Chapter 5

Plasmid Curing is a Promising Approach to Improve Thermophiles for Biotechnological Applications: Perspectives in *Archaea*

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Additional information is available at the end of the chapter

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Abstract

Thermophiles are attractive as host cells for microbial processes to produce or degrade various compounds. In these applications, it is often desirable to improve the properties of thermophiles, such as their growth rate, cell density, and protein productivity, although this is rarely achieved because of the lack of general approaches. In this chapter, we describe the elimination of the pHTA426 plasmid from a moderate thermophile, *Geobacillus kaustophilus* HTA426, and its effects on the microbial properties. This process, called plasmid curing, was simply achieved using a DNA intercalator and confirmed by phenotypic and genotypic analyses. Of note, pHTA426 curing had beneficial effects on diverse properties, probably because of the reduced energy burden in terms of plasmid replication at high temperatures. The result suggests that plasmid curing is a simple and versatile approach for improving thermophiles. In particular, this approach may be effective for archaeal thermophiles because they grow at much higher temperatures and could have the greater energy burden on plasmid replication. Data mining has also shown that plasmids are distributed in archaeal thermophiles. This chapter provides a new tip for improving archaeal thermophiles, thereby increasing the opportunities for their use in various biotechnological applications.

Keywords: genetic engineering, host improvement, plasmid curing, plasmid elimination, thermophile application

1. Introduction

Thermophiles are organisms that are capable of growing at temperatures above 55°C. Archaeal thermophiles generally grow at much higher temperatures and thus comprise most

extreme thermophiles and hyperthermophiles. These thermophiles are attractive organisms in biotechnological applications because they produce thermostable enzymes, which can be used as stable industrial catalysts even at high temperatures. Thermophile cells themselves are attractive as hosts for microbial processes at high temperatures.

High-temperature processes have several advantages compared with moderate processes using mesophiles, where an important advantage is that high temperature prevents the growth of animal pathogens, including all viruses, which are killed or at least prevented from proliferating at temperatures above 65°C [1]. High temperature also inhibits growth and/or metabolism by mesophiles, which may hinder processes of interests via involuntary reactions. The advantage is especially important for processes using crude biomass (e.g., sewage, municipal or agricultural waste, and materials from animal farms) because mesophiles and virulent pathogens are common in biomass from natural environments and they may increase during reactions performed under moderate conditions. In addition, high temperature facilitates the removal of volatile products (e.g., ethanol and butanol) while decreasing oxygen solubility; therefore, thermophiles are practical for fermentative production of alcohols [2–6]. Moreover, thermophiles often have remarkable properties useful for bioprocesses. A good example is the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1, which can use protons as an electron accepter in catabolism to generate molecular hydrogen and has been studied as a hydrogen production tool [7].

In this chapter, we will demonstrate the salutary effects of plasmid curing on thermophiles using as an example a prokaryotic thermophile that was isolated from deep sea sediments of the Mariana Trench, *Geobacillus kaustophilus* HTA426 [8, 9]. Its growth occurs at temperatures

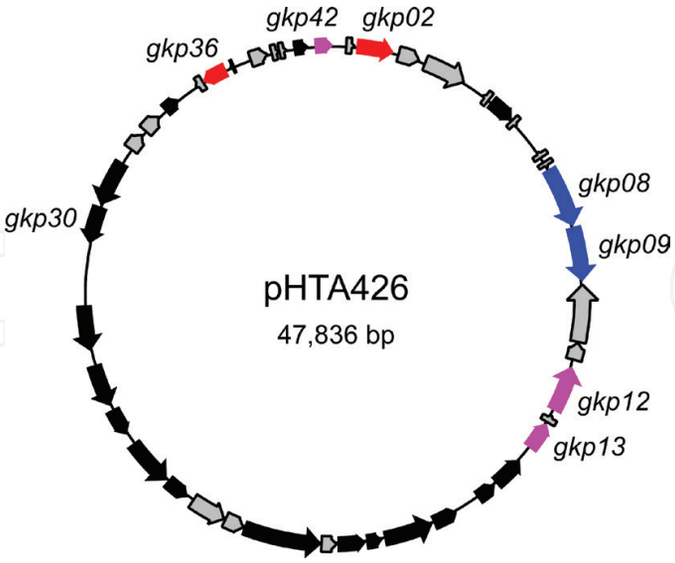

Figure 1. Structure of pHTA426. Genes for possible and hypothetical proteins are indicated by solid and faded arrows, respectively. The plasmid contains possible genes responsible for plasmid replication (*gkp02 and gkp36*), DNA restriction–modification (*gkp08 and gkp09*), and integrase/transposase (*gkp12 and gkp13*).

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ranging between 42 and 74°C, with optimal growth at 60°C, and is as rapid as *Escherichia coli* and *Bacillus subtilis*. Genetic tools are available for this strain [10–16]. The whole genome sequence has been determined [17], showing that *G. kaustophilus* HTA426 harbors the circular plasmid pHTA426 (**Figure 1**). Because the members of the genus *Geobacillus* include strains that are useful for high-temperature processes, as demonstrated by strains that are capable of degrading hydrocarbons [18], long-chain alkanes [19–21], biphenyls [22], paraffin-wax [23], or nylons [24], we have studied biotechnological applications of the genus using *G. kaustophilus* HTA426 as a model and pilot strain.

Aiming to construct a plasmid-free strain that may be useful for the genetic analysis of pHTA426, this study was originally designed to eliminate this plasmid from an HTA426 derivative, *G. kaustophilus* MK244. The resultant strain MK633 was then characterized to confirm that plasmid elimination (termed plasmid curing) had no effects on its microbial properties, but the analysis unexpectedly demonstrated that *G. kaustophilus* MK633 had advantages compared with the parent strain MK244 in terms of several properties. We suggest here that plasmid curing is a promising approach for improving diverse thermophiles.

