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# Identification of a Novel Aminopropyltransferase Involved in the Synthesis of Branched-Chain Polyamines in Hyperthermophiles

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**Longer- and/or branched-chain polyamines are unique polycations found in thermophiles. $N^4$-aminopropylspermine is considered a major polyamine in *Thermococcus kodakarensis*. To determine whether a quaternary branched penta-amine, $N^4$-bis(aminopropyl)spermidine, an isomer of $N^4$-aminopropylspermine, was also present, acid-extracted cytoplasmic polyamines were analyzed by high-pressure liquid chromatography, gas chromatography (HPLC), and gas chromatography-mass spectrometry. $N^4$-bis(aminopropyl)spermidine was an abundant cytoplasmic polyamine in this species. To identify the enzyme that catalyzes $N^4$-bis(aminopropyl)spermidine synthesis, the active fraction was concentrated from the cytoplasm and analyzed by linear ion trap–time of flight mass spectrometry with an electrospray ionization instrument after analysis by the MASCOT database. TK0545, TK0548, TK0967, and TK1691 were identified as candidate enzymes, and the corresponding genes were individually cloned and expressed in *Escherichia coli*. Recombinant forms were purified, and their $N^4$-bis(aminopropyl)spermidine synthesis activity was measured. Of the four candidates, TK1691 (BpsA) was found to synthesize $N^4$-bis(aminopropyl)spermidine from spermidine via $N^4$-aminopropylspermidine. Compared to the wild type, the *bpsA*-disrupted strain DBP1 grew at 85°C with a slightly longer lag phase but was unable to grow at 93°C. HPLC analysis showed that both $N^4$-aminopropylspermidine and $N^4$-bis(aminopropyl)spermidine were absent from the DBP1 strain grown at 85°C, demonstrating that the branched-chain polyamine synthesized by BpsA is important for cell growth at 93°C. Sequence comparison to orthologs from various microorganisms indicated that BpsA differed from other known aminopropyltransferases that produce spermidine and spermine. BpsA orthologs were found only in thermophiles, both in archaea and bacteria, but were absent from mesophiles. These findings indicate that BpsA is a novel aminopropyltransferase essential for the synthesis of branched-chain polyamines, enabling thermophiles to grow in high-temperature environments.**

Polyamines are small, positively charged aliphatic molecules containing more than two amine residues present in almost all living organisms. Putrescine [4], spermidine [34], and spermine [343] are polyamines commonly observed in the cells of various living organisms, from viruses to humans (1–4). Polyamines are important in cell proliferation and cell differentiation (5, 6), as well as contributing to adaptation to various stresses (7). Interestingly, in addition to common polyamines, thermophiles contain two types of unusual polyamines as major polyamines. One type consists of long linear polyamines such as caldopentamine [3333] and caldohexamine [33333], and the other consists of branched polyamines such as $N^4$-aminopropylnorspermidine [3(3)3], $N^4$-aminopropylspermidine [3(3)4], tetrakis-(3-aminopropyl)ammonium [3(3)(3)3], and $N^4$-bis(aminopropyl)spermidine [3(3)(3)4], where the numbers in brackets indicate the number of methylene ($CH_2$) units between $NH_2$, NH, N, or $N^+$ (8–17). Because the relative amounts of long/branched-chain polyamines in cells of (hyper)thermophiles were found to increase as growth temperatures increased, these unique polyamines are regarded as supporting the growth of thermophilic microorganisms under high-temperature conditions (18–20). An *in vitro* study indicated that long-chain and branched-chain polyamines effectively stabilized DNA and RNA, respectively (21), suggesting that these unique polyamines enhance translation efficiency under high-temperature conditions (22, 23).

Polyamines are generally synthesized from amino acids such as arginine, ornithine, and methionine (1, 5, 24). In most eukaryotes, putrescine is synthesized directly from ornithine by ornithine decarboxylase. Plants and some bacteria possess additional or alternative putrescine biosynthesis pathways, in which putrescine is synthesized from arginine via agmatine (3, 25, 26). In this pathway, agmatine is synthesized by arginine decarboxylase and then converted to putrescine by agmatine ureohydrolase or a combination of agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase. Spermidine and spermine are then produced by the addition of the aminopropyl group from decarboxylated *S*-adenosylmethionine (dcSAM). In contrast, thermophilic bacteria and archaea possess a unique polyamine biosynthetic pathway, in which spermidine is synthesized from agmatine via $N^1$-aminopropylagmatine by aminopropyltransferase, followed by ureohydrolase (18, 20, 27).

A sulfur-reducing hyperthermophilic archaeon, *Thermococcus kodakarensis* KOD1, grows at temperatures between 60 and 100°C

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**TABLE 1** Strains and primers used in this study

| Strain or primer | Relevant characteristic(s) or sequence (5′–3′)[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | $F^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE44* $\lambda^-$ *thi-1 gyrA96 relA1* | Stratagene |
| BL21-CodonPlus(DE3)–RIL | *E. coli* B $F^-$ *ompT hsdS*($r_B^-$ $m_B^-$) *dcm*$^+$ Tet$^r$ *gal* λ(DE3) *endA* Hte [*argU ileY leuW*; Cam$^r$] | Agilent Technologies |
| *T. kodakarensis* | | |
| KU216 | Δ*pyrF* | 34 |
| DAD | Δ*pdaD* Δ*pyrF* | 32 |
| DBP1 | Δ*pdaD* Δ*bpsA*::*pdaD* Δ*pyrF* | This study |
| Primers | | |
| tk0545-Fw | GGAAAACCATATGATGGCTGGAAAGGTCAG | This study |
| tk0545-Rv | GGAATTCTCAGAAGACGTTTACCTTGTCCT | This study |
| tk0548-Fw | GGAAAACCATATGATGGCGCTGAGCGACAG | This study |
| tk0548-Rv | GGAATTCTTAAACGAGCTTTTTCTCCTTCA | This study |
| tk0967-Fw | AAAAAAACATATGATGAGGATCGAAAGGCTGAA | This study |
| tk0967-Rv | AGAATTCTCAAATAAGCTCCCTCTCCG | This study |
| tk1691-Fw | AAAAAAACATATGATGAGGGAGATAATTGAGAG | This study |
| tk1691-Rv | AGAATTCTCAGGTAGTCGAGCTCTCCT | This study |
| tk1691-up1000-Fw | TTCCCCTTCTCATCGACATC | This study |
| tk1691-down1000-Rv | AATCTAGAACGTCTCCCAGATCAGC | This study |
| tkpdaD-Fw1 | AAGGATCCCGAGAATGATGTTTTAGC | This study |
| tkpdaD-Rv2 | GACTAGTTCAGTAGGGGAACATGAC | This study |
| inv-TK1691-Fw | GACTAGTGCCTTTCTGATTTATTTT | This study |
| inv-TK1691-Rv | AAGGATCCATCTCACACCTCCAGAAG | This study |
| tk1691_out_1 | GTTCTTATTTTTTTGTTTGT | This study |
| tk1691_out_2 | AAAAAAAATTAATTAGCCACGCACCCCCTAGGG | This study |
| tk1691_in_1 | GAGGCTCGCGAAGAAGAAGG | This study |
| tk1691_in_1 | ACGAATATCGCGCCCTCCTC | This study |

[a] Underlined sequences indicate restriction enzyme sites.

