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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_{GC}). The strong correlation between the typical growth temperatures of archaea and P_{GC} of their 16S rRNA genes suggests that *H. hispanica* may harbor different 16S rRNA genes having different P_{GC} 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_{GC}, 58.9%) and *rrsBC* (P_{GC}, 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_{GC} *rrsA* grew significantly faster than the double-mutant strain which harbors only *rrnC* with low-P_{GC} *rrsC*. These findings suggest the importance of 16S rRNAs transcribed from *rrsA* with high-P_{GC} in maintaining rapid growth of this halophilic archaeon at raised growth temperatures.

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

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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 *Methanothermobacter thermoautotrophicus*, exhibit 16S rRNA gene polymorphisms displaying as much as 0.1% divergence, whereas some halophilic archaea harbor 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 archaea belonging to class *Halobacteria*, have mainly been 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*, two strains closely related to *H. hispanica*, and *H. marismortui*, 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 (P_{GC}) 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-P_{GC} 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 P_{GC} of their 16S rRNA sequences: 16S rRNA genes of hyperthermophiles and thermophiles tend to have high P_{GC}, whereas 16S rRNA genes of mesophiles and psychrophiles have relatively low P_{GC}. On the basis of the relationship between the growth temperatures and P_{GC} of 16S rRNA gene sequences, we propose that *Haloarcula* strains express and utilize high P_{GC} of 16S rRNAs at high temperature and low P_{GC} 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 (T_{min}), optimum (T_{opt}), and maximum (T_{max}) growth temperatures were estimated based on P_{GC} 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 T_{min} of the strain) and 50°C (actual T_{max} 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 $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 1 g KCl, 200 g NaCl, 0.36 mg $FeCl_2 \cdot 4H_2O$, and 0.36 mg $MnCl_2 \cdot 4H_2O$ per liter of distilled water. After the pH of the medium was adjusted to 7.0, the medium was sterilized by filtration with a polyethersulfone 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* in exponential growth phase in pre-culture, and the cultures were incubated in the darkness with shaking at 180 rpm at 37°C. 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 proteinase 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 8aF/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 dideoxy cycle-sequencing method using a Model 3730*xl* 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_{min}, T_{opt}, and T_{max} Based on P_{GC} of 16S rRNA Genes

Because thermophilic and hyperthermophilic archaea have greater P_{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_{min}, T_{opt}, and T_{max} of cultured and not-yet cultured archaea based on P_{GC} of 16S rRNA genes. We therefore used these equations to estimate growth temperatures of *H. hispanica* based on P_{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_{GC} values for the internal sequences were calculated using Genetyx-Mac ver. 17.0.6 (Genetyx, Tokyo, Japan). T_{min}, T_{opt}, and T_{max} were calculated based on P_{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 *mir*Vana 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 RNeasy 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 rrsBCf/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

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 GTT ACG ACT T-3′	1424–1442	Prokaryotic 16S rRNA gene	Matsushita et al., 2016
109aF	5′-AMD GCT CAG TAA CAC GT-3′	83–99	Archaeal 16S rRNA gene	Matsushita et al., 2016
915aR	5′-GTG CTC CCC CGC CAA TTC CT-3′	858–877	Archaeal 16S rRNA gene	Matsushita et al., 2016
rrsAf	5′-CGT CCA GCG GAA ACT GTC CGG-3′	569–589	partial sequence of *rrsA*	This study
rrsAr	5′-CCG TCG GGT CCG TCT TCC TGA G-3′	674–695	Partial sequence of *rrsA*	This study
rrsBCf	5′-GGC GTC CGG TGG AAA CTA CAC 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

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, 45, and 50°C. OD_{660} 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 T_{max}) 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_{660} value to cell density. Briefly, wild-type *H. hispanica* was grown in Medium 307, and the cultures were diluted with sterilized medium. After OD_{660} 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 pre-blackened 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 2.1×10^9 cells ml^{-1} per OD_{660} 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 (t_1 and t_2) with an assumption of exponential growth; i.e., $\mu\ (h^{-1}) = (\ln N_{t2} - \ln N_{t1})/(t_2 - t_1)$, where N_{t1} and N_{t2} are the cell densities. On the basis of the growth rate, T_{min}, T_{opt}, and T_{max} 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 *Haloarcula* (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 P_{GC} of the internal sequences of *rrsA*, *rrsB*, and *rrsC* were 58.9, 56.5, and 56.4%, respectively (**Table 2**). The offset between the P_{GC} of *rrsA* and *rrsBC* was ∼2.5%. **Table 2** summarizes the estimated growth temperatures based on these P_{GC} values. The estimated T_{min}, T_{opt}, and T_{max} based on the P_{GC} 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 P_{GC} of *rrsB* and *rrsC*. The offsets between the estimated growth temperatures based on P_{GC} of *rrsA* and *rrsBC* was >10°C. These findings may indicate that harboring 16S rRNA genes with relatively high- and low-P_{GC} 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 2 | Actual growth temperatures, 16S rRNA genes, and estimated growth temperatures of *Haloarcula hispanica* JCM8911.

