Expression and Function of Different Guanine-Plus-Cytosine Content 16S rRNA Genes in *Haloarcula hispanica* at Different Temperatures

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The halophilic archaeon *Haloarcula hispanica* harbors three ribosomal RNA (rRNA) operons (*rrnA*, *rrnB*, and *rrnC*) that contain the 16S rRNA genes *rrsA*, *rrsB*, and *rrsC*, respectively. Although *rrsB* and *rrsC* (*rrsBC*) have almost identical sequences, the *rrsA* and *rrsBC* sequences differ by 5.4%, and they differ by 2.5% with respect to guanine-plus-cytosine content (P<sub>GC</sub>). The strong correlation between the typical growth temperatures of archaea and P<sub>GC</sub> of their 16S rRNA genes suggests that *H. hispanica* may harbor different 16S rRNA genes having different P<sub>GC</sub> to maintain rapid growth in a wide range of temperatures. We therefore performed reverse transcription-coupled quantitative PCR to assess expression levels of *rrsA* (P<sub>GC</sub>, 58.9%) and *rrsBC* (P<sub>GC</sub>, 56.4–56.5%) at various temperatures. The expression ratio of *rrsA* to *rrsBC* increased with culture temperature. Mutants with complete deletions of one or two of the three rRNA operons were constructed and their growth rates at different temperatures compared to that of the wild-type. The growth characteristics of the rRNA operon single-mutant strains were indistinguishable from the wild-type. The rRNA operon double-mutant strains maintained the same temperature range as wild-type but displayed reduced growth rates. In particular, the double-mutant strains grew much slower than wild-type at low temperature related to minimum growth temperature of the wild-type. On the other hand, at physiologically high temperatures the wild-type and the double-mutant strain which harbors only *rrnA* with high-P<sub>GC</sub> *rrsA* grew significantly faster than the double-mutant strain which harbors only *rrnC* with low-P<sub>GC</sub> *rrsC*. These findings suggest the importance of 16S rRNAs transcribed from *rrsA* with high-P<sub>GC</sub> in maintaining rapid growth of this halophilic archaeon at raised growth temperatures.

Keywords: 16S rRNA genes, guanine-plus-cytosine content, *Haloarcula*, temperature, intragenomic heterogeneity

INTRODUCTION

Ribosomal RNA (rRNA) genes, especially 16S rRNA genes, are a particularly good marker for phylogenetic analysis of prokaryotes because they are highly conserved in all prokaryotes (Staley, 2002). Many studies have examined the phylogenetic positions of prokaryotic species and microbial diversities in natural environments using 16S rRNA gene sequences (e.g., Hiraoka et al., 2016;
Kuechler et al., 2016). On the other hand, recent studies based on genome sequences have demonstrated that the genomes of certain prokaryotes harbor several divergent 16S rRNA genes. Sun et al. (2013) reported that almost half of the complete prokaryotic genomes examined contain a base or more dissimilarity in 16S rRNA genes. Furthermore, > 3% differences in 16S rRNA gene sequences have been detected in 14 of the 1690 complete genomes in domain Bacteria (Větrovský and Baldrian, 2013). Within the domain Archaea, some methanogens, e.g., Methanocaldococcus jannaschii and Methanomethanobacter thermoautotrophicus, exhibit 16S rRNA gene polymorphisms displaying as much as 0.1% divergence, whereas some halophilic archaean more divergent 16S rRNA gene copies (4.9–9.8%) in their genomes (Acinas et al., 2004; Boucher et al., 2004; Pei et al., 2010; Sun et al., 2013).

Haloarcula strains, halophilic archaean belonging to class Halobacteria, have been mainly isolated from solar salterns and salt lakes (Torreblanca et al., 1986), where large day-night and seasonal temperature variations are observed (Post, 1977; Wieland et al., 2005; Sima et al., 2013; Andrade et al., 2015). So far, 15 Haloarcula strains, including 10 strains named validly, have been reported (Javor et al., 1982; Mizuki et al., 2004; Namwong et al., 2011; Ding et al., 2014; Yun et al., 2015). Complete genome sequences of four strains, i.e., H. hispanica, have been determined and show that all strains carry three rRNA operons (rrnA, rrnB, and rrnC) (Baliga et al., 2004; Liu et al., 2011; Ding et al., 2014; Yun et al., 2015). Each rRNA operon includes the 5S rRNA gene, the 16S rRNA gene (rrsA, rrsB, or rrsC), and the 23S rRNA gene. The 5S rRNA genes have identical sequences. Although the 23S rRNA gene sequences of rrnA and rrnB are almost identical, the 23S rRNA gene sequence of rrnC is slightly different (~2%) from those of rrnA and rrnB. Among the 16S rRNA genes, the rrsB and rrsC (rrsBC) sequences are almost identical, whereas the rrsA and rrsBC sequences differ by ~6% (Dennis et al., 1998; Dennis, 1999).

Previous studies of the expression of 16S rRNA genes in Haloarcula strains under different salinity conditions suggest that variation in salinity does not affect the expression level of each 16S rRNA gene (López-López et al., 2007; Cui et al., 2009). On the other hand, López-López et al. (2007) demonstrated that H. marismortui displays different expression patterns of each 16S rRNA gene under a wide range of temperatures. They also performed cultivation experiments with wild-type H. marismortui and the rRNA operon single-mutant strain, which lacks rrnB containing low guanine-plus-cytosine content (PGC) of 16S rRNA gene, under various temperature conditions (Tu et al., 2005; López-López et al., 2007). They found that growth of the rRNA operon single-mutant strain was slower than that of wild-type at all tested temperatures. López-López et al. (2007) could not determine whether rrnB inactivation or a lower copy number of rRNA operons would affect growth of the mutant strain, because the rRNA operon double-mutant strains that harbor only one rRNA operon containing low- or high- PGC 16S rRNA gene were not constructed and examined. Therefore, the functional importance of rRNA transcribed from each rRNA operon including rrsA, rrsB, or rrsC on growth under different temperature conditions has not been well understood yet.

 previous studies have reported that 16S rRNA gene sequences are naturally inscribed with the thermal features of their prokaryotic hosts (Galtier and Lobry, 1997; Khachane et al., 2005; Kimura et al., 2007, 2010, 2013). The observation was based on a high correlation between the growth temperatures of the prokaryotes and the PGC of their 16S rRNA sequences: 16S rRNA genes of hyperthermophiles and thermophiles tend to have high PGC, whereas 16S rRNA genes of mesophiles and psychrophiles have relatively low PGC. On the basis of the relationship between the growth temperatures and PGC of 16S rRNA gene sequences, we propose that Haloarcula strains express and utilize high PGC of 16S rRNAs at high temperature and low PGC of 16S rRNAs at low temperature, respectively.

