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Genetic Manipulations of the Hyperthermophilic Piezophilic Archaeon *Thermococcus barophilus*

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In this study, we developed a gene disruption system for *Thermococcus barophilus* using simvastatin for positive selection and 5-fluoroorotic acid (5-FOA) for negative selection or counterselection to obtain markerless deletion mutants using single- and double-crossover events. Disruption plasmids carrying flanking regions of each targeted gene were constructed and introduced by transformation into wild-type *T. barophilus* MP cells. Initially, a *pyrF* deletion mutant was obtained as a starting point for the construction of further markerless mutants. A deletion of the *hisB* gene was also constructed in the UBOCC-3256 (Δ*pyrF*) background, generating a strain (UBOCC-3260) that was auxotrophic for histidine. A functional *pyrF* or *hisB* allele from *T. barophilus* was inserted into the chromosome of UBOCC-3256 (Δ*pyrF*) or UBOCC-3260 (Δ*pyrF* Δ*hisB*), allowing homologous complementation of these mutants. The piezophilic genetic tools developed in this study provide a way to construct strains with multiple genetic backgrounds that will allow further genetic studies for hyperthermophilic piezophilic archaea.

Since the discovery of deep-sea hydrothermal vents, many mesophilic, thermophilic, and hyperthermophilic *Bacteria* and *Archaea* have been described. However, only a few thermopiezophilic organisms have been described so far, mainly belonging to the domain *Archaea*: *Thermococcus barophilus* (1), *Palaeococcus pacificus* (2), *Palaeococcus ferrophilus* (3), *Marinitoga piezophila* (4), *Methanopyrus kandleri* (5), and *Pyrococcus yayanosii* (6, 7). *P. yayanosii* is the first and only known obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. The genomes of *M. kandleri*, *T. barophilus*, *P. yayanosii*, and *M. piezophila* are now available (8–11), but the development of genetic tools for the above species is lagging.

T. barophilus strain MP was the first true hyperthermophilic piezophilic archaeon isolated, in 1993 (1); it grows in rich medium from 48°C to 100°C, with an optimum at 85°C, and within a pressure range of 0.1 to 85 MPa, with an optimum of 40 MPa (1). *T. barophilus* is an obligate piezophile for temperatures over 95°C.

Genetic manipulations in nonpiezophilic members of the *Thermococcales*, such as *Thermococcus kodakarensis* and *Pyrococcus furiosus*, which are phylogenetically related to *T. barophilus*, have been described (12–14). For some archaeal genetic systems, such as *T. kodakarensis*, many selectable markers have been described, allowing selection according to prototrophic markers (arginine/citrulline, tryptophan, agmatine, or uracil) or antibiotics (simvastatin or mevinolin) (15); simvastatin or mevinolin can be used to screen for transformed cells. Such cells can overexpress the *P. furiosus hmg* gene (hmg_{Pf}), which encodes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a pivotal enzyme for archaeal membrane lipid biosynthesis (16), so that the hmg_{Pf} gene can then be used as a positive selection marker (17). Among prototrophic selective markers, arginine/citrulline-based selection is used when strains are able to transform aspartate into arginine using citrulline supplied by the culture medium and thus achieve arginine prototrophy (15). Agmatine, a polyamine produced by decarboxylation of arginine, is used as a marker for positive selection for agmatine prototrophs in strains from which the *argD* gene encoding arginine decarboxylase has been deleted (18). Agmatine auxotrophy is lethal, even in rich medium, which facilitates the isolation of transformants overnight on rich medium, in contrast to the several days required when transformants are selected on defined medium. As for other types of prototrophic selection, tryptophan-based selection is limited to defined medium and plasmid introduction into a specific strain from which the *trpE* gene, encoding the large subunit of anthranilate synthase, has previously been deleted (12).

The uracil marker is used in all model organisms for genetics in *Archaea* (17). The *pyrEF* genes enable complementation of the uracil auxotrophy, and 5-fluoroorotic acid (5-FOA) can be used as a counterselection marker; the uracil marker is functional for both positive selection and counterselection (negative selection) for constructing markerless deletion mutants. A similar counterselective strategy is also offered by 6-methyl purine (6-MP) (18), which must be paired with a positive selection marker because it cannot be used for positive selection itself. All these markers have at least one disadvantage, such as limitation to use in defined medium, supplement requirement, spontaneous resistance providing a high background, limited host range, and/or no consecutive positive selection and counterselection (15).

Thus, the development of genetic manipulations of the deep-sea hydrothermal vent strain *T. barophilus* MP, proposed in this study, was derived from what we knew about gene deletion systems established in the shallow hydrothermal vent species *T. kodakarensis*. A suicide vector was constructed to be used as a tool for

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gene disruption by homologous recombination in *T. barophilus*. We showed that 1 kb can be efficiently integrated directly into the chromosome by using circular DNA fragments. A strategy was developed to generate deletion mutants by PCR amplification and vector cloning to select marker replacement events with subsequent disruption, or "pop-out," of the selected marker. The genetic manipulations in *T. barophilus* established here rely on uracil auxotrophy and simvastatin resistance as selectable markers, using the pop-in/pop-out method. We constructed multiple gene mutants and tested the possibility of complementation in *trans*.