Actual growth temperature[a]			16S rRNA gene			Estimated growth temperature[b]		
T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)	Accession no.	Type	P_{GC} (%)	T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)
25	45	50	LC085245	*rrsA*	58.9	32.6 ± 16.7	51.6 ± 11.8	59.7 ± 13.1
			LC085246	*rrsB*	56.5	22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8
			LC085247	*rrsC*	56.4	21.7 ± 16.4	39.3 ± 11.5	47.6 ± 12.8

[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).*

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 gene *rrsA* and low-P_{GC} 16S rRNA genes *rrsBC* may be regulated in response to culture temperature.

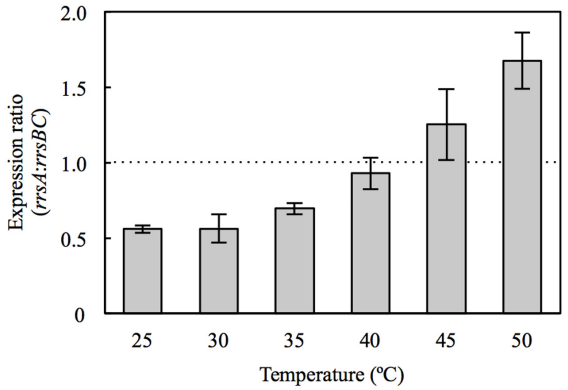

FIGURE 1 | Expression ratio of *rrsA* to *rrsBC* in *Haloarcula hispanica* cultured at 25, 30, 35, 40, 45, and 50°C. Error bars denote standard deviation of mean values for triplicate measurements. The dashed line indicates *rrsA*:*rrsBC* ratio of 1.0.

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 rRNA 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 rRNA gene *rrsA* may be important for growth under the high temperature conditions.

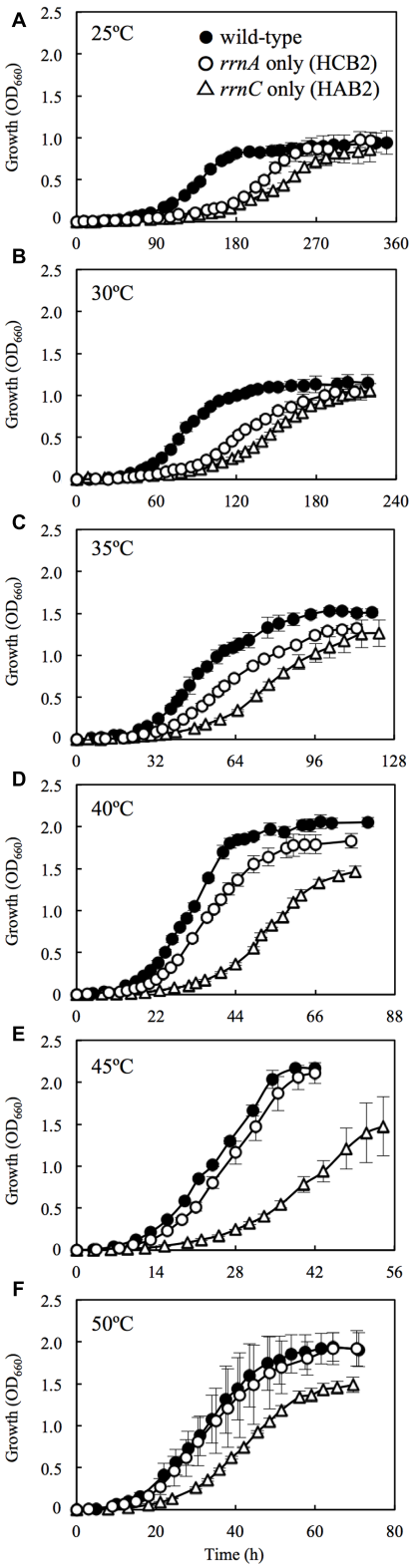

FIGURE 2 | Growth curves for wild-type *H. hispanica* and the rRNA operon double-mutant strains at 25 (A), 30 (B), 35 (C), 40 (D), 45 (E), and 50°C (F). Error bars denote standard deviation of mean values for quadruplicate or quintuplicate measurements.