but optimally at 85°C (28–31). Our previous study found that *T. kodakarensis* PdaD (TK0149) catalyzed the synthesis of agmatine, the first step in polyamine biosynthesis, and was essential for cell growth (32). Agmatine is also a precursor in the synthesis of agmatidine, an agmatine-conjugated cytidine found at the anticodon wobble position of archaeal tRNA$^{Ile}$ (33). Our genetic study revealed that TK0147 and TK0882 encode $N^1$-aminopropylagmatine synthase and $N^1$-aminopropylagmatine ureohydrolase, respectively, in the production of spermidine (20). Interestingly, larger quantities of agmatine accumulated in strain DAT, in which TK0147 is disrupted, than in the parental KU216 strain. An *in vitro* study also revealed that TK0147 encodes $N^1$-aminopropylagmatine synthase rather than spermidine synthase. Moreover, this pathway by which spermidine is synthesized via $N^1$-aminopropylagmatine is also found in a thermophilic bacterium *Thermus thermophilus*, suggesting that this pathway is characteristic of (hyper) thermophiles (18). The mechanism underlying the synthesis of further branched-chain polyamines is unclear, although these branched-chain polyamines are likely functionally important at higher temperatures. Slight amounts of branched-chain polyamines were produced by the TK0147 disruptant strain DAT, with these amounts increased by the addition of spermidine, suggesting that branched-chain polyamines are synthesized *in vivo* by an as-yet-unidentified aminopropyltransferase other than TK0147. Based on sequence similarity with known aminopropyltransferases, including spermidine and thermospermine synthases, no suitable candidates other than TK0147 were found in *T. kodakarensis*. In the present study, we identified a novel aminopropyltransferase that produced branched-chain polyamines from a *T. kodakarensis* extract. Since $N^4$-bis(aminopropyl)spermidine cannot be distinguished from $N^4$-aminopropylspermine [3(3)43] by high-pressure liquid chromatography (HPLC) analysis (17), the conditions were modified to separate these two isomers by HPLC. In addition, cytoplasmic polyamines were reanalyzed by gas chromatography (GC) and GC-mass spectrometry (MS) to determine whether the quaternary branched penta-amine $N^4$-bis(aminopropyl)spermidine, an isomer of $N^4$-aminopropylspermine, was present in *T. kodakarensis*.

## MATERIALS AND METHODS

**Microorganisms and media.** *T. kodakarensis* KOD1 (28) and its derivatives were cultivated anaerobically in a nutrient-rich medium (ASW-YT) containing 2.0 g of elemental sulfur (ASW-YT-S$^0$) or pyruvate (ASW-YT-Pyr) liter$^{-1}$ (29). For solid medium, 1% Gelrite (Wako, Osaka, Japan) was added. The stains used in the present study are summarized in Table 1. *Escherichia coli* strains were routinely cultivated at 37°C in Luria-Bertani (LB) medium, with ampicillin (50 μg ml$^{-1}$) and/or chloramphenicol (25 μg ml$^{-1}$) added to the medium when needed.

**Polyamine analysis.** *T. kodakarensis* strain KU216 (Δ*pyrF*) (34) was cultivated in ASW-YT-S$^0$ medium at 85°C until reaching the log phase and then harvested. Cells were disrupted in cold 1.5 M perchloric acid (PCA) by sonication for HPLC, GC, and GC-MS analyses. For HPLC analysis, caldohexamine [33333] was added to the mixture (final concentration, 3 mM) as an internal standard to control for extraction and separation losses. The mixture was centrifuged, and the supernatant was filtered with a 0.45-μm-pore-size Millex-LH filter (Millipore, Bedford, MA). Each supernatant (100 μl) was analyzed by HPLC on a CK-10S



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cation-exchange column (6.0 mm [inner diameter] by 50 mm; GL Science, Tokyo, Japan). The column was equilibrated with a modified elution buffer (100 mM potassium citrate monohydrate, 2.0 M KCl, 650 mM 2-propanol, 2.4 mM Brij35 [Wako]; pH 3.2 [adjusted by adding 65.0 ml of 3 M HCl per liter]) at a flow rate of 1.0 ml $min^{-1}$ at 70°C. The eluted polyamines were automatically mixed with a detection buffer composed of 400 mM boric acid, 400 mM NaOH, 4.9 mM Brij35, 7.5 mM *o*-phthalaldehyde, 171 mM ethanol, and 28 mM 2-mercaptoethanol at a flow rate of 0.5 ml $min^{-1}$ at 70°C and monitored with a fluorescence detector (GL-7453A; GL Science). dcSAM was kindly provided by Akira Shirahata, Faculty of Pharmaceutical Sciences, Josai University.

Polyamines were analyzed by GC and GC-MS as described, with slight modifications (17). PCA extracts were loaded onto a Dowex 50WX8 column to concentrate polyamines. After heptafluorobutyrization of the purified polyamine samples, GC was performed on a Shimadzu GC-17A equipped with a capillary column of Inert Cap 1MS (0.32 mm [inner diameter] by 30 m; GL Sciences), and GC-MS was performed on a JEOL JMS-700 equipped with a capillary column of Inert Cap 1MS. The heptafluorobutyryl derivatives of the polyamines were identified by GC-MS. Spermidine [34] and spermine [343] were purchased from Sigma (St. Louis, MO). Caldohexamine [33333] and $N^4$-aminopropylspermidine [3(3)4] were synthesized as described previously (35, 36). $N^4$-Bis(aminopropyl)spermidine [3(3)(3)4] was synthesized with a slight modification of the previous procedure (36). $N^4$-Aminopropylspermine [3(3)43] was prepared using a similar protocol reported in the literature (37). A detailed description of the synthesis of the latter two polyamines will be published elsewhere. Polyamines were analyzed by HPLC as reported above, with slight modifications.

**Measurement of aminopropyltransferase activity.** Aminopropyltransferase activity was measured as described with slight modifications (20), with the products of enzymatic reactions analyzed by HPLC. Each reaction mixture (200 μl) contained 100 mM acceptor substrate (spermidine, spermine, and $N^4$-aminopropylspermidine), 100 mM donor substrate dcSAM, and a crude extract of *T. kodakarensis* KU216 in 10 mM CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid)-NaOH buffer (pH 9.0). After incubation at 70°C for 5 min, 200 μl of each reaction mixture was filtered using a 0.45-μm-pore-size Millex-LH filter and analyzed by HPLC according to the procedure described above for analyzing polyamines. The quantities of enzyme products were calculated by measuring the peak areas on the chromatograms. As standards, various amounts of spermidine [34], spermine [343], $N^1$-aminopropylagmatine, $N^4$-aminopropylspermidine [3(3)4], $N^4$-aminopropylspermine [3(3)43], and $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] were analyzed by HPLC, and the peak areas on the chromatograms were measured.

**Protein fractionation for nano-LC-MS/MS analysis.** *T. kodakarensis* KU216 was cultivated in 15 liters of ASW-YT-$S^0$ liquid medium at 85°C until reaching the log phase. The harvested cells were suspended in 20 ml of buffer A (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol) and disrupted by sonication on ice. After the supernatant was obtained by centrifugation, ammonium sulfate was added to 40% saturation. The supernatant was obtained by centrifugation, and ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation, dissolved in buffer A, and dialyzed against buffer A. The solution was applied to a 100-ml Super Q anion-exchange column (Tosoh, Tokyo, Japan), followed by elution with a stepwise gradient of 250, 300, 350, 400, 450, and 500 mM NaCl in Tris-HCl buffer (pH 7.5). The fractions eluted by 250 mM NaCl with enzymatic activity for the production of $N^4$-bis(aminopropyl)spermidine from dcSAM and spermidine were collected and dialyzed against buffer A. This sample was applied to a 5-ml HiTrap Q anion-exchange column (GE Healthcare, WI), followed by elution with a linear gradient of NaCl (0 to 1.0 M). The fractions with enzymatic activity were collected, dialyzed against buffer A, reapplied to the same HiTrap Q column, and eluted with a linear gradient of NaCl (200 to 400 mM). Fractions with $N^4$-bis(aminopropyl)spermidine synthesis activity were collected, dialyzed against 20 mM phosphate buffer (pH 6.5), and applied to a 5-ml HiTrap SP cation-exchange column (GE Healthcare). The unbound flow-through fractions were collected, concentrated with an Amicon Ultra-3K device (Millipore), applied to a Superdex 200 HR 10/30 gel filtration column (GE Healthcare), and eluted with buffer A containing 200 mM NaCl. Fractions with enzymatic activity were collected and concentrated. Chromatography on the gel filtration column was repeated twice under the same conditions. The active fractions were concentrated and applied to an SDS-PAGE preparatory gel. The thick bands were cut out, dehydrated in acetonitrile, and alkylated by incubation with 55 mM iodoacetamide for 90 min. The product was digested with trypsin (Trypsin Gold; Promega, WI) overnight at 37°C, desalted by Zip-Tip (Millipore), and subjected to linear ion trap–time of flight mass spectrometry (LIT-TOFMS; Nano Frontier LD; Hitachi High Technologies, Tokyo, Japan) with an electrospray ionization (ESI) trap instrument. The results were analyzed by the MASCOT database (Matrix Science, SC) using the following criteria: database, *T. kodakarensis* genome; enzyme, trypsin; missed cleavage, 1; fixed modification, carbamideomethyl; protein mass, no restriction; peptide mass tolerance, ±0.5 Da; and fragment mass tolerance, ±0.5 Da.

