Frequent Transposition of Multiple Insertion Sequences in Geobacillus kaustophilus HTA426

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Geobacillus kaustophilus HTA426 is a thermophilic bacterium whose genome harbors numerous insertion sequences (IS). This study was initially conducted to generate mutant genes for thermostable T7 RNA polymerase in G. kaustophilus; however, relevant experiments unexpectedly identified that the organism transposed multiple IS elements and produced derivative cells that expressed a silent gene via transposition. The transposed elements were diverse and included members of the IS\textsuperscript{4}, IS\textsuperscript{701}, IS\textsuperscript{1634}, and IS\textsuperscript{Lre2} families. The transposition was relatively active at elevated temperatures and generated 4–9 bp of direct repeats at insertion sites. Transposition was more frequent in proliferative cells than in stationary cells but was comparable between both cells when sigX, which encodes an extra-cytoplasmic function sigma factor, was forcibly expressed. Southern blot analysis indicated that IS transposition occurred under growth inhibitory conditions by diverse stressors; however, IS transposition was not detected in cells that were cultured under growth non-inhibitory conditions. These observations suggest that G. kaustophilus enhances IS transposition via sigX-dependent stress responses when proliferative cells were prevented from active propagation. Considering Geobacillus spp. are highly adaptive bacteria that are remarkably distributed in diverse niches, it is possible that these organisms employ IS transposition for environmental adaptation via genetic diversification. Thus, this study provides new insights into adaptation strategies of Geobacillus spp. along with implications for strong codependence between mobile genetic elements and highly adaptive bacteria for stable persistence and evolutionary diversification, respectively. This is also the first report to reveal active IS elements at elevated temperatures in thermophiles and to suggest a sigma factor that governs IS transposition.

Keywords: extra-cytoplasmic function sigma factor, IS\textsuperscript{4}, IS\textsuperscript{701}, IS\textsuperscript{1634}, IS\textsuperscript{Lre2}, stress-induced transposition, thermophile, transposable element

INTRODUCTION

Insertion sequences (IS) are a simple class of mobile genetic elements that propagate themselves or change the position in the host's genetic material via replicative or non-replicative transposition, respectively (Vandecraen et al., 2018). An IS element is flanked by short inverted repeats and encodes a transposase that catalyzes transposition. In addition, the IS element may carry regulatory
genes essential for transposition but does not carry accessory genes. A total of 32 IS families are classified, and most encode DDE-type (named for a conserved amino acid triad) transposases (Siguier et al., 2015). Transposition potentially causes deleterious mutations; therefore, IS elements were initially considered parasitic and selfish factors that multiply without conferring a survival advantage to the host organism (Schrader and Schmitz, 2019). However, it is now known that IS transposition can provide evolutionary adaptation for their hosts via gene inactivation and/or modulated expression of the neighboring genes (Vandecraen et al., 2018).

Insertion sequences that employ DDE-type transposases can achieve either replicative transposition or non-replicative transposition (Bouuaert and Chalmers, 2010). Non-replicative transposition results from cut-and-paste mechanisms where the transposase expressed from an IS element excises the IS from the original site and integrates it into another site. Replicative transposition is performed by either copy-and-paste (donor-primed replication) or copy-in (target-primed replication) mechanisms. In the former mechanism, the transposase releases a single-stranded and circular IS element from the original site. The IS element undergoes replication and eventually integrates into another site. The latter mechanism uses transposases to nick IS termini and directly ligate them with another site. This results in formation of a Shapiro intermediate followed by the distribution of their endospores (Zeigler, 2014). However, it is now known that IS transposition can achieve either replicative transposition or non-replicative transposition (Kobayashi et al., 2015a,b; Wada et al., 2016). This strain is remarkable in its capacity to reproduce in the respective habitats (Suzuki, 2018). Pan-genomic analysis suggests that IS transposition potentially causes deleterious inactivation and/or modulated expression of the neighboring genes. A total of 32 IS families are classified, and most encode DDE-type (named for a conserved amino acid triad) transposases (Siguier et al., 2015). Transposition potentially causes deleterious mutations; therefore, IS elements were initially considered parasitic and selfish factors that multiply without conferring a survival advantage to the host organism (Schrader and Schmitz, 2019). However, it is now known that IS transposition can provide evolutionary adaptation for their hosts via gene inactivation and/or modulated expression of the neighboring genes (Vandecraen et al., 2018).

MATERIALS AND METHODS

Genetic Tools

Plasmid pGAM46 was previously constructed for marker-free gene integration into the amyA gene in G. kaustophilus (Suzuki et al., 2012). Plasmid pGKE75 (Kobayashi et al., 2015a) was used for foricable gene expression under the control of the gk704 promoter (Pgk704) of G. kaustophilus (Suzuki et al., 2013b). Plasmid pGKE74 was constructed from pGKE75 via elimination of the Pgk704 region. Plasmids were introduced into G. kaustophilus using conjugative plasmid transfer from Escherichia coli (Suzuki et al., 2013a). Chromosomal gene replacement was performed using the procedure previously described (Suzuki et al., 2012). The primer sequences are summarized in the Supplementary Table 1.