2. Experimental procedures

2.1. Bacterial strains and culture conditions

The bacterial strains employed are summarized in **Table 1**. *G. kaustophilus* MK244 was previously constructed from *G. kaustophilus* HTA426 [14]. If not specified otherwise, *G. kaustophilus* strains were grown at 60°C in Luria–Bertani (LB) and minimal media (MM) with rotary shaking at 180 rpm. MM comprised 0.3 g l^{-1} K_2SO_4, 2.5 g l^{-1} $Na_2HPO_4{\cdot}12H_2O$, 1 g l^{-1} NH_4Cl, 0.4 g l^{-1} $MgSO_4$, 3 mg l^{-1} $MnCl_2{\cdot}4H_2O$, 5 mg l^{-1} $CaCl_2{\cdot}2H_2O$, 7 mg l^{-1} $FeCl_3{\cdot}6H_2O$, 0.1% trace element solution [25], 10 mM Tris-HCl (pH 7.0), 1 g l^{-1} casamino acids, and 10 g l^{-1} D-glucose. The media also contained 5 mg l^{-1} kanamycin, 10 mg l^{-1} uracil, 50 mg l^{-1} 5-fluoroorotic acid, 1 g l^{-1} yeast extract, and/or 50 mg l^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), as necessary. *E. coli* strains were grown at 37°C in LB medium supplemented with appropriate antibiotics (50 mg l^{-1} ampicillin, 50 mg l^{-1} kanamycin, 13 mg l^{-1} chloramphenicol, and/or 7 mg l^{-1} tetracycline). The optical cell density at 600 nm was monitored automatically using an OD-MonitorA instrument (Taitec, Saitama, Japan).

2.2. Plasmids

The *E. coli-Geobacillus* shuttle plasmids are summarized in **Table 2**. To construct the pGKE25-*bgaB* plasmid, a *gkp09* downstream region of pHTA426 was amplified using the primers 5′-GGAGTTTGCCAAACTCCGGATCCAGCTTGGATTTATC-3′ (*Bam*HI site underlined) and 5′-CACAAGCTTGGGGCTGGATGTAATG-3′ (*Hin*dIII site underlined), and a *gkp09* upstream region was amplified using the primers 5′-GGCGGATCCTTCCGATTAGGTTCCCATGC-3′ (*Bam*HI site underlined) and 5′-GGCGAATTCGGCCTTTTCGCATTAC-3′ (*Eco*RI site

Strain	Relevant description	Reference
E. coli		
BR397	Conjugation helper strain; F⁻ e14⁻ (*mcrA*⁻) Δ(*mrr-hsdRMS-mcrBC*)114::*IS10* Δ*dcm*::*lacZ* Δ*dam*::*metB* pUB307 pIR207	[18]
BR398	Conjugation helper strain; F⁻ e14⁻ (*mcrA*⁻) Δ(*mrr-hsdRMS-mcrBC*)114::*IS10* Δ*dcm*::*lacZ* pUB307 pIR207	[18]
BR408	Conjugation helper strain; F⁻ e14⁻ (*mcrA*⁻) Δ(*mrr-hsdRMS-mcrBC*)114::*IS10* Δ*dcm*::*lacZ* pUB307 pIR408	[18]
G. kaustophilus		
MK244	Derivative of the wild-type strain HTA426; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) pHTA426	[20]
MK244′	Derivative of the MK244 strain; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) pHTA426 (Δ*gkp09*::P_{sigA}-*bgaB*)	This study
$MK244_{bgaB}$	Derivative of the MK244 strain; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) *gk0707*::P_{gk704}-*bgaB* pHTA426	This study
MK633	Derivative of the MK244 strain; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*)	This study
$MK633_{bgaB}$	Derivative of the MK633 strain; Δ*pyrF* Δ*pyrR* Δ$hsdM_1S_1R_1$ Δ($mcrB_1$-$mcrB_2$-$hsdM_2S_2R_2$-*mrr*) *gk0707*::P_{gk704}-*bgaB*	This study

Table 1. Bacterial strains used in this study. pUB307 mediates the conjugative transfer of *oriT*-containing plasmids. This plasmid contains kanamycin and tetracycline resistance genes. pIR207 is a chloramphenicol resistance plasmid derived from pACYCDuet-1 (Merck KgaA, Darmstadt, Germany). The plasmid was used to construct pIR408, which contains the $hsdM_1S_1$ and $hsdM_2S_2$ genes from *G. kaustophilus* HTA426, and thus, it is responsible for heterologous DNA methylation in *E. coli* [18]. *G. kaustophilus* MK244 lacks genes related to pyrimidine biosynthesis (*pyrF* and *pyrR*) and DNA restriction–modification ($hsdM_1S_1R_1$, $mcrB_1$, $mcrB_2$, $hsdM_2S_2R_2$, and *mrr*). P_{sigA}-*bgaB* and P_{gk704}-*bgaB* denote *bgaB* expression cassettes under the control of P_{sigA} [19] and P_{gk704} promoters [21], respectively.

Plasmid	Relevant description	Reference
pGAM47-*bgaB*	pUC19 derivative; pUC replicon, *oriT, bla, pyrF*, P_{sigA}-*bgaB* flanked by *gk0707* upstream and downstream regions	[19]
pGAM48-*bgaB*	pUC19 derivative; pUC replicon, *oriT, bla, pyrF*, P_{gk704}-*bgaB* flanked by *gk0707* upstream and downstream regions	[21]
pGKE25	pUC19 derivative; pUC replicon, *oriT, bla, pyrF*	[20]
pGKE25-*bgaB*	pGKE25 derivative; pUC replicon, *oriT, bla, pyrF*, P_{sigA}-*bgaB* flanked by *gkp09* upstream and downstream regions	This study
pUCG18T	pUC18 derivative; pUC and pBST1 replicons, *oriT, bla, TK101*	[18]

Table 2. *E. coli-Geobacillus* shuttle plasmids used in this study. *bla* and *TK101* genes confer resistance to ampicillin in *E. coli* and kanamycin in *G. kaustophilus*, respectively. *pyrF* encodes orotidine-5′-phosphate decarboxylase in *G. kaustophilus* HTA426. *oriT* is the conjugative transfer origin. P_{sigA}-*bgaB* and P_{gk704}-*bgaB* denote *bgaB* expression cassettes under the control of P_{sigA} [19] and P_{gk704} promoters [21], respectively. The pUC and pBST1 replicons allow autonomous plasmid replication in *E. coli* and *G. kaustophilus*, respectively.

