acetogens based on the acetogen's mode of energy conservation (2). However, biochemical evidence for this notion is lacking. The thermophilic acetogenic species *Thermoanaerobacter kivui* is considered a model for the Ech-containing acetogens and thus a candidate to address this hypothesis.

Here, we describe the development and application of a genetic toolkit for the thermophilic acetogenic bacterium *T. kivui*. This bacterium also belongs to the phylum *Firmicutes*, and it grows optimally at 66°C, being therefore the most thermophilic acetogenic bacterium characterized yet (12). Comparably short doubling times of ~2.5 h during growth on $H_2 + CO_2$, the lack of requirement for vitamins (12), and the recent discovery that the organism can be adapted to grow on CO (13) make the bacterium a potential candidate for bioengineering approaches toward synthesis gas fermentation. Analysis of the genome sequence revealed that energy conservation during autotrophic growth is different than that in the mesophilic acetogenic species *A. woodii* and *C. ljungdahlii*. While the genome contains the Wood-Ljungdahl pathway for CO_2 fixation, genes encoding the Rnf complex are absent (14); however, energy may instead be conserved through one of two Ech hydrogenase complexes. Interestingly, the substrate range of *T. kivui* is rather restricted compared to that of other, nonacetogenic species within the genus *Thermoanaerobacter* (15) but also compared to that of other acetogens. For example, unlike the thermophilic species *M. thermoacetica*, *T. kivui* is not able to metabolize alcohols, and unlike mesophilic *Sporomusa* species or *A. woodii*, it cannot use methylated compounds such as methanol, methylamines, or glycine betaine (4). Besides $H_2 + CO_2$ and CO, *T. kivui* has been described to only use pyruvate, formate, and the sugars glucose, fructose, and mannose to mainly produce acetate (12). These observations indicate that the pathways of substrate oxidation, acetate formation from $H_2 + CO_2$, and energy conservation in *T. kivui* differ from those in other acetogens. This prompted us to develop genetic tools to study its metabolism. In this article, we describe the ability of *T. kivui* for DNA uptake, the generation of a uracil-auxotrophic *pyrE* deletion mutant strain, and the application of *pyrE* as a selective marker for markerless deletions on the chromosome of *T. kivui*.

RESULTS AND DISCUSSION

***T. kivui* is naturally competent for DNA uptake.** Our aim was to develop methods for genetic modification of the thermophilic acetogenic bacterium *T. kivui*, including both the uptake and replication of plasmids and the generation of mutations on the chromosome. Initially, we developed a protocol to grow *T. kivui* on solid media, enabling the selection of single colonies, which was complicated by the requirement

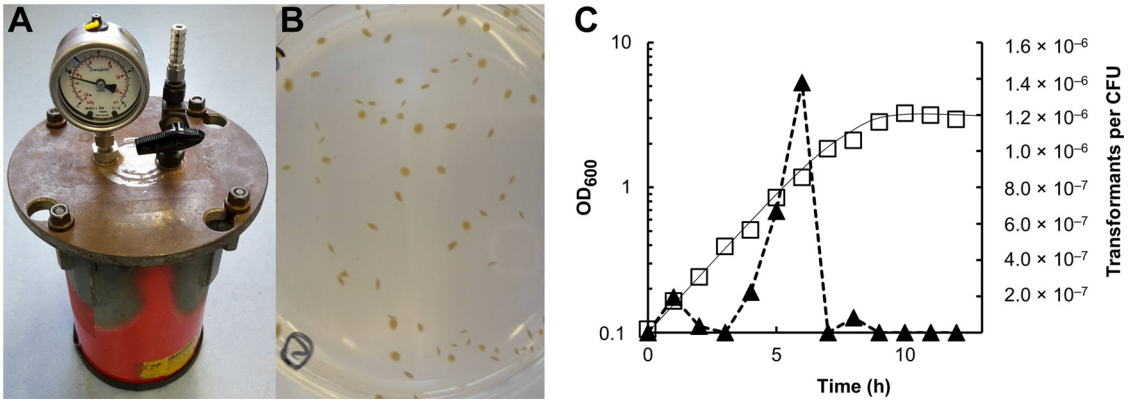

FIG 1 Growth of *T. kivui* in solid medium and natural competence for DNA uptake. (A) Sealed metal jar for anaerobic incubation of *T. kivui* at 65°C. (B) *T. kivui* embedded in agar medium formed yellow to brownish disc-like colonies. (C) Natural competence of *T. kivui* during growth on complex medium with glucose at 65°C. One-ml subsamples of two growing cultures were incubated with 250 ng of plasmid pMU131 (16), and then embedded in complex medium with or without 200 $\mu g \cdot ml^{-1}$ kanamycin. Squares, representative growth curve; triangles, corresponding transformants per CFU.

for anoxic conditions at elevated temperatures. To address this requirement, custom-made jars made from fire extinguishers already available to the lab were filled with an oxygen-free atmosphere (approximately 1% H_2 in N_2/CO_2 [80/20, vol/vol]) and proved to keep anoxic conditions during incubation at 65°C (Fig. 1A). Plating of *T. kivui* was initially performed inside an anoxic glove box; however, we found that *T. kivui* tolerates short exposure to oxygen at room temperature (~22°C), likely because the strain is thermophilic, with no growth or metabolic activity much below 50°C (12). Maintaining sterile conditions and general handling are much easier outside the anoxic glove box; therefore, we decided to routinely pour the plates at ambient temperature and air and subsequently transferred them to an anoxic glove box, allowing the agar medium to solidify before incubation in the jars. *T. kivui* formed yellowish to brownish convex colonies when embedded in the agar medium (Fig. 1B), as observed by Leigh et al. (12). Regularly, 5% to 10% of the cells from the liquid medium formed colonies, with a maximal observed plating efficiency of ~70%. Streaking a culture on the surface of solidified medium, as performed with aerobes, also worked for *T. kivui* as described before (12), but colonies were less defined, and the plating efficiency was lower.