In the present study, Haloarcula hispanica, for which the complete genome sequence has been determined, was used. The 16S rRNA genes of H. hispanica were sequenced, and the minimum (Tmin), optimum (Top), and maximum (Tmax) growth temperatures were estimated based on PGC of the 16S rRNA genes using the microbial molecular thermometer proposed by Kimura et al. (2013). Additionally, expression levels of rrsA and rrsBC between 25°C (actual Tmin of the strain) and 50°C (actual Tmax of the strain) were determined by reverse transcription-coupled quantitative PCR (qPCR) using specific primer sets. Moreover, we constructed rRNA operon single-mutant strains that lack rrnA, rrnB, or rrnC by using wild-type H. hispanica. We further constructed rRNA operon double-mutant strains that harbor only rrnA or rrnC by using the single-mutant strains and assessed their growth in a wide temperature range. The combined results from both gene expression and mutation experiments provide insight into the physiological advantage of harboring 16S rRNA genes of different sequence with respect to the growth of H. hispanica.

**MATERIALS AND METHODS**

**Strain and Cultivation for DNA Extraction**

Haloarcula hispanica JCM8911 was obtained from the Japan Collection of Microorganisms (JCM, Tsukuba, Ibaraki, Japan). The strain was grown in Medium 307, which contained 2 g casamino acid (BD, Franklin Lakes, NJ, USA), 2 g Bacto yeast extract (BD), 1 g sodium glutamate, 3 g trisodium citrate, 10 g MgSO4·7H2O, 1 g CaCl2·2H2O, 1 g KCl, 200 g NaCl, 0.36 mg FeCl2·4H2O, and 0.36 mg MnCl2·4H2O per liter of distilled water. After the pH of the medium was adjusted to 7.0, the medium was sterilized by filtration with a polysulfone membrane filter (pore size, 0.22 μm; Thermo Fisher Scientific, Waltham, MA, USA) and autoclaving at 121°C for 20 min. Exactly 20 ml of the medium was injected into autoclaved 60-ml screw-cap test tubes and inoculated with cells of H. hispanica JCM8911. The cultures were centrifuged at 6230 × g for 3 min. The pelleted cells were stored at −25°C until DNA extraction.
Cloning and Sequencing of 16S rRNA Genes

In order to make standards for qPCR described below, we performed cloning and sequencing of 16S rRNA genes of *H. hispanica*. Bulk DNA was extracted from *H. hispanica* cells grown in Medium 307 with modifications (Tchinda et al., 2016). Briefly, the pelleted cells were lysed with lysozyme and protease K solution. Then the genomic DNA was extracted with successive phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol steps and precipitated with ethanol. Next, 16S rRNA genes were amplified from the bulk DNA using the archaea-specific primer set 8F/1512uR (Table 1). PCR products were purified with a MicroSpin S-400 HR column (GE Healthcare, Little Chalfont, UK) and cloned using the Zero Blunt TOPO PCR Cloning kit (Life Technologies, Carlsbad, CA, USA). The PCR products were ligated into vector pCR4Blunt-TOPO (Life Technologies). *Escherichia coli* TOP10 cells (Life Technologies) were transformed with the ligated plasmid to construct a clone library. Insert DNA from selected recombinant colonies was sequenced by the dyeodeoxy cycle-sequencing method using a Model 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). The most similar 16S rRNA gene sequence was determined by the BLAST program (Altschul et al., 1990). The 16S rRNA gene sequences obtained in this study were deposited in the DDBJ/EMBL/GenBank database under accession numbers LC085245, LC085246, and LC085247.

Estimating $T_{\text{min}}$, $T_{\text{opt}}$, and $T_{\text{max}}$ Based on $P_{\text{GC}}$ of 16S rRNA Genes

Because thermophilic and hyperthermophilic archaea have greater $P_{\text{GC}}$ values for 16S rRNA genes compared with psychrophilic and mesophilic archaea (Galtier and Lobry, 1997; Khachane et al., 2005; Kimura et al., 2006, 2007, 2010), Kimura et al. (2013) proposed linear regression equations to infer $T_{\text{min}}$, $T_{\text{opt}}$, and $T_{\text{max}}$ of cultured and not-yet cultured archaea based on $P_{\text{GC}}$ of 16S rRNA genes. We therefore used these equations to estimate growth temperatures of *H. hispanica* based on $P_{\text{GC}}$ value of each 16S rRNA gene.

Kimura et al. (2013) used partial 16S rRNA gene sequences (ca. 800 bp) between the archaea-specific primers 109aF and 915aR in order to estimate their growth temperatures (Table 1). Thus, we manually selected the internal sequences from the 16S rRNA gene sequences determined in this study. $P_{\text{GC}}$ values for the internal sequences were calculated using Genetyx-Mac ver. 17.0.6 (Genetyx, Tokyo, Japan). $T_{\text{min}}$, $T_{\text{opt}}$, and $T_{\text{max}}$ were calculated based on $P_{\text{GC}}$ of the respective sequence using Kimura’s equations.

Culture Experiment to Assess the Expression of 16S rRNA Genes

*Haloarcula hispanica* cells in exponential growth phase in pre-culture were inoculated into 60-ml screw-capped tubes containing 20 ml of Medium 307. The cultures were incubated in the dark with shaking at 180 rpm at 25, 30, 35, 40, 45, and 50°C. The optical density at 660 nm (OD$_{660}$) of the culture was monitored using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific) with sterilized medium as the negative control. When the cultures reached the early exponential growth phase (OD$_{660}$ = 0.25–0.50), the cultures were centrifuged at 6230 g for 3 min. The pelleted cells were mixed with 100 μl RNAlater (Life Technologies) and stored at −85°C until RNA extraction.