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underlined). In addition, a *bgaB* expression cassette encoding thermostable β-galactosidase under the control of P_{sigA} promoter was amplified from the pGAM47-*bgaB* plasmid [13] using the primers 5′-AAGATCTCTTCGCCTCATCCGCACGATTTC-3′ and 5′-GCCAGATCTCTAAACCTTCCCGGCTTCATC-3′ (*Bgl*II site underlined). The downstream region was cloned between the *Bam*HI and *Hin*dIII sites of the pGKE25 plasmid [14], and the upstream region was cloned between the *Bam*HI and *Eco*RI sites. The *bgaB* expression cassette was then cloned in the *Bam*HI site of the resulting plasmid to yield pGKE25-*bgaB* for replacing the *gkp09* gene in pHTA426 with the *bgaB* cassette. The pGAM48-*bgaB* plasmid [15] was used to integrate a *bgaB* expression cassette under the control of the P_{gk704} promoter at the *gk0707* locus in the *G. kaustophilus* chromosome. The pUCG18T plasmid [12] was used to assess the transformation efficiency of *G. kaustophilus*.

2.3. Plasmid introduction into *G. kaustophilus*

Plasmids were introduced into *G. kaustophilus* by conjugative plasmid transfer from *E. coli* BR408 [12]. Briefly, an *E. coli* donor (10 ml) and a *G. kaustophilus* recipient (100 ml) were grown in LB media. The cells were subsequently mixed, centrifuged, and spotted onto LB plates. After incubation at 37°C for 20 h, the resultant cells were collected and incubated at 60°C on appropriate media to isolate *G. kaustophilus* transformants. The transformation efficiency, as the number of transformants per 10^6 recipients, was determined as described previously [12]. Data were expressed as the mean ± standard error (n = 3).

2.4. pHTA426 curing from *G. kaustophilus* MK244

The *gkp09* gene in pHTA426 was replaced by a *bgaB* expression cassette using pGKE25-*bgaB* with *pyrF*-based counterselection [13]. The resultant clone, strain MK244′, was successively cultured three times in LB media supplemented with 20 μM acridine orange to facilitate pHTA426 curing. In each culture, an aliquot (10^3 cells) was grown on LB plates supplemented with X-gal to identify candidates from which pHTA426 was eliminated along with the *bgaB* cassette. The candidates were purified using LB plates with X-gal.

2.5. Southern blot

Total DNA (25 μg) was digested using *Eco*RV and separated on an agarose gel by electrophoresis. DNA was transferred onto a nylon membrane and hybridized with digoxigenin-labeled DNA probes to detect the *bgaB* and *gkp30* regions. Probes were synthesized using PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) using the primers 5′-GCCGGATCCTGTTATCCTCAATTTGTTAC-3′ and 5′-GCCGGATCCTGTTATCCTCAATTTGTTAC-3′ (for *bgaB* probe) and 5′-CCGATATAGGCTGAGAACGC-3′ and 5′-CAGCTGGTAGACATGGGG-3′ (for *gkp30* probe). Hybridized DNA was detected with the chromogenic method using DIG Nucleic Acid Detection Kit (Roche).

2.6. Construction of *G. kaustophilus* $MK244_{bgaB}$ and $MK633_{bgaB}$

A *bgaB* expression cassette under the control of the P_{gk704} promoter was integrated at the *gk0707* locus in *G. kaustophilus* using pGAM48-*bgaB*, as described previously [15]. *G. kaustophilus*

MK244 and MK633 were subjected to this process to generate strains $MK244_{bgaB}$ and $MK633_{bgaB}$, respectively.

2.7. BgaB assay

G. kaustophilus $MK244_{bgaB}$ and $MK633_{bgaB}$ were cultured for 4 h in MM containing yeast extract but not D-glucose or casamino acids, and then for 20 h in the presence of 10 g l^{-1} maltose. Cells were subsequently harvested, sonicated in 50 mM sodium phosphate (pH 6.0), and clarified by centrifugation to obtain a lysate. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 6.0), 2 mM *p*-nitrophenyl-β-D-galactopyranoside, and the lysate. The mixture was incubated at 60°C to react and then diluted with ice-cold 2 M sodium carbonate (900 μl) to terminate the reaction. *p*-Nitrophenol liberated in the mixture was quantified based on the absorbance at 405 nm and using an experimentally derived standard curve. One unit was defined as the amount of enzyme required to generate 1 μmol of *p*-nitrophenol per min. Proteins were quantified by the Bradford method using a protein assay kit (Nacalai Tesque, Kyoto, Japan). Data were expressed as the mean ± standard error (n = 4–5).

2.8. Plasmid stability assay

G. kaustophilus harboring pUCG18T was precultured in LB medium with kanamycin until the optical cell density at 600 nm reached 0.5. An aliquot (200 μl) was then cultured in LB medium (20 ml) without kanamycin until the stationary phase. The resultant cells were incubated on LB plates with or without kanamycin to determine the concentrations of kanamycin-resistant and viable cells, respectively. The plasmid retention rate was defined as the number of kanamycin-resistant cells per viable cells. Data were expressed as the mean ± standard error (n = 3).