Construction of Plasmids

Table 1 summarizes relevant plasmids. T7 promoter (P7) was amplified from pET-16b (Merck KGaA, Darmstadt, Germany) using the primers T7-250F and T7R. The pyrF gene encoding for orotidine 5′-phosphate decarboxylase of G. kaustophilus was amplified using the primers pyrF0F and pyrFTR. The P7 and pyrF fragments were cloned between the HindIII and SphI sites of pGAM46 and cloned between the SphI and BamHI sites, respectively, of pGAM46 to give pGAM46P7-pyrF carrying the P7-pyrF cassette. The gene for T7 RNA polymerase (T7R) was amplified from E. coli BL21(DE3) using the primers T7R0F and T7RTR and cloned between the SphI and BamHI sites of pGKE75 to give pGKE75-T7RP, which carried the Pgk704-T7RP cassette. To construct the Pgk704-sigB cassette, Pgk704 was amplified from pGKE75 using the primers gk704-250F and sigB0R; sigB encoding for sigma factor B (SigB) of G. kaustophilus was amplified using the primers sigB0F and sigBTR. These fragments were combined using fusion PCR and cloned between the SphI and BamHI sites to give pGKE74P-gk704-sigB. To construct the Pgk704-rsbV cassette, Pgk704 was amplified using the primers gk704-250F and rsbV0R; rsbV encoding for anti-SigB antagonist (RsbV) of G. kaustophilus was amplified using the primers rsbV0F and rsbVTR. These fragments were combined and cloned between the HindIII and BamHI sites of pGKE74 to give pGKE74P-gk704-rsbV. To construct the Pgk704-sigX cassette, Pgk704 was amplified using the primers gk704-250F and sigX0R; sigX encoding for sigma factor X (SigX) of G. kaustophilus was amplified using the primers sigX0F and sigXTR. These fragments were combined and cloned between the HindIII and BamHI sites to give pGKE74P-gk704-sigX.

Bacterial Strains

Table 1 summarizes thermophilic strains used in this study. G. kaustophilus strains MK242 and MK480 were previously constructed from G. kaustophilus HTA426 (Suzuki et al., 2015). The Pgk704-bgaB cassette in MK242 and MK480 was...
Strains lack genes for pyrimidine biosynthesis (pyrF and pyrR) and restriction–modification systems (hsdM).

The T7 and gk704 promoters are abbreviated as P7 and Pgk704, respectively. The pyrF gene encodes for a pyrimidine biosynthetic enzyme. Plasmids pGKE74Pgk704-rsbV, pGKE74Pgk704-sigX, and pGKE75-T7RP direct production of anti-sigma factor B antagonist (RsbV), sigma factor B (SigB), sigma factor X (SigX), and T7 RNA polymerase (T7RP), respectively, under the Pgk704 control in G. kaustophilus. These plasmids carry kanamycin-resistant gene for thermophiles. Most strains lack genes for pyrimidine biosynthesis (pyrF and pyrR) and restriction–modification systems (hsdM S R1, mcrB1, mcrB2, mcrC, mrr, mfd).

| Strain or plasmid | Relevant description | References |
|-------------------|---------------------|------------|
| HTA426            | pGAM46 derivative used to integrate P7- pyrF cassette at G0707 locus | Takami et al., 2004b |
| gk704             | pGAM46 derivative used to integrate P7- pyrF cassette at G0707 locus | This study |
| pGKE75            | pGKE75 derivative used for forcible T7RP expression | Kobayashi et al., 2015a |
| T7RP              | pGKE75 derivative without Pgk704 | This study |
| pGKE74Pgk704-rsbV | pGKE74 derivative carrying Pgk704-rsbV cassette for rsbV expression | This study |
| pGKE74Pgk704-sigB | pGKE74 derivative carrying Pgk704-sigB cassette for sigB expression | This study |
| pGKE74Pgk704-sigX | pGKE74 derivative carrying Pgk704-sigX cassette for sigX expression | This study |

The T7 and pgk704 promoters are abbreviated as P7 and Pgk704, respectively. The pyrF gene encodes for a pyrimidine biosynthetic enzyme. Plasmids pGKE74Pgk704-rsbV, pGKE74Pgk704-sigX, and pGKE75-T7RP direct production of anti-sigma factor B antagonist (RsbV), sigma factor B (SigB), sigma factor X (SigX), and T7 RNA polymerase (T7RP), respectively, under the Pgk704 control in G. kaustophilus. These plasmids carry kanamycin-resistant gene for thermophiles. Most strains lack genes for pyrimidine biosynthesis (pyrF and pyrR) and restriction–modification systems (hsdM S R1, mcrB1, mcrB2, mcrC, mrr, mfd).

Error-prone strains also lack genes for DNA repair (mutS, mutL, mutY, ung, and mfd).

### Culture Conditions

*Geobacillus kaustophilus* was cultured in Luria–Bertani (LB; Nacalai Tesque, Kyoto, Japan) or semisynthetic (MM, MU, MC, or MN) media. The MM medium contained inorganic salts (K2SO4, 0.3 g/L; Na2HPO4·12H2O, 2.5 g/L; NH4Cl, 1 g/L; MgSO4, 0.4 g/L; MnCl2·4H2O, 3 mg/L; CaCl2·2H2O, 5 mg/L; and FeCl3·6H2O, 7 mg/L), 0.1% trace element solution (Armature et al., 1991), Tris–HCl (10 mM, pH 7.5), uracil (10 mg/L), casamino acids (1 g/L), and d-glucose (10 g/L). The other semisynthetic media were based on MM medium; however, MU medium lacked uracil. The MC medium lacked both casamino acids and d-glucose, and MN lacked casamino acids and NH4Cl. Solid media contained agar (20 g/L). Kanamycin (5 mg/L) was added when necessary. The optical density at 600 nm (OD600) was monitored using an infrared-dependent detector (OD-Monitor A; Taitec, Saitama, Japan).