RNA Extraction and Complementary DNA (cDNA) Synthesis

Cells were thawed on ice, and the RNAlater was removed and discarded. Total RNA was extracted from the cells using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). Contaminating genomic DNA in the extracted RNA samples was removed using the TURBO DNA-Free kit (Life Technologies). Total RNA was purified with the RNasy MinElute Cleanup kit (Qiagen, Hilden, Germany). The quality and concentration of RNA were verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoVue Plus spectrophotometer (GE Healthcare). Single-strand complementary DNA (cDNA) was synthesized from the purified total RNA using the SuperScript III first strand synthesis system (Life Technologies) as following manufacturer’s protocol. The cDNA was purified using QIAquick PCR Purification kit (Qiagen). The purified cDNA was stored at −25°C until qPCR analysis.

qPCR

The specific primer sets rrsAf/rrsAr for rrsA and rrsB Cf/rrsBCr for rrsBC were designed using Primer Express 2.0 software ver. 2.0 (Applied Biosystems) (Table 1). To test the specificity of these primer sets, qPCR was performed with PCR products of rrsA, rrsB, and rrsC that were amplified from the clones in the 16S rRNA gene-clone library described above. The PCR products were purified with QIAquick PCR purification kit (Qiagen). The qPCR was performed on an ABI Prism 7300 Real Time PCR System (Applied Biosystems) with PowerUP SYBR Green master mix (Life Technologies). Since the sequences of rrsB and rrsC are nearly identical, it was not possible to design primer sets for specifically amplifying rrsB and rrsC, independently.

Next, rrsA and rrsBC in the cDNA were quantified by qPCR using the ABI Prism 7300 Real Time PCR System. Each PCR mixture contained 2 μl of diluted cDNA template, 2 μl of each designed primer set (each 300 nM), 10 μl of PowerUP SYBR Green PCR master mix (Life Technologies), and 4 μl of nuclease-free water (Ambion). The PCR conditions included an initial step of 50°C for 2 min and 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 1 min. The standard curves were prepared from diluted PCR products (1/10, 1/100, 1/1000, 1/10000) of rrsA, rrsB, and rrsC that were amplified from the clones in the 16S rRNA gene-clone library described above. The PCR reactions were performed in triplicate for technical repeats and four individuals for biological repeats.

Construction and Cultivation of rRNA Operon Double-Mutant Strains

To assess any advantage to *H. hispanica* of having multiple distinct 16S rRNA genes, we disrupted the three rRNA operons
by using a mutation method (See Supplementary Materials and Methods for details). Briefly, an rRNA operon in wild-type *H. hispanica* was replaced with novobiocin resistance gene to construct rRNA operon single-mutant strains (HA2, HB2, or HC2) that lack *rrnA*, *rrnB*, or *rrnC*, respectively (Supplementary Table 1 and Figure 1). Furthermore, *rrnB* in HC2, *rrnA* in HC2 (or *rrnC* in HA2), and *rrnB* in HA2 were replaced with mevinolin resistance genes to construct the rRNA operon double-mutant strains HCB2, HCA2 (or HAC2), or HAB2 that contain only *rrnA*, *rrnB*, or *rrnC*, respectively (Supplementary Table 1 and Figure 2). Disruption of operons *rrnA*, *rrnB*, and *rrnC* in these mutant strains was confirmed by PCR amplification using primer sets, AVF/ACR, BVF/BCR, or CVF/CCR (Supplementary Table 2). The rRNA operon double-mutant strains harboring only *rrnB* (HCA2 and HAC2) could not be constructed despite repeated attempts (Supplementary Table 1).

The rRNA operon single- and double-mutant strains and wild-type strain were inoculated into Medium 307 and incubated in the dark with shaking at 180 rpm at 25, 30, 35, 40, and 50°C. OD660 of the cultures was monitored using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific), and growth curves were drawn based on the values. The culture experiments were performed in quadruplicate or quintuplicate. The culture experiment at 50°C (actual Tmax) was carried out twice to confirm the growth of the double-mutant and wild-type strains.

To calculate the maximum growth rates at each temperature, we determined a cell number factor to convert from OD to cell density. Briefly, wild-type *H. hispanica* was grown in Medium 307, and the cultures were diluted with sterilized medium. After OD660 of the cultures was measured using Spectronic 200 spectrophotometer (Thermo Fisher Scientific), the cells in the cultures were fixed in formaldehyde (final concentration 7%) for 16 h at 4°C as described previously (Antón et al., 1999). The cultures were filtered using preblackened polycarbonate filters (pore size, 0.2 µm; diameter, 25 mm) (Millipore, Billerica, MA, USA). The cells collected on the filters were stained with SYBR Green I (1:100 dilution) (Life Technologies). The cells were observed under a model BX51 epifluorescence microscope equipped with a U-MNIB3 fluorescence filter (Olympus, Tokyo, Japan), and 50 microscopic fields were counted for each sample. A cell number factor of 2.1 × 10⁹ cells ml⁻¹ per OD660 determined in this study was used to determine cell density in the cultures (Supplementary Figure 3). Growth rate (µ) was calculated between the individual incubation periods (t1 and t2) with an assumption of exponential growth; i.e., µ (h⁻¹) = (ln Nt2 – ln Nt1) / (t2 − t1), where Nt1 and Nt2 are the cell densities. On the basis of the growth rate, Tmin, Topt, and Tmax of the strains were determined.

### RESULTS AND DISCUSSION

#### 16S rRNA Gene Sequences and Growth Temperature Estimation

A total of 16 clones were randomly selected from a clone library of *H. hispanica* strain JCM8911, and the sequences of 16S rRNA genes were determined (1440 bp). Three types of 16S rRNA genes were identified and matched rrsA, rrsB, and rrsC in the genome sequence as determined by Liu et al. (2011). The sequences of rrsB and rrsC were 99.6% identical, whereas the sequences of rrsA and rrsBC were 94.6–94.9% identical. These results confirm previous reports of intragenomic polymorphism of 16S rRNA genes in *Halobacula* (e.g., Cui et al., 2009).