MATERIALS AND METHODS

Media and growth conditions. *T. barophilus* strain MP was isolated from chimney samples harvested from the Snake Pit hydrothermal site, at a depth of 3,550 m on the mid-Atlantic ridge, in July 1993 (1). *T. barophilus* cultures were grown under anaerobic conditions at 85°C in *Thermococcales* rich medium (TRM) (7) or *Thermococcales* amino acid (TAA) medium (A. Cario and P. Oger, unpublished data) supplemented with sulfur. The TAA medium composition is as follows: 23 g NaCl, 3.3 g PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 3 g $MgCl_2 \cdot 6H_2O$, 2 g $C_2H_3NaO_2$, 1 g $(NH_4)_2SO_4$, 0.5 g KCl, 0.05g NaBr, 0.02 g $SrCl_2 \cdot 6H_2O$, vitamin mixture, modified Wolfe's trace minerals, 1 ml resazurin, and 0.1 g per liter of each of 19 amino acids (except histidine). After cell transformation, the mutants were selected on TRM supplemented with 2.5 μg/ml simvastatin (Sigma) or 8 mg/ml 5-FOA (5-fluoroorotic acid hydrate; Euromedex). Auxotrophic growth assays were performed on TAA medium with the addition of uracil in the presence or absence of histidine, as appropriate. After filtration (Millipore filter, 0.45 μm), the liquid medium was dispensed anaerobically into 50-ml vials which were sealed with butyl-rubber stoppers, and the medium was reduced with 0.1 ml of a 10% (wt/vol) sterile $Na_2S \cdot 9H_2O$ solution just before inoculation. Unless stated otherwise, the experiments were carried out in triplicate in the presence of sulfur.

A pressure ranging from 0.1 MPa to 70 MPa was used to monitor the growth of mutants in TRM and TAA medium; cultivation under hydrostatic pressure was performed in sterile syringes, and cultures were incubated in a high-hydrostatic-pressure (HHP), high-temperature incubator (Top Industrie), as previously described (7).

Growth was monitored by cell counting using a Thoma chamber and photonic microscopy at a magnification of ×40 (Olympus) or using flow cytometry (CyFlow Space; Partec). Cells were fixed with 2.5% glutaraldehyde and counted by one of the two previously described methods.

The genetic manipulations, DNA preparation, and transformation protocols were conducted under atmospheric pressure (0.1 MPa). *Escherichia coli* strain DH5α was used for general DNA manipulation, *E. coli* was cultured in LB medium (liquid or solid) at 37°C, and the presence of plasmids was selected for by adding 100 μg/ml ampicillin to the medium.

Construction of suicide vectors. The pUD plasmid was kindly provided by the Imanaka lab (19). This plasmid bears the ampicillin resistance gene and the *T. kodakarensis pyrF* gene with its putative promoter region; it is a replicative plasmid in *E. coli* but not in *Thermococcales* strains.

The hmg_{Pf} gene was obtained by PCR amplification on the pLC70 plasmid (20) with the primers SalI-HMG-CoA-Up and KpnI-HMG-CoA-Do (see Table S1 in the supplemental material); the resulting PCR product bears the KpnI and SalI restriction enzyme sites at its extremities. The hmg_{Pf} amplicon was cloned in the pUD plasmid digested by the restriction enzymes KpnI and SalI, and after ligation between the pUD plasmid and the hmg_{Pf} PCR-amplified gene (using T4 ligase; Promega), a plasmid named pUDH was constructed (Fig. 1).

In the second step of this work, the pUDH plasmid was digested by XhoI and SmaI enzymes. This digestion linearized the plasmid, allowing the excision of the *T. kodakarensis pyrF* gene but not its promoter region.

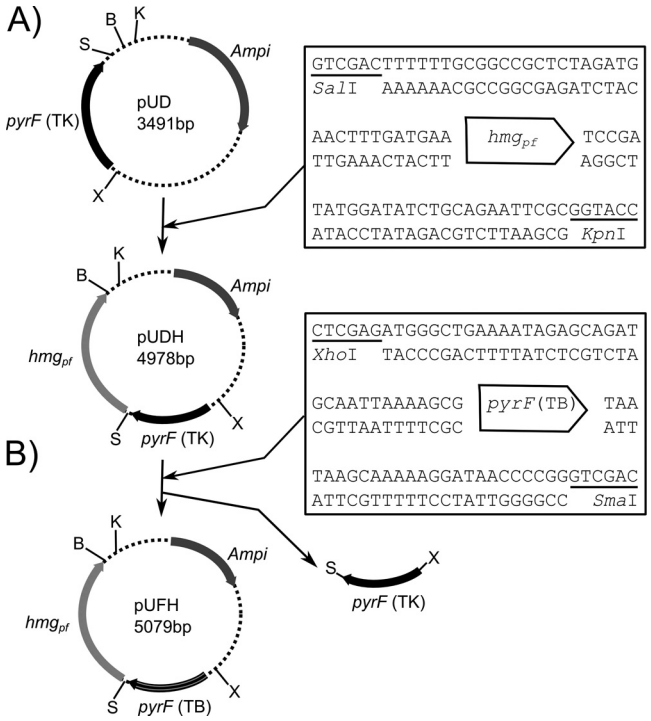

FIG 1 Construction of pUDH and pUFH plasmids. (A) Primers SalI-HMG-CoA-Up and KpnI-HMG-CoA-Do were used to amplify hmg_{Pf} from the vector pLC70 (20). pUD and HMG-CoA were digested by SalI (S) and BamHI (B) and were then ligated to obtain the plasmid pUDH. (B) Primers XhoI-pyrF-TB-Up and XmaISmaI-pyrF-TB-Do were used to amplify the *T. barophilus pyrF* gene. pUDH and pyrF(TB) were digested by XhoI (X) and SmaI (S) and were then ligated to obtain the plasmid pUFH. In pUDH and pUFH, the restriction enzyme sites BamHI (B) and KpnI (K) were conserved to enable cloning of the homologous regions in these plasmids.

The linearized plasmid was ligated with the PCR-amplified *T. barophilus pyrF* gene obtained with the primers XhoI-*pyrF*-TB-Up and SmaI-*pyrF*-TB-Do from genomic DNA, and the resulting plasmid was named pUFH (Fig. 1).