Having established a method for growth on solid medium, the next step was to find a method to insert foreign DNA into the cells. The ability to take up plasmid DNA by natural competence has been described for several *Thermoanaerobacter species* and for the related *Thermoanaerobacterium saccharolyticum* (16). Therefore, we decided to obtain a plasmid, pMU131, that was used by Shaw and colleagues (16) and that contains a native origin of replication for *Thermoanaerobacterium*, a PUC origin for replication in *Escherichia coli*, and Amp^r for ampicillin selection in *Escherichia coli*. The plasmid also contained a thermostable kanamycin resistance cassette from *Staphylococcus aureus* (Kan^r) that obviously conferred resistance to higher concentrations of kanamycin (>100 $\mu g \cdot ml^{-1}$) in several *Thermoanaerobacter* species (16). That observation was valuable to us, as thermostable antibiotics and proteins conferring mediating resistance to antibiotics at higher temperatures are scarce, and only a few selectable markers for kanamycin, hygromycin, bleomycin (17, 18), and thiamphenicol (18) resistance are available for the genetic manipulation of thermophilic bacteria. The MIC of kanamycin that did not allow growth of *T. kivui* in liquid and solid agar medium was determined to be 200 $\mu g \cdot ml^{-1}$, which is in the range of reported values for other *Thermoanaerobacter* species (16). Subsequently, growing cultures of *T. kivui* were incubated in the presence of plasmid pMU131 (0.5 $\mu g \cdot ml^{-1}$) until the stationary phase was reached (optical density at 600 nm [OD_{600}], ≥2.5), and then plated. As observed for other *Thermoanaerobacter* species, after 2 to 3 days of anoxic incubation at 65°C, kanamycin-resistant colonies only occurred on plates with *T. kivui* that had been grown

previously in the presence of plasmid pMU131. Colonies were transferred to liquid medium containing 200 $\mu g \cdot ml^{-1}$ kanamycin, and after growth had been observed, the presence of the plasmid in the kanamycin resistant isolates was verified by reisolation of the plasmid, followed by PCR or by restriction digest analysis (data not shown). This showed that *T. kivui* is naturally competent for DNA uptake, as reported for other *Thermoanaerobacter* species (16). Unlike in the related species *Thermoanaerobacterium saccharolyticum*, where the highest transformation frequencies were observed in the early exponential growth phase (16), DNA uptake in *T. kivui* mainly occurred in the mid-exponential growth phase (Fig. 1C). Accordingly, transcript levels of genes annotated as *comEA* (TKV_RS04530), *comEC1* (TKV_RS04705), *comEC2* (TKV_RS11745), and *recA* (TKV_RS06255), which are involved in natural competence for DNA uptake in other species, e.g., in the related Gram-positive *Bacillus subtilis* (19) or in *T. saccharolyticum* (16), were increased by factors of $\sim 2.5 \pm 0.4$, 2.1 ± 0.4, 2.4 ± 0.8, and 2.1 ± 1.0 in the mid-exponential ($OD_{600} = 1.3$) versus the early exponential ($OD_{600} \sim 0.3$) growth phases. Yet, the molecular mechanism of DNA uptake in *T. kivui* has not been studied, and may or may not be similar to what has been published for other Gram-positive anaerobes. A transformation frequency—the fraction of living cells that took up plasmid pMU131—of up to 3.9×10^{-6} was observed, which is in the range of that reported for other *Thermoanaerobacter* species (16). In the mid-exponential growth phase ($\sim 4 \times 10^8$ cells $\cdot$ ml^{-1}), about 10^3 cells were transformed per μg plasmid DNA (9.1×10^2 μg^{-1}, 0.25 μg DNA provided per ml culture). A high transformation efficiency was also observed at a DNA concentration of 0.8 $\mu g \cdot ml^{-1}$ (7.1×10^2 μg^{-1}), while the transformation efficiencies slightly decreased if 1.0 $\mu g \cdot ml^{-1}$ or more DNA was provided ($\leq 3.3 \times 10^2$ μg^{-1}). Taken together, we demonstrated that *T. kivui* is naturally competent for DNA uptake, and a thermostable kanamycin resistance cassette, Kan^r, can be used as selective marker.

Generation of a *pyrE* deletion mutant. Only a few genetic systems for markerless mutants have been reported for anaerobic thermophiles. The most commonly used selection mechanism is based on the requirement for uracil for the biosynthesis of pyrimidines (17). On the one hand, incubation with 5-fluoroorotate (5-FOA) allows selection against the presence of either gene (*pyrE* or *pyrF*) encoding orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase, respectively, as 5-fluoroorotic acid is converted to toxic 5-fluorouracil in the presence of both enzymes. On the other hand, reintroduction of the respective gene complements the uracil auxotrophy, again leading to a 5-FOA-sensitive phenotype. Genetic systems based on uracil auxotrophy and sensitivity to 5-FOA have been developed, e.g., for the hyperthermophilic archaeon *Thermococcus kodakarensis* (20) and for the extreme thermophilic *Thermoanaerobacter*-related plant biomass degrader *Caldicellulosiruptor bescii* (21, 22). Many *Thermoanaerobacter* species, however, require the addition of yeast extract, which contains uracil, to the medium. Hence, for some species, kanamycin resistance cassettes have been integrated to disrupt genes (23), or alternative genetic markers such as thymidine kinase have been used (24).

As mentioned above, *T. kivui* does not depend on the addition of yeast extract or even on vitamins (12), enabling us to use *pyrE* as a genetic marker. This was fortunate, as our goal was the development of a markerless mutagenesis system in *T. kivui*. Initially, to enforce integration of DNA into the *T. kivui* genome, a basic plasmid (pMBTkv001) was generated that contained Kan^r but not the putative *Thermoanaerobacterium* origin of replication. As expected, transformation with pMBTkv001 *per se* did not result in any kanamycin-resistant colonies. We then fused ~1-kbp upstream and downstream regions (UFR and DFR, respectively) flanking the *pyrE* gene of *T. kivui* into the multiple cloning site of pMBTkv001, resulting in plasmid pMBTkv002b (Fig. 2A). This enabled homologous recombination at the UFR or DFR site, and integration of pMBTkv002b into the genome resulted in kanamycin-resistant colonies of *T. kivui*. When we isolated DNA from these colonies and verified by PCR the integration of the plasmid into the genome, we also observed an additional smaller DNA fragment that