The sequence regions between the archaea-specific primers 109Af and 915Ar (795 bp) were selected from the 16S rRNA gene sequences. The PGC of the internal sequences of rrsA, rrsB, and rrsC were 58.9, 56.5, and 56.4%, respectively (Table 2). The offset between the PGC of rrsA and rrsBC was ~2.5%. Table 2 summarizes the estimated growth temperatures based on these PGC values. The estimated Tmin, Topt, and Tmax based on the PGC of rrsA were 32.6 ± 16.7, 51.6 ± 11.8, and 59.7 ± 13.1°C, which are much higher than those calculated from the PGC of rrsB and rrsC. The offsets between the estimated growth temperatures based on PGC of rrsA and rrsBC were >10°C. These findings may indicate that harboring 16S rRNA genes with relatively high- and low-PGC values allows *H. hispanica* to maintain rapid growth over a wide temperature range.

#### Survey of Expression of 16S rRNA Genes of *H. hispanica*

To check the selectivity of the specific primers designed in this study, qPCR was performed with PCR products of rrsA, rrsB, and rrsC that were amplified from the clones in the 16S rRNA gene-clone library. The rrsA-specific primer set, rrsAf/rrsAr, provided the proper products from only diluted PCR products of rrsA as

| Table 1: Primers targeting 16S rRNA genes for PCR, reverse transcription-coupled qPCR, and sequencing in this study. |
|------------------|------------------|------------------|------------------|
| Name | Sequence | Position | Target | Reference |
| 8Af | 5′-TCY GGT TGA TCC TGC C-3′ | 3-18 | Archaeal 16S rRNA gene | Burggraf et al., 1991 |
| 1512uR | 5′-GGT TAC CTT GGT ACG ACT T-3′ | 1424-1442 | Prokaryotic 16S rRNA gene | Matsushita et al., 2016 |
| 109Af | 5′-AMG GCT CAG TAA CAC GT-3′ | 83-99 | Archaeal 16S rRNA gene | Matsushita et al., 2016 |
| 915Ar | 5′-GGT CTC CCC CGG CAA TTC CT-3′ | 858-877 | Archaeal 16S rRNA gene | Matsushita et al., 2016 |
| rrsAf | 5′-GCG GAA GAA ACT GTC CCG-3′ | 569-589 | partial sequence of rrsA | This study |
| rrsAr | 5′-CGG TGG GGT CGG TCT TCC TCA G-3′ | 674-695 | Partial sequence of rrsA | This study |
| rrsBCf | 5′-GGG GTC CGG TGG TGG AAA CTA CAG AG-3′ | 567-589 | Partial sequence of rrsBC | This study |
| rrsBCr | 5′-CAC TGT CGG GTC CGG TCT CTC AAC-3′ | 674-697 | Partial sequence of rrsBC | This study |
templates at annealing temperature of 58°C, whereas the rrsBC-specific primer set, rrsBCf/rrsBCr, provided the proper products from only diluted PCR products of rrsB and rrsC at annealing temperature of 58°C. These results indicated that these primer sets were sufficiently selective to detect and quantify rrsA and rrsBC, respectively.

Our survey of 16S RNA gene expression demonstrated that rrsA and rrsBC expression varied with temperatures. In particular, the expression ratio of rrsA to rrsBC (rrsA:rrsBC) increased with culture temperature (Figure 1). The ratios at 45 and 50°C exceeded 1.0, which were significantly greater than those in the 25–35°C range (P < 0.05 by Student's t-test). On the other hand, the ratios at 25, 30, and 35°C were below 1.0. Especially, the ratios at 25 and 30°C were 0.56, which means that total expression of rrsB and rrsC was almost as twice as that of rrsA at the low temperatures. Our results suggest that transcription of high-P_GC 16S rRNA genes rrsA and low-P_GC 16S rRNA genes rrsBC may be regulated in response to culture temperature.

Construction and Culture of rRNA Operon Double-Mutants

We constructed rRNA operon single-mutant strains (HA2, HB2, and HC2) that lack rrnA, rrnB, or rrnC, respectively (Supplementary Table 1). We further constructed rRNA operon double-mutant strains, namely HCB2 harboring only rrnA and HAB2 harboring only rrnC (Supplementary Table 1). Operon deletion was confirmed by PCR with specific primer sets and by electrophoresis of the PCR products (Supplementary Figure 4).

The wild-type *H. hispanica* was able to grow at temperature ranging from 25 to 50°C, with optimum growth at 45°C (Table 2). The fastest growth was 0.16 h⁻¹ at the *T_opt* of 45°C (Supplementary Figure 5). The growth characteristics of the wild-type strain at various temperatures were almost the same as those of the rRNA operon single-mutant strains (data not shown). In the culture experiments using the double-mutant strains, the wild-type grew faster than HCB2 and HAB2 at all tested temperatures (Figure 2). HCB2 and HAB2 were able to grow within the same temperature range (i.e., 25–50°C) as the wild-type strain (Supplementary Figure 5). The HCB2 that harbors only rrnA containing high-P_GC rrsA grew optimally at 45°C. On the other hand, optimum growth of HAB2, which harbors only rrnC containing low-P_GC rrsC, was slightly shifted to low temperature of 40°C.

At 25°C, the wild-type *H. hispanica* grew significantly faster than HAB2 and HCB2 (Figure 2A). Condon et al. (1995) used *Escherichia coli* with multiple rRNA operons inactivated by antibiotic cassettes to demonstrate that the number of rRNA operons in the genome affects cell proliferation rate. Yano et al. (2013) found that the existence of multiple rRNA operons underlies the high growth rate of *Bacillus subtilis*. Another study reported that the copy number of rRNA operons on the genomes is correlated with growth rate of the prokaryotes under optimal culture condition (Vieira-Silva and Rocha, 2010; Roller et al., 2016). Our results support these studies and suggest that the number of rRNA operons influences cell proliferation rate at low temperatures close to actual *T_min* (25°C).

At 30°C, HCB2 had slightly higher growth rate than HAB2 (Figure 2B), and this difference increased in cultures at 35 and 40°C (Figures 2C,D). At 45 and 50°C, the growth curves for HCB2 were almost identical with those of the wild-type strain (Figures 2E,F). These findings suggest that the rRNAs transcribed from rrnA, including the high-P_GC 16S RNA gene rrsA, result in more rapid growth of *H. hispanica* at high temperatures of 45 and 50°C. The expression survey in this study also showed that expression of rrsA was higher than those of rrsBC at 45 and 50°C (Figure 1), which also supports that rRNAs transcribed from rrnA including high-P_GC 16S RNA gene rrsA may be important for growth under the high temperature conditions.