The homologous regions encompassing the target genes to be excised were amplified by two successive PCR amplification steps, in a process known as splicing by overlap extension (21, 22). After the first amplification, two fragments of 1 kb encompassing the targeted genes were obtained; the primers used for this first amplification were named 1Up/1Do and 2Up/2Do (see Table S1 in the supplemental material). The two fragments were used as the matrix for the second PCR amplification, by using the primers 1Up and 2Do (see Table S1 in the supplemental material), and the resulting PCR product of the second step was a DNA fragment of 2 kb composed of the two merged homologous regions. The different primers used for amplified homologous regions were Δ*pyrF*-1Up, Δ*pyrF*-1Do, Δ*pyrF*-2Up, and Δ*pyrF*-2Do for deletion of *pyrF* (*TERMP_01290*); Δ*hisB*-1Up, Δ*hisB*-1Do, Δ*hisB*-2Up, and Δ*hisB*-2Do for deletion of *hisB* (*TERMP_00437*); and Δ*TERMP_00005-1Up*, Δ*TERMP_00005-1Do*, Δ*TERMP_00005-2Up*, and Δ*TERMP_00005-2Do* for the deletion of *TERMP_00005* (see Table S1 in the supplemental material). By using this approach, three homologous-region fragments were created: the Δ*pyrF*, Δ*hisB*, and Δ*TERMP_00005* fragments. The fragment *TERMP_01289*(HR1)-*TERMP_01291*(HR2) was cloned into the plasmid pUDH using the restriction sites KpnI and BglII (on the amplification)/BamHI (on the plasmid), and the fragments *TERMP_00436*(HR1)-*TERMP_00438*(HR2) and *TERMP_00004*(HR1)-*TERMP_00006*(HR2) were cloned into the plasmid pUFH using the restriction sites KpnI and BglII/BamHI; we thus obtained the suicide vectors pUDH-1, pUFH-1, and pUFH-2 (Table 1).

TABLE 1 *T. barophilus* plasmids used and constructed in this study

Plasmids	Resistance	Parent plasmid	Description	Reference
pUD	Ampicillin			19
pUDH	Ampicillin, simvastatin	pUD	Cloning of HMG-CoA reductase-encoding gene in pUD	This work
pUFH	Ampicillin, simvastatin	pUDH	Replacement of *pyrF* (*T. kodakarensis*) by *pyrF* (*T. barophilus*)	This work
pUDH-1	Ampicillin, simvastatin	pUDH	Cloning of homologous regions flanking *pyrF*	This work
pUFH-1	Ampicillin, simvastatin	pUFH	Cloning of homologous regions flanking *hisB*	This work
pUFH-2	Ampicillin, simvastatin	pUFH	Cloning of homologous regions flanking *TERMP_00005*	This work
pUHH	Ampicillin, simvastatin	pUFH-2	Replacement of *pyrF* by *hisB*	This work

In order to complement the *hisB* mutant, the *pyrF* gene present in pUFH but not its promoter region was replaced by the *T. barophilus hisB* gene; the *hisB* gene was amplified using the primers Verif-hisB-Up and Verif-hisB-Do (see Table S1 in the supplemental material). The *hisB* PCR product and pUFH vector were digested by XhoI and SmaI and then ligated (using T4 ligase; Promega), and the resulting plasmid was named pUHH (Table 1).

Transformation of *T. barophilus*. The $CaCl_2$ method for *Methanococcus voltae* PS (23) was modified for the transformation of *T. barophilus*. The $CaCl_2$ cell treatment was not a prerequisite for the transformation. Cells were cultivated in liquid TRM with sulfur for 16 h at 85°C at atmospheric pressure, and an aliquot of 1 ml of this overnight culture was introduced into 50 ml of fresh TRM without sulfur and incubated for 6 h at 85°C. Cells were harvested by centrifugation (8,000 × *g*, 6 min), concentrated in 1 ml of fresh TRM without sulfur, and kept on ice for 30 min under an anaerobic tent. An aliquot of 4 to 5 μg of plasmid DNA was added to 200 μl of concentrated cells, and the mixture was incubated on ice for 1 h. A heat shock at 85°C was carried out for 10 min, followed by incubation for 10 min on ice. The transformants were then used to inoculate 20 ml of fresh TRM with sulfur and incubated at 85°C for 18 h. The cells were harvested by centrifugation (8,000 × *g*, 6 min), resuspended in 100 μl of fresh TRM, and spread on plated TRM containing simvastatin (final concentration of 2.5 μg/ml) or 5-FOA (8 mg/ml) under anaerobic conditions. Medium solidification was achieved using Phytagel (Sigma) at a concentration of 10 g/liter, and the plates were incubated for 5 days at 85°C. The resulting Sim^r strains were analyzed by PCR using genomic DNA as a matrix.

Complementation of *pyrF* and *hisB* mutants. The pUFH-2 and pUHH plasmids were introduced, respectively, into UBOCC-3256 (Δ*pyrF*) and UBOCC-3260 (Δ*pyrF* Δ*hisB*). Many transformants that were resistant to simvastatin were obtained, and after PCR screening, all clones had undergone single-crossover recombination ($pyrF^+$ or $hisB^+$) at one or the other of the homologous regions (*TERMP_00004* or *TERMP_00006*) carried by both plasmids (data not shown).

Pop-out recombination. After the transformation of *T. barophilus* by a suicide vector (Fig. 2), two steps were needed to perform the excision of the targeted gene, a pop-in (vector insertion) by one crossover event and a pop-out recombination. For this purpose, a strain with a suicide vector inserted was streaked onto TRM plates supplemented with 5-FOA at 8 mg/ml. In principle, the strains that are able to grow on this medium are obviously resistant to 5-FOA, sensitive to simvastatin, and Δ*pyrF*; however, we observed several false positives at this step. To address this problem, the colonies that grew on TRM–5-FOA plates were restreaked on TRM-simvastatin plates to eliminate the false positives that were still simvastatin resistant. The strains which were simvastatin sensitive and 5-FOA resistant were kept; the pop-out recombination was then obtained, and a PCR amplification was performed to check whether the pop-out recombination resulted in a targeted gene deletion or wild type (WT) genotype recurrence (Fig. 2).