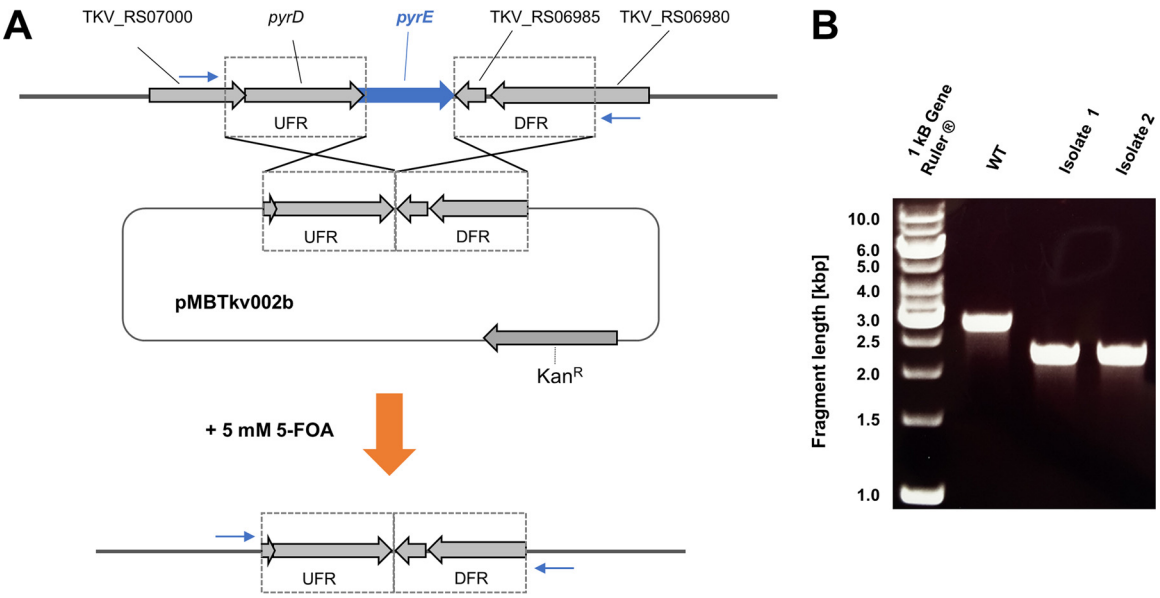

FIG 2 Deletion of *pyrE*. (A) *T. kivui* was transformed with plasmid pMBTkv002b, which was constructed for the deletion of the *pyrE* gene via double homologous recombination using 1-kbp upstream and downstream flanking regions (UFR and DFR). After transformation, *T. kivui* was plated in the presence of 5 mM 5-fluoroorotic acid (5-FOA). (B) The loss of *pyrE* (573 bp) was verified by PCR using primers binding outside the flanking regions (blue arrows in panel A). Shown is the electrophoretic separation of the DNA fragments from the PCRs using genomic DNA of *T. kivui* DSM2030 (wild type, WT) and of two 5-FOA-resistant isolates.

already indicated loss of *pyrE* in a subpopulation. Therefore, we concluded that plasmid DNA does not only integrate into the genome by a single homologous recombination event but also via double homologous recombination (see also Fig. 4B). This prompted us to switch our strategy. *T. kivui* was then transformed with plasmid pMBTkv002b and immediately subjected to 5-FOA in the presence of uracil to select for the loss of *pyrE*. Seven isolates were obtained that were resistant to 5-FOA but dependent on uracil, and which lacked *pyrE*, as verified by PCR (Fig. 2B). We selected one of them and verified the clean and markerless deletion of *pyrE* by sequencing the PCR product. Unlike the wild-type strain, the respective *T. kivui* Δ*pyrE* isolate, TKV002, no longer grew in the absence of uracil in minimal medium (Fig. 3A). However, uptake of plasmid pKOM1 and pKOM2 containing *pyrE* either under the control of the promoter of the Kan^r cassette employed, P_{kan} (Fig. 3A), or under the control of the gyrase promoter from *Thermoanaerobacter* sp. strain X514, $P_{gyrX514}$, respectively, fully restored growth of *T. kivui* TKV002 in minimal medium without uracil.

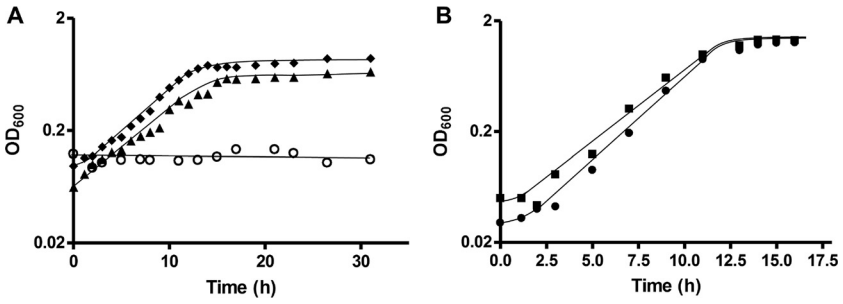

FIG 3 Physiology of the *T. kivui* Δ*pyrE* strain TKV002. (A) Growth of the wild type (DSM 2030, filled triangles), *T. kivui* TKV002 (open circles), and *T. kivui* TKV002 containing plasmid pKOM1 encoding *pyrE* under the control of P_{kan} (filled diamonds), without the addition of uracil. (B) Growth of *T. kivui* TKV002 with P_{kan}-*pyrE* reinserted into the genome in between open reading frames (ORFs) TKV_c24500 and TKV_c24520 (filled squares), and growth of *T. kivui* TKV002 in the presence of 40 μM uracil (closed circles). All experiments were performed in minimal medium with glucose (25 mM) at 65°C. Shown is one representative experiment out of three independent biological replicates.