**Expression and purification of the candidate proteins.** The genes examined here are located at the following sites on the *T. kodakarensis* genome: *TK0545*, bp 465352 to 466569 (+); *TK0548*, bp 467635 to 468804 (−); *TK0967*, bp 844599 to 845645 (+); and *TK1691*, bp 1488064 to 1489119 (−). The *TK0545*, *TK0548*, *TK0967*, and *TK1691* genes were amplified by using the primer pairs tk0545-Fw/tk0545-Rv, tk0548-Fw/tk0548-Rv, tk0967-Fw/tk0967-Rv, and tk1691-Fw/tk1691-Rv, respectively (Table 1). These amplified fragments were separately cloned into the NdeI/EcoRI sites of pET21a, yielding plasmids pTK0545, pTK0548, pTK0967, and pTK1691, respectively. These plasmids were used to transform *E. coli* BL21-CodonPlus(DE3)–RIL cells, which were grown in LB medium containing 100 μg of ampicillin $ml^{-1}$ at 37°C for 6 h. After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h, the cells were harvested by centrifugation, resuspended in buffer A, and disrupted by sonication. Cell debris was removed by centrifugation, and each supernatant was incubated at 70°C for 30 min and then centrifuged again. Each resultant supernatant was applied to a 5-ml HiTrap Q anion-exchange column and eluted with a linear gradient of NaCl (0 to 1.0 M) in buffer A. Each purified protein was dialyzed against buffer A. To purify TK1691, ammonium sulfate was added to the soluble fraction to give 70% saturation. The precipitate was collected by centrifugation, dissolved in buffer A, dialyzed against the same buffer, and applied to a 5-ml HiTrap Q anion-exchange column. The column was eluted with a linear gradient of NaCl (0 to 1.0 M) in buffer A. Fractions containing TK1691 (in 500 to 550 mM NaCl) were collected and applied to a Superdex 200 HR10/30 gel filtration column (GE Healthcare) in buffer A containing 200 mM NaCl. The protein concentration was determined by a Bradford dye-binding assay, using bovine serum albumin as a standard (38).

**Construction of a *TK1691* deletant.** The principles underlying the disruption of specific genes in *T. kodakarensis* have been described (see Fig. 5A) (39). The vector for disrupting the *TK1691* gene through double-crossover homologous recombination was constructed using the following procedures. Using *T. kodakarensis* genomic DNA as a template, the *TK1691* gene, along with its 5′- and 3′-flanking regions (ca. 1,000 bp each), was PCR amplified using the primers tk1691-up1000-Fw and tk1691-down1000-Rv. The resulting DNA fragment was cloned into the EcoRV/XbaI sites of pUD2, resulting in the plasmid pUD2-TK1691. Similarly, the *pdaD* gene, along with 100 bp of its 5′-franking region, was PCR amplified from *T. kodakarensis* genomic DNA using the primers tkpdaD-Fw1 and tkpdaD-Rv1. The region encoding *TK1691* in pUD2-TK1691 was removed by inverse PCR with the primers inv-tk1691-Fw and inv-tk1691-Rv, and the resultant PCR-amplified DNA fragment was cloned into the SpeI/BamHI sites of the PCR-amplified DNA fragment containing the *pdaD* gene and its 5′-flanking region.

The resulting disruption vector, pUD2-Δtk1691::pdaD, was used to delete the *TK1691* gene from the host strain, yielding *T. kodakarensis* DAD

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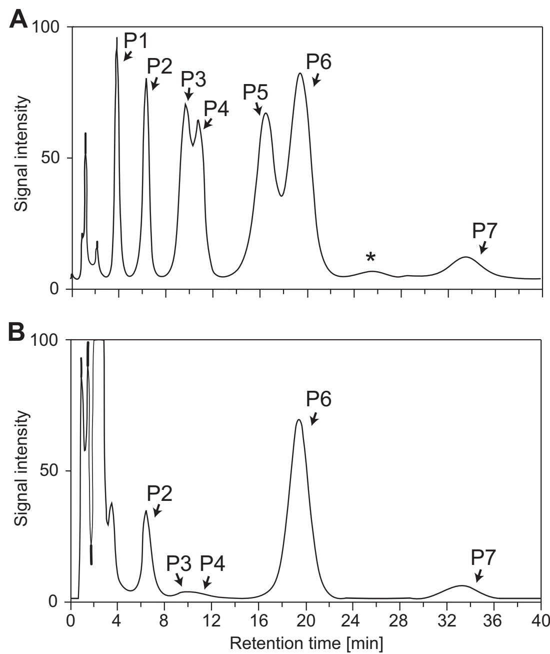


**FIG 1** Intracellular polyamines in *T. kodakarensis* analyzed by HPLC. (A) Peak standard; (B) Intracellular polyamines in *T. kodakarensis*. The *T. kodakarensis* KU216 strain was cultivated in ASW-YT-S$^0$ medium at 85°C until reaching mid-logarithmic phase. The intracellular composition of polyamines in the trichloroacetic acid was analyzed by HPLC. Abbreviations: P1, putrescine [4]; p2, spermidine [34]; P3, $N^4$-aminopropylspermidine [3(3)4]; P4, spermine [343]; P5, $N^4$-aminopropylspermine [3(3)43]; P6, $N^4$-bis(aminopropyl)spermidine [3(3)(3)4]; P7, caldohexamine [33333]. The numbers in brackets represent the number of methylene $CH_2$ chain units between $NH_2$, NH, N, and $N^+$. An asterisk indicates an unknown peak.

($\Delta pdaD$ $\Delta pyrF$) (32). Gene deletion was confirmed by nucleotide sequencing.