DNA purification and extraction. Plasmid DNA was extracted from an *E. coli* DH5α strain with a plasmid extraction kit supplied by Thermofisher. *E. coli* DH5α cultures (5 ml) at exponential growth phase were harvested after centrifugation (6,000 × *g*, 6 min, 4°C) and then used to extract DNA vectors with a final concentration of 160 to 200 ng/μl.

Genomic DNA extraction of *T. barophilus* was performed using a phenol-chloroform-isoamyl alcohol (PCI) extraction method, as follows. A 20-ml portion of cell culture at exponential growth phase (approximately 10^8 cells/ml) was centrifuged at 7,500 × *g* for 15 min at 4°C. The cell pellet was resuspended in 800 μl TE buffer (100 mM Tris HCl [pH 8], 50 mM NaCl, 50 mM EDTA [pH 8]). To ensure cell lysis, 100 μl of 10% SDS, 100 μl of 10% Sarkosyl, and 50 μl of proteinase K (20 mg/ml) were added, and the cell suspension was then incubated for 1 h at 55°C; 1 ml phenol-chloroform-isoamyl alcohol (PCI) (25:24:1) was added, and after centrifugation at 14,000 × *g* for 15 min at 4°C, the aqueous phase containing the total DNA was recovered, to which 1 ml of chloroform was then added to eliminate all phenol traces. The DNA was subsequently precipitated by the addition of 0.7 ml isopropanol for 1 h at −20°C and recovered after centrifugation (14,000 × *g* for 15 min at 4°C). The DNA was then washed with 75% ethanol, and after centrifugation (14,000 × *g* for 15 min at 4°C), it was resuspended in 200 μl 10 mM Tris-HCl (pH 8) buffer; RNase (50 mg/ml) was added to eliminate residual RNA in each sample.

The DNA was quantified by NanoDrop, and the quality of extraction was checked by electrophoresis on a 1% agarose gel containing ethidium bromide at a final concentration of 0.5 mg/ml (in a bath of 40 mM Tris [pH 8], 40 mM acetate, 1 mM EDTA [pH 8] [1× TAE]). The separation was performed at 85 V for 40 min, with a 1-kb ladder (Promega) as the size marker.

PCR conditions. hmg_{Pp}, *pyrF*, *hisB*, and all homologous regions were amplified using *Pfu* polymerase (Promega). Routine tests by PCR amplification were performed using *Taq* Polymerase (Promega), and PCR was performed as follows: 94°C for 5 min; 30 cycles of 94°C for 60 s, 50 to 58°C for 60 s, and 72°C for 1 to 4 min; 72°C for 10 min.

RESULTS

Effects of different simvastatin and 5-FOA concentrations on the growth of *T. barophilus*. To select the markers that could be used for positive-selection integration and counterselection excision from the *T. barophilus* genome, the sensitivity of *T. barophilus* to different drugs and antibiotics was assessed using 5-FOA, 6-MP, simvastatin, and mevinolin. Surprisingly, *T. barophilus* was insensitive to the drug 6-MP, even though it possesses a gene (*TERMP_00517*) (9) annotated as encoding a hypoxanthine-guanine phosphoribosyl-transferase. *TERMP_00517* is a homologue of the *TK0664* gene in *T. kodakarensis* (80% of identity), the inactivation of which results in 6-MP resistance in *T. kodakarensis* (18). In contrast to *T. kodakarensis*, the *T. barophilus* MP strain is auxotrophic for tryptophan, and it is not amenable to tryptophan-based positive selection, because this WT species possesses only one gene (*TERMP_00157*) encoding a Trp synthase-like β subunit and lacks six genes (among them *trpE*) encoding enzymes that transform chorismate to tryptophan (9, 24).

T. barophilus MP seems to be sensitive to 5-FOA concentrations ranging from 5 to 8 mg/ml, but only during 24 h of growth on solid rich medium and less than 10 h of growth on liquid rich medium (see Fig. S2A in the supplemental material), beyond