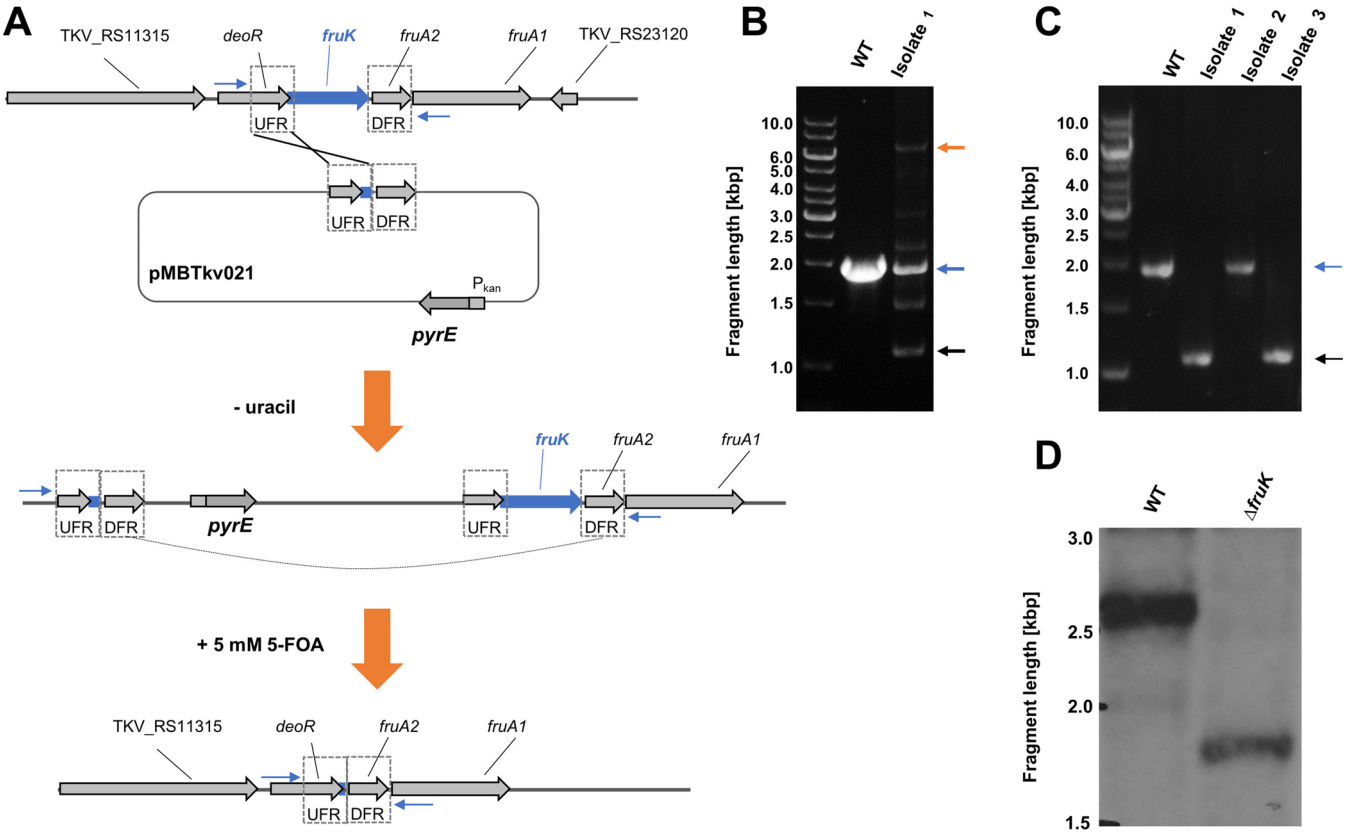

FIG 4 Deletion of *fruK*. (A) Strategy for deletion of *fruK* using plasmid pMBTkv021 via two independent homologous recombination events. (B) DNA fragments separated by agarose gel electrophoresis after PCR amplification of the *fruK* locus from genomic DNA of wild type (WT) *T. kivui* and of uracil-auxotrophic isolates after selection against uracil auxotrophs in the first round of selection and (C) 5-FOA resistant isolates after the second round of selection. Putative plasmid integration, orange arrow; wild type allele, blue arrow; *fruK* deletion (black arrow). (D) Southern blot analysis of the *fruK* locus with NsiI-digested DNA from *T. kivui* DSM2030 (wild type, WT; expected fragment size, 2,668 bp) and the Δ*fruK* strain (expected fragment size, 1,735 bp).

Subsequently, we aimed to complement the uracil auxotrophy of strain TKV002 by reintroducing *pyrE* into the genome. Purposely, to avoid polar effects, this was not performed at its original locus. Instead, we found a region between the convergent genes TKV_c24500 and TKV_c24520 that may not be transcribed. Subsequently, plasmid pMBTkv007 was manufactured for the reintegration of the P_{kan}-*pyrE* cassette into the genome between these two genes. The resulting strain with P_{kan}-*pyrE* integrated between the genes TKV_c24500 and TKV_c24520 was indeed able to grow again on minimal medium without uracil (Fig. 3B). One the one hand, this enabled us to use *pyrE* as a genetic marker for markerless deletions. On the other hand, as we did not observe adverse effects on growth of the resulting strain, the region between the convergent genes TKV_c24500 and TKV_c24520 may be useful for integration of genes or metabolic pathways in bioengineering approaches. For example, similarly, genes for a metabolic pathway to produce 3-hydroxypropionate have been inserted into a region with little transcriptional activity in the genome of the hyperthermophilic archaeon *Pyrococcus furiosus* (25).

***fruK* deletion mutant.** As described above, *T. kivui* grows with fructose as sole carbon and energy source, with an observed approximate doubling time of 1.3 h (see also Fig. 5A). The genome of *T. kivui* contains a gene cluster encoding a putative phosphotransferase uptake system (genes *fruA1* and *fruA2*) and a putative 1-phosphofructokinase (14). Additionally, the gene cluster includes a gene for a *deoR*-type regulator (Fig. 4A). The organization of the gene cluster is similar to what has been described in *Lactococcus lactis* (26), where fructose-1-phosphate has been demonstrated to accumulate in the cell and to act as an effector molecule. While a detailed study of fructose metabolism and its regulation in *T. kivui* was not the primary subject

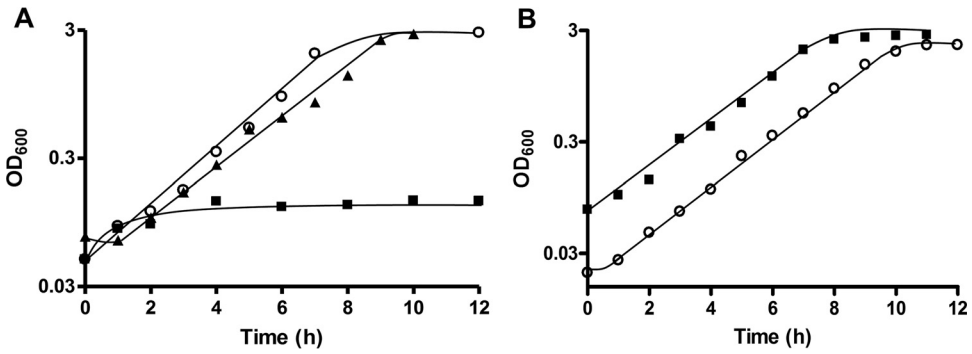

FIG 5 FruK is essential for fructose metabolism. (A) Growth of the *T. kivui* strain Δ*fruK* strain TKV003 (closed squares) and the wild-type strain DSM 2030 (open circles) in the presence of 40 μM uracil. Growth of *T. kivui* TKV003 with plasmid pKOM3 (with *fruK* under the control of P_{kan}) in the absence of uracil (closed triangles). All experiments were performed on complex medium with 25 mM fructose at 65°C. (B) Growth of *T. kivui* TKV003 (closed squares) in the presence of uracil and the wild-type strain DSM 2030 (open circles) on complex medium with 25 mM glucose. Shown are data from one representative experiment out of three independent biological replicates.

of this study, we aimed for a markerless deletion of *fruK* to demonstrate the use of *pyrE* as selective marker. *T. kivui* TKV002 was transformed with plasmid pMBTkv021, which contained *pyrE* and the UFR and DFR of *fruK*. In the first round of selection, we obtained uracil-prototroph colonies that contained the plasmid (Fig. 4B) and that were subjected to 5 mM 5-FOA in the second round of selection (Fig. 4A). 5-FOA-resistant colonies that did not contain *pyrE* anymore partly reverted to the parent strain TKV002, but some did not contain *fruK* (Fig. 4C) anymore. The clean and markerless deletion of *fruK* was verified by PCR, by sequencing, and by Southern blot analysis (Fig. 4D). The resulting uracil-dependent Δ*fruK* strain, TKV003, did not grow anymore on complex medium with fructose as sole source of energy, carbon, and electrons (Fig. 5A), even after prolonged incubation time (>3 days), while its growth with glucose (Fig. 5B) or mannose remained unaffected. Finally, the uptake of a plasmid (pKOM3) containing *fruK* under the control of P_{kan} restored the growth of strain TKV003 on fructose (doubling time [t_D], 1.7 h). While biochemical evidence that *T. kivui fruK* encodes a 1-phosphofructokinase is pending, the presented phenotype clearly demonstrated the essential role of the gene product of *fruK* in fructose metabolism. Moreover, the deletion of *fruK* and its complementation represents a promising proof-of-concept for future genetic manipulations of the thermophilic acetogenic bacterium *Thermoanaerobacter kivui*, toward understanding of its energy metabolism, and potentially toward its biotechnological application.