### Table 2 | Actual growth temperatures, 16S rRNA genes, and estimated growth temperatures of *Haloarcula hispanica* JCM8911.

| Actual growth temperature | Accession no. | 16S rRNA gene | Estimated growth temperature |
|---------------------------|---------------|---------------|-----------------------------|
| **T_min (°C)** | **T_opt (°C)** | **T_max (°C)** | **T_min (°C)** | **T_opt (°C)** | **T_max (°C)** |
| 25 | 45 | 50 | LO085245 | rsA | 58.9 | 32.6 ± 16.7 |
|  |  |  | LO085246 | rsB | 56.5 | 22.2 ± 16.4 |
|  |  |  | LO085247 | rsC | 56.4 | 21.7 ± 16.4 |

* TABLE 2 | Actual growth temperatures, 16S rRNA genes, and estimated growth temperatures of *Haloarcula hispanica* JCM8911.

| Accession no. | Type | P_GC (%) |
|---------------|------|----------|
| LO085245 | rsA | 58.9 |
| LO085246 | rsB | 56.5 |
| LO085247 | rsC | 56.4 |

* a Actual growth temperatures were determined in this study.

* b Estimated growth temperatures were calculated from P_GC of rrsA, rrsB, and rrsC using the microbial molecular thermometer proposed by Kimura et al. (2013).


**Survival Strategy of Haloarcula Strains**

*Haloarcula hispanica* has been identified in a number of solar salterns and desert salt lakes (e.g., Aralal et al., 1996; Pašič et al., 2005; Tapilatu et al., 2010; Mani et al., 2012). These hyper-saline habitats generally have large daily temperature fluctuations, i.e., temperature can vary by $>10^\circ C$ (Post, 1977; Wieland et al., 2005; Sima et al., 2013; Andrade et al., 2015). Because of this habitat feature, it is predicted that *H. hispanica* would express the rRNA operon with high-$P_{GC}$ 16S rRNA gene to grow faster in the daytime, when environmental temperatures rise to around $T_{\text{max}}$ (50°C). On the other hand, *H. hispanica* would express all of the three rRNA operons to grow in the nighttime and/or early morning, when environmental temperatures drop to around $T_{\text{min}}$ (25°C).

Fifteen *Haloarcula* strains have been isolated from hyper-saline environments worldwide (e.g., Juez et al., 1986; Ihara et al., 1997; Oren et al., 1999; Yang et al., 2007). Except for *H. aidinensis*, 14 *Haloarcula* strains so far examined harbor several different 16S rRNA gene sequences in the genome (Supplementary Table 3). Thus, intragenomic 16S rRNA gene heterogeneity seems to be common feature in the genus *Haloarcula*. We further confirmed that 9 of the 14 *Haloarcula* strains for which sequence was available show a $>2.0\%$ difference in $P_{GC}$ among the 16S rRNA genes. The estimated growth temperatures based on $P_{GC}$ values of the respective 16S rRNA genes suggested $>10^\circ C$ differences as well as *H. hispanica* (Supplementary Table 3). Additionally, previous study using a *Haloarcula* strain suggested that the sequences of putative promoter regions were obviously different among upstream regions of rRNA operons (Dennis et al., 1998; Dennis, 1999; López-López et al., 2007). These findings suggest that *Haloarcula* strains may regulate the expression of these 16S rRNA genes in response to culture temperature conditions, and this can be tested in future studies.

**CONCLUSION**

In this study, we determined the sequences and $P_{GC}$ values of 16S rRNA genes in the genome of the halophilic archaeon *H. hispanica*, and growth temperatures of *H. hispanica* were estimated based on the $P_{GC}$ values. The estimated growth temperatures of cells carrying the high-$P_{GC}$ 16S rRNA gene (*rrsA*) were approximately $10^\circ C$ higher than those carrying the low-$P_{GC}$ 16S rRNA genes (*rrsB* and *rrsC*), suggesting that *H. hispanica* harbors different 16S rRNA genes of different $P_{GC}$ to maintain rapid growth in a wide range of temperatures.

We characterized the expression of *rrsA* and *rrsBC* of *H. hispanica* at different growth temperatures. We found that *rrsA* was expressed at significantly higher levels than *rrsBC* at higher temperatures such as 45 and 50°C. Our results indicate the importance of a high-$P_{GC}$ 16S rRNA gene at raised growth temperatures in the *Haloarcula* species. We further constructed rRNA operon double-mutant strains of *H. hispanica*. Culture experiments showed that the wild-type strain grew faster than the mutant strains at temperatures between 25 and 40°C.
At 45 and 50°C, the double-mutant strain harboring only rRNA (including rrsA) grew much faster than the double-mutant strain harboring only rRNA (including rrsC), and the growth rate was similar to that of the wild-type strain. These findings suggest that the copy number of rRNA operons affects the growth rate of *H. hispanica* under low temperature conditions and that rRNAs transcribed from rRNA, which contains the high-PGC 16S RNA gene rrsA, function to promote rapid growth under high temperature conditions.

**AUTHOR CONTRIBUTIONS**

YS and HK conceived this study. YS performed all the experiments and drafted the manuscript. TF helped YS to construct the mutant strains. All authors confirmed and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00482/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Expression and function of different guanine-plus-cytosine content 16S rRNA genes in *Haloarcula hispanica* at different temperatures

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Supplementary Material and Methods

Disruption of rRNA operons in *Har. hispanica*

Standard protocols for handling *E. coli* and *Hfx. volcanii* DNA were followed, with slight modifications, and the double-integration method was adapted to construct *rrn*-deleted mutants (Dyall-Smith, 2009; Sambrook and Russell, 2001; Tu et al., 2005). First, specific PCR primer sets were designed using Genetyx Mac ver. 17.0.6 (Genetyx) and NCBI's Primer Blast to amplify each operon, including the regions ~1000 bp upstream and downstream (Supplementary Figures 1 and 2). The primer sets were named AUF/ADR for the region containing *rrnA*, BUF/BDR for the region containing *rrnB*, and CUF/CDR for the region containing *rrnC* (Supplementary Table 2). Additionally, primer sets were designed to amplify only the upstream and downstream regions of each rRNA operon with the intervening vector sequence (Supplementary Figures 1 and 2). These primer sets were named ADF/AUR, BDF/BUR, and CDF/CUR.