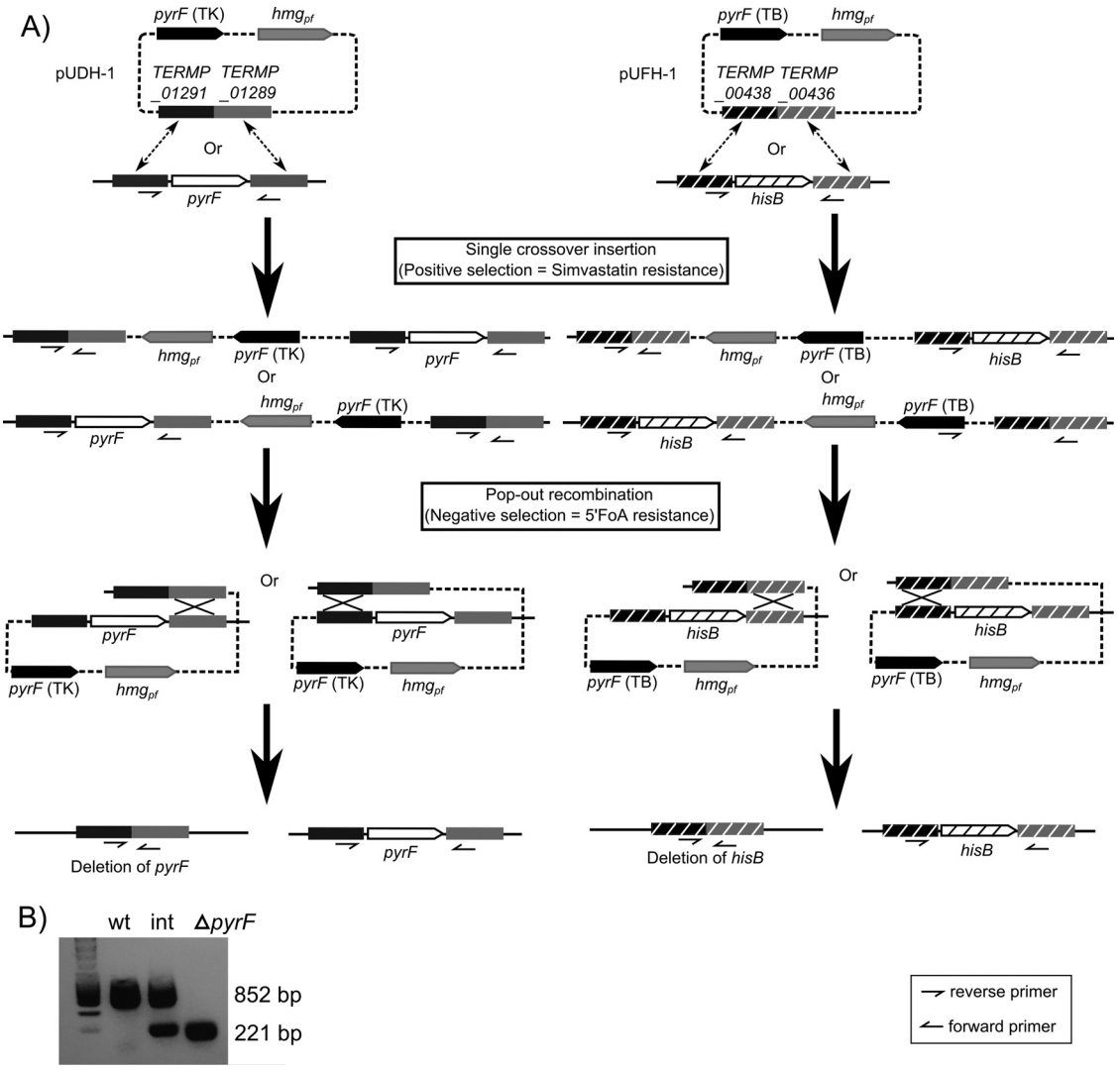

FIG 2 Deletion pathway of *pyrF* and *hisB* genes. (A) Two suicide vectors were constructed to ligate homologous-region amplification (*TERMP_01289*, *TERMP_01291*, *TERMP_00436*, and *TERMP_00438*) with pUFH or pUDH. The plasmids used were pUDH-1 and pUFH-1. After transformation, the plasmid was integrated into the genome by a first crossover event in the homologous-region fragment. The second step was the pop-out recombination (or excision) event. There are two possibilities: a recombination between the other homologous fragments, resulting in the deletion of the targeted gene, or a recombination between the same homologous fragments of the first recombination, which gives the WT genotype. *pyrF* was deleted from strain UBOCC-3107 (WT), and *hisB* was deleted from strain UBOCC-3256 (Δ*pyrF*). (B) To verify the different genotype configurations, PCR amplification was performed with the primers matching the HR1 and HR2 regions: XhoI-pyrF-TB-Up and SmaI-pyrF-TB-Do for *pyrF* deletion and Verif-hisB-Up and Verif-hisB-Do for *hisB* deletion (data not shown).

which cell growth was observed, probably due to uracil contamination or drug thermal degradation, which gives high backgrounds, as was reported for *T. kodakarensis* and other *Archaea* (15, 17). The uracil marker is functional in *T. barophilus*, and a concentration as high as 8 mg/ml was chosen, at least for negative selection.

In comparison with *T. kodakarensis*, sensitivity to simvastatin was assessed in *T. barophilus* using drug concentrations ranging from 1 to 10 μg/ml. As previously described, 5 μg/ml of simvastatin was needed to observe a total inhibition of *T. kodakarensis* growth (13). In contrast, 2.5 μg/ml of simvastatin was sufficient to inhibit the growth of *T. barophilus* cells for at least 5 days of incubation on both solid and liquid media (see Fig. S2B in the supplemental material), indicating that this concentration would be suitable and sufficient for selecting Sim^r transformants and that simvastatin could be used for positive selection.

Construction of gene deletion plasmids. To achieve gene disruption in *T. barophilus*, two plasmids, pUDH and pUFH, were designed for double-crossover events (Fig. 1) and constructed using the plasmid pUD (19) (see Materials and Methods). The pUDH plasmid contains a marker cassette containing the *pyrF* gene from *T. kodakarensis* and an *hmg$_{Pf}$* cassette (Fig. 1A), whereas the pUFH plasmid contains the same *hmg$_{Pf}$* cassette and the *pyrF* gene from *T. barophilus* (Fig. 1B). These two plasmids were replicative in *E. coli* and conferred ampicillin resistance but were not replicative in *T. barophilus*. The strategy for construction of a targeted gene excision strain is shown in Fig. 1. The pop-in/pop-out method was used in *T. barophilus*; by this method, inte-

TABLE 2 *T. barophilus* strains used and constructed in this study

Strain	Genotype	Parent strain	Genome region(s) deleted from parent strain	Reference
UBOCC-3107	Wild type	*T. barophilus* MP		1
UBOCC-3256	Δ*pyrF*	UBOCC-3107	*TERMP_01290* (nucleotides 1122862–1123491)	This work
UBOCC-3260	Δ*pyrF* Δ*hisB*	UBOCC-3256	*TERMP_00437* (nucleotides 362319–362849)	This work
UBOCC-3262	Δ*pyrF TERMP_00006*::*pUFH-2*	UBOCC-3256		This work
UBOCC-3265	Δ*pyrF* Δ*hisB TERMP_00004*::*pUHH*	UBOCC-3260		This work

gration of constructs into the genome (pop-in) is selected for by transformation to simvastatin resistance, and intramolecular recombinants that have lost the plasmid (pop-out) are counterselected using 5-FOA.