MATERIALS AND METHODS

Growth conditions. *T. kivui* strain LKT-1 (DSM2030), referred to as wild type, and all derived mutant strains (Table 1) were routinely cultivated under strict anoxic conditions at 65°C. Complex medium contained 2 g · liter^{-1} yeast extract and was prepared as described previously (13). Defined minimal medium was prepared as described previously (13), with the following slight modifications. It contained 60 mM $NaHCO_3$, 3.7 mM KH_2PO_4, 7.5 mM NH_4Cl, 1.6 mM $MgCl_2 \cdot 6H_2O$, 6.8 mM NaCl, 0.34 mM $CaCl_2 \cdot 2H_2O$, 3 mM cysteine-HCl, 10 ml · liter^{-1} trace element solution DSM141, 10 ml · liter^{-1} vitamin solution DSM141, and 4 μM resazurin. The medium was flushed with N_2/CO_2 (80/20 vol/vol) before autoclaving. All gases, including the N_2/CO_2 and H_2/CO_2 gas mixtures were purchased from Praxair Deutschland GmbH (Düsseldorf, Germany). Unless denoted otherwise, glucose from a sterile anoxic stock solution was added as growth substrate to a final concentration of 25 mM after autoclaving.

All growth experiments were carried out under strict anoxic conditions using 20-ml Hungate glass tubes or in 100-ml serum bottles filled with 10 ml or 50 ml medium, respectively, and sealed with butyl

TABLE 1 Strains used in this study

Strain	Deleted gene(s) (locus)	Parent strain	Reference or source
DSM 2030	NA[a]	NA	12
TKV002	*pyrE* (*TKV_c14380*)	DSM 2030	This study
TKV003	*pyrE*, *fruK* (*TKV_c23150*)	TKV002	This study

[a]NA, not applicable.

rubber stoppers. The gas phase contained a mixture of N_2 and CO_2 (80/20, vol/vol; at 110 kPa). Growth in liquid medium was monitored by measuring the optical density at 600 nm.

Agar medium was supplemented with 1.5% Bacto agar (BD Difco, BD Life Sciences, Heidelberg, Germany). After autoclaving, the medium was allowed to cool to below 60°C before addition of substrates, kanamycin, uracil, or 5-fluoroorotic acid (5-FOA), if needed. Routinely, cell suspensions (100 μl to 650 μl) were mixed with 8 to 25 ml agar-containing medium and poured onto petri dishes, which were immediately transferred to an anoxic glove box (Coy Laboratory Products, Grass Lake, MI) and allowed to solidify at ambient temperature. Alternatively, cells were also transferred by an inoculation loop onto the surface of agar-containing medium which had been allowed to solidify at ambient air. The solid agar plates were subsequently transferred to a custom-made jar (Fig. 1A) containing palladium catalyst (Oxoid, Hampshire, England). The jar was sealed inside the anoxic glove box and the gas atmosphere inside the jar was adjusted so that it finally contained approximately 1% H_2 in N_2/CO_2 (80/20, vol/vol) at 50 kPa overpressure. The jar containing the plates was then transferred to incubation at 65°C for 2 to 3 days. Before the jar was opened to evaluate growth on solid agar medium, it was allowed to cool to room temperature. Screening for colonies and transfer of colonies to liquid medium was regularly performed under oxic conditions at ambient temperature.

Chemical analyses. Fructose was determined by high-performance liquid chromatography (HPLC) (P680 pump, ASI-100 autosampler, TCC-100 column compartment; Dionex, Idstein, Germany). Samples were centrifuged and the supernatant was passed through a 0.45-μm filter to remove particles. Sugars were separated on a HyperREZ XP Carbohydrate Ca^{2+} column at 80°C (ThermoFisher Scientific, Waltham, USA) using degassed and filtered water as eluent at a flow rate of 0.6 ml · min^{-1}. Compounds were detected using a RefractoMax 512 refractive index detector (ThermoFisher Scientific, Waltham, USA). Organic acids, including acetate and short-chain alcohols such as ethanol, were detected by gas chromatography using a Clarus 580 GC gas chromatograph (PerkinElmer, Waltham, MA, USA) as described previously (13).

Transformation of *T. kivui*. DNA uptake by *T. kivui* was routinely achieved by natural transformation. Therefore, 1 to 2.5 μg of plasmid DNA was added to a freshly inoculated 5-ml culture of *T. kivui*. Cells were incubated at 65°C overnight (~16 h) and subsequently, 100 to 500 μl of the dense culture (OD_{600} $\geq$2.5 for complex medium, OD_{600} $\geq$0.8 for defined medium) was embedded in agar medium as described above. Transformation efficiency was studied using plasmid pMU131 (16), which confers resistance to kanamycin (200 μg · ml^{-1}). To test growth-phase dependency of plasmid DNA uptake, 1-ml subsamples of a growing *T. kivui* culture were taken and incubated with 250 ng plasmid pMU131 for a period of 1 h at 65°C and then embedded in agar medium containing kanamycin. As no DNA uptake was observed in agar medium, no DNase treatment was necessary to remove excess DNA before plating.

For the determination of the optimal amount of DNA for uptake, 1-ml subsamples of a growing *T. kivui* culture from the mid-exponential phase were incubated with different amounts of plasmid pMU131 for a period of 1 h and then plated. Plasmid DNA was routinely isolated from kanamycin-resistant *T. kivui* isolates, and the presence of pMU131 was verified by PCR or by restriction analysis. Plasmid DNA for transformation of *T. kivui* for sequencing and restriction analysis was isolated with the GenElute miniprep kit (Sigma-Aldrich, Munich, Germany).

Gene expression analysis. The 10-ml cultures were rapidly cooled to 20°C or lower in an ice-water bath. Cells were pelleted by centrifugation and subsequently resuspended in 1 to 2 ml 1× salt solution containing 8.9 g · $liter^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 7.8 g · $liter^{-1}$ $NaH_2PO_4 \cdot 2H_2O$, 0.22 g · $liter^{-1}$ KH_2PO_4, 0.22 g · $liter^{-1}$ K_2HPO_4, 0.31 g · $liter^{-1}$ $(NH_4)Cl$, 0.22 g · $liter^{-1}$ $(NH_4)_2SO_4$, 0.45 g · $liter^{-1}$ NaCl, 0.09 g · $liter^{-1}$ $MgSO_4$, 0.006 g · $liter^{-1}$ NaCl, 0.002 g · $liter^{-1}$ $FeSO_4$. Lysozyme (3 mg · ml^{-1}) was added, and the suspension was incubated for 30 min at room temperature. Afterwards, approximately 30 glass beads, 350 μl Lysis Solution R (InviTrap Spin Cell RNA minikit 0113; Stratec Molecular GmbH, Berlin, Germany), and 5 μl mercaptoethanol were added to the suspension, and cells were lysed in a cell disruptor (MM301; Retsch, Haan, Germany) for 5 min at 50 Hz. After separation of the cell debris by centrifugation (14,000 × *g*, 5 min, 4°C), RNA was isolated from the supernatant using the InviTrap Spin Cell RNA minikit according to the manufacturer's protocol. Ten μg of eluted RNA was subjected to a DNase treatment with TurboDNAse (Ambion Thermo Fisher Scientific, Waltham, USA). RNA was subsequently precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate at −20°C, pelleted by centrifugation, washed with 70% ethanol, and subsequently eluted in RNase-free water. cDNA synthesis was carried out with 1 μg of DNA-free RNA using murine leukemia virus (M-MLV) reverse transcriptase according to the manufacturer's protocol (Promega, Madison, USA). Relative transcript analysis was performed with cDNA from three independent biological replicates in a Rotor Gene RG-3000 quantitative PCR (qPCR) cycler (Corbett Research, Cambridge, UK) using SYBR green qPCR Kits (Fisher Scientific GmbH, Schwerte, Germany) according to the manufacturer's protocol. Primers used for amplification were MB_IG_0007 and MB_IG_0008 (*comEA*, TKV_c09400/TKV_RS04530), MB_IG_0033 and MB_IG_0034 (*comEC1*, TKV_c09780/TKV_RS04705), MB_IG_0011 and MB_IG_0012 (*comEC2*, TKV_c24050/TKV_RS11745), and MB_IG_0013 and MB_IG_0014 (*recA*, TKV_c12920/TKV_RS06255). Relative transcript levels were calculated using the housekeeping gene gyrase (TKV_c00100TKV_RS00050) amplified with primers gyrB_for_qRT/gyrB_rev_qRT as reference. Sequences of all primers used in this study are listed in Table 2.