The genomic regions containing rRNA operons were amplified by PCR using the primer sets AUF/ADR, BUF/BDR, and CUF/CDR. The PCR products were ligated into vector pCR4Blunt-TOPO (Life Technologies). The ligated plasmids were transformed into *E. coli* TOP10 cells (Life Technologies), and clone libraries were constructed as described above. Next, the regions other than the rRNA operon in the plasmid, i.e., the downstream and upstream regions and vector, were amplified using the primer sets ADF/AUR, BDF/BUR, and CDF/CUR. A novobiocin-resistance gene derived from pMDS2 was ligated with the PCR products, generating plasmids pNA, pNB, and pNC (Holmes and Dyall-Smith, 1991; Supplementary Table 1). Additionally, a mevinolin (a statin)-resistance gene derived from pWL102 was ligated with the PCR products, generating pMA, pMB, and pMC (Lam and Doolittle, 1989; Supplementary Table 1). The resistance gene–ligated plasmids were transformed into *E. coli* TOP10 cells (Life Technologies), and clone libraries were constructed as described above. The plasmids were extracted from individual clones and then purified for transformation.

Transformation and isolation of mutant strains were performed on Medium 168, which contained 2 g casamino acid, 2 g Bacto yeast extract, 1 g sodium glutamate, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 2 g KCl, 200 g NaCl, 36 mg FeCl₂·4H₂O, 0.36 mg MnCl₂·4H₂O, and 20 g agar per liter of distilled water. Plasmid pNA, pNB, or pNC was introduced into the wild-type *Har. hispanica* by the PEG600 method (Cline and Doolittle, 1992; Dyall-Smith, 2009). Pop-in strains, indicated by colonies on Medium 168 supplemented with 10 µg ml⁻¹ of novobiocin (Wako, Osaka,
Japan), were collected. The obtained colonies were designated as HA1 (for pNA), HB1 (for pNB), and HC1 (for pNC) (Supplementary Table 1 and Supplementary Figure 1). HA1, HB1, and HC1 were streaked on Medium 168 without novobiocin for a second homologous recombination event. Pop-out colonies appearing on Medium 168 were collected. Actual disruption of \textit{rrnA}, \textit{rrnB}, and \textit{rrnC} was confirmed by PCR amplification using primer sets AVF/ACR, BVF/BCR, and CVF/CCR, which are complementary to primer sets AUR/ADF, BUR/BDF, and CUR/CDF, respectively (Supplementary Table 2). The mutant strains, which lacked \textit{rrnA}, \textit{rrnB}, and \textit{rrnC}, were designated as HA2, HB2, and HC2, respectively (Supplementary Table 2).

We further constructed rRNA operon double-mutant strains harboring only one of the three operons, \textit{rrnA}, \textit{rrnB}, or \textit{rrnC}. For disruption of the second rRNA operon, pMB, pMA (or pMC), and pMB was introduced into HC2, HC2 (or HA2), and HA2 with PEG600 as described above (Supplementary Figure 2). These strains were grown on Medium 168 supplemented with 100 µg ml\(^{-1}\) of simvastatin (another statin; Wako). The pop-in strains with colonies appearing on the medium were designated HCB1, HCA1 (or HAC1), and HAB1 (Supplementary Table 1). Each strain was separately streaked on Medium 168 without simvastatin for the second homologous recombination. The colonies appeared on the medium were collected, and disruption of operons \textit{rrnA}, \textit{rrnB}, and \textit{rrnC} was confirmed by PCR amplification using primer sets AVF/ACR, BVF/BCR, and CVF/CCR, respectively (Supplementary Table 2). The rRNA operon double-mutant strains harboring only \textit{rrnA}, \textit{rrnB}, or \textit{rrnC} were designated as HCB2, HCA2 (or HAC2), and HAB2, respectively (Supplementary Table 1).
## Supplementary Table 1. Plasmids and *Haloarcula hispanica* strains used in this study.

| Plasmid or strain | Description or genotype | Source or reference |
|-------------------|-------------------------|----------------------|
| **Plasmid**       |                         |                      |
| pCR4Blunt-TOPO    | Plasmid with ampicillin-resistance gene and kanamycin-resistance gene | Invitrogen           |
| pMDS2             | Plasmid with novobiocin-resistance gene (Nov<sup>R</sup>) | Holmes and Dyall-Smith (1991) |
| pWL102            | Plasmid with mevinolin-resistance gene (Mev<sup>R</sup>) | Lam and Doolittle (1989) |
| pNA               | Plasmid with rrnA adjacent fragment of *H. hispanica* and nov<sup>R</sup> for disruption of rrnA | This study            |
| pNB               | Plasmid with rrnB adjacent fragment of *H. hispanica* and nov<sup>R</sup> for disruption of rrnB | This study            |
| pNC               | Plasmid with rrnC adjacent fragment of *H. hispanica* and nov<sup>R</sup> for disruption of rrnC | This study            |
| pMA               | Plasmid with rrnA adjacent fragment of *H. hispanica* and mev<sup>R</sup> for disruption of rrnC | This study            |
| pMB               | Plasmid with rrnB adjacent fragment of *H. hispanica* and mev<sup>R</sup> for disruption of rrnB | This study            |
| pMC               | Plasmid with rrnC adjacent fragment of *H. hispanica* and mev<sup>R</sup> for disruption of rrnC | This study            |
| **Strain**        |                         |                      |
| JCM8911           | Wild-type strain        | JCM8911              |
| HA1               | *rrnA*:::[Δ*rrnA-nov<sup>R</sup>]* | JCM8911, pNA pop-in | This study |
| HA2               | Δ*rrnA* including nov<sup>R</sup> | JCM8911, pNA pop-out | This study |
| HB1               | *rrnB*:::[Δ*rrnB-nov<sup>R</sup>]* | JCM8911, pNB pop-in | This study |
| HB2               | Δ*rrnB* including nov<sup>R</sup> | JCM8911, pNB pop-out | This study |
| HC1               | *rrnC*:::[Δ*rrnC-nov<sup>R</sup>]* | JCM8911, pNC pop-in | This study |
| HC2               | Δ*rrnC* including nov<sup>R</sup> | JCM8911, pNC pop-out | This study |
| HCB1              | Δ*rrnCrrnB*:::[Δ*rrnB-mev<sup>R</sup>]* | HC2, pMB pop-in | This study |
| HCB2              | Δ*rrnCΔrrnB* including nov<sup>R</sup> and mev<sup>R</sup> | HC2, pMB pop-out | This study |
| HCA1              | Δ*rrnC* *rrnA*:::[Δ*rrnA-mev<sup>R</sup>]* | HC2, pMA pop-in | This study |
| HCA2              | Δ*rrnCΔrrnA* including nov<sup>R</sup> and mev<sup>R</sup> | HC2, pMA pop-out | Not obtained |
| HAC1              | Δ*rrnA* *rrnC*:::[Δ*rrnC-mev<sup>R</sup>]* | HA2, pMC pop-in | This study |
| HAC2              | Δ*rrnAΔrrnC* including nov<sup>R</sup> and mev<sup>R</sup> | HA2, pMC pop-out | Not obtained |
| HAB1              | Δ*rrnA* *rrnB*:::[Δ*rrnB-mev<sup>R</sup>]* | HA2, pMB pop-in | This study |
| HAB2              | Δ*rrnAΔrrnB* including nov<sup>R</sup> and mev<sup>R</sup> | HA2, pMB pop-out | This study |