Construction of *pyrF* and *hisB* mutants. The new constructs pUDH and pUFH were used to clone the flanking regions of the targeted genes *pyrF* and *hisB*, respectively, and the resulting plasmids were named pUDH-1 and pUFH-1 (Fig. 2A). These plasmids were used to transform *T. barophilus* MP using simvastatin as the resistant marker (see Materials and Methods). The transformation efficiency was approximately 10^2 transformants per μg plasmid DNA. The Sim^r transformants containing plasmids integrated into the *T. barophilus* chromosome were checked by PCR, and as shown in Fig. 2B, this led to an amplification of two bands: a large one (852 bp), corresponding to the WT allele, and a small one (221 bp), corresponding to the deleted gene (Fig. 2B). After PCR verification, selected clones were spread on solid TRM with 5-FOA (8 mg/ml) and incubated for 4 days at 85°C. About 100 to 300 colonies per plate were obtained, some of which were restreaked on solid TRM containing simvastatin (2.5 μg/ml); 70 to 90% of these were Sim^r, which demonstrated that the pop-out event had not occurred in these cases. At least 10% of the colonies were Sim^s and were checked by PCR amplification of a 221-bp fragment, demonstrating that the pop-out event had occurred. (Fig. 2B). One Δ*pyrF* strain mutant (UBOCC-3256) (Table 2) was selected and will serve as a starting point for further genetic manipulations.

In order to delete the *hisB* gene, the plasmid pUFH-1 was used to transform the strain UBOCC-3256 by following the strategy described above for pUDH-1. Similar frequencies of pop-in and pop-out events were obtained, and at least three Sim^s mutants were verified by PCR to confirm the excision of the *hisB* gene (data not shown). One strain, named UBOCC-3260 (Table 2), was selected for further experiments.

Characterization of mutants. A comparison of the growth rates and yields of the *T. barophilus* wild type and *T. barophilus* strains UBOCC-3256 and UBOCC-3260 in TAA medium in the presence or absence of uracil and/or uracil plus histidine is shown in Fig. 3. Growth experiments were performed at 0.1 MPa, 40 MPa (Fig. 3), and 70 MPa (see Fig. S1 in the supplemental material). Strain UBOCC-3256 (Δ*pyrF*) did not grow in the absence of uracil but did grow in defined medium supplemented with uracil and showed a growth rate and yield comparable to those of the wild type (Fig. 3). Strain UBOCC-3260 (Δ*pyrF* Δ*hisB*) was not able to grow on TAA medium with or without uracil. The growth of this strain resumed when defined medium was supplemented with uracil and histidine (Fig. 3), and the growth rate and yield were similar to those of the wild-type strain. These data demonstrated that UBOCC-3256 and UBOCC-3260 are auxotrophic for uracil and for uracil plus histidine, respectively. The mutants and the WT strains grew better in a defined medium at 40 MPa (Fig. 3), which corresponds to the optimal growth pressure for *T. barophilus* MP (1), and they also showed growth capabilities that were comparable at 0.1 and 70 MPa (Fig. 3; also, see Fig. S1 in the supplemental material) but slightly lower than at 40 MPa (Fig. 3). The growth yield of the mutants was comparable to that of the WT when cells were grown in TRM regardless of the hydrostatic pressure applied (see Table S2 in the supplemental material). These results demonstrated that *pyrF* and *hisB* gene deletion did not show pleiotropic effects in *T. barophilus*.

Complementation of the Δ*pyrF* and *hisB* strains by wild-type *pyrF* and *hisB* alleles restores uracil and histidine prototrophy. Several attempts to transform *T. barophilus* MP with the pLC70 plasmid (20) were unsuccessful. This shuttle vector, known to replicate and to express genes in both *T. kodakarensis* and *E. coli*, was constructed by ligating the pTN1 plasmid (25) from *Thermococcus nautilus* to the commercial vector pCR2.1-TOPO with addition of selectable markers (hmg_{Pf} and *trpE*) (20). Maintenance of the pLC70 plasmid in *T. kodakarensis* depends on a likely functional replication protein, Rep74, of pTN1 (25), but this plasmid could not be propagated in *T. barophilus* even though it carries an hmg_{Pf} gene, which confers simvastatin resistance to *T. barophilus* cells. Neither an *E. coli*/*T. barophilus* shuttle vector nor a plasmid replicative in *T. barophilus* is yet available, and these should be constructed in order to expand the genetic toolbox for this species. An alternative complementation strategy was used by introducing pUFH-2 into UBOCC-3256 (Δ*pyrF*) (Table 1). This plasmid carries the flanking regions of the gene *TERMP_00005* and a copy of the *pyrF* gene from *T. barophilus*. The construct was integrated into the chromosome of the UBOCC-3256 strain, and transformants were selected for their resistance to simvastatin. Many clones were screened by PCR, and all had undergone single-crossover recombination ($pyrF^+$) at one or the other of the homologous regions (data not shown). One clone was selected and named strain UBOCC-3262 (Table 2). The chromosome of this strain was checked using the primers Verif-int-comp-2Up and Verif-int-comp-2Do (see Table S1 in the supplemental material), and this showed that the plasmid pUFH-2 was inserted in *TERMP_00006* gene (data not shown). We examined the growth of *T. barophilus* MP and its derivatives in TAA medium. The growth rate of UBOCC-3262 was comparable to that of the WT, while UBOCC-3256 could not grow in the absence of uracil (Fig. 4A).

The *T. barophilus pyrF* gene of pUFH-2 plasmid was replaced by the *T. barophilus hisB* gene, and the resulting plasmid, pUHH (Table 1), was introduced into UBOCC-3260 (Δ*pyrF* Δ*hisB*). A number of transformants were selected for their resistance to simvastatin, and after PCR screening and growth assays (data not shown), one clone was selected and named UBOCC-3265 (Table 2).