## RESULTS

**Composition of intracellular polyamines in *T. kodakarensis*.** Since previous HPLC analysis was unable to distinguish $N^4$-aminopropylspermine from its quaternary branched penta-amine isomer, $N^4$-bis(aminopropyl)spermidine (17), the latter may be present in *T. kodakarensis* cells. $N^4$-bis(aminopropyl)spermidine and $N^4$-aminopropylspermine, however, were clearly separated with a modified buffer, which had a more acidic pH and higher KCl concentration than the previous buffer (20) (Fig. 1A). Using these conditions, $N^4$-bis(aminopropyl)spermidine was found to be a major polyamine of *T. kodakarensis* (Fig. 1B); however, a peak corresponding to $N^4$-aminopropylspermine was not detected. To confirm that $N^4$-bis(aminopropyl)spermidine is a major polyamine in *T. kodakarensis*, acid-extracted cytoplasmic polyamines were analyzed by GC and GC-MS. Two major peaks, corresponding to $N^4$-aminopropylnorspermidine and $N^4$-aminopropylspermidine, were detected (Fig. 2A). Since $N^4$-bis(aminopropyl)spermidine is converted to $N^4$-aminopropylnorspermidine and $N^4$-aminopropylspermidine during GC and GC-MS analyses (40), peaks 1 and 2 in Fig. 2A correspond to $N^4$-aminopropylnorspermidine and $N^4$-aminopropylspermidine (Fig. 2B), respectively, indicating that $N^4$-bis(aminopropyl)spermidine is a major polyamine in *T. kodakarensis*, whereas $N^4$-aminopropylspermine is not. The peak identification in the previous study was incorrect. The amounts of

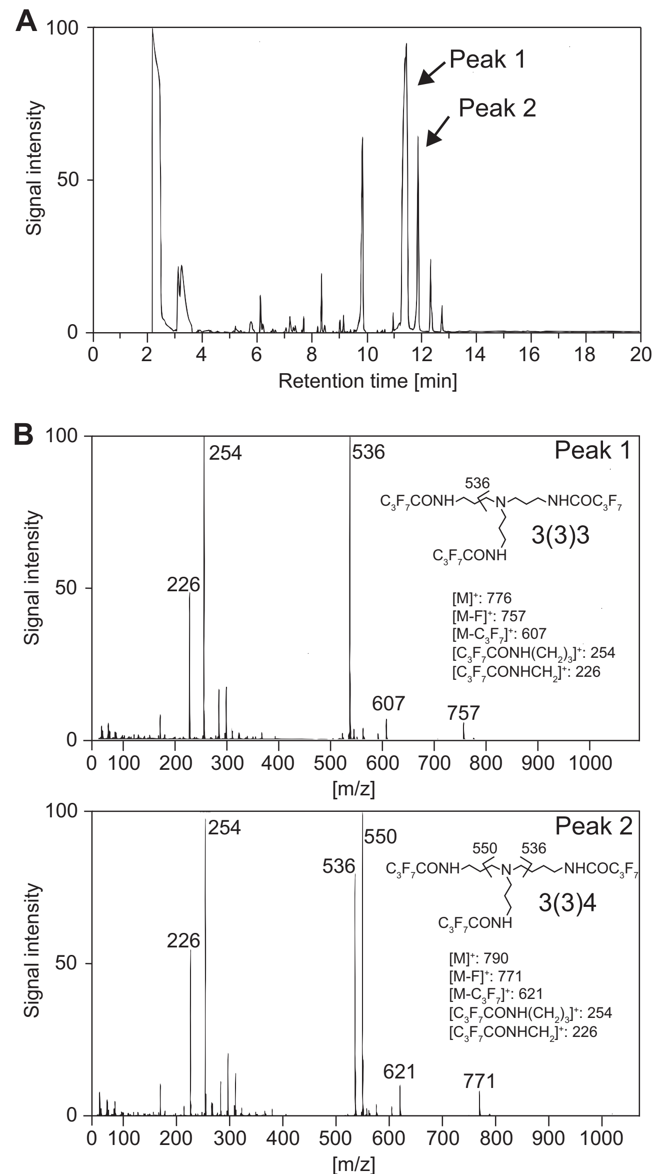


**FIG 2** GC and GC-MS analyses of a *T. kodakarensis*. (A) GC of a *T. kodakarensis* KU216 cell extract after derivatization to heptafluorobutyl compounds. (B) GC-MS analysis of peaks 1 (upper panel) and 2 (lower panel), eluted at 11.5 and 11.9 min, respectively. Polyamines identified by GC-MS are indicated in abbreviated forms by the numbers of methylene chain units. The molecular weights of the heptafluorobutylated polyamine, $[M-F]^+$, $[M-C_3F_7]^+$, and two other fragments corresponding to major MS peaks are shown in each panel.

major intracellular polyamines, spermidine [34] (shown as peak P2 in Fig. 1B), $N^4$-aminopropylspermidine [3(3)4] (peak P3), spermine [343] (peak P4), and $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] (peak P6) were 2.19, 1.00, 0.91, and 3.32 $\mu$mol g$^{-1}$ in wet cells, respectively.

**Identification of $N^4$-bis(aminopropyl)spermidine synthase.** To identify the enzyme that catalyzes $N^4$-bis(aminopropyl)spermidine synthesis, *T. kodakarensis* KU216 cells cultivated at 85°C were disrupted by sonication, and the cytoplasmic fraction was concentrated by ammonium sulfate precipitation, anion- or cation-exchange chromatography, and gel filtration. The fractions

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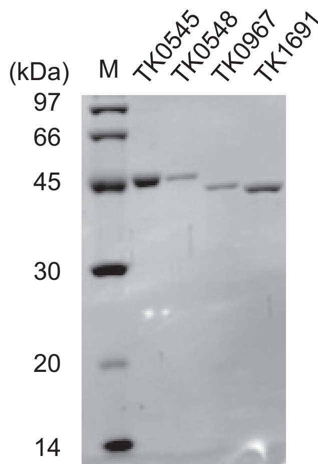


**FIG 3** SDS-PAGE with Coomassie brilliant blue staining of purified recombinant proteins. Purified recombinant proteins TK0545, TK0548, TK0967, and TK1691 are shown in their respective lanes. Lane M, molecular mass markers.

containing the enzyme were identified by monitoring $N^4$-bis(aminopropyl)spermidine synthesis activity on HPLC. These fractions were applied to SDS-PAGE, and protein bands were sliced out. The stained gel particles were dehydrated in acetonitrile and then alkylated, desalted, and digested with trypsin. The fractions were applied to LIT-TOFMS with an ESI instrument, with the results analyzed by MASCOT relative to the *T. kodakarensis* genome database. We identified four proteins—TK0545 as *S*-adenosylmethionine synthetase, TK0548 as aspartate aminotransferase, TK1691 as a hypothetical protein, and TK0967 as Xaa-Pro aminopeptidase—using the MASCOT database. The MASCOT scores for the TK0545, TK0548, TK1691, and TK0967 proteins were 334, 300, 256, and 145, respectively. To determine the protein with $N^4$-bis(aminopropyl)spermidine synthase activity, the four genes were separately cloned into the expression plasmid pET21a and the recombinant proteins expressed in *E. coli* and purified (Fig. 3). Assessment of their $N^4$-bis(aminopropyl)spermidine synthase activities by HPLC showed that TK1691 catalyzed the synthesis of $N^4$-bis(aminopropyl)spermidine from spermidine (Fig. 4D). In contrast, the three other purified proteins, TK0545, TK0548, and TK0967, did not show $N^4$-bis(aminopropyl)spermidine synthesis activity (Fig. 4A and B). HPLC showed that, when spermidine was the substrate, most of the product was $N^4$-bis(aminopropyl)spermidine, with a slight amount of $N^4$-aminopropylspermidine. When $N^4$-aminopropylspermidine was used, the substrate, $N^4$-bis(aminopropyl)spermidine was produced. The specific activity of enzyme using either spermidine or $N^4$-aminopropylspermidine as the substrate was approximately 0.34 μmol $min^{-1}$ $mg^{-1}$. These findings indicated that TK1691 catalyzed the production of $N^4$-bis(aminopropyl)spermidine via $N^4$-aminopropylspermidine. In contrast, when spermine was the substrate, only $N^4$-aminopropylspermine was produced, with the specific activity of enzyme using spermine being ~0.12 μmol $min^{-1}$ $mg^{-1}$. Taken together, these findings indicate that TK1691 is a bifunctional enzyme, which acts on linear tri- and tetra-amines, as well as on tertiary tetra-amines.