JCM, Japan Collection of Microorganisms
Supplementary Table 2. Designed primers used for construction of mutant strains in this study

| Name | Description | Position on two chromosomes | Target |
|------|-------------|-----------------------------|--------|
| AUF  | 5’-GAA CCG GTA CGT GAT GGC TTC AA-3’ | I; 212084-212106 | upstream region of *rrnA* |
| ADR  | 5’-ACC GTC CGG AGA TAC ACA GGT TGA-3’ | I; 219338-219315 | downstream region of *rrnA* |
| BUF  | 5’-CAC CCA CCT GTT CGA GTA TCT-3’ | I; 1774700-1774720 | upstream region of *rrnB* |
| BDR  | 5’-GTA CGC TGA TCT CGG TGT CA-3’ | I; 1782143-1782124 | downstream region of *rrnB* |
| CUF  | 5’-TAT CAG AGC CAG ACC TGA GT-3’ | II; 7556-7575 | upstream region of *rrnC* |
| CDR  | 5’-CTG TCG GCA GGT GAA ATA GT-3’ | II; 14991-14972 | downstream region of *rrnC* |
| ADF  | 5’-GAA CGA ATT CAC ACT CCT CGA-3’ | I; 218315-218338 | downstream region of *rrnA* |
| AUR  | 5’-GTT AGG CGG GAT CAC ACC CAT AT-3’ | I; 213140-213117 | upstream region of *rrnA* |
| BDF  | 5’-ATT CCA TCT TGA CGG CGG AC-3’ | I; 1781031-1781050 | downstream region of *rrnB* |
| BUR  | 5’-ATT CCA GTC TAT GCG GCA GA-3’ | I; 1775752-1775733 | upstream region of *rrnB* |
| CDF  | 5’-ACT GAC CCA CTC AGT GAA CA-3’ | II; 13928-13947 | downstream region of *rrnC* |
| CUR  | 5’-TAC ACG AAC ACA AAG CCT CA-3’ | II; 8686-8667 | upstream region of *rrnC* |
| AVF  | 5’-TAT ATG GGT GTG ATC CCG CCT AAC-3’ | I; 213117-213140 | *rrnA* or drug resistance gene |
| ACR  | 5’-TCG AGG AGT GCT GTG AAT CCT TTC-3’ | I; 218338-218315 | *rrnA* or drug resistance gene |
| BVF  | 5’-TCT GCC GCA TAG ACT GGA AT-3’ | I; 1775733-1775752 | *rrnB* or drug resistance gene |
| BCR  | 5’-GTC CGC CGT CAA GAT GGA AT-3’ | I; 1781050-1781031 | *rrnB* or drug resistance gene |
| CVF  | 5’-TGA GGC TTT GTG TGC TGC TA-3’ | II; 8667-8686 | *rrnC* or drug resistance gene |
| CCR  | 5’-TGT TCA CTG AGT GGG TCA AT-3’ | II; 13947-13928 | *rrnC* or drug resistance gene |
## Supplementary Table 3. $P_{GC}$ of 16S rRNA genes ($rrs$) and estimated growth temperatures for *Haloarcula* strains.