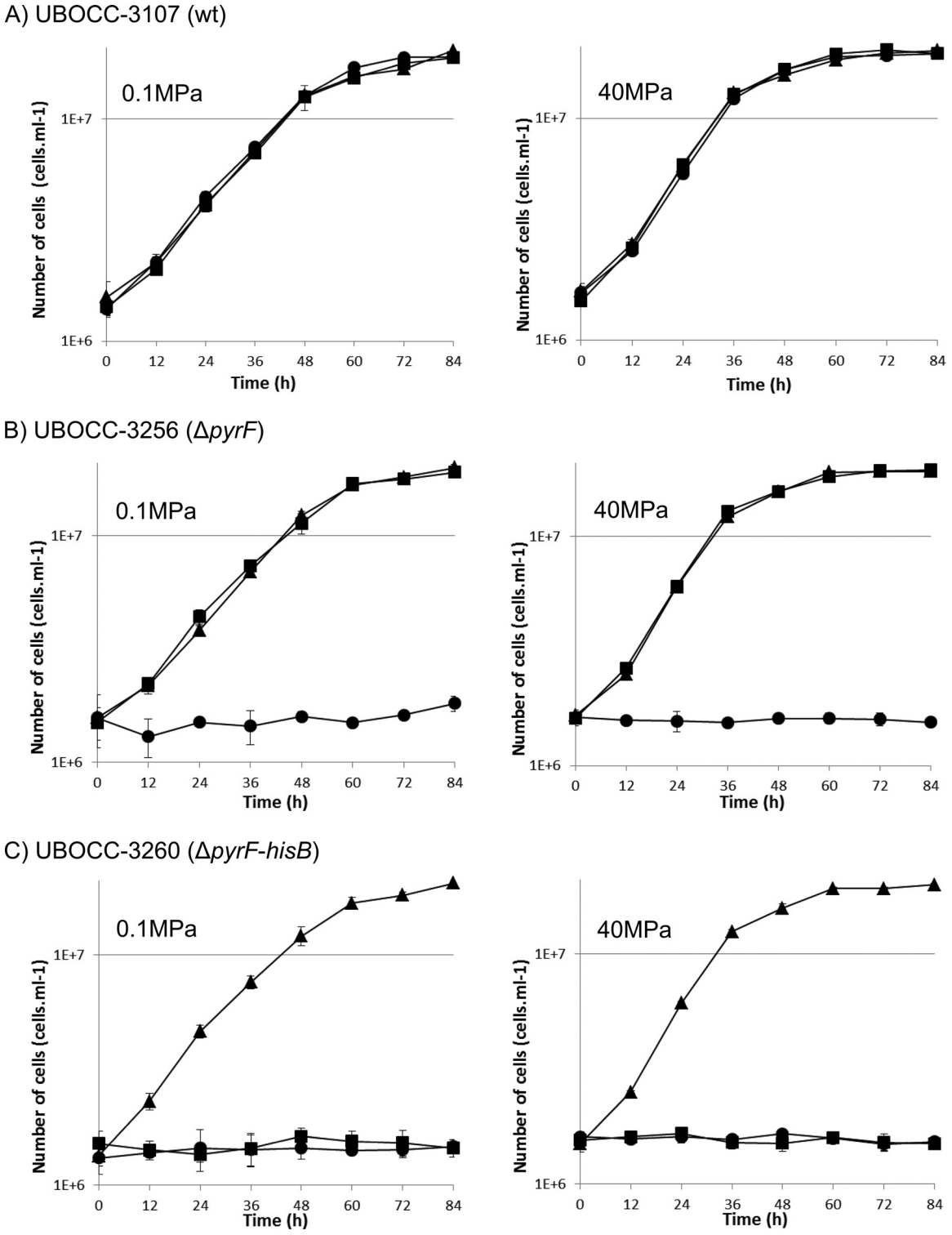

FIG 3 Characterization of UBOCC-3256 (Δ*pyrF*) and UBOCC-3260 (Δ*pyrF* Δ*hisB*) mutants at 0.1 MPa and 40 MPa. Growth assays were carried out in TAA medium at 85°C, without uracil (■), with uracil (●), and with uracil and histidine (▲). These growth experiments were carried out at hydrostatic pressures of 0.1 MPa and 40 MPa.

The chromosome of this strain was checked using the primers Verif-int-comp-1Up and Verif-int-comp-1Do (see Table S1 in the supplemental material), and this showed that the plasmid pUHH was inserted in the *TERMP_00004* gene (data not shown). The growth rate and yield of UBOCC-3265 were comparable to those of the WT strain, while UBOCC-3260 could not grow in the absence of histidine (Fig. 4B).