**Effect of *T. kodakarensis bpsA* disruption on cell growth.** To examine the physiological roles of the *TK1691* gene, which we

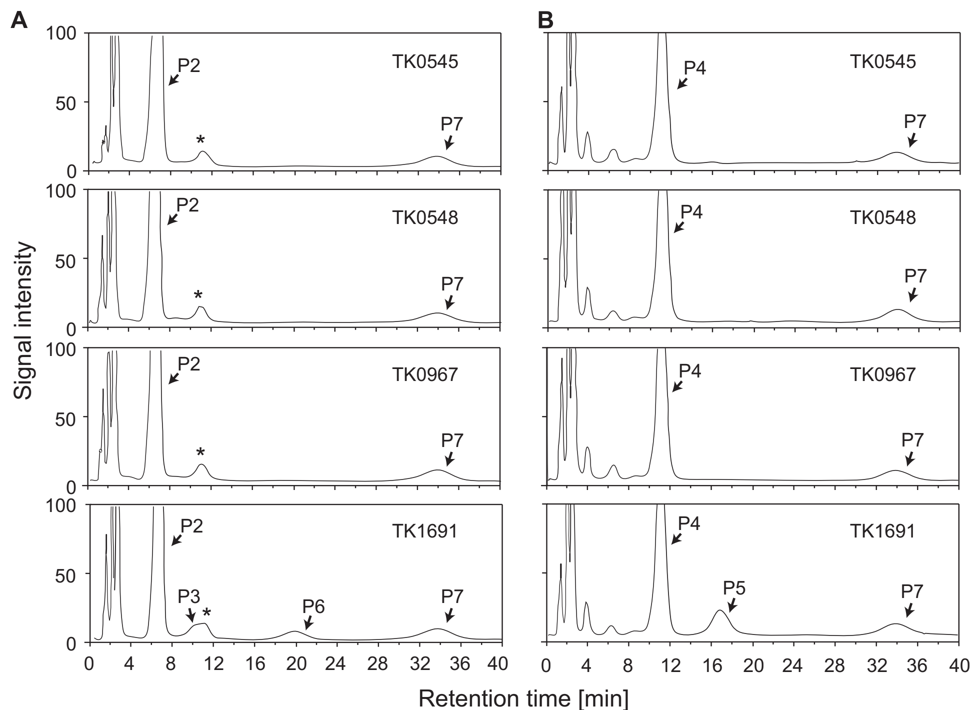


**FIG 4** Aminopropyltransferase activity of recombinant proteins. Enzymatic assays were performed at 70°C using purified proteins (1.6 μg) as described in Materials and Methods. HPLC profiles of reaction mixtures of each purified recombinant enzyme—TK0545, TK0548, TK0967, and TK1691—using substrates spermidine [34] (A) and spermine [343] (B) are shown. Abbreviations: P2, spermidine [34]; P3, $N^4$-aminopropylspermidine [3(3)4]; P6, $N^4$-bis(aminopropyl) spermidine [3(3)(3)4]; P7, caldohexamine [33333]. Asterisks indicate unknown peaks.

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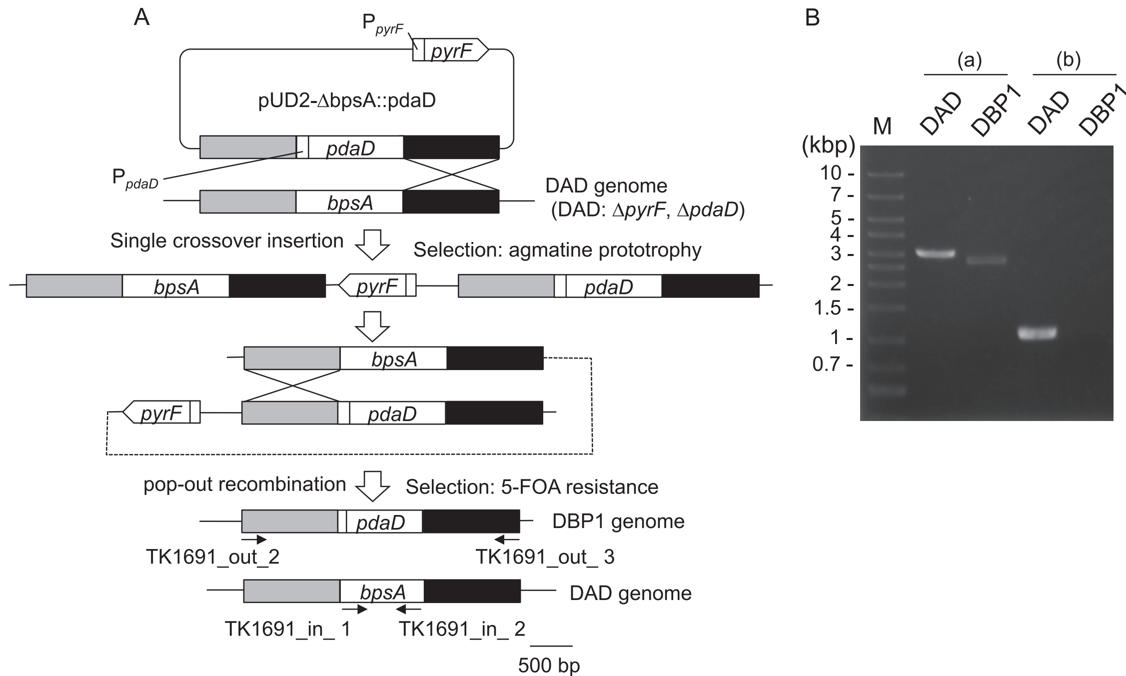


**FIG 5** Strategy for the targeted disruption of the *bpsA* gene by homologous recombination. (A) Construction of a *bpsA* disruptant of *T. kodakarensis*. Introduction of a disruption plasmid, pUD2-ΔbpsA::pdaD, into the parental DAD strain resulted in the disruption of the chromosomal *bpsA* by homologous recombination. The positions of the primer-annealing sites on PCR are indicated with arrows. (B) Agarose gel electrophoresis of PCR products from genomic DNA of strains DBP1 and DAD. PCR amplification, using the primers tk1691_out_1 and tk1691_out_2, of fragments obtained from the 5′- and 3′-flanking regions of *bpsA* yielded DNA fragments of 3.1 kbp for DAD and 2.6 kbp for DBP1 (a). PCR fragments (1 kbp) were obtained from the genomic DNA of DAD, but not of DBP1 (b). DNA size markers are shown in lane M.

termed the *bpsA* (branched-chain polyamine synthase A) gene, in *T. kodakarensis*, a *bpsA* deletion mutant (disruptant) DBP1 (Δ*pyrF* Δ*pdaD* Δ*bpsA*::*pdaD*) was constructed by replacing the *bpsA* gene with the *pdaD* gene (Fig. 5A). A *pdaD* gene encoding arginine decarboxylase, which catalyzes the synthesis of agmatine, is essential for the growth of *T. kodakarensis* (32). The plasmid pUD2-ΔbpsA::pdaD was introduced into the strain DAD (Δ*pyrF* Δ*pdaD*). Candidate mutants which showed agmatine prototrophy were isolated following pop-in recombination of the *pdaD* gene and pop-out recombination of the *pyrF* marker gene. The mutant genotype was confirmed by PCR amplification with the primers tk1691_out_1 and tk1691_out_2, which annealed outside the target region, confirming the expected change in length (2.6 bp) of the amplified DNA fragments (Fig. 5Ba). The internal primers tk1691_in_1 and tk1691_in_2, which annealed within the *bpsA* coding region, amplified a 1.1-kb fragment in wild-type DNA but not in mutant DNA (Fig. 5Bb), indicating that *bpsA* had been successfully disrupted.

