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 (ΔpyrF) 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 (ΔpyrF) or UBOCC-3260 (ΔpyrF ΔhisB), 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 yanoensis* (6, 7). *P. yanoensis* 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. yanoensis*, 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<sub>B</sub>), which encodes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a pivotal enzyme for archaeal membrane lipid biosynthesis (16), so that the *hmg<sub>B</sub>* gene can then be used as a positive selection marker (17). Among prototrophic selectable 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 *repE* 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...
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 MgCl₂·6 H₂O, 2 g C₂H₃NaO₂, 1 g (NH₄)₂SO₄, 0.5 g KCl, 0.05 g NaBr, 0.02 g SrCl₂·6 H₂O, 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; Euremedex). 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% (vol/vol) sterile Na₂S·9H₂O 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. kodakarenensis pyrF* gene with its putative promoter region; it is a replicative plasmid in *E. coli* but not in *Thermococcales* strains.

The *hmgPf* 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 *hmgPf* amplicon was cloned in the pUD plasmid digested by the restriction enzymes KpnI and SalI, and after ligation between the pUD plasmid and the *hmgPf* 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 Xhol and SmaI enzymes. This digestion linearized the plasmid, allowing the excision of the *T. kodakarenensis pyrF* gene but not its promoter region.

The linearized plasmid was ligated with the PCR-amplified *T. barophilus pyrF* gene obtained with the primers Xhol-pyrF-TB-Up and SmaI-pyrF-TB-Do to generate deletion mutants by PCR amplification, two fragments of 1 kb encompassing the targeted genes were obtained (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 and 1Do (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 ΔpyrF-1Up, ΔpyrF-1Do, ΔpyrF-2Up, and ΔpyrF-2Do for deletion of *pyrF* ([TERM_01290]; ΔhisB-1Up, ΔhisB-1Do, ΔhisB-2Up, and ΔhisB-2Do for deletion of *hisB* ([TERM_00437]); and ΔTERM_00005-1Up, ΔTERM_00005-1Do, ΔTERM_00005-2Up, and ΔTERM_00005-2Do for the deletion of *TERM_00005* (see Table S1 in the supplemental material). By using this approach, three homologous-region fragments were created: the ΔpyrF, ΔhisB, and ΔTERM_00005 fragments. The fragment [TERM_01289(HR1)]-[TERM_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 [TERM_00436(HR1)]-[TERM_00438(HR2)] and [TERM_00004(HR1)]-[TERM_00006(HR2)] were cloned into the plasmid pUDH using the restriction sites KpnI and BglII/BamHI; we thus obtained the suicide vectors pUDH-1, pUDH-1, and pUDH-2 (Table 1).
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 VeriR-hisB-Up and VeriL-hisB-Down (see Table S1 in the supplemental material). The hisB PCR product and pUFH vector were digested by XhoI and SmaI and then inserted was streaked onto TRM plates supplemented with 5-FOA at 8 April 2014 Volume 80 Number 7.

Transformation resulted in a targeted gene deletion or wild type (WT) genotype. PCR amplification was performed to check whether the pop-out recombinants were wild type or the other of the homologous regions (*termp*). The colonies that grew on TRM–5-FOA plates were restreaked on TRM-simvastatin plates to eliminate the false positives that were still simvastatin sensitive to simvastatin. Many transformants that were resistant to simvastatin were obtained, and after PCR screening, all clones were harvested after centrifugation (6,000 g, 6 min, 4°C) and then used for positive-selection integration and counterselection excision from the *T. barophilus* genome, the sensitivity of *T. barophilus* to 6-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

| TABLE 1 *T. barophilus* plasmids used and constructed in this study |
|-----------------|-----------------|-----------------|-----------------|
| Plasmids        | Resistance      | Parent plasmid   | Description                                      | Reference |
| pUD             | Ampicillin      | pUD             | Cloning of HMG-CoA reductase-encoding gene in pUD | This work |
| pUDH            | Ampicillin, simvastatin | pUDH          | Replacement of pyrF (*T. kodakarenis*) by pyrF (*T. barophilus*) | This work |
| pUFH            | 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      | pUFH-2         | Replacement of pyrF by hisB                       | This work |