2.9. Cell density assay

G. kaustophilus cells were cultured in LB medium until the stationary phase. Cells were harvested by centrifugation and analyzed to determine the wet weight. Data were expressed as the mean ± standard error (n = 4–5).

2.10. Mutation frequency assay

The frequency of spontaneous mutations was assessed based on the generation of rifampicin- and streptomycin-resistant cells via *rpoB* and *rpsL* genes, respectively [26]. *G. kaustophilus* (10^3 cells) was cultured at 60°C in LB medium until the stationary phase. The resultant cells were then incubated on LB plates with or without efficacious rifampicin or streptomycin (10 mg l^{-1}) to determine the concentrations of mutant (rifampicin- or streptomycin-resistant) and viable cells, respectively. The colonies were counted to calculate the ratio of mutant cells relative to the viable cells incubated, which was defined as the mutation frequency. Data were expressed as the mean ± standard error (n = 3).

2.11. Nucleotide stability assay

Deoxyribonucleoside triphosphates (1 mM) were incubated for 24 h in 20 mM sodium phosphate (pH 7.0) at 30, 60, 80, and 90°C. The residual nucleotides in samples (5 μl) were

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analyzed using reversed-phase high-performance liquid chromatography. The chromatography system comprised solvent delivery units (LC-10AT; Shimadzu, Kyoto, Japan), an ultraviolet absorption detector (SPD-10Avp; Shimadzu), a reverse-phase column (Cosmosil 2.5C_{18}-MS-II; Nacalai Tesque), and a column bath at 30°C. Solvents A and B comprised 5 mM tetrabutylammonium bromide in 20 mM sodium phosphate (pH 7.0) and 90% (v/v) acetonitrile in water, respectively. After injecting the sample into a column that had been equilibrated with 15% solvent B, the column was isocratically developed at a flow rate of 0.5 ml min^{-1} for 1 min and then at a linear gradient of 15–60% solvent B over 15 min. The chromatogram was obtained by detection at 260 nm.

2.12. Genome data mining

Genome data were collected from the GenBank database (https://www.ncbi.nlm.nih.gov/genome) in December 2016. The collection was performed for bacterial thermophiles (*Geobacillus* spp.), bacterial mesophiles (*B. subtilis*), archaeal thermophiles (*Pyrobaculum, Pyrococcus, Sulfolobus*, and *Thermococcus* spp.), archaeal mesophiles (*Haloarcula, Halococcus, Haloferax*, and *Halorubrum* spp.), and archaeal methanogens (*Methanobacterium, Methanobrevibacter, Methanocaldococcus, Methanocella, Methanococcoides, Methanococcus, Methanocorpusculum, Methanoculleus, Methanofollis, Methanogenium, Methanohalophilus, Methanolacinia, Methanolinea, Methanolobus, Methanomassiliicoccus, Methanomethylovorans, Methanomicrobium, Methanoplanus, Methanoregula, Methanosaeta, Methanosalsum, Methanosarcina, Methanosphaerula, Methanothermobacter, Methanothermococcus, Methanothermus, Methanotorris*, and *Methermicoccus* spp.). The growth temperatures of archaeal methanogens were based on the Methanogens database (http://metanogen.biotech.uni.wroc.pl). Genome sizes were expressed as the mean ± standard deviation.

3. Results

3.1. Genetic features of the pHTA426 plasmid

The pHTA426 sequence suggested that the plasmid was a large circular plasmid (47.9 kb) comprising 1.3% of the circular chromosome of *G. kaustophilus* HTA426 (3.54 Mb) and that it encoded possible proteins for plasmid replication (*gkp02*) and plasmid partition (*gkp36*). A partition system has a role in the stable transmission of single-copy plasmids during cell division, so pHTA426 appeared to be present as a single copy in *G. kaustophilus* HTA426. In addition, the plasmid contained genes for a type II restriction–modification system, which was homologous to the *Alw*I restriction-modification system (*gkp08* encoding methyltransferase and *gkp09* encoding endonuclease). A type II restriction-modification system generally comprises an endonuclease and methyltransferase, where the endonuclease cuts exogenous DNA at specific sites, but not endogenous DNA that has been methylated by methyltransferase. In the *Alw*I system, *Alw*I methyltransferase is responsible for 5′-GG^{6m}ATC-3′ and 5′-G^{6m}ATCC-3′ methylation (^{6m}A, N^6-methyladenin), whereas *Alw*I endonuclease cuts 5′-GGATC-3′ and 5′-GATCC-3′ sites but not 5′-GG^{6m}ATC-3′ and 5′-G^{6m}ATCC-3′ sites. Because plasmids carrying a type II restriction-modification system have greater segregational stability [27], it is likely that the *gkp08–gkp09* system contributes to the stable maintenance of pHTA426.

3.2. Construction of *G. kaustophilus* MK633

Figure 2A shows the process employed to eliminate pHTA426 from *G. kaustophilus* MK244. To facilitate plasmid curing and readily identify positive clones from which pHTA426 was eliminated, the *gkp09* gene was preliminarily replaced by a *bgaB* expression cassette using pGKE25-*bgaB*. The resultant strain MK244′ was then cultured successively in the presence of a DNA intercalator, and we screened for positive clones by using the X-gal degradation assay. Fortunately, one positive clone was obtained from the first culture but not from the second culture. From the third culture, 24 positive clones were identified, which suggests that three successive rounds of culture were effective for pHTA426 curing. The positive clone obtained from the first culture was designated as *G. kaustophilus* MK633.