### Generation Assay of Uracil Prototrophs From *G. kaustophilus* MK536

*Geobacillus kaustophilus* MK536 was precultured overnight at 60°C in LB medium (5 mL). The cells were collected by centrifugation (14,000 × g, 10 s) and suspended in sterile water (1 mL) to remove medium elements. Cells were collected again by centrifugation and resuspended in sterile water (0.15 mL). The suspension was used to prepare a dilution series in sterile water, which was plated on MM plates and then incubated at 65°C for 72 h to obtain uracil prototrophs. The dilution series was also inoculated on MM plates for 24 h to determine viable cell concentrations. The generation frequency of uracil prototrophs was defined as the ratio of generated uracil prototrophs to incubated viable cells (10^3–10^6 cfu).

### Isolation of Genomic DNA

Mixtures were vigorously agitated during each addition of reagents. *G. kaustophilus* was cultured at 60°C in LB medium (30 mL). Cells were collected by centrifugation (4,400 × g, 5 min) and suspended in TEG buffer (3 mL) that contained Tris–HCl (25 mM, pH 8.0), ethylenediaminetetraacetic acid (10 mM), d-glucose (50 mM), lysozyme (1 mg/mL), and ribonuclease A (1 μg/mL). Following incubation at 37°C for 30 min, the suspension was mixed with sodium dodecyl sulfate (10%, 0.3 mL) and proteinase K (4 μg) and then incubated at 60°C for 30 min. The homogenate was supplemented with NaCl...
(5 M, 0.3 mL), cetyltrimethylammonium bromide (5%, 0.3 mL), and phenol/chloroform/isoamyl alcohol (25:24:1, 0.3 mL). After centrifugation (4,400 \( \times \) g, 10 min), the aqueous supernatant was transferred to a conical tube and mixed with an equal volume of ethanol. The tube was repeatedly inverted to precipitate genomic DNA, which was washed twice with ethanol (70%, 1 mL) and dissolved in TE buffer (1 mL) that contained Tris–HCl (10 mM, pH 7.5) and ethylenediaminetetraacetic acid (1 mM). For next-generation sequencing, genomic DNA was further purified using a NucleoSpin gDNA Clean-up (Takara Bio, Otsu, Japan).

### Sequencing Analysis

The pyrF upstream region in uracil prototrophs was amplified using the primers pyrF\(_{320R}\) and amyA\(_{1300R}\). The amplicons were purified using the GenElute Agarose Spin Columns (Sigma Aldrich, St. Louis, MO, United States) and sequenced with Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, United States). Cycle sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with the primers amyA\(_{240F}\), amyA\(_{400F}\), amyA\(_{480R}\), amyA\(_{800R}\), amyA\(_{1300R}\), and/or pyrF\(_{320R}\). The library for next-generation sequencing was constructed using an NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, United States) and validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). Sequences were obtained as 150 bp pair-end reads on a NovaSeq 6000 system (Illumina, San Diego, CA, United States). The assembly was first performed using Velvet (Zerbino and Birney, 2008) with default parameters. Based on the assembly, sequencing reads were aligned and assembled into contigs using SSPACE (Boetzer et al., 2011) and GapFiller (Boetzer and Pirovano, 2012). The draft sequence was compared with the complete sequence of \textit{G. kaustophilus} HTA426 (Takami et al., 2004b) using BLAST\(^1\) to identify IS elements that had transposed. The read sequences were also mapped to the genome sequence of \textit{G. kaustophilus} HTA426 using Burrows–Wheeler Aligner (Li and Durbin, 2010) and analyzed using Integrative Genomics Viewer (Thorvaldsdottir et al., 2013) to confirm the IS transposition. The IS elements transposed in \textit{G. kaustophilus} MK536\(_{up}\) were amplified using the following primers: is25F and is25R (at GK3299 locus); is28F and is28R (between GK1097 and GK1098 loci); is72F and is72R (at GK0885 locus); and is87F and is87R (between GK0301 and GK0302 loci). Amplicons were sequenced to determine the intact sequences.

### Transcription Analysis

\textit{Geobacillus kaustophilus} strains MK536 and MK536\(_{up}\) were cultured in MM medium at 60°C. Cells were collected at OD\(_{600}\) = 1, and RNA was purified using an RNPurify Bacteria Reagent and RNEasy Mini Kit (Qiagen, Venlo, Netherlands) with gDNA Eraser (Takara Bio). The pyrF transcript was detected using endpoint reverse transcription-polymerase chain reaction (RT-PCR). The RT reaction was performed using a PrimeScript RT reagent Kit (Takara Bio) with the PyrF\(_{TR}\) primer, whereas PCR was performed using Quick Taq HS DyeMix (Toyobo, Osaka, Japan) with two sets of primers: pyrF\(_{TR}\) and pyrF\(_{600R}\) (primer A) and is701\(_{520F}\) and pyrF\(_{200R}\) (primer B). Thermal cycles comprised 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min. The reaction without reverse transcriptase was used as negative control. The transcription of rpoB, which encodes for RNA polymerase β subunit, was detected as positive control using the primers rpoB\(_{2800F}\) and rpoB\(_{3800R}\).