Disruptions in the genes encoding the enzymes agmatine ureohydrolase (TK0882) and spermidine synthase (TK0147), both of which are involved in spermidine biosynthesis, decreased the rate of *T. kodakarensis* growth at 85°C and severely decreased growth at 93°C compared to the wild type (20), suggesting that branched polyamines and spermidine support *T. kodakarensis* growth at higher temperatures. To assess the effect of *bpsA* disruption on cell growth at different temperatures, the parental host strain KU216 and the disruptant strain DBP1 were cultivated at 85 and 93°C. At 85°C, the growth curve of DBP1 showed a slightly extended lag phase compared to that of KU216 (Fig. 6A). In contrast, at 93°C,

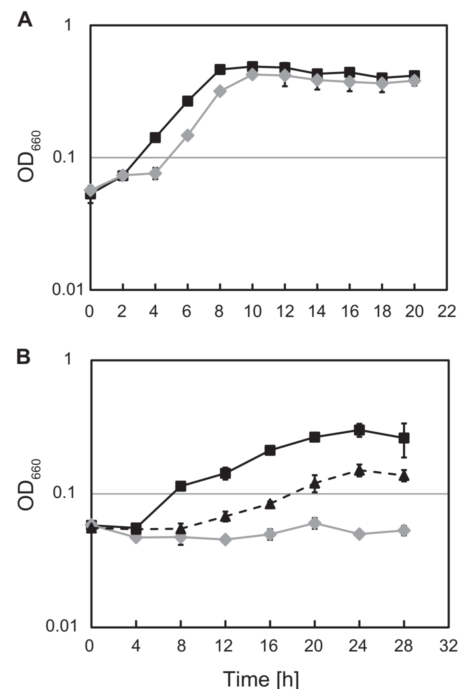


**FIG 6** Representative growth phenotypes of KU216 and DBP1 strains at two different temperatures. Wild-type (black lines) and DBP1 (gray lines) cells were separately cultivated at 85°C (A) and 93°C (B) in an ASW-YT-$S^0$ medium. Broken line in panel B represents the growth curve of DBP1 strain grown in the presence of 1 mM $N^4$-bis(aminopropyl)spermidine [3(3)(3)4]. Error bars represent the standard deviations from three independent experiments.

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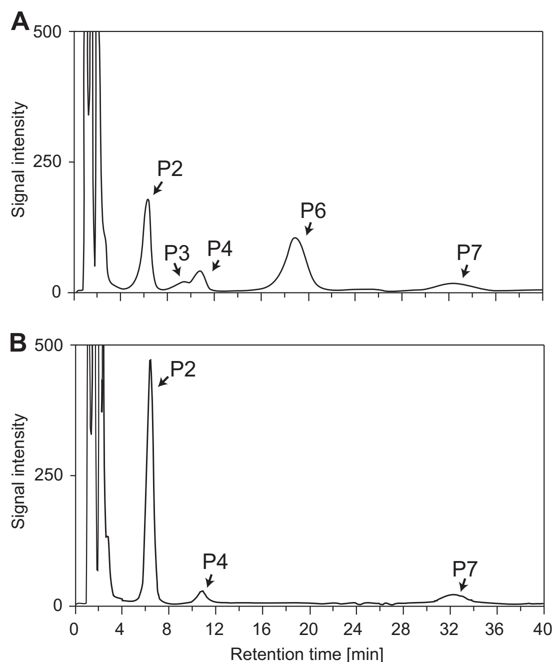


**FIG 7** Polyamine composition in *T. kodakarensis* DBP1 cells. *T. kodakarensis* strains KU216 (A) and DBP1 (B) were separately cultivated in ASW-YT-$S^0$ media at 85°C until mid-logarithmic phase. The intracellular polyamine composition of each perchloroacetic acid-precipitated extract of these cells was analyzed by HPLC.

there was no cell growth of the disruptant strain DBP1 (Fig. 6B), indicating that the *bpsA* gene is required for growth at the higher temperature. The growth defect of DBP1 at 93°C was partially restored by the addition of 1 mM $N^4$-bis(aminopropyl)spermidine to the medium (Fig. 6B). The obtained results show that $N^4$-bis(aminopropyl)spermidine is required for cell growth of *T. kodakarensis* in a higher-temperature environment.

**Composition of cytoplasmic polyamines in strain DBP1.** To analyze the changes in polyamine composition resulting from disruption of the *bpsA* gene, DBP1 cells were cultivated at 85°C and extracted with PCA, and the extracted fraction was analyzed by HPLC. $N^4$-bis(aminopropyl)spermidine and $N^4$-aminopropylspermidine were both absent from the DPB1 cell extract (Fig. 7), indicating that the synthesis of these branched-chain polyamines is catalyzed by BpsA *in vivo*. Two major peaks corresponding to spermidine and spermine were observed at retention times of 7.1 and 11.5 min, respectively. Interestingly, the amount of spermidine was ~2.5-fold higher in DPB1 cells than in KU216 cells (5.63 versus 2.25 μmol $g^{-1}$ in wet cells) (Fig. 7). In contrast, both cell types contained similar amounts of spermine (ca. 1.15 μmol $g^{-1}$ in wet cells). These results indicated that $N^4$-bis(aminopropyl) spermidine was produced from spermidine by the sequential reactions catalyzed by BpsA via $N^4$-aminopropylspermidine.

## DISCUSSION

Polyamines are organic polycations present in the cells of various living organisms. Generally, polyamines interact with nucleic acids (21, 41, 42) and are involved in cell proliferation and differentiation (5, 6). Common polyamines include putrescine, spermidine, and spermine (1, 5, 24). In addition, thermophiles, including hyperthermophiles, have unique polyamines, including long- and/or branched-chain polyamines (8–17). Although *T. kodakarensis* was found to contain the branched-chain polyamine $N^4$-aminopropylspermine (20), it was unclear whether these cells also contained its isomer, $N^4$-bis(aminopropyl)spermidine. Both of these isomers were reported to appear at the same position on HPLC, suggesting that these molecules cannot be distinguished by HPLC (17). Using HPLC analysis performed with modified separation conditions, together with precise GC and GC-MS analyses, we found that $N^4$-bis(aminopropyl)spermidine is a major polyamine in *T. kodakarensis*.

The *T. kodakarensis* enzyme TK0147 was found to be a $N^1$-aminopropylagmatine synthase, catalyzing the transfer of an aminopropyl group from dcSAM to agmatine. However, TK0147 was unable to synthesize $N^4$-aminopropylspermidine or $N^4$-bis(aminopropyl)spermidine from spermidine *in vitro* (20). Both of these polyamines were synthesized by the *TK0147* deletant *in vivo* (20), however, indicating that other, as-yet-unknown, aminopropyltransferases catalyze the production of branched-chain polyamines in *T. kodakarensis*. Indeed, the results presented here showed that the *TK1691* gene encodes an as-yet-unidentified novel aminopropyltransferase, which was found to act as a branched-chain polyamine synthase (BpsA). Biochemical and genetic studies showed that BpsA is a bifunctional enzyme, which catalyzes the sequential condensation of spermidine with the aminopropyl groups of dcSAMs to produce $N^4$-bis(aminopropyl) spermidine via $N^4$-aminopropylspermidine. This result was confirmed by the polyamine composition of DBP1, which showed the accumulation of spermidine in the cytoplasm. The *TK1691* gene is therefore essential for the production of branched-chain polyamines in *T. kodakarensis*. The $N^4$-bis(amionopropyl)spermidine biosynthetic pathway predicted here is outlined in Fig. 8.