Survival Strategy of *Haloarcula* Strains

Haloarcula hispanica has been identified in a number of solar salterns and desert salt lakes (e.g., Arahal et al., 1996; Pašić 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°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_{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_{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°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°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 *rrnC* (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-P_{GC} 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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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 $MgSO_4 \cdot 7H_2O$, 2 g KCl, 200 g NaCl, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 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 *rrnA*, *rrnB*, and *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 *rrnA*, *rrnB*, and *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, *rrnA*, *rrnB*, or *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 *rrnA*, *rrnB*, and *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 *rrnA*, *rrnB*, or *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		Invitorgen
pMDS2	Plasmid with novobiocin-resistance gene (Nov^R)		Holmes and Dyall-Smith (1991)
pWL102	Plasmid with mevinolin-resistance gene (Mev^R)		Lam and Doolittle (1989)
pNA	Plasmid with *rrnA* adjacent fragment of *Har. hispanica* and nov^R for disruption of *rrnA*		This study
pNB	Plasmid with *rrnB* adjacent fragment of *Har. hispanica* and nov^R for disruption of *rrnB*		This study
pNC	Plasmid with *rrnC* adjacent fragment of *Har. hispanica* and nov^R for disruption of *rrnC*		This study
pMA	Plasmid with *rrnA* adjacent fragment of *Har. hispanica* and mev^R for disruption of *rrnA*		This study
pMB	Plasmid with *rrnB* adjacent fragment of *Har. hispanica* and mev^R for disruption of *rrnB*		This study
pMC	Plasmid with *rrnC* adjacent fragment of *Har. hispanica* and mev^R for disruption of *rrnC*		This study
Strain			
JCM8911	Wild-type strain	JCM8911	JCM
HA1	*rrnA*+::[Δ*rrnA*-nov^R]	JCM8911, pNA pop-in	This study
HA2	Δ*rrnA* including nov^R	JCM8911, pNA pop-out	This study
HB1	*rrnB*+::[Δ*rrnB*-nov^R]	JCM8911, pNB pop-in	This study
HB2	Δ*rrnB* including nov^R	JCM8911, pNB pop-out	This study
HC1	*rrnC*+::[Δ*rrnC*-nov^R]	JCM8911, pNC pop-in	This study
HC2	Δ*rrnC* including nov^R	JCM8911, pNC pop-out	This study
HCB1	Δ*rrnC rrnB*+::[Δ*rrnB*-mev^R]	HC2, pMB pop-in	This study
HCB2	Δ*rrnC*Δ*rrnB* including nov^R and mev^R	HC2, pMB pop-out	This study
HCA1	Δ*rrnC rrnA*+::[Δ*rrnA*-mev^R]	HC2, pMA pop-in	This study
HCA2	Δ*rrnC*Δ*rrnA* including nov^R and mev^R	HC2, pMA pop-out	Not obtained
HAC1	Δ*rrnA rrnC*+::[Δ*rrnC*-mev^R]	HA2, pMC pop-in	This study
HAC2	Δ*rrnA*Δ*rrnC* including nov^R and mev^R	HA2, pMC pop-out	Not obtained
HAB1	Δ*rrnA rrnB*+::[Δ*rrnB*-mev^R]	HA2, pMB pop-in	This study
HAB2	Δ*rrnA*Δ*rrnB* including nov^R and mev^R	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 AGC ACT CCT CGA-3'	I; 218315-218338	downstream region of *rrnA*