Deletion of *pyrE*. Genomic DNA (gDNA) was extracted after Zhou et al. (27). All molecular cloning procedures, transformation in *E. coli* DH5α, and colony screening were performed according to standard protocols (28). Initially, a suicide plasmid lacking a functional origin of replication for *T. kivui*, pMBTkv001, was developed. Therefore, the kanamycin resistance cassette Kan^r of plasmid pMU31 was amplified by PCR using primers MB_1001 and MB_1002, and then inserted into the multiple cloning site of plasmid PUC19 using SphI and HindIII. For the development of plasmid pMBTkv002b for the deletion of *pyrE*,

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)
MB_1001	CAGGC ATGCG GCTGC AGGTC GATAA ACC
MB_1002	CCCAA GCTTT TTAGA CATCT AAATC TAGGT ACTAA AAC
MB_1003	CCCGG GGATC CTTGT CTTGT ATGCG CTTGC AAAG
MB_1006	CAGGT CGACT CTAGA TAAGA GAATT AGAGG GCCTT CC
MB_1007	GTACC CGGGG ATCCA CTGTT GTCAC AAATT CTCCT C
MB_1008	CCTTT CTATC CTATT TACAT AACCA AGTTT CAAAT CCTC
MB_1009	GGTTA TGTAA ATAGG ATAGA AAGG TGAG GGAAA TGG
MB_1010	CCTGC AGGTC GACTC TAGAC AGCAA CTACC TTTTT CATAA TTAAT CC
MB_0028	AAAGT TGGCG TATAA CATAG TATCG
MB_0029	CGATA CTATG TTATA CGCCA ACTTT CCTAT TTTTA CTCTT CTTTC TGG
MB_0030	CCTTT AAAGA ACCTA TAATA TCATA TTCAC AACCT CCGCA AATTT ATCC
MB_0032	CTCTA AGACT TCTTC TCTTT CCATA TTCAC AACCT CCGCA AATTT ATCC
MB_IG_0005	CTCGT TCTTC AAACA CTTTC ATTAG G
MB_IG_0006	GGAAT GGTGA CACAA GTAAT TGAG
MB_IG_0007	CCAGT GGAA AGACC TGTAG ATAGT G
MB_IG_0008	CCTTC TTCCA TAGTA TAAAC TCCCG
MB_IG_0011	TCTCT ATGAG GATGT CCGTA GTC
MB_IG_0012	CACGG AAGTT CATCA GCATC TA
MB_IG_0013	TCTCT CCCTT GGCCT AATCT
MB_IG_0014	GGAAT TTCG AGAGA GGGAA GTC
MB_IG_0018	TTAAA ATTTC CTGCT TCCCG GCTTT AC
MB_IG_0024	CGGGA AGCAG GAAAT TTTAA TACCT AGATT TAGAT GTCT AAAA AG
MB_IG_0027	ATGGA AAGAG AAGAA GTCTT AGAG
MB_IG_0028	CTCTA AGACT TCTTC TCTTT CCATT TATTA TTTCC TTCCT CTTTT CTAC
MB_IG_0029	TTATA AATAT GCTTA TACCT TAATT TCTCA TCCCC TTTAA TCAAC C
MB_IG_0030	GGATG AGAAA TTAAG GTATA AGCAT ATTTA TAACT CTCAT TG
MB_IG_0031	GCATA CAAGA CAAGG ATCCC CGGGT ACCGA GCTC
MB_IG_0032	CTAAT TCTCT TATCT AGAGT CGACC TGCAG GCATG C
MB_IG_0033	GGGCA TGCCG TTGAC ATTAT AG
MB_IG_0034	CTTAG CATCT CTTTC GGGCA TTG
gyrB_for_qRT	CCAGT TGTGC TTCCT TCTCG ATTTC C
gyrB_rev_qRT	GCGAC AATGC CATCT ATGAC TTCTC C
LH009	GAGGA AGGAA ATAAT AAATG GAAAG AGAAG AAGTC TTAGA G
LH010	ATCTA GGTAT TAAAA TTTCC TGCTT CCCGG CTTTA C
LH013	GTACC CGGGG ATCCC CACAC ACCTC ACCTT AA
LH014	CTCTA AGACT TCTTC TCTTT CCATA GAGGA ATGTA AAAGA AATC
LH016	CAATT ATATC CATAA ATTAA AATTT CCTGC TTCCC GGC
LH017	GCAGG AAATT TTAAT TTATG GATAT AATTG TTATG ATTGC AAAAC
LH018	CATGC CTGCA GGTCG ACTCT AGATA TCCTG CTT
LH024	GCTTT AACCA GAAAG AAGAG TAAAA ATAGG AGAGG AATGT AAAAG AAATC AAAAT AAAAA AC
LH025	GATTT CTTTT ACATT CCTCT CCTAT TTTTA CTCTT CTTTC TGGTT AAAGC AATAC TACAT TTCC
LH028	CAGGC TGTGA TAATT TGAGA A
LH029	GGTCA CGATT TAAAG GACTT A
LH038	GGTAC GACTA CTCTT GAAAT T
LH039	CGCCA TATAG GTATG AGCAA
LH054	GAATT TAGTA TGGTC AGCCA
LH055	CTGGA GGAAA AGGGT TTCAT
LH056	GAGGA AGGAA ATAAT AAATG ATAAC GACGG TAACG GC
LH057	CTTTT TAGAC ATCTA AATCT AGGTA TCACC TTTCT ATCCT TTCTA
LH058	GTCGT TATCA TTTAT TATTT CCTTC CTCTT TTCTAC AG
LH059	GGTGA TACCT AGATT TAGAT GTCTA AAAAG CTTGG CGTAA T

