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HAL Id: hal-01111536
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Submitted on 6 Feb 2015

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Genetic Manipulations of the Hyperthermophilic Piezophilic Archaeon Thermococcus barophilus

Axel Thiel,a,b,c Grégoire Michoud,a,b,c Yann Moalic,a,b,c Didier Flament,a,b,c Mohamed Jebbara,a,b,c

Université de Bretagne Occidentale, UMR 6197-Laboratoire de Microbiologie des Environnements Extrêmes, Institut Universitaire Européen de la Mer, Plouzané, France; CNRS, UMR 6197-Laboratoire de Microbiologie des Environnements Extrêmes, Institut Universitaire Européen de la Mer, Plouzané, France; Ifremer, UMR 6197-Laboratoire de Microbiologie des Environnements Extrêmes, Technopôle Brest-Iroise, Plouzané, France

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 (∆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 (∆pyrF) or UBOCC-3260 (∆pyrF ∆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 yanyanosii (6, 7). P. yanyanosii 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. yanyanosii, 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 kodakarenis and Pyrococcus furiosus, which are phylogenetically related to T. barophilus, have been described (12–14). For some archaeal genetic systems, such as T. kodakarenis, 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 (hmg59), which encodes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a pivotal enzyme for archaeal membrane lipid biosynthesis (16), so that the hmg59 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 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 counterselection 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. kodakarenis. 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 · 6H2O, 2 g C2H3NaO2, 1 g (NH4)2SO4, 0.5 g KCl, 0.05g NaBr, 0.02 g SrCl2 · 6H2O, 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 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. kodakarenisis pyrF gene with its putative promoter region; it is a replicative plasmid in E. coli but not in Thermococcales strains.

The hmgO gene was obtained by PCR amplification on the pLC70 plasmid (20) with the primers Sall-HMG-CoA-Up and Kpn1-HMG-CoA-Do (see Table S1 in the supplemental material); the resulting PCR product bears the Kpn1 and Sall restriction enzyme sites at its extremities. The hmgO amplicon was cloned in the pUD plasmid digested by the restriction enzymes Kpn1 and Sall, and after ligation between the pUD plasmid and the hmgO 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 Sma1 enzymes. This digestion linearized the plasmid, allowing the excision of the T. kodakarenisis 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-5'-Up and XmaISmaI-pyrF-5'-Do to amplify the T. barophilus pyrF gene. pUDH and pyrF(TB) were digested by Xhol (X) and Smal (S) and were then ligated to obtain the plasmid pUFH. In pUDH and pUFH, the restriction enzyme sites BamHI (B) and Kpn1 were conserved to enable cloning of the homologous regions in these plasmids.

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 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 Kpn1 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 pUFH using the restriction sites Kpn1 and BglII/BamHI; we thus obtained the suicide vectors pUDH-1, pUFH-1, and pUFH-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 Verif-hisB-Up and Verif-hisB-Do (see Table S1 in the supplemental material). The PCR product and pUFH vector were digested by XhoI and Smal and then ligated (using T4 ligase; Promega), and the resulting plasmid was named pUFH-H (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 anaerobic conditions. 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, simvastatin (0.5 mg/ml), and various concentrations of simvastatin and 5-FOA from 5 to 8 mg/ml, but only during 24 h of growth of 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_000517) (9) annotated as encoding a hypoxanthine-guanine phosphoribosyl-transferase. TERMP_00517 is a homologue of the TK0664 gene in T. kodakarense (80% of identity), the inactivation of which results in 6-MP resistance in T. kodakarense (18). In contrast to T. kodakarense, 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_000517) encoding a Trp synthase-like β subunit and lacks six genes (among them trpE) encoding enzymes that transform chorismate to tryptophan (9, 24).

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. kodakarense (80% of identity), the inactivation of which results in 6-MP resistance in T. kodakarense (18). In contrast to T. kodakarense, 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_000517) 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

| 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. kodakarense) 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      | pUHH-2          | Replacement of pyrF by hisB                               | This work        |
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 chosen, as 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-

**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).
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 10⁵ 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 90% of these were Sim, which demonstrated that the pop-out formation efficiency was approximately 10² 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 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. (Fig. 2B). One ΔpyrF 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 Thermo- coccus nautilis to the commercial vector pCR2.1-TOPO with addition of selectable markers (hmgAp 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 hmgF 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_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 (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>T. barophilus MP</td>
<td>TAA medium</td>
<td></td>
</tr>
<tr>
<td>UBOCC-3256</td>
<td>ΔpyrF</td>
<td>UBOCC-3107</td>
<td>TERMP_01290 (nucleotides 1122862–1123491)</td>
<td>This work</td>
</tr>
<tr>
<td>UBOCC-3260</td>
<td>ΔpyrF ΔhisB</td>
<td>UBOCC-3256</td>
<td>TERMP_00457 (nucleotides 362319–362849)</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
Gene Deletion in Hyperthermophilic Piezophilic Archaea

A) Complementation of pyrF deletion

![Graph showing complementation of pyrF deletion.](image)

FIG 4 Complementation of pyrF and hisB mutations. Growth assays of strains UBOCC-3107 (WT) (●), UBOCC-3256 (ΔpyrF) (■), and UBOCC-3262 (ΔpyrF TERMP_00006:pUFH-2) (▲) (A) and of strains UBOCC-3107 (WT) (●), UBOCC-3260 (ΔpyrF-ΔhisB) (■), and UBOCC-3265 (ΔpyrF ΔhisB 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 10^7 transformants per μg DNA, which is comparable to that reported for T. kodakarensis (10^7 to 10^8 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 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 counterselection 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 ΔpyrF 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 of adaptation to HHP.
into the mechanisms that have evolved to allow *T. barophilus* to cope with HHP conditions.

### ACKNOWLEDGMENTS

We thank Jacques Oberto, Tadayuki Imanaka, Haruyuki Atomi, and Thomas J. Santangelo for helpful discussions and/or for providing vectors and protocols and Mickael Beauregger for his advice and help with performing growth experiments under high hydrostatic pressure. We are indebted to Helen McCombie for helpful language improvement. We also thank Nadège Quintin for the deposition of strains described in this work in the UBO culture collection (http://www.univ-brest.fr/souchothque/Collection+LM2E).

This work was supported by the Agence Nationale de la Recherche (ANR-10-BLAN-1725 01-Living deep). A.T. was supported by a postdoctoral fellowship from the Conseil Général 29 and from Ifremer. G.M. was supported by a Ph.D. fellowship from the Conseil Régional de Bretagne.

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