### Southern Blot Analysis of IS\textit{Gka1}/IS\textit{Gka2} Transposition

\textit{Geobacillus kaustophilus} MK536 was precultured in LB medium (5 mL) at 60°C. The cells were washed with sterile water (see above), and an aliquot (10\(^5\)–10\(^6\) cfu) was incubated at 65°C on MU plates for 72 h. Generated colonies were purified on MU plates and used as uracil prototrophs, whereas background cells (without colony formation) were purified on LB plates and used as uracil auxotrophs. Washed cells were incubated at 65°C for as long as possible using media that prevent active cell growth: MC and MN media and LB medium supplemented with kanamycin (5 mg/L) or chloramphenicol (10 mg/L). Cells were recovered on LB plates and screened using MM and MU plates to distinguish between uracil prototrophs and auxotrophs. In addition, washed cells were incubated at 65°C for 24 h. An aliquot of the culture (1 mL) was inoculated in fresh medium and further incubated under the same conditions. This process was repeated an additional five times. Subsequently, cells were colonized on LB plates. Respective clones were analyzed by Southern blotting to detect IS elements of \textit{G. kaustophilus} (IS\textit{Gka1} and IS\textit{Gka2}). Genomic DNA (25 μg) was digested with \textit{DraI} and \textit{MunI}. The products were separated on an agarose gel (0.9%) by electrophoresis and transferred onto a nylon membrane to hybridize with a digoxigenin-labeled DNA probe. The probe was synthesized using a PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) with the primers is701\(_{520F}\) and is701\(_{800R}\). Hybridized DNA was detected using a DIG Nucleic Acid Detection Kit (Roche).

### Generation Assay of Uracil Prototrophs and Rifampicin-Resistant Mutants From MK536 Derivatives

\textit{Geobacillus kaustophilus} strains MK536\(_{p74}\), MK536\(_{rsbV}\), MK536\(_{sigB}\), and MK536\(_{sigX}\) were precultured at 60°C in LB medium (20 mL). After the culture had reached proliferative phase (OD\(_{600}\) = 1) and stationary phase (plus four additional hours of incubation), the cells were collected and washed with sterile water (see above). An aliquot (10\(^5\)–10\(^6\) cfu) was incubated at 65°C for 96 h on MU plates to obtain uracil prototrophs. The aliquot was also incubated on MM plates for 24 h to determine viable cell concentrations. The generation frequency of uracil prototrophs was defined as the ratio of generated uracil prototrophs to incubated viable cells (10\(^5\)–10\(^6\) cfu). In addition,
proliferative and stationary cells were spread on LB plates supplemented with rifampicin (10 mg/L) and incubated at 65°C for 24 h to obtain rifampicin-resistant mutants. The aliquots were also incubated on LB plates for 24 h to determine viable cell concentrations. The generation frequency of rifampicin-resistant mutants was defined as the ratio of generated rifampicin-resistant mutants to incubated viable cells (10⁵–10⁸ cfu).

Statistical and Bioinformatic Analyses
Statistical significance was analyzed using unpaired Student’s t-tests (one-tailed) with Microsoft Excel 2016. The sequence comparison was performed using ClustalW², and IS elements were predicted using ISsaga (Varani et al., 2011).

RESULTS
Unexpected Generation of Uracil Prototrophs From G. kaustophilus MK536
Geobacillus kaustophilus MK536 lacks the pyrF gene essential for pyrimidine biosynthesis. Although the strain harbors intact pyrF under the P_T7 control (Figure 1), the gene is theoretically silent in the absence of T7RP that functions at elevated temperatures. Therefore, G. kaustophilus MK536 is auxotrophic for uracil; however, this strain unexpectedly produced uracil prototrophs when incubated on minimum medium without uracil (MU) at 65°C. The prototrophs appeared following incubation for >48 h (Figure 2A). When a prototroph (termed MK536_up1) was randomly selected and again incubated on MU plates, the majority of cells (>95%) formed colonies within 24 h as similar to those formed by the wild-type strain (HTA426). The prototrophy was stable and remained so throughout subculturing. The generation of uracil prototrophs was more rapid at 70°C but was most frequent at 65°C with prolonged incubation (Figure 2B). The optimal growth temperature of G. kaustophilus is 65°C (Suzuki, 2017); thus, uracil prototrophs were actively generated at temperatures where G. kaustophilus efficiently propagates. Prototrophs were not generated from a control strain lacking the P_T7-pyrF cassette (MK242).

Uracil Prototrophs Carry IS Elements in the pyrF Upstream Region
For uracil prototrophy, it was hypothesized that P_T7 mutations were responsible for T7RP-independent pyrF expression because uracil prototrophs were not generated from G. kaustophilus MK242; thus, we sequenced upstream of pyrF in 49 prototrophs including G. kaustophilus MK536_up1. All the sequences lacked mutations but did contain different IS elements that encoded for DDE-type transposases (Figure 1). The IS elements were precisely flanked by DR that originated from the insertion site; therefore, it was probable that the event resulted from transposition but not from heterologous recombination. Among the 49 elements, 31 and 14 were completely identical with the IS elements at GK0302, GK2085, and GK2942 loci (termed IS_Gka1; Supplementary Figure 1) and those at GK0390 and GK1725.
loci (ISGka2; Supplementary Figure 1), respectively. The ISGka2 sequence was highly homologous to the element at GK0778 locus but was distinguishable via a mutation. The other four sequences were completely identical to the IS element at GK0169 locus (ISGka3; Supplementary Figure 2) or those at GK0015, GK2451, and GK3431 loci (ISGka4; Supplementary Figure 3). The homologs of ISGka3 and ISGka4 were further identified at three (GK1006, GK1161, and GK1712) and five (GK0785, GK0875, GK1016, GK1720, and GKP33) loci, respectively. Based on sequence similarities, ISGka1 and ISGka2 were together classified to the IS701 family, whereas ISGka3 and ISGka4 were classified to the IS4 and ISLre2 families, respectively.