AUR	5'-GTT AGG CGG GAT CAC ACC CAT ATA-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 TCG 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 TTC GTG TA-3'	II; 8667-8686	*rrnC* or drug resistance gene
CCR	5'-TGT TCA CTG AGT GGG TCA GT-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[a]	16S rRNA gene					Estimated growth temperature[d]			
	Type	Accession number	Sequnece difference[b] (%)	P_{GC} (%)	P_{GC} offset[c] (%)	T_{min} (ºC)	T_{opt} (ºC)	T_{max} (ºC)	T_{opt} offset[e] (ºC)
Haloarcula hispanica JCM8911	*rrsA*	LC085245	5.4	58.9	2.5	32.6 ± 16.7	51.6 ± 11.8	59.7 ± 13.1	12.3
	rrsB	LC085246		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
	rrsC	LC085247		56.4		21.7 ± 16.4	39.3 ± 11.5	47.6 ± 12.8	
Haloarcula marismrotui	*rrsA*	AY596297	5.6	58.6	2.4	31.7 ± 16.7	50.2 ± 11.8	58.4 ± 13.0	11.9
	rrsB	AY596298		56.2		20.9 ± 16.4	38.3 ± 11.5	46.6 ± 12.8	
	rrsC	AY596297		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
"*Haloarcula* sp. CBA1115"	*rrsA*	CP010529	5.4	59.0	2.9	33.1 ± 16.8	52.2 ± 11.8	60.3 ± 13.1	14.4
	rrsB	CP010529		56.1		20.4 ± 16.4	37.8 ± 11.5	46.1 ± 12.8	
	rrsC	CP010529		56.1		20.4 ± 16.4	37.8 ± 11.5	46.1 ± 12.8	
"*Haloarcula hispanica* N601"	*rrsA*	CP006884	5.2	58.9	2.4	32.6 ± 16.7	51.6 ± 11.8	59.7 ± 13.1	11.9
	rrsB	CP006884		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
	rrsC	CP006885		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
Haloarcula amylolytica	*rrsA*	DQ826512	5.6	58.5	2.4	30.9 ± 16.7	49.7 ± 11.8	57.9 ± 13.0	11.9
	rrsB	DQ826513		56.1		20.4 ± 16.4	37.8 ± 11.5	46.1 ± 12.8	
	rrsC	DQ826518		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
Haloarcula japonica	*rrsA*	EF645684	5.0	58.4	2.2	30.5 ± 16.7	49.2 ± 11.7	57.4 ± 13.0	10.9
	rrsB	EF645685		56.2		20.9 ± 16.4	38.3 ± 11.5	46.6 ± 12.8	
	rrsC	EF645686		56.4		21.7 ± 16.4	39.3 ± 11.5	47.6 ± 12.8	
"*Haloarcula rubripromontorii* SL3"	*rrsA*	KU198854	4.4	58.9	2.2	32.6 ± 16.7	51.6 ± 11.8	59.7 ± 13.1	10.8
	rrsB	KU198855		56.7		23.0 ± 16.4	40.8 ± 11.6	49.0 ± 12.8	
Haloarcula quadrata	*rrsA*	AB010965	5.0	58.6	2.1	31.7 ± 16.7	50.2 ± 11.8	58.4 ± 13.0	10.4
	rrsB	AB010964		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
Haloarcula argentinensis	*rrsA*	EF645680	4.8	58.5	2.0	30.9 ± 16.7	49.7 ± 11.8	57.9 ± 13.0	9.9
	rrsB	EF645681		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
"*Haloarcula californiae*"	*rrsA*	AB477985	5.4	58.4	1.7	30.5 ± 16.7	49.2 ± 11.7	57.4 ± 13.0	8.4
	rrsB	AB477984		56.7		23.0 ± 16.4	40.8 ± 11.6	49.0 ± 12.8	
Haloarcula vallismortis	*rrsA*	EF645687	5.3	58.4	1.3	30.5 ± 16.7	49.2 ± 11.7	57.4 ± 13.0	6.4
	rrsB	EF645688		57.1		24.8 ± 16.5	42.8 ± 11.6	51.0 ± 12.9	
"*Haloarcula sinaiiensis*"	*rrsA*	D14130	2.7	57.4	1.0	26.1 ± 16.5	44.3 ± 11.6	52. 5± 12.9	5.0
	rrsB	D14129		56.4		21.7 ± 16.4	39.3 ± 11.5	47.6 ± 12.8	
Haloarcula tradensis	*rrsA*	FJ429313	3.9	57.8	0.7	27.9 ± 16.6	46.2 ± 11.7	54.4 ± 13.0	3.4
	rrsB	FJ429314		57.1		24.8 ± 16.5	42.8 ± 11.6	51.0 ± 12.9	
	rrsC	FJ429316		57.4		26.1 ± 16.5	44.3 ± 11.6	52.5 ± 12.9	
Haloarcula salaria	*rrsA*	FJ429318	1.7	56.8	0.3	23.5 ± 16.5	41.3 ± 11.6	49.6 ± 12.8	1.5
	rrsB	FJ429317		56.5		22.2 ± 16.4	39.8 ± 11.6	48.1 ± 12.8	
"*Haloarcula aidinensis*"	*rrsA*	AB771438	0	58.6	0	31.7 ± 16.7	50.2 ± 11.8	58.4 ± 13.0	0
	rrsB	AB771439		58.6		31.7 ± 16.7	50.2 ± 11.8	58.4 ± 13.0	