These results demonstrated that *pyrF* or *hisB* gene deletion in

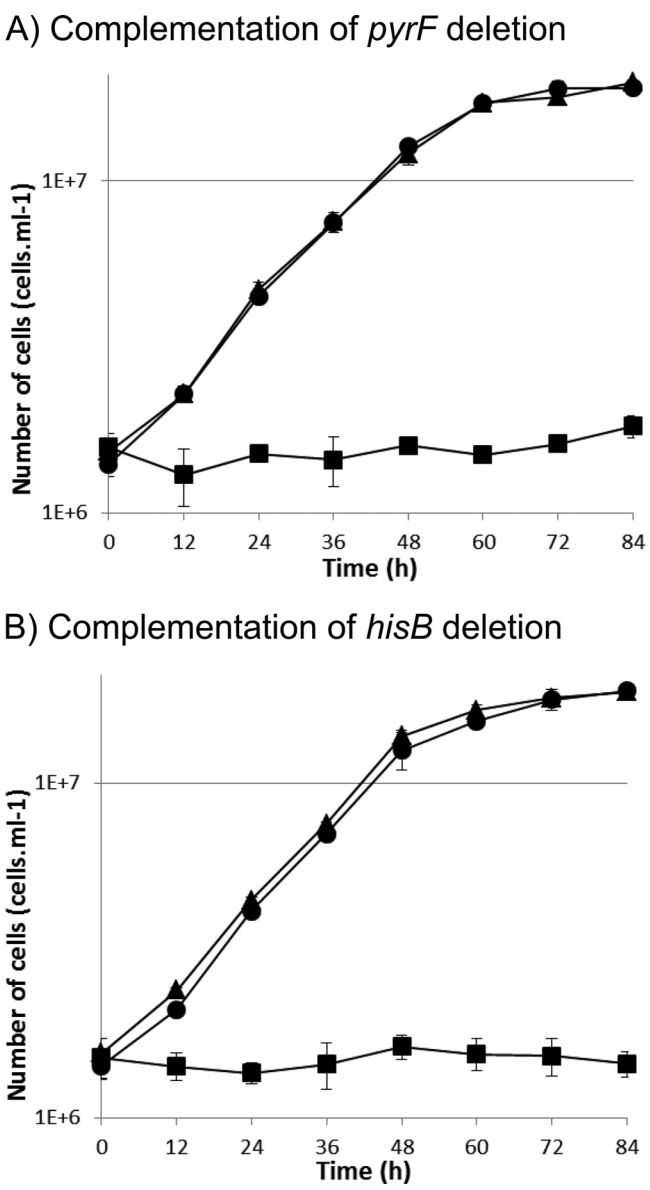

FIG 4 Complementation of *pyrF* and *hisB* mutations. Growth assays of strains UBOCC-3107 (WT) (●), UBOCC-3256 (Δ*pyrF*) (■), and UBOCC-3262 (Δ*pyrF TERMP_00006*::pUFH-2) (▲) (A) and of strains UBOCC-3107 (WT) (●), UBOCC-3260 (Δ*pyrF*-Δ*hisB*) (■), and UBOCC-3265 (Δ*pyrF* Δ*hisB TERMP_00004*::pUHH) (▲) (B) were carried out at 85°C in TAA medium without (A) or with (B) uracil. These growth experiments were performed at 0.1 MPa.

T. barophilus can be complemented by ectopic integration of a *pyrF* or *hisB* allele from *T. barophilus*.

DISCUSSION

In this study, we report the successful disruption of the *pyrF* locus in *T. barophilus*, a hyperthermophilic piezophilic archaeon, and the development of a gene deletion system based on resistance against simvastatin and 5-FOA. Simvastatin was used for positive-selection transformants in nutrient-rich medium for hyperthermophilic and halophilic archaea (13, 26–29). *T. barophilus* appeared to be more sensitive to this drug than *T. kodakarensis* (13) and *P. furiosus* (28). The MIC for the untransformed host was low (1 μg/ml), and the sensitivity of *T. barophilus* to 5-FOA is comparable to that of *T. kodakarensis* (19). As in *T. kodakarensis* and *P. furiosus*, an effective gene disruption system has been established in *T. barophilus*, and the ability to use this method to generate single and multiple deletions in the same strain will help analyze and decipher the mechanisms of adaptation to HHP in this important hyperthermophilic piezophilic archaeon. Using circular DNA containing 1 kb of homologous regions, the frequency of transformation for *T. barophilus* is estimated at 10^2 transformants per μg DNA, which is comparable to that reported for *T. kodakarensis* (10^1 to 10^2 transformants per μg DNA) (12) but less than that observed for *P. furiosus* (10^5 transformants per μg of DNA) (30). The advantage of the method developed here to generate gene deletion in *T. barophilus* is that the selection and counterselection steps can be performed in rich medium. Even through no spontaneous Sim^r mutants were generated in *T. barophilus*, in contrast to what was reported for the genetic manipulation of *T. kodakarensis* and *P. furiosus* (13, 15, 30), it is necessary to enrich Sim^r cells in liquid medium after transformation prior to direct isolation of Sim^r colonies on plates.

Uracil prototrophic selection can be used for simultaneous transformation and gene deletion in *T. barophilus*, but the limitation of this selectable marker is the interference caused by background growth of the Δ*pyrF* strain on solid medium. Simvastatin-based selection is useful for positive selection in *T. barophilus*, whereas uracil-based negative selection or counterselection suffers from a lack of efficiency in *T. barophilus*. This pattern has already been observed in other hyperthermophiles: such a counterselective strategy is also available for a 6-methyl purine-based marker, which provides a reliable counterselective pressure in *T. kodakarensis* and *P. furiosus* strains from which the *xgprt* gene (*TK0664* and *PF1950*), encoding a hypoxanthine-guanine phosphoribosyltransferase (20, 31), has been deleted. *T. barophilus* is insensitive to 6-MP despite the presence of *TERMP_00517*, which is orthologous to *TK0664* (80% identity) and *PF1950* (77% identity), in its genome.

As a proof of concept of the efficiency of these genetic tools, the *hisB* gene, encoding imidazole glycerol-phosphate dehydratase, was deleted in UBOCC-3256. The corresponding mutant showed impaired growth in defined medium in the absence of histidine, and the growth resumed in defined medium where histidine was supplied. Moreover, we introduced a nonreplicative plasmid carrying the *pyrF* allele from *T. kodakarensis* or *T. barophilus*, which was integrated into the Δ*pyrF T. barophilus* strain by a single crossover into the homologous genomic region of *TERMP_00005*, and we demonstrated that *T. barophilus* mutants can be complemented using this strategy until a shuttle vector that can stably replicate and express selectable phenotypes in both *T. barophilus* and *E. coli* becomes available.

The nutritional markers targeted in this study provide nutrition selection, and the resulting mutants were auxotrophic regardless of the culture hydrostatic pressure. These genetic tools developed for *T. barophilus* will help to study the adaptation of *T. barophilus* to deep-sea hydrothermal-vent conditions, notably HHP. Indeed, transcriptomic studies have highlighted HHP-responsive genes involved in hydrogen production, amino acid uptake and metabolism, sugar uptake and metabolism, and CO assimilation (24). Deletions of genes involved in these pathways *in vivo* are in progress to examine the roles of related enzymes in hydrostatic pressure adaptation. This will provide greater insight

into the mechanisms that have evolved to allow *T. barophilus* to cope with HHP conditions.

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