**N**¹-Aminopropylagmatine, a New Polyamine Produced as a Key Intermediate in Polyamine Biosynthesis of an Extreme Thermophile, *Thermus thermophilus* *

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**In the extreme thermophile *Thermus thermophilus*, a disruption of the gene homologous to speB (coding for agmatinase = agmatine ureohydrolase) resulted in the disappearance of N¹-aminopropylagmatine (N¹-amidino-1,8-diamino-4-azaoctane, N¹-amidinospermidine), a new compound, whereas all other polyamines produced by the wild-type strain were absent from the cells.**

Double disruption of speB and speE (polyamine aminopropyltransferase) resulted in the disappearance of N¹-aminopropylagmatine and the accumulation of agmatine. These results suggested the following. 1) N¹-Aminopropylagmatine is produced from agmatine by the action of an enzyme coded by speE. 2) N¹-Aminopropylagmatine is a metabolic intermediate in the biosynthesis of unique polyamines found in the thermophile. 3) N¹-Aminopropylagmatine is a substrate of the SpeB homolog. They further suggest a new biosynthetic pathway in *T. thermophilus*, by which polyamines are formed from agmatine via N¹-aminopropylagmatine. To confirm our speculation, we purified the expression product of the speB homolog and confirmed that the enzyme hydrolyzes N¹-aminopropylagmatine to spermidine but does not act on agmatine.

Polyamines play important roles in cell proliferation and cell differentiation. Common polyamines such as putrescine, spermidine, and spermine are distributed ubiquitously in cells and tissues at relatively high concentrations (1, 2).

*Thermus thermophilus*, of which the genome project was completed using two strains, HB8 and HB27 (Structural-Biological Whole Cell Project at www.srg.harima.riken.go.jp/thermus/i_index.htm and see Ref. 3, respectively), produces a variety of polyamines including unusually long polyamines and branched ones (4) (see Fig. 9C). These long and branched polyamines have a marked effect of protecting and stabilizing nucleic acids (5, 6) and of activating cell-free polypeptide synthesis at high temperature (7–9).

In many organisms, such as bacteria, yeast, animals, and plants, the first step of polyamine biosynthesis is production of putrescine by decarboxylation of L-ornithine (see Fig. 9A). An additional or alternative pathway of putrescine biosynthesis that is often seen in plants and sometimes in bacteria is decarboxylation of L-arginine followed by hydrolysis of agmatine. Agmatine ureohydrolase or agmatinase, coded by the speB gene, catalyzes this second reaction. The next step is production of spermidine and spermine by the addition of an aminopropyl group to putrescine and spermidine, respectively. This reaction is catalyzed by spermidine or spermine synthase (putrescine/spermidine aminopropyltransferase) coded by the speE gene (1).

To investigate the polyamine biosynthetic pathway in *T. thermophilus*, we constructed a disruption strain of the speE gene homolog of *T. thermophilus*. Disruption of the speB gene homolog resulted in drastic reduction of triamines, longer and branched polyamines without accumulation of agmatine, and in accumulation of an unknown compound. Double disruption of speB and speE gene homologs resulted in disappearance of this compound and accumulation of agmatine in the cells. The new compound was identified as N¹-aminopropylagmatine (N¹-amidino-1,8-diamino-4-azaoctane, N¹-amidinospermidine) by comparison with the chemically synthesized authentic compound. *In vitro* reactions revealed that SpeE is responsible for the production of N¹-aminopropylagmatine, and SpeB converts N¹-aminopropylagmatine to spermidine.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**

The strains of *T. thermophilus* and Escherichia coli and plasmids used in this study are listed in Table I. The rich growth and minimum media for *T. thermophilus* were as described previously (10). Leucine, isoleucine, and uracil (50 μg/ml each) were included in the minimum medium. Media were solidified as described (11).

**Construction of Plasmids**

All nucleotide sequences of *T. thermophilus* HB8 used in this study were kindly provided by Dr. Seiki Kuramitsu of Osaka University. Construction of pSBKm and pSEPE is shown in Fig. 1. PCR was carried out for 25 cycles (94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min) with LA Taq in GC buffer (Takara Bio) using pairs of oligonucleotide primers listed in Table II. Two pairs of primers, speB(KpnI)-speB(HinDIII) and speBEcoDw5-speBEcoDw3, were used to construct pSBKm; and an additional two pairs, speE(KpnI)-speE(HindIII) and speEEdw3-speEEdw3′, were used to construct pSEPE. Genomic DNA of *T. thermophilus* HB8 was used as the PCR template. PCR products were digested with restriction endonucleases listed in Table II and cloned into pBluescript SK− or pHVTK (14) to construct pSBKm, pSBD, pSEUKm, and pSED. The 3'-half of speB and the downstream
region of speE were inserted into pSBUKm and pSEUKm to prepare
pSBKm and pSEKm, respectively. The HTK region of pSEKm was
replaced with the pyrE gene to construct pSEPE.