G. kaustophilus MK244′ degraded X-gal to form blue colonies on LB plates with X-gal, whereas strains MK244 and MK633 did not (**Figure 2B**). This suggests that strain MK633 lacked the *bgaB* gene. The MK633 chromosome is resistant to *Dpn*I (which digests 5′-G^{6m}ATC-3′ but not 5′-GATC-3′) but sensitive to *Alw*I (see above), in contrast to the chromosomes from strains MK244 and MK244′ (**Figure 2C**), so it is likely that strain MK633 lacked the *gkp08* gene encoding an *Alw*I methyltransferase homolog. Southern blot analysis (**Figure 2D**) confirmed that strain MK633 lacked

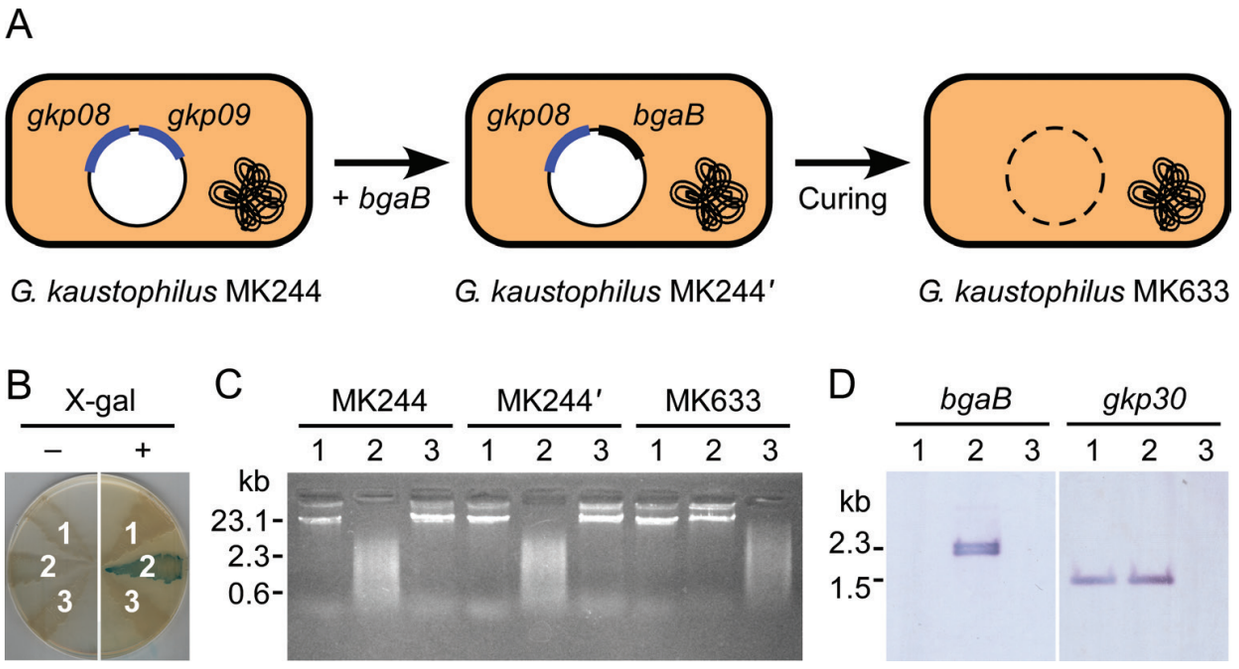

Figure 2. Construction of *G. kaustophilus* MK633. (A) Schematic representation of pHTA426 curing from *G. kaustophilus* MK244. The *gkp09* gene in pHTA426 was replicated by the *bgaB* expression cassette (P_{sigA}-*bgaB*) to generate strain MK244′. The plasmid pHTA426 (Δ*gkp09*::P_{sigA}-*bgaB*) in strain MK244′ was subsequently eliminated using acridine orange. The positive clones from which pHTA426 was eliminated, including strain MK633, were identified using the X-gal degradation assay. (B) X-gal degradation assay. *G. kaustophilus* strains MK244 (1), MK244′ (2), and MK633 (3) were grown at 60°C on LB plates with (+) or without (−) X-gal. (C) DNA methylation assay of MK244, MK244′, and MK633 chromosomes. Total DNA from the strains was digested with restriction enzymes (1, none; 2, *Dpn*I; 3, *Alw*I) and analyzed by agarose gel electrophoresis. (D) Southern blot analysis of pHTA426. Total DNA from strains MK244 (1), MK244′ (2), and MK633 (3) was digested with *Eco*RV, and detected by *bgaB* (left panel) and *gkp30* probes (right panel).

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the *bgaB* and *gkp30* genes, which are located on opposite sides of pHTA426 (Δ*gkp09*::P_{sigA}-*bgaB*). Based on these results, we concluded that *G. kaustophilus* MK633 lacked pHTA426.

3.3. Microbial properties of *G. kaustophilus* MK244 and MK633

G. kaustophilus MK244 and MK633 were characterized in detail (**Table 3**). Both strains grew in LB and MM with comparable doubling times. The difference in their mutation frequencies was also not significant. However, strain MK633 was more transformed efficiently with pUCG18T than strain MK244 and it maintained the plasmid with higher stability. Moreover, strain MK633 grew at higher cell densities than strain MK244. The cell density of *G. kaustophilus* MK244' was lower than that of strain MK633 at 60°C (0.30 ± 0.03 g wet), but the pUCG18T retention rate was comparable (43 ± 14%).

When cultured at 60°C, strain $MK633_{bgaB}$ produced 220 ± 20 units of BgaB, whereas strain $MK244_{bgaB}$ produced 140 ± 10 units (**Figure 3A**). BgaB was also produced more abundantly by $MK633_{bgaB}$ at 50 and 70°C. Moreover, *G. kaustophilus* $MK633_{bgaB}$ had advantages in terms of the cell yield per culture (**Figure 3B**), protein yield per culture (**Figure 3C**), and BgaB-specific activity (**Figure 3D**). The higher specific activity suggests that $MK633_{bgaB}$ enhanced the BgaB productivity per cell.

