A previous report described the presence of a self-splicing group I intron in a flagellin gene from a thermophilic Bacillus species. Here, we present evidence that the splicing reaction of the flagellin intron is dependent on temperature. Furthermore, a complementation analysis using a Bacillus subtilis flagellin-deficient mutant indicated that the intron-containing flagellin gene significantly restored the motility of the mutant at higher temperatures.

Key words: ribozyme; group I self-splicing intron; flagellin; bacterial intron; thermophilic Bacillus

The intron is a nucleotide sequence that interrupts a gene and is not translated into the amino acid sequence although it is transcribed into mRNA. To express a functional gene, splicing that involves intron excision with post-transcriptional processing is required. Many functions of introns have been reported for eukaryotic genomes, and it is possible that some introns are essential for mRNA diversity (alternative splicing) and the proper timing of gene expression.1–3) The functions of most bacterial introns, however, have not yet been clarified. All eubacterial introns are classified into two types of self-splicing ribozymes (group I and, group II introns), which are able to excise themselves from precursor mRNA transcripts without any other enzymes. Group I introns are known to be widely distributed in both prokaryotes and eukaryotes.4) They are characterized by conserved RNA secondary structures that are essential for splicing reactions in vivo and in vitro.5) Bacterial group I introns are found more abundantly in non-protein coding genes, such as tRNA or rRNA.6,7) In contrast, only a few genes have been reported to have intron-containing protein coding genes of genomic DNA. These are notably housekeeping elements involved in DNA metabolism and repair functions, except in the case of flagellin.8–11) Flagellin is an essential protein for bacterial motility by the flagellar motor. The principal component of the bacterial flagellum is a long helical filament composed of 20,000 monomeric subunits of flagellin that functions as a propeller to push the cell forward. Bacterial motility is important in obtaining the proper timing of gene expression.1–3) The functions of introns have been reported for eukaryotic genomes, and it is possible that some introns are essential for splicing reactions in vivo and in vitro.4) They are characterized by conserved RNA secondary structures that are essential for splicing reactions in vivo and in vitro.5) Bacterial group I introns are found more abundantly in non-protein coding genes, such as tRNA or rRNA.6,7) In contrast, only a few genes have been reported to have intron-containing protein coding genes of genomic DNA. These are notably housekeeping elements involved in DNA metabolism and repair functions, except in the case of flagellin.8–11) Flagellin is an essential protein for bacterial motility by the flagellar motor. The principal component of the bacterial flagellum is a long helical filament composed of 20,000 monomeric subunits of flagellin that functions as a propeller to push the cell forward. Bacterial motility is important in obtaining optimal environmental conditions. However, even in the absence of the flagellin gene, cells can grow in an artificial optimized medium. The results of a database search with BLASTN, indicated that group I introns and corresponding sequences in the flagellin genes have been found largely in gram-positive thermophiles of Firmicutes.11) Here, these flagellin introns must be stable and must be spliced in vivo at normal growth temperatures, which are between 50 and 70 °C. Our previous study found that Geobacillus steaerothermophilus (NBRC 12550, type strain) produces a mature flagellin ORF by an intron-splicing reaction in vivo, and that this intron showed splicing independently under non-physiological conditions (e.g., 25 mM HEPES pH 7.5, 15 mM MgCl₂, 0.2 mM GTP, 1.0 mM KCl, 60 °C) in vitro.11) We focused on the group I introns of flagellin genes from two gram-positive thermophilic bacteria, Geobacillus steaerothermophilus and Bacillus sp. Kps3 (DDBJ accession nos. AB453704 and AB453702). These introns were specific for the bacterial flagellin genes, rather than the homing endonuclease genes, which also form part of the mobile genetic element.12) In the present study, we found that the in vitro self-splicing reaction of the flagellin group I intron was promoted by temperature, and that a flagellin gene interrupted by an intron produced a functional flagellin protein in Bacillus subtilis.