To make speE overexpression plasmid, PCR was carried out for 25
cycles of 98 °C for 0.5 min and 72 °C for 3 min with PfuTurbo DNA
polymerase (Stratagene) by using primers of sE5/H11032 NdeI and sE3/H11032 Hind.

To make speB overexpression plasmid, PCR was carried out for 25
cycles (94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min) with LA
Taq in GC buffer using primers of sB5’NdeI and sB3’Hind (Table II).

Each PCR product was digested with NdeI and HindIII and was cloned
between the NdeI and HindIII site of expression plasmid pET21c to
make pESE8 and pESB8, respectively.

All cloned nucleotide sequences were determined to verify the fidelity
of the amplified product to the original sequence using BigDye Termi-
nator Ready Reaction Premix (PerkinElmer Life Sciences) and an ABI
PRISM 377 DNA Sequencing System (PerkinElmer Life Sciences).

FIG. 1. Construction of plasmids
used for gene disruption of speB and
speE. See “Experimental and Procedures”
for details. A, construction of pSBKm. B,
construction of pSEPE.
Overexpression and Purification of speE and speB Gene Products

E. coli BL21-CodonPlus(DE3)-RP cells carrying pESE8 or pESB8 were grown in 10 liters of 2YT medium at 37 °C. When apparent absorbance at 600 nm reached 0.3, isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.5 mM, and incubation was continued for an additional 2 h. For purification of SpeE, cells were collected and suspended in 10 ml of 20 mM Tri-HCl (pH 7.5), 1 mM EDTA followed by cell disruption with a sonicator. After removing the cell debris by centrifugation (30,000 rpm, 20 min), the cell extract was heated for 15 min at 75 °C, and the denatured protein was removed by centrifugation (30,000 rpm, 20 min). Heat treatment was repeated twice. The proteins precipitated by 50% saturated ammonium sulfate were collected by centrifugation and suspended in 20 ml of 50 mM Tri-HCl (pH 7.5), 1 mM EDTA. Then the enzyme was further purified by a Hitrep QXL column (Amersham Biosciences) and by Resource HIC PHE column (Amersham Biosciences). SpeE thus obtained was homogeneous on SDS-PAGE and was stored at 4 °C as a suspension in 60% saturated ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0) until use.

For purification of SpeB, cells were corrected and suspended in 10 ml of 50 mM Tri-HCl (pH 7.5), 1 mM MgCl₂, 5 mM dithiothreitol, 40 μM pyridoxal phosphate, and 1 mM agmatine) and were disrupted by sonication. After removing the cell debris by centrifugation (30,000 rpm, 20 min), the cell extract was heated for 15 min at 75 °C, and the denatured protein was removed by centrifugation (30,000 rpm, 20 min). Heat treatment was repeated twice. The supernatant was fractionated by 30–50% saturated ammonium sulfate. The precipitate collected by centrifugation was suspended in 50 mM sodium phosphate buffer (pH 7.0) until use.

To confirm the reaction product of SpeE, a reaction mixture consisting of 100 mM HEPES-NaOH (pH 7.4), 5 mM MgCl₂, 5 mM dithiothreitol, 2 mM agmatine, 337.5 μM dCAP, and 32 μg of purified SpeE in a final volume of 2 ml was incubated at 37 °C overnight. Polyamines were isolated by using Dowex 50W-X4 (Muromachki Technos Co. Ltd.) column chromatography. Fractions containing polyamines that react with o-phthalaldehyde were collected and concentrated.

For identification of the chemical reaction catalyzed by SpeE, a reaction mixture consisting of 100 mM HEPES-NaOH (pH 7.4), 5 mM MgCl₂, 5 mM dithiothreitol, 40 μM pyridoxal phosphate, 50 μg of partially purified SpeE, and 40 μg of dried reaction product of SpeE described above or 50 μg of chemically synthesized N²-aminoagmatine in a final volume of 50 μl was incubated at 65 °C for 1 h. In another experiment, 40 μg of agmatine was added instead of N²-aminoagmatine to investigate whether or not SpeB possesses agmatinase activity. The reaction product was analyzed by HPLC.