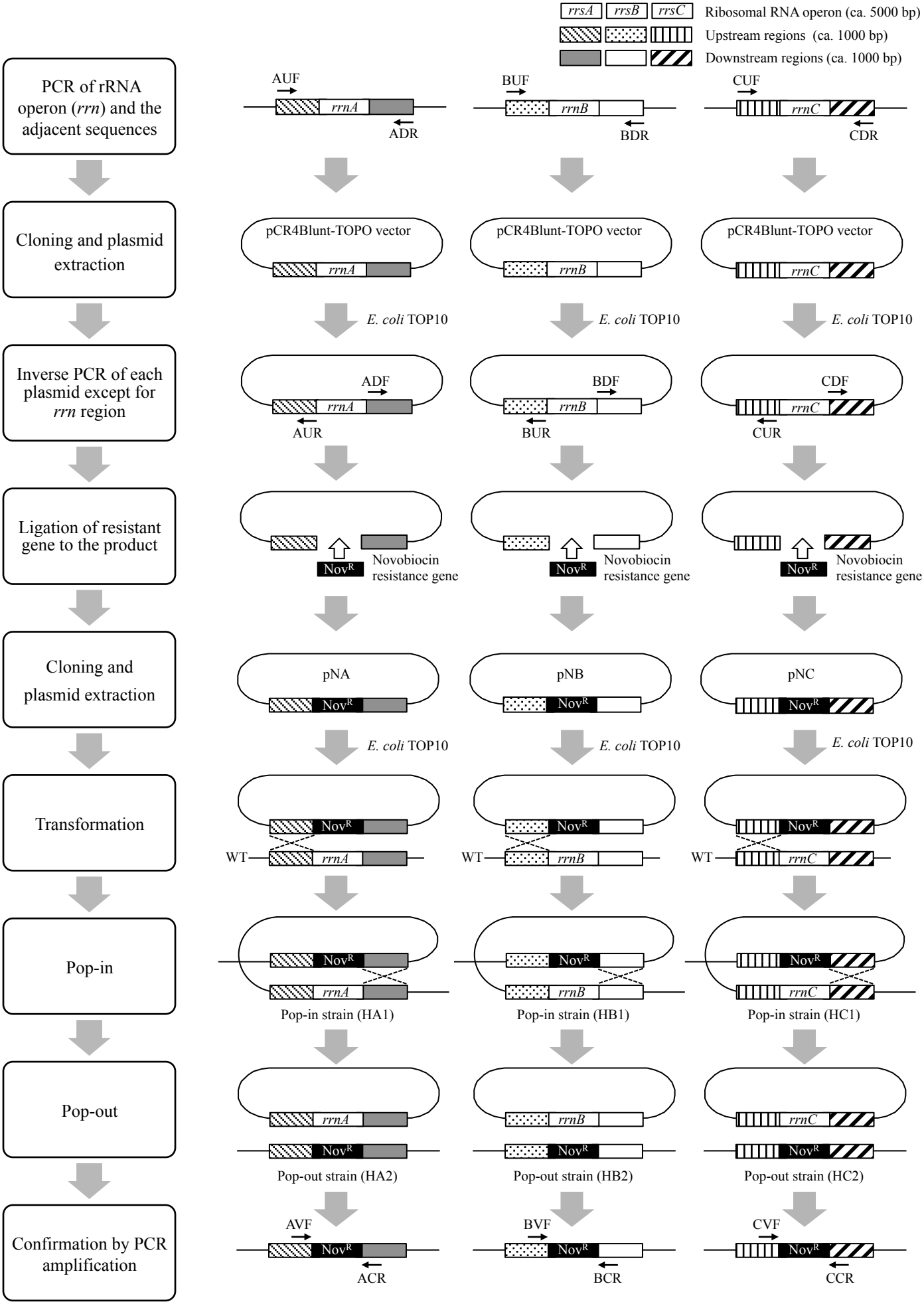
[a]Quotation marks represent six strains that their names have not been published validly.