**Insertion Features of the IS Elements**

Table 2 summarizes the classification, position, direction, and DR sequence of IS elements identified at the pyrF upstream site. The ISGka3 sequence was inserted in the parallel direction with the transposase and pyrF genes, whereas ISGka4 was inserted in the opposing direction. In agreement with observations for IS4 and ISLre2 families (Siguier et al., 2015), ISGka3 and ISGka4 generated 9 bp of DR. The ISGka1 and ISGka2 sequences were inserted in both directions and occasionally at distant locations from the pyrF gene. Their transposition generated 4–9 bp of DR in disagreement with the observations of another set of IS701 members, which are known to generate 4 or 5 bp of DR (Siguier et al., 2015). Hot spots were observed immediately upstream of the pyrF gene and were favored by ISGka1 and ISGka2. Consensus sequences were not identified around insertion sites, although hot spots were abundant in adenine and thymine positions (Figure 1B).

**Transcription of pyrF in G. kaustophilus MK536 
MK536up1**

Endpoint RT-PCR analysis showed that the pyrF gene was positively transcribed in MK536up1 but not in MK536; there was a continuous transcript from ISGka1 upstream to pyrF (Figure 2C). Although faint bands were detected for MK536up1 samples without RT via genomic DNA contamination, band signals were stronger for samples with RT. These observations suggested that G. kaustophilus MK536up1 became prototrophic for uracil by pyrF expression via leaky and read-through transcription of the transposase gene and/or via active transcription from another promoter in ISGka1.

**Table 2 | Insertion sequences identified at pyrF upstream in uracil prototrophs.**

| IS element | Insertion site | Direction | Direct repeat | Clone number |
|------------|---------------|-----------|--------------|-------------|
| ISGka2     | −1415/−1409   | Parallel  | 5′-TCAATGA-3′ | 1           |
| ISGka3     | −758/−750     | Parallel  | 5′-GTTGAAAC-3′ | 1           |
| ISGka3     | −461/−453     | Parallel  | 5′-CTTCGAGT-3′ | 1           |
| ISGka1     | −233/−226     | Parallel  | 5′-TAATGTGA-3′ | 1           |
| ISGka4     | −40/−32       | Opposite  | 5′-CTAGAAATA-3′ | 2           |
| ISGka1     | −33/−27       | Parallel  | 5′-TAATTTT-3′ | 2           |
| ISGka1     | −33/−27       | Opposite  | 5′-TAATTTT-3′ | 1           |
| ISGka1     | −27/−21       | Parallel  | 5′-TGTTTAA-3′ | 2           |
| ISGka1     | −23/−20       | Parallel  | 5′-TAAC-3′    | 1           |
| ISGka1     | −23/−16       | Parallel  | 5′-TAACCTTA-3′ | 1           |
| ISGka1     | −23/−16       | Opposite  | 5′-TAACCTTA-3′ | 1           |
| ISGka1     | −23/−15       | Parallel  | 5′-TAACCTTA-3′ | 5           |
| ISGka2     | −23/−17       | Parallel  | 5′-TAACCTTA-3′ | 1           |
| ISGka2     | −23/−16       | Parallel  | 5′-TAACCTTA-3′ | 3           |
| ISGka2     | −23/−14       | Parallel  | 5′-TAACCTTA-3′ | 2           |
| ISGka1     | −14/−9        | Parallel  | 5′-GAAGAGA-3′ | 1 (MK536up1) |
| ISGka1     | −14/−8        | Parallel  | 5′-GAAGAGA-3′ | 3           |
| ISGka1     | −14/−7        | Parallel  | 5′-GAAGAGA-3′ | 3           |
| ISGka2     | −14/−7        | Parallel  | 5′-GAAGAGA-3′ | 5           |
| ISGka2     | −14/−7        | Opposite  | 5′-GAAGAGA-3′ | 1           |
| ISGka1     | −13/−7        | Parallel  | 5′-GAAGAGA-3′ | 2           |

Uracil prototrophs were generated from G. kaustophilus MK536, and the pyrF upstream in 49 prototrophs was sequenced to identify IS elements. The insertion site indicates possible sites where the element was inserted. The position corresponds to the upstream and downstream locations of the sequence that resulted in direct repeats. The position number is based on the original sequence (Figure 1B). The direction indicates that the element was inserted in the parallel or opposite direction for transposase and pyrF genes. An element was inserted between the −23 and −14 positions without direct repeats (ND). G. kaustophilus MK536up1 carries ISGka1 with the parallel direction.
Genome-Wide IS Transposition in *G. kaustophilus* MK536<sub>up1</sub>

Next-generation sequencing of *G. kaustophilus* MK536<sub>up1</sub> provided 8 × 10<sup>6</sup> reads and 169 contigs with 318 depth, which were compared with the complete genome sequence of *G. kaustophilus* HTA426 (Takami et al., 2004b). In addition to ISGka1 that transposed to the *pyrF* upstream region, ISGka2 and an IS element of the IS1634 family were inserted at two and one loci, respectively, (Figure 3). The ISGka2 sequence was flanked by 7 or 8 bp of DR. The IS1634 member was identical to the IS elements at GK0145 and GK3302 loci (termed ISGka5; Supplementary Figure 4) and generated 6 bp of DR. The PCR assays confirmed that these elements were absent at the respective loci in *G. kaustophilus* MK536. Although another IS1634 member was also identified at GK3299 locus, the element already existed in *G. kaustophilus* MK536; thus, it was likely that this transposed during construction of *G. kaustophilus* MK536 from *G. kaustophilus* HTA426. IS deletion was not identified in the genome sequence, which suggested that these elements achieved replicative transposition. Many mutations (134 single nucleotide variants, 50 deletions, and 81 insertions) were identified; however, it was unclear whether these mutations occurred during generation of MK536<sub>up1</sub> from MK536 or during construction of MK536 from HTA426.