3.4. Nucleotide stability

In bacteria and *Archaea*, DNA replication proceeds in cytosol (approximately at pH 7) using deoxyribonucleoside triphosphates as the building blocks. To assess their thermolability in cytosol, deoxyribonucleoside triphosphates (i.e., dATP, dCTP, dGTP, and dTTP) were treated at high temperatures and analyzed to determine residual amounts relative to those after incubation at 30°C. Most nucleotides were instable at 60°C (residual ratio: dATP, 68%; dCTP, 70%; dGTP, 71%; and dTTP, >99%). All of the nucleotides were clearly degraded into other forms when incubated at 80°C (dATP, 10%; dCTP, 26%; dGTP, 12%; and dTTP, 15%) and were completely degraded at 90°C (residual ratio, <0.2%). This suggests that the deoxyribonucleoside triphosphates are physicochemically unstable in the thermophiles.

3.5. Archaeal thermophiles: Smaller genomes

Genomic data were analyzed to compare the genome sizes of thermophiles (capable of growing at > 55°C) and mesophiles (capable of growing at 20–55°C). The genomes of thermophilic bacteria *Geobacillus* spp. (3.4 ± 0.3 Mb; n = 57) were smaller than those of phylogenetically related mesophiles, e.g., *B. subtilis* (4.1 ± 0.3 Mb; n = 100). The results were similar for archaeal methanogens, in which thermophiles had smaller genomes (1.7 ± 0.6 Mb; n = 20) than mesophiles (2.6 ± 0.8 Mb; n = 57). Archaeal thermophiles, such as *Pyrobaculum* (2.2 ± 1.6 Mb; n = 4), *Pyrococcus* (1.8 ± 0.1 Mb; n = 5), *Sulfolobus* (2.5 ± 0.2 Mb; n = 5), and *Thermococcus* (2.0 ± 0.1 Mb; n = 18) members, have much smaller genomes compared with archaeal mesophiles, such as *Haloarcula* (3.6 ± 1.2 Mb; n = 9), *Halococcus* (3.6 ± 0.4 Mb; n = 7), *Haloferax* (3.7 ± 0.4 Mb; n = 6), and *Halorubrum* (3.1 ± 0.8 Mb; n = 13) members. These data suggest that the thermophiles, especially archaeal thermophiles, tend to have smaller genomes than mesophiles.

	MK244	MK633
Transformation efficiency (per 10^6 recipients)		
E. coli BR397 donor	<1	300 ± 75
E. coli BR398 donor	55 ± 17	22 ± 3
E. coli BR408 donor	87 ± 14	34 ± 14
Mutation frequency (per 10^6 cells)		
Rifampicin resistance	12 ± 2	42 ± 8
Streptomycin resistance	7 ± 4	7 ± 2
Doubling time in LB medium (min)		
50°C	47 ± 6	42 ± 2
60°C	23 ± 2	21 ± 3
70°C	38 ± 7	32 ± 5
Doubling time in MM medium (min)		
50°C	100 ± 9	130 ± 20
60°C	83 ± 6	89 ± 2
70°C	ND	ND
pUCG18T retention rate (%)		
50°C	6 ± 3	58 ± 7
60°C	6 ± 4	79 ± 9
70°C	1 ± 1	64 ± 2
Cell yield (g wet weight per 20 ml culture)		
50°C	0.41 ± 0.01	0.41 ± 0.01
60°C	0.30 ± 0.03	0.36 ± 0.01
70°C	0.38 ± 0.03	0.41 ± 0.01

Table 3. Microbial properties of *G. kaustophilus* MK244 and MK633. Analyses were repeated more than three times. Data represent the mean ± standard error. ND, growth was not observed within 2 days.

3.6. Distribution of plasmids in archaeal thermophiles

Data mining showed that many thermophiles harbored plasmids, although not the majority. In *Archaea*, plasmids were identified frequently in *Sulfolobus* spp. (14 strains) and *Thermococcus* spp. (11 strains): pARN3 (26.2 kb), pARN4 (26.5 kb), pHEN7 (7.8 kb), pHVE14 (35.4 kb), pING1 (24.6 kb), pKEF9 (28.9 kb), pLD8501 (26.6 kb), pRN1 (5.4 kb), pRN2 (7.0 kb), pSOG1 (29.0 kb), pSOG2 (26.0 kb), pSSVx (5.7 kb), pXZ1 (7.0 kb), and pYN01 (42.2 kb) in *Sulfolobus islandicus*; pIT3 (5.0 kb), pMGB1 (28.0 kb), and pSSVi (5.7 kb) in *Sulfolobus solfataricus*; pTBMP1 (54.2 kb) in *Thermococcus barophilus*; an unnamed plasmid (3.6 kb) in *Thermococcus eurythermalis*; pTN1 (3.6 kb), pTN2 (13.0 kb), and pTN3 (18.3 kb) in *Thermococcus nautili*; an unnamed plasmid