initially, 1-kb upstream (UFR) and downstream (DFR) regions flanking *pyrE* (TKV_c14380; TKV_RS06990) were amplified by PCR using primer pairs MB_1003/MB_IG_0029 and MB_IG_0030/MB_1006, respectively. Subsequently, the UFR and DFR DNA fragments were fused by overlap extension PCR using primers MB_1003/MB_1006, and plasmid pMBTkv_001 was amplified using primers MBIG_0031 and MB_IG_0032. Finally, the UFR-DFR fusion was inserted into pMBTkv_001 using a Gibson Assembly Mastermix (NEB, Frankfurt am Main, Germany), and *E. coli* DH5α was transformed with the reaction mix. The plasmid was retrieved from *E. coli* DH5α and transformed into *T. kivui*. Colonies resistant to kanamycin (200 μg · ml^{-1}) were transferred to liquid complex medium containing kanamycin (200 μg · ml^{-1}). In the second round, selection for loss of the plasmid and of *pyrE* was performed using 5 mM 5-fluoroorotic acid, (5-FOA), while the medium contained 40 μM uracil to complement auxotrophic *pyrE* mutants. Alternatively, selection for the loss of *pyrE* without integration of the whole plasmid was directly performed using 5 mM 5-FOA and 40 μM uracil. Integration into the *pyrE* locus and loss of *pyrE* was

verified by PCR using primers MB_IG_0006 and MB_IG_0005. Deletion of *pyrE* was additionally verified by sequencing the locus.

To generate replicating plasmids pKOM1 and pKOM2 for complementation of the uracil auxotrophy of strain TKV002, the kanamycin resistance cassette in plasmid pMU131was replaced with *pyrE*. For plasmid pKOM1 the plasmid backbone of pMU131, excluding Kan^r but including the promoter P_{kan}, was amplified by PCR using primers MB_IG_0024 and MB_IG_0028 and fused to *pyrE* of *T. kivui*, which was amplified by primers MB_IG_0018 and MB_IG_0027 using the Gibson Assembly Mastermix. For the construction of plasmid pKOM2, the promoter of gyrase $P_{gyrX514}$ from *Thermoanaerobacter* sp. strain X514 was amplified using primers MB_0029 and MB0032. The *pyrE* gene fragment was amplified as for plasmid pKOM1, and the plasmid backbone of pMU131 was amplified using primers MB_0028 and MB_IG_024. All three products were fused using the Gibson Assembly Mastermix to generate plasmid pKOM2. pKOM1 and pKOM2 were transformed into *T. kivui* as described above, although minimal medium without uracil was used to allow for selection of uracil prototrophs.

Plasmid pMBTkv007 was constructed to reintroduce *pyrE* into the genome between the convergent genes TKV_c24500 and TKV_c24520. The UFR and DFR (~1000 bp each) of that genome region were amplified by PCR using primer pairs LH013/LH024 and LH017/LH018, respectively. $P_{gyrX514}$ from *Thermoanaerobacter* species strain X514 and *pyrE* from *T. kivui* genomic DNA were amplified by PCR using primer pairs LH025/MB_0032 and MB_IG_0027/LH016, respectively. The four PCR products were fused by PCR, digested with BamHI and XbaI, and ligated into plasmid pMBTkv001. *T. kivui* was transformed with plasmid pMBTkv007 and selection for transformants with *pyrE* was performed using minimal medium without uracil. Integration of *pyrE* was verified by PCR, with primers LH029 and LH028 binding outside the locus, and by subsequent sequencing of the PCR product.

Deletion of *fruK*. Initially, a basic plasmid, pMBTkv005, was constructed for generating markerless deletions in the genome of *T. kivui*. pMBTkv005 is derived from pMBTkv001, except that Kan^r was replaced with *T. kivui pyrE*, amplified by PCR using primers LH009 and LH010, and fused to the pMBTkv001 backbone (which was amplified by PCR using primers MB_IG_0024 and MB_IG_0028) by Gibson Assembly Mastermix. pMBTkv005 lacked an origin of replication that was functional in *T. kivui*.

Plasmid pMBTkv021 for the deletion of *fruK* (TKV_RS11305; old locus tag TKV_c23150) was generated from plasmid pMBTkv005. Initially, 0.5-kbp upstream (UFR) and downstream (DFR) regions flanking *fruK* were amplified using the primer pairs MB_1007/MB_1008 and MB_1009/MB_1010, respectively. The flanking regions were fused by overlap extension PCR using primers MB_1007 and MB_1010, digested with BamHI and XbaI, and ligated into the multiple cloning site of pMBTkv005. The resulting plasmid pMBTkv021 was transformed into *T. kivui*. Selection for transformants containing the plasmid integrated into the genome was performed using minimal medium without uracil. Integration of the plasmid was verified by PCR using primer LH038 and LH039. In the second round, selection for loss of the plasmid and of *pyrE* was performed using 5 mM 5-fluoroorotic acid, while the minimal medium contained 40 μM uracil. Uracil-auxotrophic isolates were screened by PCR for the loss of *fruK* using primers LH038 and LH039. Deletion of *pyrE* was additionally verified by sequencing the locus and by Southern blot analysis (see below).

Replicating plasmid pKOM3 was constructed to complement the deficiency to grow on fructose. Therefore, *fruK* was amplified by PCR using primer pair LH056/LH057 and the backbone of plasmid pKOM1 was amplified by PCR using primer pair LH058/LH059, and both fragments were fused using the Gibson Assembly Mastermix.

Southern blot analysis. The genotypes of wild type and mutants in the *fru* gene cluster were analyzed by hybridization with digoxigenin (DIG)-labeled DNA according to standard procedures (28), with the following modifications. A 506-bp-long probe for the *fruK* locus was generated using primers LH054 and LH055, in a PCR with 20 μM labeled UTP and a deoxynucleoside triphosphate (dNTP) mixture (0.24 mM dGTP, 0.24 mM dCTP, 0.16 mM dATP, and 0.03 mM dTTP). *T. kivui* genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). One μg of genomic DNA was digested with NsiI. DNA fragments were separated on a 1% agarose gel and subsequently transferred to a charged nylon membrane (Genescreen Plus hybridization transfer membrane; PerkinElmer, Waltham, MA, USA) by capillary transfer. The membrane was hybridized with the probe at 50°C overnight. Binding of the probe to the anti-DIG antibody (Roche, Basel, Switzerland) and detection of DIG-labeled DNA fragments with CDP-Star (Roche) was performed according to the manufacturer's recommendations.

ACKNOWLEDGMENTS

We thank Dan Olson and Lee Lynd (Dartmouth College, NH, USA) for providing us with plasmid pMU131. We are indebted to Michael Rother (TU Dresden, Germany) for the development of the metal jars used for the anaerobic incubation of *T. kivui* on solid medium.