| Strain*       | Type  | Accession number | Sequence difference$^b$ (%) | $P_{GC}$ (%) | $P_{GC}$ offset$^c$ (%) | $T_{min}$ ($^\circ$C) | $T_{opt}$ ($^\circ$C) | $T_{max}$ ($^\circ$C) | $T_{opt}$ offset$^c$ (%) |
|---------------|-------|------------------|-----------------------------|--------------|--------------------------|----------------------|------------------|----------------------|--------------------------|
| *Haloarcula hispanica* JCM8911 | $rrs$A | LC085245         | 5.4                         | 58.9         | 2.5                      | 32.6 ± 16.7          | 51.6 ± 11.8       | 59.7 ± 13.1          | 12.3                     |
|               | $rrs$B | LC085246         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
|               | $rrs$C | LC085247         | 56.4                        | 21.7         | 39.3 ± 11.5              | 47.6 ± 12.8          |                 |                      |                          |
| *Haloarcula marismortui* | $rrs$A | AY596297         | 5.6                         | 58.6         | 2.4                      | 31.7 ± 16.7          | 50.2 ± 11.8       | 58.4 ± 13.0          | 11.9                     |
|               | $rrs$B | AY596298         | 56.2                        | 20.9         | 38.3 ± 11.5              | 46.6 ± 12.8          |                 |                      |                          |
|               | $rrs$C | AY596297         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
| “*Haloarcula sp. CBA1115*” | $rrs$A | CP010529         | 5.4                         | 59.0         | 2.9                      | 33.1 ± 16.8          | 52.2 ± 11.8       | 60.3 ± 13.1          | 14.4                     |
|               | $rrs$B | CP010529         | 56.1                        | 20.4         | 37.8 ± 11.5              | 46.1 ± 12.8          |                 |                      |                          |
|               | $rrs$C | CP010529         | 56.1                        | 20.4         | 37.8 ± 11.5              | 46.1 ± 12.8          |                 |                      |                          |
| *Haloarcula amylolytica* | $rrs$A | DQ826512         | 5.6                         | 58.5         | 2.4                      | 30.9 ± 16.7          | 49.7 ± 11.8       | 57.9 ± 13.0          | 11.9                     |
|               | $rrs$B | DQ826513         | 56.1                        | 20.4         | 37.8 ± 11.5              | 46.1 ± 12.8          |                 |                      |                          |
|               | $rrs$C | DQ826518         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
| *Haloarcula japonica* | $rrs$A | EF645684         | 5.0                         | 58.4         | 2.2                      | 30.5 ± 16.7          | 49.2 ± 11.7       | 57.4 ± 13.0          | 10.9                     |
|               | $rrs$B | EF645685         | 56.2                        | 20.9         | 38.3 ± 11.5              | 46.6 ± 12.8          |                 |                      |                          |
|               | $rrs$C | EF645686         | 56.4                        | 21.7         | 39.3 ± 11.5              | 47.6 ± 12.8          |                 |                      |                          |
| “*Haloarcula rubripromontorii SL3*” | $rrs$A | KU198854         | 4.4                         | 58.9         | 2.2                      | 32.6 ± 16.7          | 51.6 ± 11.8       | 59.7 ± 13.1          | 10.8                     |
|               | $rrs$B | KU198855         | 56.7                        | 23.0         | 40.8 ± 11.6              | 49.0 ± 12.8          |                 |                      |                          |
| *Haloarcula quadrata* | $rrs$A | AB010965         | 5.0                         | 58.6         | 2.1                      | 31.7 ± 16.7          | 50.2 ± 11.8       | 58.4 ± 13.0          | 10.4                     |
|               | $rrs$B | AB010964         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
| *Haloarcula argentensis* | $rrs$A | EF645680         | 4.8                         | 58.5         | 2.0                      | 30.9 ± 16.7          | 49.7 ± 11.8       | 57.9 ± 13.0          | 9.9                      |
|               | $rrs$B | EF645681         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
| “*Haloarcula californiae*” | $rrs$A | AB477984         | 5.4                         | 58.4         | 1.7                      | 30.5 ± 16.7          | 49.2 ± 11.7       | 57.4 ± 13.0          | 8.4                      |
|               | $rrs$B | AB477984         | 56.7                        | 23.0         | 40.8 ± 11.6              | 49.0 ± 12.8          |                 |                      |                          |
| *Haloarcula vallismortis* | $rrs$A | EF645687         | 5.3                         | 58.4         | 1.3                      | 30.5 ± 16.7          | 49.2 ± 11.7       | 57.4 ± 13.0          | 6.4                      |
|               | $rrs$B | EF645688         | 57.1                        | 24.8         | 42.8 ± 11.6              | 51.0 ± 12.9          |                 |                      |                          |
| “*Haloarcula sinaiensis*” | $rrs$A | DI4130           | 2.7                         | 57.4         | 1.0                      | 26.1 ± 16.5          | 44.3 ± 11.6       | 52.5 ± 12.9          | 5.0                      |
|               | $rrs$B | DI4129           | 56.4                        | 21.7         | 39.3 ± 11.5              | 47.6 ± 12.8          |                 |                      |                          |
| *Haloarcula tradensis* | $rrs$A | FJ429313         | 3.9                         | 57.8         | 0.7                      | 27.9 ± 16.6          | 46.2 ± 11.7       | 54.4 ± 13.0          | 3.4                      |
|               | $rrs$B | FJ429314         | 57.1                        | 24.8         | 42.8 ± 11.6              | 51.0 ± 12.9          |                 |                      |                          |
|               | $rrs$C | FJ429316         | 57.4                        | 26.1         | 44.3 ± 11.6              | 52.5 ± 12.9          |                 |                      |                          |
| *Haloarcula salaria* | $rrs$A | FJ429318         | 1.7                         | 56.8         | 0.3                      | 23.5 ± 16.5          | 41.3 ± 11.6       | 49.6 ± 12.8          | 1.5                      |
|               | $rrs$B | FJ429317         | 56.5                        | 22.2         | 39.8 ± 11.6              | 48.1 ± 12.8          |                 |                      |                          |
| “*Haloarcula aidinensis*” | $rrs$A | AB771438         | 0                           | 58.6         | 0                        | 31.7 ± 16.7          | 50.2 ± 11.8       | 58.4 ± 13.0          | 0                       |
|               | $rrs$B | AB771439         | 58.6                        | 31.7         | 50.2 ± 11.8              | 58.4 ± 13.0          |                 |                      |                          |

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*Quotation marks represent six strains that their names have not been published validly.

$^b$Sequence difference between $rrsA$ and $rrsB$ or $rrsC$.

$^c$Offsets between $P_{GC}$ of $rrsA$ and $P_{GC}$ of $rrsB$ or $rrsC$.

$^d$Growth temperatures estimated from $P_{GC}$ of $rrsA$, $rrsB$, and $rrsC$ based on microbial molecular thermometer of Kimura et al. (2013).

$^e$Offsets between optimum growth temperatures estimated from $P_{GC}$ of $rrsA$ and that estimated from $P_{GC}$ of $rrsB$ or $rrsC$, respectively.
Supplementary Figure 1. Flow chart of the first rRNA operon (rrn) deletion method. Mutant strains, plasmids, and primer sets designed in this study are summarized in Supplementary Tables 1 and 2.
Supplementary Figure 2. Flow chart of the second rRNA operon (rrn) deletion method. Mutant strains, plasmids, and primer sets designed in this study are summarized in Supplementary Tables 1 and 2.
Supplementary Figure 3. Correlation between cell density and $\text{OD}_{660}$ value in *Haloarcula hispanica*. 

Cell density $= 2.1 \times 10^9 (\text{OD}_{660})$
Supplementary Figure 4. Confirmation of rRNA operon deletion in mutant strains of *Har. hispanica*. (a) Location of PCR amplification and the length of each PCR product (scale bars). (b) Electrophoresis results for the PCR products of deletion sites from both wild-type (W) and mutant strains HCB2 (ΔCB) and HAB2 (ΔAB). N: negative control. M: markers, 1-kb DNA ladder (Promega, Madison, WI, USA).
Supplementary Figure 5. Maximum growth rates (\(\mu\)) of wild-type, HCB2, and HAB2 at each temperature. Error bars denote standard deviation of mean values for quadruplicate or quintuplicate measurements.
Supplementary References

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