Our previous study confirmed that the flagellin intron of G. steaerothermophilus has the same exon-intron boundaries between in vivo and in vitro splicing products.11) The flagellin intron of Bacillus sp. Kps3 is highly homologous to that of G. steaerothermophilus with regard to insertion site, intron sequences, and predicted intron folding (Fig. 1A–C). To confirm the splicing of the Bacillus sp. Kps3 flagellin intron, we attempted isolate the total RNA from late-log phase culture, and cDNA was synthesized by RT-PCR in accordance with our previous study.11) The PCR products using flagellin specific primer pairs (FLG_D1 and FLG_D1_Gst, FLG_D1 and FLG_D1_Kps3) from genomic DNA and cDNA were resolved on an agarose gel. Two disparate bands corresponding to the flagellin gene fragment containing the intron (unspliced) and the approximately 360-bp shorter fragment (spliced) were detected in both flagellin genes. The absence of the unspliced fragment was also confirmed in the RT-PCR product (Fig. 1D). These results indicate that the group I introns of these bacteria were almost completely spliced in vivo.

These flagellin introns must always be subjected to high temperatures, because under natural environmental
conditions the host microorganisms grow preferentially between 60 and 70 °C. The introns must be stable at these temperatures for an efficient self-splicing reaction to occur. To determine at what temperatures the group I introns could be self-spliced, in vitro splicing was carried out at various temperatures (20, 30, 40, 50, 60, 70, 80, 90, and 100 °C). First we constructed plasmids pEThagGst[+int] and pEThagKps3[+int] as in our previous study.11) These plasmids were based on plasmid pET41b(+) (Novagen, Madison, WI), and the intron-containing flagellin genes from *G. stearothermophilus* and *Bacillus sp. Kps3* were cloned into plasmid pET41b(+) under the control of a T7 promoter. The RNAs used in the self-splicing assays were synthesized with an *in vitro* Transcription T7 Kit (Takara Bio Inc., Shiga, Japan) from XhoI-digested fragments of these plasmids. The splicing reaction was carried out as in the previous study with slight modification in reaction temperature.11)

The *in vitro* spliced products were confirmed by RT-PCR with the primers used for *in vivo* splicing analysis. Both the resulting RT-PCR products from *G. stearothermophilus* and *Bacillus sp. Kps3* indicated that the splicing reaction occurred at a wide range of temperatures, and that splicing efficiency increased with rising temperatures (maximum efficiency at 70 °C) (Fig. 2A).

We also analyzed the nucleotide sequences of each RT-PCR product and confirmed that all the reactions occurred at the same position as those of the *in vivo* splicing product (data not shown). Additionally, to determine whether high temperature is a prerequisite for the complete splicing reaction of the flagellin intron, the production of the joined exon from the *Bacillus* sp. Kps3 flagellin gene was monitored (Fig. 2B). The splicing
reaction was completed at 40°C in 3 h, but 30°C, it was not completed for the lower reactivity.

In order to evaluate functional gene expression in the Bacillus sp. Kps3 flagellin gene with or without the intron, we used a heterologous expression system using Bacillus subtilis.13,14) The Bacillus sp. Kps3 flagellin gene was PCR-amplified with primers FLG01 and FLG04Kps3 from genomic DNA and cloned into plasmid pBHP101,14) which was derived from Escherichia coli-Bacillus subtilis shuttle vector pHP13.15) Using plasmid pBHP101, the gene could be expressed under the control of the Bacillus subtilis flagellin promoter. The resulting plasmid was labeled pBHPagKps3[+int], and the intron-less version, pBHPagKps3[−int], was constructed from the RT-PCR product with the same primer pair. These plasmids were introduced into Bs/C1hag (the flagellin knockout Bacillus subtilis strain), and the resulting transformants of single colonies were spotted onto a soft LB agar plate. Because it has been suggested that flagellin glycosylation is indispensable for G. stearothermophilus flagellar filament formation, it was not possible to use the G. stearothermophilus flagellin gene in the Bs/C1hag complementation assay.13,14) The results for motility complementation at three different temperatures are shown in Fig. 3. Swarming assays were repeated three times independ-
At present, the role of the flagellin intron has not been determined, and the presence of an intron in the flagellin gene expression or motility of the host species does not affect Bacillus species does not affect the flagellin gene expression or motility of the host microorganisms. In view of heterologous flagellin gene expression, it has been suggested that the splicing activity might be related to regulation of flagellin gene expression that occurs under lower temperatures (Fig. 3). Understanding the characteristics of these introns should improve our knowledge of thermophilic bacterial splicing and may help eventually in identifying the functions of these genes.

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References