Southern Blot Analysis of ISGka1/ISGka2 Transposition

*Geobacillus kaustophilus* MK536 was incubated at 65°C for 72 h on MU plates to isolate uracil prototrophs, and eight clones were analyzed by Southern blotting that collectively detects ISGka1 and ISGka2 (Figure 4A). Six bands were present in the MK536 samples. The band lengths corresponded to the theoretical ones predicted from the sequences around the loci G0778 (7.5 kb), G0285 (2.8 kb), G0302 (2.7 kb), G0390 (2.4 kb), G1725 (2.2 kb), and G2942 (2.0 kb). The original bands remained present in the seven prototrophs; thus, ISGka1 and ISGka2 generally achieved replicative transposition that provided new information on the IS701 family. One prototroph lost the band at GK3020 locus. Because an IS element uses either replicative or non-replicative mechanism, the loss seemed to result from a band shift via IS insertion around the locus, as observed for *G. kaustophilus* MK536<sub>up1</sub> (Figure 3A). A band was further shared at 4.2 kb, which was attributable to the *pyrF* upstream region that carries ISGka1 or ISGka2 on the basis of the theoretical length. Additional bands were also detected in four prototrophs. This supported IS transposition occurring in a genome-wide manner. In addition to uracil prototrophs, background cells that remained uracil auxotrophs were recovered from MU plates and analyzed by Southern blotting to show that the six clones increased the band signals at diverse lengths but not at 4.2 kb (Figure 4B). Notably, successive cultures under non-inhibitory growth conditions in LB medium resulted in undetectable ISGka1/ISGka2 transposition (Figure 4C). These observations implied that ISGka1/ISGka2 transposition extensively occurred in cells (>75%) on MU plates and that cells became uracil prototrophs when an IS element fortuitously transposed to the *pyrF* upstream region.

Culture Conditions for ISGka1/ISGka2 Transposition

*Geobacillus kaustophilus* MK536 was incubated in liquid MU at 65°C for 48 h and then grown on LB plates. Any subsequent colonies were classified as uracil prototrophs or auxotrophs, and eight clones were analyzed by Southern blotting to detect ISGka1 and ISGka2. The signals were changed in five prototrophs and two auxotrophs (Table 3), which suggested that ISGka1/ISGka2 transposition was enhanced not only on MU plates but also in liquid MU. The 4.2 kb signal was not detected in the prototrophs; however, PCR analysis confirmed that the *pyrF* upstream region had lengthened, suggesting that these clones might carry an IS element other than ISGka1 or ISGka2 at the *pyrF* upstream site. Similarly, MK536 cells were incubated in diverse media to prevent the cells from active propagation and then eight clones were recovered on LB plates to analyze ISGka1/ISGka2 transposition. Incubation was performed for 72 h; however, the incubation time was shortened to 48 h when cells were not recovered. Table 3 summarizes the incubation conditions and the number of clones that achieved ISGka1/ISGka2 transposition. Transposition was detected for cells that underwent growth inhibition by kanamycin, chloramphenicol, or carbon or nitrogen starvation. These observations suggested that IS transposition was enhanced under growth inhibitory conditions and may be regulated via a stress response pathway.

Expression of *sigX* Enhances Generation of Uracil Prototrophs From *G. kaustophilus* MK536

To see whether stress response regulators govern IS transposition in *G. kaustophilus*, we constructed MK536 derivatives that forcibly expressed *rsbV* (MK536<sub>rsbV</sub>), *sigB* (MK536<sub>sigB</sub>), and
sigX (MK536<sub>sigX</sub>). The cells were precultured until they reached proliferative and stationary growth phases and then incubated at 65°C on MU plates to determine the generation frequency of uracil prototrophs (Figure 5A). As with G. kaustophilus MK536, the control strain (MK536<sub>p74</sub>) generated prototrophs following incubation for >48 h. Similar observations were also made for MK536<sub>rsbV</sub> and MK536<sub>sigB</sub>. In these strains, the generation frequency was higher in proliferative cells than in stationary cells. Although the stationary cells of MK536<sub>rsbV</sub> and MK536<sub>sigB</sub> exhibited lower frequency than did those of MK536<sub>p74</sub>, the difference was not substantial. In contrast, MK536<sub>sigX</sub> more rapidly and frequently generated uracil prototrophs than did the other strains. Moreover, the generation frequency was comparable between proliferative and stationary cells at >48 h. These observations suggested that IS transposition was enhanced via sigX-dependent stress responses and that the response was stronger in proliferative cells.