### Chemical Synthesis

**Mono(benzylaminoxy-carbonyl)-1,4-butanediame Hydrochloride (3)—**

This compound was prepared as a chloride by the method of Lawson et al. (20), except for the use of 1,4-butanediamidine (1) instead of 1,3-propandiamine to prepare bis(benzylaminoxy-carbonyl)-1,4-butanediamine (2) (1.00 g, 3.87 mmol) and N³-bromopropylphthalimide (1.04 g, 3.87 mmol) in 13 ml of acetonitrile was refluxed at 40 °C for 19 h in the presence of NaCl (0.6 g). The product was filtrated, washed with ether, and dried. The residue was treated with 10 ml of hydrobromic acid (30–32%) in acetic acid for 1 h at room temperature and then added to dry ether. The 1-phthalimidopropyl-1,4-butanediamine hydrobromide was washed with dry ethyl ether and dried to yield a white solid (0.90 g, 53%). This solid was hydroscopic and was recrystallized from methanol. For ¹H NMR (CD3OH), the values were 7.80–7.89 (4H, m), 3.79–3.84 (2H, t), 2.98–3.14 (6H, m), 1.78–1.80 (4H, m).

**1-Phthalimidoprolyl-1,4-butanediame Dihydrobromide (4)—**

A stirred solution of mono(benzylaminoxy-carbonyl)-1,4-butanediamin hydrobromide (3) (1.00 g, 3.87 mmol) and N³-bromopropylphthalimide (1.04 g, 3.87 mmol) in 13 ml of acetonitrile was refluxed at 40 °C for 19 h in the presence of KF-Celite (0.6 g). The product was filtered, washed with ether, and dried. The residue was treated with 10 ml of hydrobromic acid (30–32%) in acetic acid for 1 h at room temperature, and then added to dry ether. The 1-phthalimidopropyl-1,4-butanediamine hydrobromide was washed with dry ether and dried to yield a white solid (0.90 g, 53%). This solid was hydroscopic and was recrystallized from methanol. For ¹H NMR (CD3OH), the values were 7.80–7.89 (4H, m), 3.79–3.84 (2H, t), 2.98–3.14 (6H, m), 2.07–2.13 (2H, m), and 1.78–1.80 (4H, m).

**1-Guanyl-3,5-dimethyl Pyrazole Hydrobromide (5)—**

This compound was prepared as a nitrate by the method of Bannard et al. (21), except that a larger amount of hydrobromic acid was added to the reaction mixture prior to crystallization by adding ether. The yield was 68%. For ¹H NMR (CD3Cl), the values were 9.25 (2H, bs), 7.77 (2H, bs), 6.15 (1H, s), 2.82 (3H, s), and 2.26 (3H, s).

**Aminopropylagmatine Pentahydrochloride (6)—**

A stirred solution of 1-phthalimidopropyl-1,4-butanediamin dihydrobromide (4) (0.09 g, 0.21 mmol) and 1-guanyl-3,5-dimethyl pyrazole hydrobromide (5) (0.045 g, 0.21 mmol) in 2 ml of dry ethanol was refluxed for 11 h in the presence of NaOH (0.025 g, 0.63 mmol). After removal of NaBr, the reaction product was washed with ether and dried. The residue was refluxed for 24 h with 5 ml of concentrated hydrochloric acid. The reaction mixture was applied to a small column of Dowex 50W-X4 (H⁺ form) and eluted with 6 ml hydrochloric acid. Fractions of 30 ml were collected and examined by analytical HPLC, and those containing the desired compound were combined and evaporated to dryness under reduced pressure at about 40 °C. A pale yellow oil was obtained (0.06 g, 77%). For ¹H NMR (400 MHz, CD₃OD) δ, the values were 1.69 (2H, m), 1.80 (2H, m), 2.13 (2H, m), 3.14 (6H, m), 3.25 (2H, t). For ¹³C NMR (75 MHz, CD₃OD), δ, the values were 22.88, 23.78, 25.10, 36.60, 40.49, 44.52, 47.32, and 156.77. For high resolution mass spectrometry, the value calculated for C₁₈H₂₆N₄O₂H+ (M+H) was 325.1757, and the value found was 186.1886; the value calculated for C₁₈H₂₆N₄O₂ (M+2H) was 189.1953, and the value found was 189.1953; the value calculated

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; HTK, highly thermostable kanamyacin nucleotidyltransferase; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; dC, decarboxylated S-adenosylmethionine.