[b]Sequence difference between *rrsA* and *rrsB* or *rrsC*.

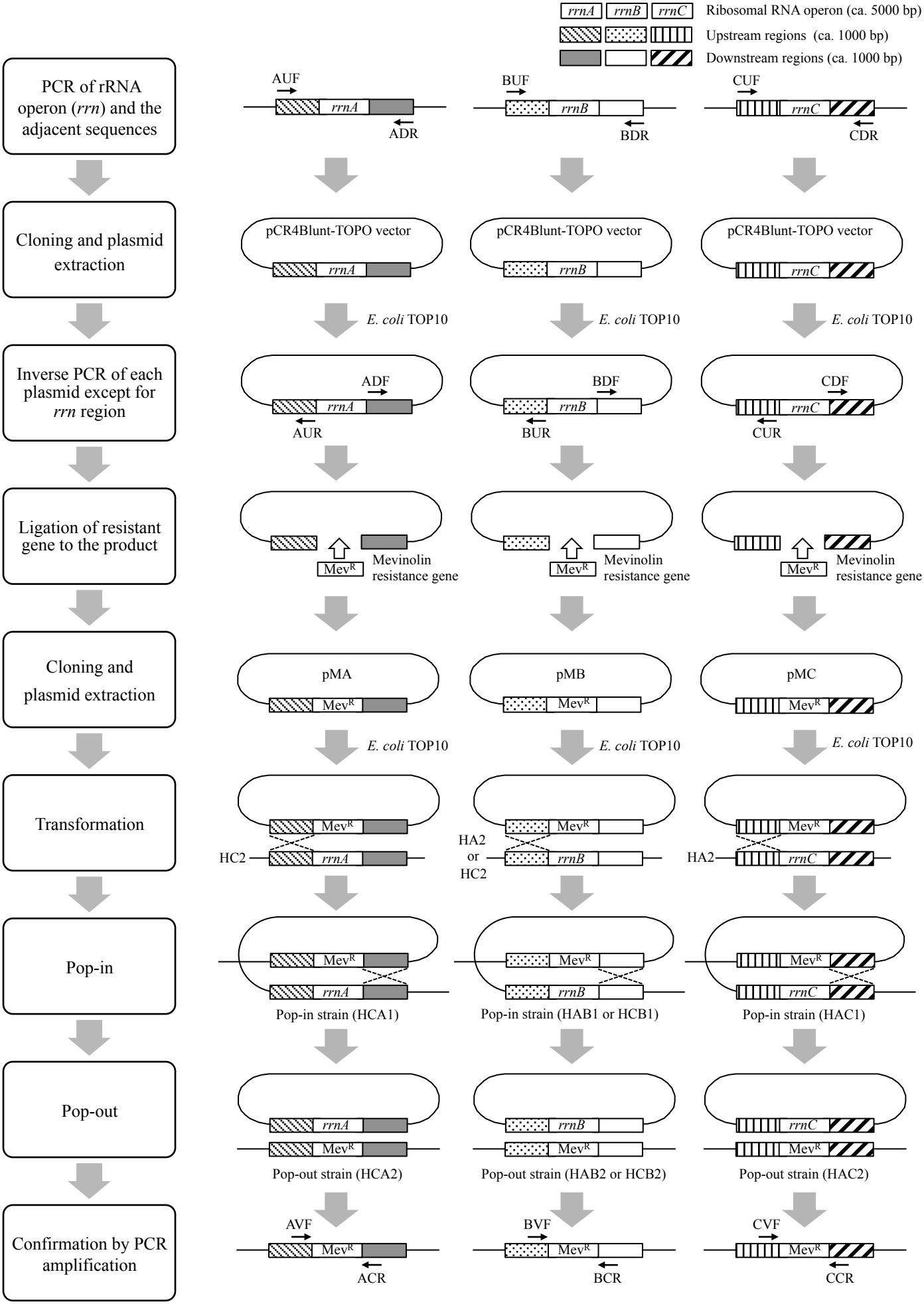
[c]Offsets between P_{GC} of *rrsA* and P_{GC} of *rrsB* or *rrsC*.

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

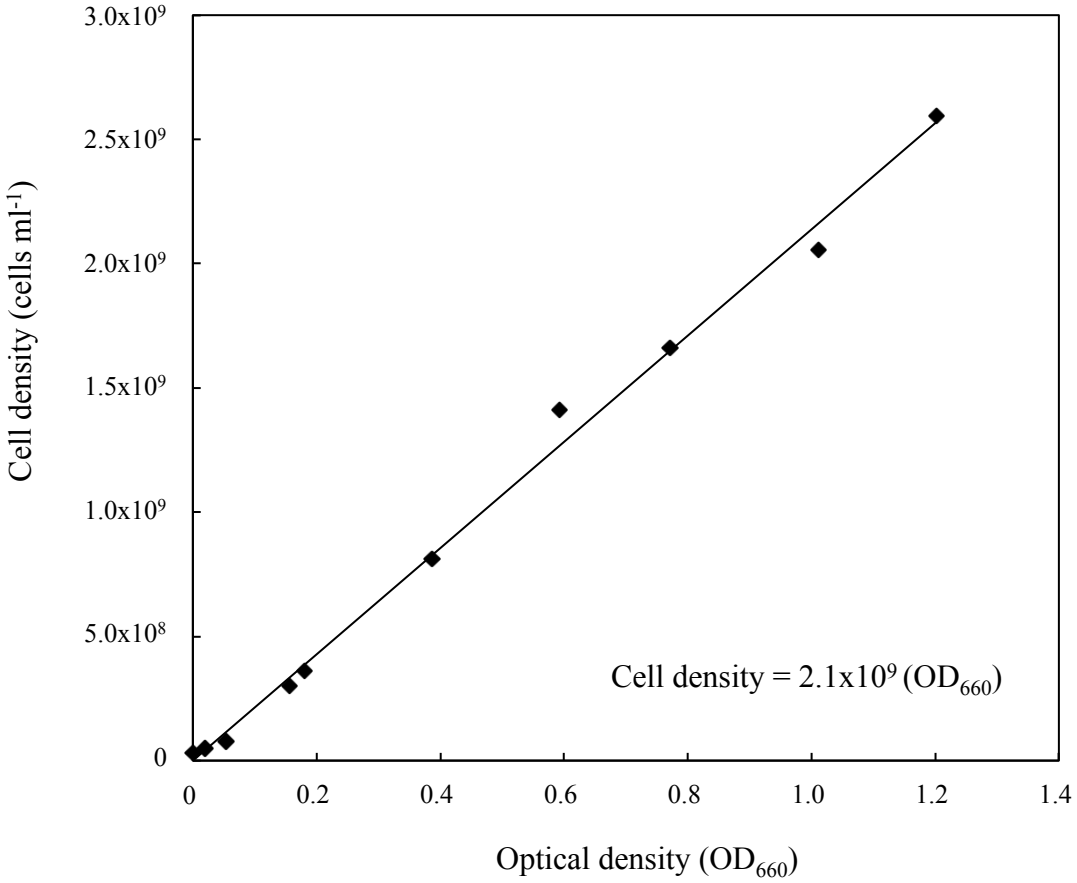
[e]Offsets between optimum growth temperatures estimated from P_{GC} of *rrsA* and that estimated from P_{GC} of *rrsB* or *rrsC*, respectively.