We found that disruption of the *bpsA* gene caused a severe growth defect in *T. kodakarensis* at 93°C. However, the growth rate and final cell yield at 85°C were similar in DBP1 and KU216 strains. In contrast, our previous study showed that disruption of the *TK0882* gene in the DUH8 strain and disruption of the *TK0147* gene in the DAT strain led to their decreased growth rates at 85°C compared to the parental strain. The differences in growth properties at 85°C may be explained by the intracellular polyamine compositions of these strains. Spermidine and spermine were identified as major polyamines in DBP1 cells grown at 85°C. The amount of spermidine was 2.5-fold greater in the DBP1 than in the WT strain, whereas the amount of spermine in the two strains was similar. In contrast, the amounts of spermidine were ~100-fold lower in the DUH8 and DAT than in the DBP1 stain. The addition of 1 mM spermidine to the medium partially restored the growth rates of the DUH8 and DAT strains (20). In addition, the amounts of spermine in the DUH8, DAT, and DBP1 strains were similar. Taken together, these findings suggest that the accumulation of larger amounts of spermidine in the DBP1 strain enables these cells to grow at 85°C. It is noteworthy that the growth defect of DBP1 cells at 93°C was partially restored by the addition of $N^4$-bis(aminopropyl)spermidine (Fig. 6B). This result suggests that *T. kodakarensis* possesses a transport system for $N^4$-bis(aminopropyl)spermidine.

Since branched-chain polyamines are unique to thermophiles, the distribution of *T. kodakarensis* BpsA orthologs was expected to be limited to thermophiles. The phylogenetic tree of *T. kodakarensis* BpsA orthologs constructed with known spermidine, spermine, and thermospermine synthases over all domains of life

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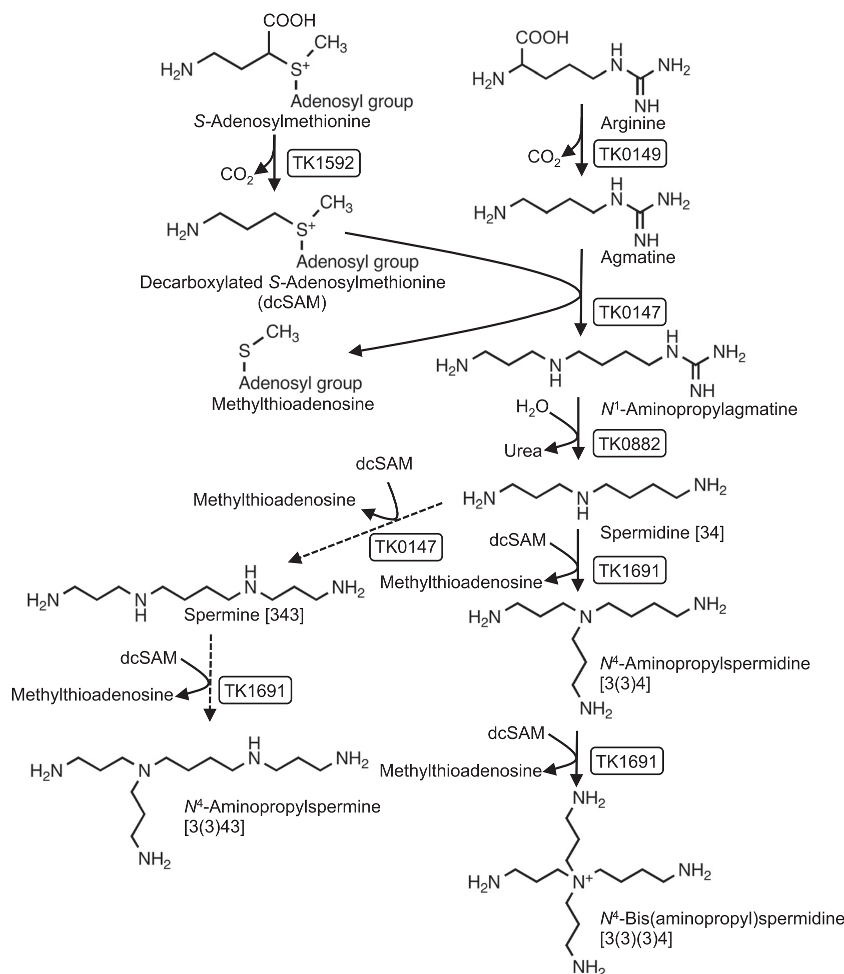


**FIG 8** Proposed pathway for the biosynthesis of polyamines in *T. kodakarensis*. The proposed biosynthetic pathway in *T. kodakarensis* is shown, along with the enzymes pyruvoyl-dependent arginine decarboxylase proenzyme (TK0149), agmatine ureohydrolase (TK0882), pyruvoyl-dependent *S*-adenosylmethionine decarboxylase proenzyme (TK1592), and aminopropyltransferases (TK0147 and TK1691). The solid arrows represent the major reaction pathway for producing $N^4$-bis(aminopropyl)spermidine [3(3)(3)4]. The broken arrows show a pathway confirmed by *in vitro* studies.

showed that the BpsA orthologs were conserved only in (hyper) thermophiles in the phylum Euryarchaeota and bacteria (Fig. 9). No BpsA orthologs were not found in hitherto known members of the phylum Crenarchaeota, consisting with the fact that the occurrence of branched polyamines has never been reported in Crenarchaeota (16). In contrast, aminopropyltransferases that produce spermidine, thermospermine, and spermine synthase homologs, including *E. coli* SpeE (43), *Arabidopsis thaliana* At5g19530 (44), and *T. kodakarensis* TK0147 (20), have been identified in various organisms of bacteria and plants. Furthermore, *T. kodakarensis* BpsA and its orthologs were distinct from other known aminopropyltransferases that produce the linear polyamines, thermospermine, spermidine, and spermine. The *T. kodakarensis* BpsA orthologs, previously designated *S*-adenosylmethionine-dependent methyltransferases, lack the dcSAM- and general polyamine-binding motifs found in *E. coli* SpeE (45) and *Thermotoga maritima* PAPT (46). The conserved GGG(E/D)G motif has been reported in known aminopropyltransferases that synthesize the production of linear polyamines (46, 47). The carboxy group of the “E/D” residue of GGG(E/D)G interacts with the amino group of dcSAM, preventing *S*-adenosylmethionine (SAM) binding by steric and electrostatic interference with the carboxy group of SAM. Indeed, the GGG(E/D)G motif is found in TK0147 and its orthologs but not in *T. kodakarensis* BpsA and its orthologs. While *T. kodakarensis* BpsA accepts linear-chain (e.g., spermidine and spermine), dcSAM, and branched-chain (e.g., $N^4$-aminopropylspermidine) polyamines as the substrates, the conserved amino acid residues essential for aminopropyltransferase activity were not present in *T. kodakarensis* BpsA and its orthologs, suggesting that the branched-chain polyamines are synthesized by a novel catalytic mechanism involving aminopropyl transfer.

Phylogenetically, aminopropyltransferases can be classified into four groups. One group (group A) includes the thermophilic *T. kodakarensis* BpsA orthologs (PF1111 from *P. furiosus*, Metig0730 from *Methanotorris igneus*, and TTHC0171 from *T. thermophilus*). A second group (group B) consists of the TK0147 orthologs, including *E. coli* SpeE (43) and *P. furiosus* PF0127 (48). Group C consists of several other aminopropyltransferases, which act as thermospermine synthases, including PAE1203 from *Pyrobaculum aerophilum* (49), Hbut0057 and Hbut0383 from *Hyperthermus butylicus* (49), and At5g19530 from *Arabidopsis thaliana* (44). *T. kodakarensis* BpsA orthologs (group A) are unique to ther-