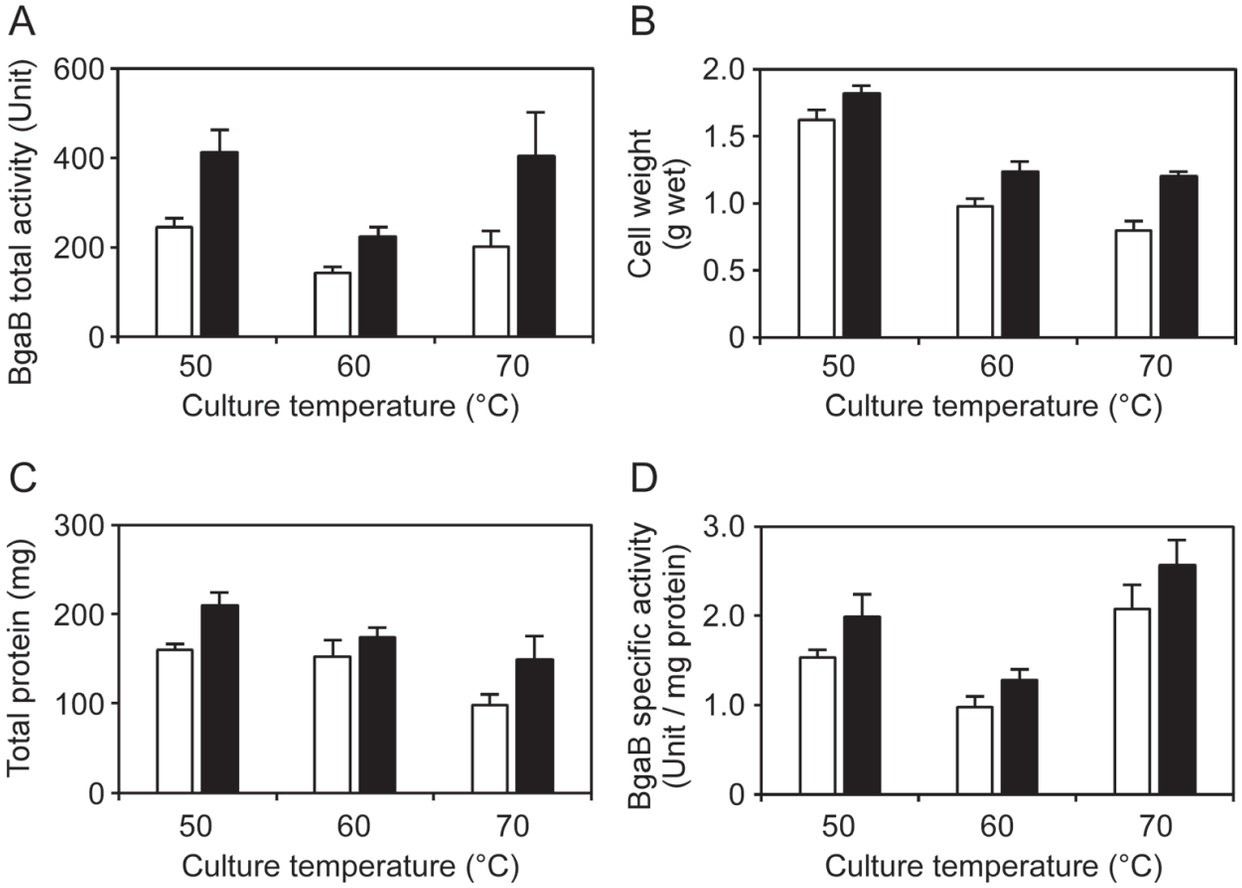

Figure 3. BgaB production by *G. kaustophilus* MK244$_{bgaB}$ and MK633$_{bgaB}$. These strains were cultured at 50, 60, and 70°C in medium (100 ml) and analyzed to determine the total activity in terms of intracellular BgaB (A), cell weight (B), intracellular total protein (C), and BgaB-specific activity (D). Data are expressed as the mean ± standard error (n = 4–5).

(49.1 kb) in *Thermococcus peptonophilus*; and pAMT7 (8.6 kb), pAMT11 (20.5 kb), pCIR10 (13.3 kb), pEXT9a (10.6 kb), pIRI33 (11.0 kb), pIRI48 (13.0 kb), and pT26-2 (21.6 kb) in *Thermococcus* spp. The other plasmids identified in archaeal thermophiles are as follows: pDSM2661_1 (58.4 kb) and pDSM2661_2 (16.6 kb) in *Methanocaldococcus jannaschii*; pMTBMA4 (4.4 kb) in *Methanothermobacter marburgensis*; pFV1 (13.5 kb), pFZ1 (11.0 kb), pME2001 (4.4 kb), and pME2200 (6.2 kb) in *Methanothermobacter thermautotrophicus*; pMETOK01 (14.9 kb) in *Methanothermococcus okinawensis*; pGT5 (3.4 kb) in *Pyrococcus abyssi*; and pTA1 (15.7 kb) in *Thermoplasma acidophilum*. In addition, several cryptic plasmids have been identified in archaeal thermophiles [28].

Plasmids are also distributed in bacterial thermophiles. In *Geobacillus* spp., seven strains of *Geobacillus* spp. harbored plasmids: pGS18 (plasmid length, 62.8 kb), pSTK1 (1.9 kb), pTB19 (11.9 kb), and an unnamed plasmid (21.7 kb) in *Geobacillus stearothermophilus*; pLW1071 (57.7 kb) in *Geobacillus thermodenitrificans*; pLDW-1 (48.7 kb) in *Geobacillus thermoleovorans*; and pBt40 (39.7 kb) in *Geobacillus* sp. In *Thermus* spp., 12 strains were identified as plasmid carriers. Their plasmids include pTA14 (14.4 kb), pTA16 (16.6 kb), pTA69 (69.9 kb), and pTA78 (78.7 kb) in *Thermus aquaticus*; pTB1 (342.8 kb) and pTB2 (10.3 kb) in *Thermus brockianus*; pTHEOS01 (271.7 kb) and pTHEOS02 (57.2 kb) in *Thermus oshimai*; pTP143 (143.3 kb) in *Thermus parvatiensis*; pTSC8 (8.4 kb) in *Thermus scotoductus*; and pTF62 (10.4 kb), pTHTHE1601 (440.0 kb), pTT8

(9.3 kb), pTT27 (232.6 kb), pTTJL1801 (265.9 kb), pTTJL1802 (142.7 kb), and pVV8 (81.2 kb) in *Thermus thermophilus*. In *Parageobacillus thermoglucosidans*, pGEOTH01 (80.8 kb), pGEOTH02 (19.6 kb), pNCI001 (83.9 kb), and pNCI002 (47.9 kb) were identified.