We are grateful to Deutsche Forschungsgemeinschaft (DFG) for financial support.

REFERENCES

1. Drake HL, Gössner AS, Daniel SL. 2008. Old acetogens, new light. Ann N Y Acad Sci 1125:100–128. https://doi.org/10.1196/annals.1419.016.
2. Schuchmann K, Müller V. 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. Nat Rev Microbiol 12:809–821. https://doi.org/10.1038/nrmicro3365.
3. He Y, Li M, Perumal V, Feng X, Fang J, Xie J, Sievert SM, Wang F. 2016. Genomic and enzymatic evidence for acetogenesis among multiple lineages of the archaeal phylum Bathyarchaeota widespread in marine sediments. Nat Microbiol 1:16035. https://doi.org/10.1038/nmicrobiol.2016.35.

4. Schuchmann K, Müller V. 2016. Energetics and application of heterotrophy in acetogenic bacteria. Appl Environ Microbiol 82:4056–4069. https://doi.org/10.1128/AEM.00882-16.
5. Schink B, Stams A. 2006. Syntrophism among prokaryotes, p 309–336. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (ed), The prokaryotes: a handbook on the biology of bacteria, 3rd ed, vol 2. Springer Science+Business Media, Berlin, Germany.
6. Wood HG, Ragsdale SW, Pezacka E. 1986. The acetyl-CoA pathway of autotrophic growth. FEMS Microbiol Lett 39:345–362. https://doi.org/10.1111/j.1574-6968.1986.tb01865.x.
7. Ljungdahl LG. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu Rev Microbiol 40:415–450. https://doi.org/10.1146/annurev.mi.40.100186.002215.
8. Weiss MC, Sousa FL, Mrnjavac N, Neukirchen S, Roettger M, Nelson-Sathi S, Martin WF. 2016. The physiology and habitat of the last universal common ancestor. Nat Microbiol 1:16116. https://doi.org/10.1038/nmicrobiol.2016.116.
9. Schuchmann K, Müller V. 2013. Direct and reversible hydrogenation of CO_2 to formate by a bacterial carbon dioxide reductase. Science 342:1382–1385. https://doi.org/10.1126/science.1244758.
10. Biegel E, Müller V. 2010. Bacterial Na^+-translocating ferredoxin: NAD^+ oxidoreductase. Proc Natl Acad Sci U S A 107:18138–18142. https://doi.org/10.1073/pnas.1010318107.
11. Hedderich R, Forzi L. 2005. Energy-converting [NiFe] hydrogenases: more than just H_2 activation. J Mol Microbiol Biotechnol 10:92–104. https://doi.org/10.1159/000091557.
12. Leigh JA, Mayer F, Wolfe RS. 1981. *Acetogenium kivui*, a new thermophilic hydrogen-oxidizing, acetogenic bacterium. Arch Microbiol 129:275–280. https://doi.org/10.1007/BF00414697.
13. Weghoff MC, Müller V. 2016. CO metabolism in the thermophilic acetogen *Thermoanaerobacter kivui*. Appl Environ Microbiol 82:2312–2319. https://doi.org/10.1128/AEM.00122-16.
14. Hess V, Poehlein A, Weghoff MC, Daniel R, Müller V. 2014. A genome-guided analysis of energy conservation in the thermophilic, cytochrome-free acetogenic bacterium *Thermoanaerobacter kivui*. BMC Genomics 15:1139. https://doi.org/10.1186/1471-2164-15-1139.
15. Onyenwoke RU, Wiegel J. 2015. *Thermoanaerobacter*. *In* Whitman WB (ed), Bergey's manual of systematics of Archaea and Bacteria. John Wiley & Sons, Hoboken, NJ.
16. Shaw AJ, Hogsett DA, Lynd LR. 2010. Natural competence in *Thermoanaerobacter* and *Thermoanaerobacterium* species. Appl Environ Microbiol 76:4713–4719. https://doi.org/10.1128/AEM.00402-10.
17. Zeldes BM, Keller MW, Loder AJ, Straub CT, Adams MWW, Kelly RM. 2015. Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals. Front Microbiol 6:1209. https://doi.org/10.3389/fmicb.2015.01209.
18. Olson DG, Maloney M, Lanahan AA, Hon S, Hauser LJ, Lynd LR. 2015. Identifying promoters for gene expression in *Clostridium thermocellum*. Metab Eng Commun 2:23–29. https://doi.org/10.1016/j.meteno.2015.03.002.
19. Chen I, Dubnau D. 2004. DNA uptake during bacterial transformation. Nat Rev Microbiol 2:241–249. https://doi.org/10.1038/nrmicro844.
20. 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. https://doi.org/10.1128/JB.185.1.210-220.2003.
21. Chung D, Cha M, Farkas J, Westpheling J. 2013. Construction of a stable replicating shuttle vector for *Caldicellulosiruptor* species: use for extending genetic methodologies to other members of this genus. PLoS One 8:e62881. https://doi.org/10.1371/journal.pone.0062881.
22. Lipscomb GL, Conway JM, Blumer-Schuette SE, Kelly RM, Adams MW. 2016. A highly thermostable kanamycin resistance marker expands the tool kit for genetic manipulation of *Caldicellulosiruptor bescii*. Appl Environ Microbiol 82:4421–4428. https://doi.org/10.1128/AEM.00570-16.
23. Yao S, Mikkelsen MJ. 2010. Identification and overexpression of a bifunctional aldehyde/alcohol dehydrogenase responsible for ethanol production in *Thermoanaerobacter mathranii*. J Mol Microbiol Biotechnol 19:123–133. https://doi.org/10.1159/000321498.
24. Shao X, Zhou J, Olson DG, Lynd LR. 2016. A markerless gene deletion and integration system for *Thermoanaerobacter ethanolicus*. Biotechnol Biofuels 9:1–8. https://doi.org/10.1186/s13068-015-0423-8.
25. Keller MW, Schut GJ, Lipscomb GL, Menon AL, Iwuchukwu IJ, Leuko TT, Thorgersen MP, Nixon WJ, Hawkins AS, Kelly RM, Adams MWW. 2013. Exploiting microbial hyperthermophilicity to produce an industrial chemical, using hydrogen and carbon dioxide. Proc Natl Acad Sci U S A 110:5840–5845. https://doi.org/10.1073/pnas.1222607110.
26. Barrière C, Veiga-da-Cunha M, Pons N, Guédon E, van Hijum SAFT, Kok J, Kuipers OP, Ehrlich DS, Renault P. 2005. Fructose utilization in *Lactococcus lactis* as a model for low-GC Gram-positive bacteria: its regulator, signal, and DNA-binding site. J Bacteriol 187:3752–3761. https://doi.org/10.1128/JB.187.11.3752-3761.2005.
27. Zhou JZ, Fries MR, Cheesanford JC, Tiedje JM. 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. Int J Syst Bacteriol 45:500–506. https://doi.org/10.1099/00207713-45-3-500.
28. Sambrook J, Russell DW (ed). 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.