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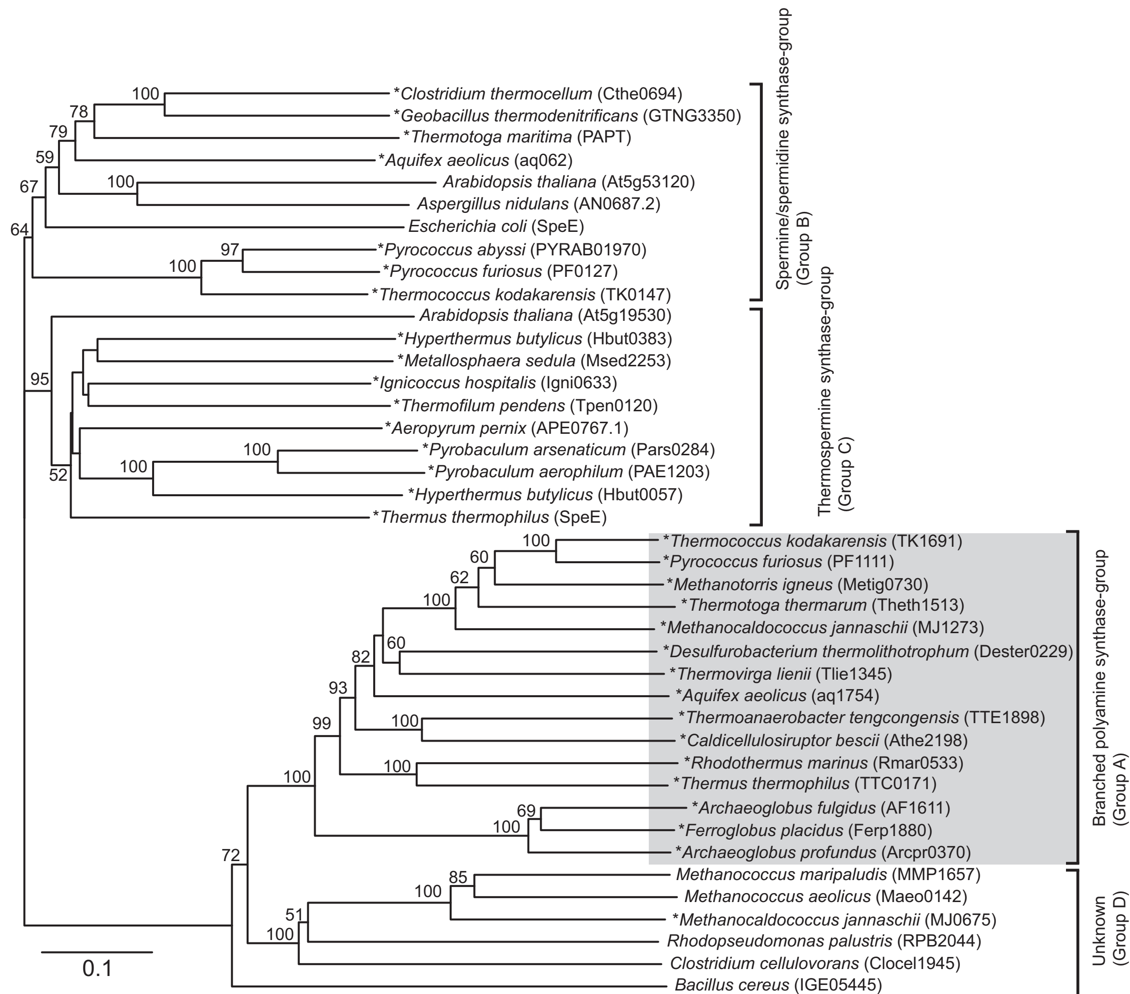


**FIG 9** Phylogenetic tree of aminopropyltransferases involved in polyamine synthesis. Phylogenetic analysis was performed using the neighbor-joining method of the ClustalX program. A Gonnet-series protein weight matrix was used with a gap opening penalty of 10.0 and a gap extension penalty of 0.05. The scale bar represents one substitution per 10 amino acids. Bootstrap values of >50 to >100 trials are shown. BpsA orthologs are shown in the shaded square. Swiss-Prot or GenBank accession numbers for the sequences are indicated in parentheses as follows: *Clostridium thermocellum* (Cthe0694, YP_001037122.1), *Geobacillus thermodenitrificans* (GTNG3350, YP_001127432), *Thermotoga maritima* (PAPT [TM0654], Q9WZC2), *Arabidopsis thaliana* (At5g53120, Q94BN2; At5g19530, Q9S7X6), *Aspergillus nidulans* (AN0687.2, XP_658291), *Escherichia coli* (SpeE, P09158), *Pyrococcus abyssi* (PYRAB01970, Q9V277), *Hyperthermus butylicus* (Hbut0057, YP_001012280; Hbut0383, YP_001012598), *Metallosphaera sedula* (Msed2253, YP_001192315), *Ignicoccus hospitalis* (Igni0633, YP_001435222), *Thermofilum pendens* (Tpen0120, YP_919533), *Aeropyrum pernix* (APE0767.1, Q9YE02); *Pyrobaculum arsenaticum* (Pars0284, YP_001152549.1), *Pyrobaculum aerophilum* (PAE1203, Q8ZXM4), *Thermococcus kodakarensis* (TK1691 [BpsA], Q5JIZ3; TK0147, Q5JFG9), *Pyrococcus furiosus* (PF1111, Q8U1U4; ACAPT [PF0127], Q8U4G1), *Methanotorris igneus* (Metig0730, YP_004484345), *Thermotoga thermarum* (Theth1513, YP_004660662), *Methanocaldococcus jannaschii* (MJ1273, Q58669; MJ0675, Q58088), *Desulfurobacterium thermolithotrophum* (Dester0229, YP_004280945), *Thermovirga lienii* (Tlie1345, YP_004933171), *Aquifex aeolicus* (aq1754, O67635; aq062, O66473), *Thermoanaerobacter tengcongensis* (TTE1898, Q8R8U3), *Caldicellulosiruptor bescii* (Athe2198, YP_002574046); *Rhodothermus marinus* (Rmar0533, YP_003289823), *Thermus thermophilus* (TTC0171, Q72L89; SpeE, Q72K55), *Archaeoglobus fulgidus* (AF1611, O28662), *Ferroglobus placidus* (Ferp1880, YP_003436295), *Archaeoglobus profundus* (Arcpr0370, YP_003400112), *Methanococcus maripaludis* (MMP1657, Q6LWQ1), *Methanococcus aeolicus* (Maeo0142, YP_001324346), *Rhodopseudomonas palustris* (RPB2044, Q2IYF9), *Clostridium cellulovorans* (Clocel1945, YP_003843453), and *Bacillus cereus* (IGE05445, EJV74907).

mophiles, whereas branched molecules are also present in mesophiles (group D in Fig. 9). Moreover, *M. jannaschii*, an archaeal hyperthermophile, has two *T. kodakarensis* BpsA orthologs, MJ1273 and MJ0675. MJ1273 is highly homologous to *T. kodakarensis* BpsA and belongs to aminopropyltransferase group A. MJ1273 is regarded as the enzyme responsible for the synthesis of branched-chain polyamines. Indeed, $N^4$-bis(aminopropyl)spermidine was found to be synthesized in an *M. jannaschii* extract (16). In contrast, MJ0675 is located on a different branch of the phylogenetic tree and has been tentatively designated as belonging to group D. *Methanococcus maripaludis* MMP1657 is homologous to MJ0675, with both predicted to be RNA methylases. Methyltransferases transfer a methyl group from SAM to an acceptor; these enzymes and aminopropyltransferases are thought to be derived from a common ancestor. The apparent lack of any branched-chain polyamines in *M. maripaludis* extract (16) suggests that enzymes, including MMP1657, belonging to aminopropyltransferase group D are not likely to be responsible for the synthesis of branched-chain polyamines.

Primitive hyperthermophiles likely require long- and/or



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branched-chain polyamines to stabilize DNA and/or RNA at high temperatures (21). A universal phylogenetic tree based on 16S and 18S rRNA gene and theoretical studies suggested that life originated with (hyper)thermophiles (50–52). By adapting to lower-temperature environments, these microorganisms may have lost their ability to synthesize group A enzymes during the course of evolution because branched-chain polyamines are not required for cell growth at lower temperatures. Thus, branched-chain polyamines appear to be molecules key for survival in high-temperature environments.

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