4. Discussion

In this study, we analyzed the effects of plasmid curing on thermophiles by characterizing *G. kaustophilus* MK244 and MK633 (**Table 3** and **Figure 3**). Both strains exhibited comparable growth at 50–70°C in LB and MM; therefore, pHTA426 had no positive effects on cell growth under standard conditions. The mutation frequencies were largely comparable between strains MK244 and MK633, although *G. kaustophilus* MK633 lacked the *gkp08* gene responsible for *dam*-like methylation (5′-GG^{6m}ATC-3′and 5′-G^{6m}ATCC-3′) and the *dam* methylation (5′-G^{6m}ATC-3′) is essential for DNA mismatch repair in *E. coli* [29]. In *B. subtilis* 168, DNA mismatch repair only involves *mutS* and *mutL* products, which do not depend on DNA methylation [30, 31], thereby suggesting that *G. kaustophilus* may use a mismatch repair system similar to the *B. subtilis* system rather than the *E. coli* system. In fact, the *G. kaustophilus* genome [17] contains *mutS* and *mutL* but not *mutH*, as found in *B. subtilis* 168.

A restriction-modification system generally protects the host microbe from transformation with exogenous DNA because the system cuts exogenous DNA that is not methylated by methyltransferase. However, a microbe can accept exogenous DNA that imitates the methylation pattern because a restriction-modification system is unable to cut this exogenous DNA [32]. In a previous study [12], we constructed *E. coli* strains BR397, BR398, and BR408 for conjugative plasmid transfer into *G. kaustophilus* HTA426. *E. coli* BR408 produces DNA that imitates the methylation pattern in *G. kaustophilus* HTA426. *E. coli* BR398 produces DNA with *dam* methylation, whereas *E. coli* BR397 produces methyl-free DNA. Although *G. kaustophilus* MK244 could not accept the pUCG18T plasmid transferred from the *dam*$^-$ strain *E. coli* BR397, *G. kaustophilus* MK633 accepted pUCG18T from *E. coli* BR397 as well as strains BR398 and BR408. These results can be explained by the elimination of *gkp09* from strain MK633 because the *gkp09* product digests methyl-free DNA but not DNA with *dam* methylation (5′-G^{6m}ATC-3′, which covers *gkp08* methylation (5′-GG^{6m}ATC-3′ and 5′-G^{6m}ATCC-3′. In addition, *G. kaustophilus* MK633 maintained pUCG18T with higher stability than strain MK244. This observation is also attributable to the elimination of *gkp09* because the *gkp09* product can occasionally digest endogenous plasmids that have not undergone *gkp08* methylation immediately after plasmid replication. This hypothesis is supported by the fact that *G. kaustophilus* MK244′ maintained pUCG18T as stably as strain MK633. Thus, *G. kaustophilus* MK633 acquired advantages compared with strain MK244 in terms of plasmid transformation and plasmid stability due to the elimination of a restriction-modification system along with pHTA426 curing.

An intriguing observation was that *G. kaustophilus* MK633 had a higher cell density in the stationary phase. In contrast to the advantages in terms of plasmid transformation and plasmid stability, this observation cannot be explained by the elimination of *gkp09* because *G. kaustophilus* MK244′ grew less efficiently than strain MK633. It was also interesting that strain MK633$_{bgaB}$ produced higher amounts of BgaB than MK244$_{bgaB}$ (**Figure 3A**). This observation is

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attributable mainly to the higher cell density of MK633$_{bgaB}$ (**Figure 3B**), as observed with strain MK633, and thus the higher protein yields from strain MK633$_{bgaB}$ (**Figure 3C**). In addition, this observation can be attributed to the higher BgaB productivity per cell because strain MK633$_{bgaB}$ had a higher BgaB specific activity (**Figure 3D**). Thus, these results suggest that the elimination of pHTA426 improved the cell density per culture and BgaB productivity per cell, thereby remarkably enhancing the production of BgaB.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that the crude extracts from *G. kaustophilus* MK244 and MK633 had negligible differences in terms of their protein compositions (data not shown). Therefore, it is unlikely that *G. kaustophilus* MK633 lost a nonessential protein that was abundantly synthesized from pHTA426, thereby reducing the energy burden to enhance the cell yield and BgaB productivity. Instead, these enhancements may be attributable to the reduced energy burden for plasmid replication. In fact, evidence suggests that plasmid maintenance places burdens on cell growth and/or metabolism [33–36]. Given that pHTA426 replicates as a single copy, this plasmid accounts for only 1.3% of total DNA; however, deoxyribonucleoside triphosphates are more unstable at higher temperatures, thereby suggesting that plasmid replication may place a considerable energy burden on thermophiles even though the plasmid is not extremely large or present in high copy numbers. This hypothesis is consistent with our genome data analysis, which showed that thermophiles have relatively smaller genomes than mesophiles, and the negative correlation between genome size and growth temperature reported by Sabath *et al* [37]. Overall, we consider that the lower energy burden incurred for DNA replication can explain why pHTA426 curing improved the cell density per culture and BgaB productivity per cell.

In conclusion, we demonstrated that pHTA426 curing was effective for improving the performance of a moderate thermophile, *G. kaustophilus* MK244. The cell density and protein productivity were presumably improved by the reduced amounts of energy required for DNA replication at high temperatures, so plasmid curing may be a simple approach for improving thermophiles in terms of these properties. In particular, this approach may be effective for archaeal thermophiles because they grow at extremely high temperatures and thus could have a greater energy burden on plasmid replication. Moreover, in archaeal thermophiles, plasmids may account for larger fractions of chromosomes than in moderate thermophiles because archaeal thermophile genomes are generally smaller. Therefore, plasmid curing could remarkably reduce the energy burden in archaeal thermophiles. We note that many archaeal thermophiles harbor plasmids, such as *S. solfataricus* P2 (carrying pSSVi) and *T. barophilus* MP (carrying pTBMP1), which have been studied as model acidophilic hyperthermophiles [38] and piezophilic hyperthermophiles [39], respectively. Even if these thermophiles harbor a single copy plasmid, our results suggest that plasmid curing can improve their performance in terms of the cell density and protein productivity.

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