**Expression of sigX Has Negligible Effects on Rifampicin-Resistant Mutations**

*Geobacillus* spp. apparently induce mutagenesis when proliferative cells are exposed to rifampicin and efficiently generate rifampicin-resistant cells via *rpoB* mutations (Suzuki et al., 2018). To see whether the mutagenesis depends on sigX-dependent stress responses, MK536 derivatives (MK536<sub>p74</sub>, MK536<sub>rsbV</sub>, MK536<sub>sigB</sub>, and MK536<sub>sigX</sub>) were assessed by generation frequency assay of rifampicin-resistant mutants. Cells were precultured until they reached proliferative and stationary phases and incubated for 24 h at 65°C on LB plates supplemented with rifampicin. Growth colonies were counted to determine that the generation frequency of rifampicin-resistant mutants was higher in proliferative cells than in stationary cells for all of the strains, but substantial differences were not observed between the respective strains (Figure 5B). This suggested that the rifampicin-resistant mutations were independent from sigX-dependent stress responses.

**DISCUSSION**

Isothermal and transcription-based amplification of nucleic acids can be performed using T7RP, which is responsible for strong transcription from P<sub>7</sub> (Niemz et al., 2011). Because amplification performance is potentially improved when conducted at higher temperatures, thermostable T7RP variants are of biotechnological importance (Bouain et al., 2013). This study was originally designed to generate mutant genes for thermostable T7RP variants in *G. kaustophilus* MK534<sub>T7RP</sub>. The strain is auxotrophic for uracil by the ΔpyrF genotype but carries pyrF under control of P<sub>T7</sub> (P<sub>T7</sub>-pyrF); therefore, it could become prototrophic with functional expression of T7RP from pGKE75-T7RP. In addition, this strain lacks genes for DNA repair (*mutS, mutL, mutY, ung*, and *mfd*) and thereby could serve as an error-prone strain. We expected that *G. kaustophilus* MK534<sub>T7RP</sub> would generate mutant genes for thermostable T7RP variants via culture and that such
genes could be found in clones prototrophic for uracil at elevated temperatures. As expected, MK534$_{T7RP}$ generated uracil prototrophs at $65^\circ$C; however, similar prototrophs were intrinsically generated from a control strain that lacked pGKE75-MK242; therefore, we assumed P$_{T7}$ mutations in uracil prototrophs and unexpectedly identified diverse IS elements at the pyrF upstream region (Figure 1). IS elements are known to cause neighboring gene expression from internal promoters or via formation of hybrid promoters (Zhang and Saier, 2016; Vandecraen et al., 2018). In fact, pyrF transcription was detected in MK536$_{up1}$ in contrast to MK536 (Figure 2C). In G. kaustophilus MK536$_{up1}$, a promoter upstream of the transposase gene apparently contributes to pyrF transcription. However, considering several prototrophs carried ISGka1/ISGka2 or ISGka4 at the pyrF upstream region in the opposite direction (Table 2), they seem to harbor promoters with the opposite direction. Possible opposite promoters identified in ISGka1/ISGka2 and ISGka4 are shown in Supplementary Figures 1 and 3, respectively.

**TABLE 4** | IS elements predicted in G. kaustophilus HTA426.

| Location | IS family | Subgroup | Transposase | Copy number | Note |
|----------|-----------|----------|-------------|-------------|------|
| Chromosome | IS3 | IS150 | DDE | 3 | |
| | IS4 | IS231 | DDE | 1 | |
| | IS4 | IS4Sa | DDE | 5 | ISGka3 |
| | IS5 | IS5 | DDE | 1 | |
| | IS6 | IS6 | DDE | 4 | |
| | IS21 | | DDE | 1 | |
| | IS66 | IS6st12 | DDE | 5 | |
| | IS110 | | DEDD | 10 | |
| | IS200/IS605 | HUH/Y1 | | 5 | |
| | IS200/IS605 | IS1341 | HUH/Y1 | 7 | |
| | IS256 | | DDE | 7 | |
| | IS481 | | DDE | 8 | |
| | IS630 | | DDE | 4 | |
| | IS701 | | DDE | 7 | ISGka1/ISGka2 |
| | IS962 | | DDE | 6 | |
| | IS1634 | | DDE | 20 | ISGka5 |
| | ISL3 | | | 10 | |
| | ISLre2 | | DDE | 10 | ISGka4 |
| pHTA426 | IS6 | | DDE | 1 | |
| | IS66 | IS6st12 | DDE | 1 | |
| | ISLre2 | | DDE | 2 | |

The analysis was performed using ISsaga (Varani et al., 2011). Transposase indicates that DDE, DEDD, or HUH/Y1-type transposases are encoded in the family.
at elevated temperatures; thus, this study is the first to identify thermophilic IS elements. Mobile elements can be used for gene discovery and gene delivery (Picardreau, 2010; Narayanavari et al., 2017). Therefore, ISGka1–ISGka5 have the potential to expand the genetic tools for thermophiles.

Southern blot analysis suggested that ISGka1/ISGka2 transposition frequently occurred in a genome-wide manner. Genome-wide transposition has been supported by the MK536_up1 genome, which carries additional IS elements at four loci (Figure 3). ISGka1/ISGka2 transposition was detected not only in cells that became uracil prototrophs but also in background cells that remained uracil auxotrophs; however, transposition was not detected when cells were cultured under growth non-inhibitory conditions (Figure 4). These observations suggest that G. kaustophilus enhances IS transposition in response to pyrimidine deficiency and generated numerous mutants, including uracil prototrophs, where an element was fortunately transposed into the pyrf upstream region. The idea is consistent with growth of uracil prototrophs on MU plates after >48 h incubation. Given that prototrophs had randomly arisen during preculture, the colonies should have appeared within 24 h, as observed for G. kaustophilus strains HTA426 and MK536_up1. Uracil prototrophs identified in liquid MU supposedly carried another type of IS elements in the pyrf upstream region, which seems to perform transposition earlier than ISGka1 or ISGka2 under this condition because prototrophs generated earlier in the liquid culture rapidly grow and could become dominant throughout the subsequent phases. This observation also supports the IS transposition enhanced under pyrimidine deficient conditions.