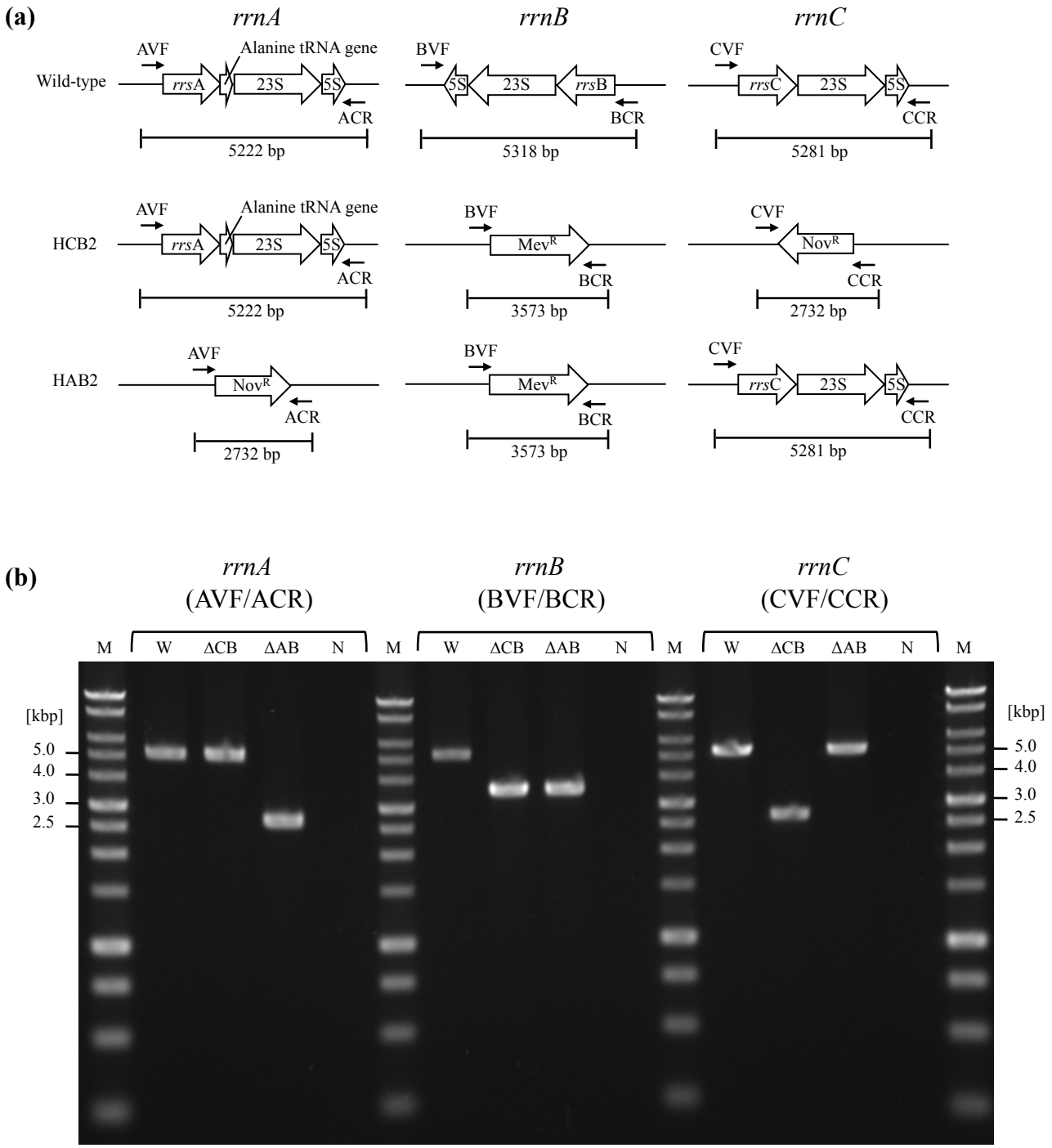
Supplementary Figure 1. Flow chart of the first rRNA operon (*rrn*) deletion method. Mutant strains, plasmids, and primer sets designed in this study are summarized in Supplementary Tables 1 and 2.