Genomic DNA extraction of *T. barophilus* was performed using a phenol-chloroform-isomyl alcohol (PCI) extraction method, as follows. A 20-ml portion of cell culture at exponential growth phase (approximately 10^6 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-isomyl 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_004*, 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 40 s, 50 to 58°C for 40 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*._000517) (9) annotated as encoding a hypoxanthine-guanine phosphoribosyl-transferase. *TERMP_00517* is a homologue of the *TK0664* gene in *T. kodakarenis* (80% of identity), the inactivation of which results in 6-MP resistance in *T. kodakarenis* (18). In contrast to *T. kodakarenis*, 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_00517*) 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. This work

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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 Simr 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 *hmgPf* cassette (Fig. 1A), whereas the pUFH plasmid contains the same *hmgPf* 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-

![Deletion pathway of pyrF and hisB genes.](image-url)

**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 (∆*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).
gration of constructs into the genome (pop-in) is selected for by transformation to simvastatin resistance, and intramolecular recombining constructs 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⁺ 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 re-screened on solid TRM containing simvastatin (2.5 μg/ml); 70 to 90% of these were Sim⁺, which demonstrated that the pop-out event had not occurred in these cases. At least 10% of the colonies were Sim⁻ and were checked by PCR amplification of a 221-bp fragment, demonstrating that the pop-out event had occurred in this strain. A 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⁺_ 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 (ΔpyrF) 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 (ΔpyrF ΔhisB) 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 ΔpyrF and ΔhisB 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 nautilis_ to the commercial vector pCR2.1-TOPO with addition of selectable markers (hmgPf and trpE) (20). Maintenance of the pLC70 plasmid in _T. kodakarensis_ depends on a functional replication protein, Rep74, of pTN1 (25), but this plasmid could not be propagated in _T. barophilus_ even though it carries an hmgPf 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 (ΔpyrF) (Table 1). This plasmid carries the flanking regions of the gene _TERMP_00006 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 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 (ΔpyrF ΔhisB). 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).

**Table 2.** _T. barophilus_ strains used and constructed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Parent strain</th>
<th>Genome region(s) deleted from parent strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBOCC-3107</td>
<td>Wild type</td>
<td><em>T. barophilus</em> MP</td>
<td>TERMP_01290 (nucleotides 1122862–1123491)</td>
<td>1</td>
</tr>
<tr>
<td>UBOCC-3256</td>
<td>ΔpyrF</td>
<td>UBOCC-3107</td>
<td>TERMP_00457 (nucleotides 362319–362849)</td>
<td>This work</td>
</tr>
<tr>
<td>UBOCC-3260</td>
<td>ΔpyrF ΔhisB</td>
<td>UBOCC-3256</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>UBOCC-3262</td>
<td>ΔpyrF TERMP_00006::pUFH-2</td>
<td>UBOCC-3256</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>UBOCC-3265</td>
<td>ΔpyrF ΔhisB TERMP_00004::pUHH</td>
<td>UBOCC-3260</td>
<td></td>
<td>This work</td>
</tr>
</tbody>
</table>
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

![Graphs showing growth rate and yield of UBOCC-3256 and UBOCC-3260 under various conditions.](image-url)

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
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 10⁸ transformants per µg DNA, which is comparable to that reported for *T. kodakarensis* (10⁸ to 10¹² transformants per µg DNA) (12) but less than that observed for *P. furiosus* (10⁷ 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 Simr 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 Simr cells in liquid medium after transformation prior to direct isolation of Simr 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 ΔpyrF 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 glyceral-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 ΔpyrF *T. barophilus* strain by a single cross-over 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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