Stress-induced transposition has been reported for several organisms by respective stressors (Zhang and Saier, 2016; Vandecraen et al., 2018; Lee et al., 2019, 2020). In G. steaothermophilus CU21, IS4 and IS21 members have been reported to achieve transposition during growth inhibition by chloramphenicol exposure (Xu et al., 1993). Notably, ISGka1/ISGka2 transposition was detected in eight clones that underwent growth inhibition by antibiotic exposure or starvation; therefore, IS transposition may be enhanced by growth inhibition regardless of stressors. Although transposition was not observed under similar starvation conditions on solid media, this was attributed to nutrient contaminants in the agar enabling minimal growth under conditions of incomplete starvation. We note that transposition enhanced by growth inhibition helps organisms achieve genetic diversification that results in environmental adaptation exclusively during the period of growth inhibition; in parallel, transposition permitted by hosts is advantageous for IS elements in terms of their propagation. Geobacillus spp. harbor numerous IS elements in their genomes (Suzuki, 2018), which may reflect codendependence relationships between Geobacillus spp. and IS elements for evolutionary diversification and stable persistence, respectively.

* Bacillus subtilis* 168 is a model bacterium that is phylogenetically related to *G. kaustophilus* (Suzuki, 2018). In *B. subtilis*, various stress responses are positively regulated by SigB where the function is repressed by RsbW (anti-SigB) via complex formation, whereas RsbW is released by RsbV (anti-SigB antagonist); therefore, SigB can be activated by RsbV (van Schaik and Abeel, 2005). In *G. kaustophilus*, homologous genes for RsbV, RsbW, and SigB have been identified at the GK3422, GK3423, and GK3424 loci, respectively, (Takami et al., 2004b). Extra-cytoplasmic function (ECF) sigma factors are also known to positively regulate stress responses (van Schaik and Abeel, 2005). Although *B. subtilis* employs multiple ECF sigma factors (e.g., SigM, SigV, SigW, SigX, SigY, and SigZ), only the homologs for sigW and sigX at their respective loci GK0150 and GK2254 have been identified in *G. kaustophilus* (Takami et al., 2004b).

Because IS transposition was apparently enhanced by growth inhibition, we focused on RsbV, SigB, SigW, and SigX as potential regulators that govern IS transposition in *G. kaustophilus* and constructed *rsbV* (MK536_up1), *sigB* (MK536_up1), and *sigX* (MK536_up1) expressers. Despite repetitive trials, neither a sigW expressor nor most deletion mutants (ΔsigW, ΔsigX, ΔrsbV, or ΔrsbW) could be constructed. Notably, uracil prototrophs were rapidly and substantially generated from MK536_up1 in comparison with the other constructs (Figure 5A); therefore, it is possible that *G. kaustophilus* enhances IS transposition via sigX-dependent stress responses. This is the first observation suggesting that a sigma factor regulates IS transposition. In *B. subtilis*, SigX-dependent promoters share tgaAACnwv and CGwCww consensus sequences at −35 and −10 regions, respectively, (Huang and Helmann, 1998). However, similar regions were not found upstream of transposable genes in ISGka1–ISGka5 (Supplementary Figures 1–4), and therefore SigX seems to indirectly regulate IS transposition rather than to directly bind to IS elements. It is also noteworthy that SigX is involved in controlling several processes related to cell envelope modification in *B. subtilis* (Souza et al., 2014). Conceivably, *G. kaustophilus* may enhance IS transposition by sensing cell surface damage driven by growth inhibition via sigX-dependent stress responses.

Uracil prototrophs were equally generated from proliferative and stationary cells of MK536_up1 in contrast to other strains (Figure 5A). This profile suggests that *G. kaustophilus* enhances IS transposition under SigX regulation when proliferative cells are prevented from active propagation, thus potentially enabling immediate adaptation via genetic diversification. *Geobacillus* spp. can form robust endospores in the stationary phase (Suzuki, 2018); therefore, IS transposition may be enhanced as an adaptation strategy specific to the proliferative phase. We previously observed that exposure of *G. kaustophilus* to rifampicin apparently induced mutagenesis to produce rifampicin-resistant mutants and the induction was stronger in proliferative cells than in stationary cells (Suzuki et al., 2018). Although this manner implies that mutagenesis may be also governed by SigX in parallel to IS transposition, rifampicin-resistant mutants were comparably generated between MK536_up1 and MK536_up1 (Figure 5B); therefore, mutagenesis is not under the SigX control. Multiple mechanisms appear to be employed to generate genetic diversification in *G. kaustophilus* and potentially other *Geobacillus* spp. This characteristic may be a primary reason why *Geobacillus* spp. are highly adaptive organisms.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA database PRJNA699136.

AUTHOR CONTRIBUTIONS

HS had conceived the experiment plan, supervised the experiment process, and wrote the original manuscript. JK examined T7RP mutations and identified IS transposition. MT and MO analyzed the transposition frequency and insertion sites of IS elements. TT performed the Southern blot and mutation assay. MO performed the transcriptome and genome analyses. TO had supervised the experiment process. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.650461/full#supplementary-material

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Conflict of Interest: 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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