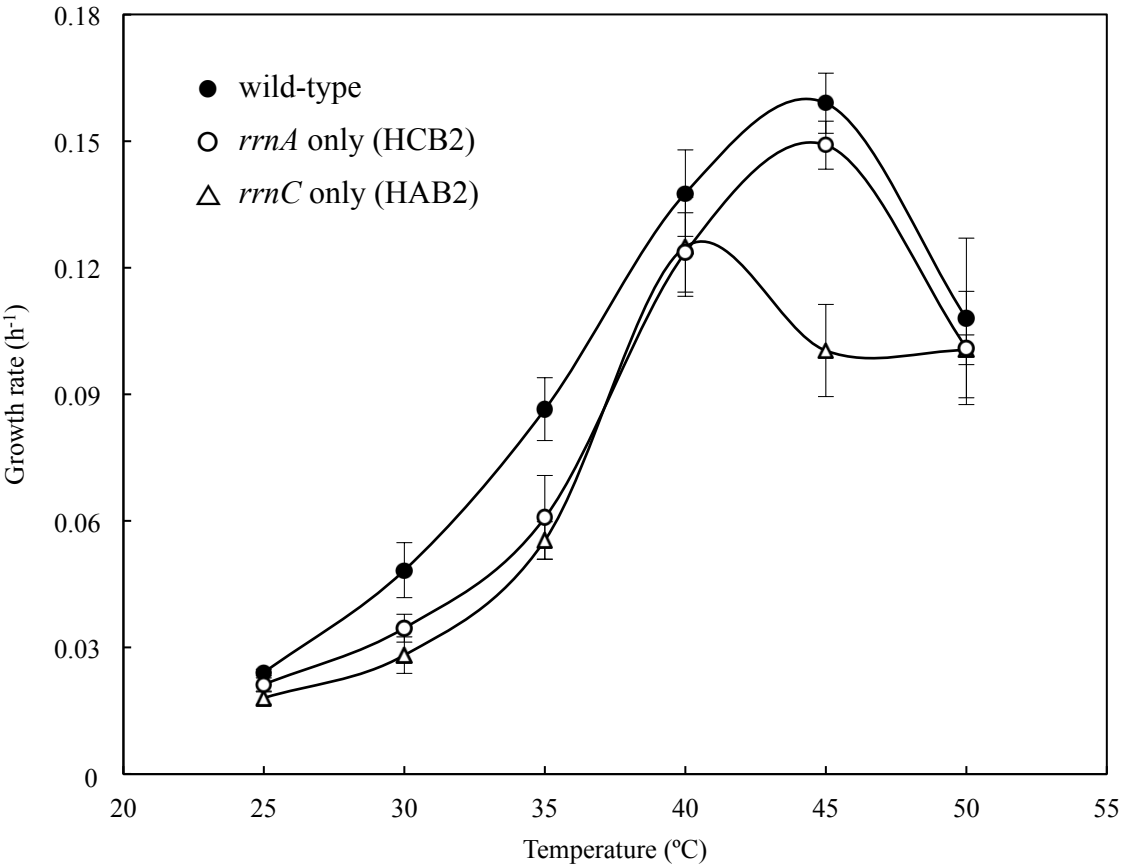
Supplementary Figure 2. Flow chart of the second rRNA operon (*rrn*) deletion method. Mutant strains, plasmids, and primer sets designed in this study are summarized in Supplementary Tables 1 and 2.



Supplementary Figure 3. Correlation between cell density and OD_{660} value in *Haloarcula hispanica*.



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 (μ) of wild-type, HCB2, and HAB2 at each temperature. Error bars denote standard deviation of mean values for quadruplicate or quintplicate measurements.

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