[2] K. Samejima, unpublished data.
for C₈H₂₄N₅ (M⁺/H₁₁O₁₃) was 190.2032, and the value found was 190.2007.

RESULTS

To elucidate the biosynthetic pathway of T. thermophilus, we searched for gene homologs of known polyamine biosynthetic enzymes in the T. thermophilus genome. Homologs of arginine decarboxylase (speA, the open reading frame numbers of T. thermophilus HB8 and HB27 (3) are TT0340 and TTC1277, respectively), agmatine ureohydrolase (speB, HB8, TT0338; HB27, TTC0764), two S-adenosylmethionine decarboxylases (speD, HB8, TT1493 and 1749; HB27, TTC0473 and TTC1093), and spermidine synthase (speE, HB8, TT0339; HB27, TTC0472) were found, but a homolog of the ornithine decarboxylase gene (speC) was not.

Alignment of the amino acid sequences of the speB gene homologs is shown in Fig. 2. The amino acid sequence deduced from the T. thermophilus speB gene homolog showed 29–32% similarity to those of other organisms. The histidine residue that is critical for catalytic activity in the E. coli enzyme (22) and the manganese ion-binding residues (23) are conserved in all amino acid sequences examined.

To ascertain whether polyamine synthesis starts from ornithine or arginine in the thermophile, we first disrupted the speB gene homolog of T. thermophilus. The disruption strain, named MOSB, was constructed by insertion of the HTK gene into the speB gene homolog of TTY1. When MOSB was cultivated in minimum medium at 70 °C, the disruption showed no effect on growth (doubling time of MOSB was 7.2 h while that of TTY1 was 4.9 h; see Fig. 3). Growth on a minimum medium plate at 75 °C also showed little difference between MOSB and TTY1 (data not shown). However, disruption of the speB gene homolog resulted in significant defect of growth at 78 °C in minimum medium (Table III). In addition, the MOSB colony was pale yellow, whereas the TTY1 colony exhibited a bright yellow color (data not shown).

Intracellular polyamine composition of MOSB grown in minimum medium at 70 °C was analyzed by HPLC. Most polyamines found in the cells of TTY1 (Fig. 4A) fell below the limit of detection by our method in MOSB (Fig. 4B). At the same time, an unknown polyamine designated as polyamine A in Fig. 4 was accumulated. Cadaverine, agmatine, and two unknown polyamines (X and Y) were concomitantly detected as minor compounds. Fig. 5A shows the results of GC analysis of polyamines in MOSB. The major peak occurred at 9.3 min, and its molecular weight was estimated as 775 based on mass numbers of molecular fragments observed on the GC-MS spectrum shown in Fig. 5B. This corresponds to the molecular weight of N₁-aminopropylagmatine bound to three heptafluorobutyryl molecules. Because the gene coding for aminopropyltransferase is present in MOSB, the major unknown polyamine of MOSB can be predicted to be N₁-aminopropylagmatine. Two additional unknown polyamines (X and Y in Fig. 4B) could not be identified because their amounts were too small.

To confirm the identity of polyamine A, authentic N₁-aminopropylagmatine and a New Polyamine Biosynthetic Pathway
propylagmatine was chemically synthesized (Fig. 6). In HPLC analyses, the retention time of the synthesized N\textsuperscript{1}-aminopropylagmatine was identical to that of polyamine A (data not shown).

By having identified polyamine A as N\textsuperscript{1}-aminopropylagmatine, we next disrupted the gene of polyamine aminopropyltransferase (speE homolog) of MOSB. To construct the speB and speE double-disruption strain, we disrupted the speB homolog by inserting the HTK gene of *T. thermophilus* MOSBE. MOSBE showed defective growth at 70 °C in minimum medium (doubling time was 12.8 h; see Fig. 3) and significantly defective growth at 78 °C (Table III). Like that of MOSB, the MOSBE colony was pale yellow (data not shown). As shown in Fig. 4, agmatine was accumulated in MOSBE cells, and N\textsuperscript{3}-aminopropylagmatine, the major polyamine of MOSB, was drastically diminished. A small amount of cadaverine was detected, but other polyamines, especially long and branched polyamines, were undetectable.

These results indicated that the major polyamine biosynthesis in *T. thermophilus* starts from arginine and not from ornithine. A new polyamine, N\textsuperscript{3}-aminopropylagmatine, produced from arginine by the speE gene homolog, plays a key role as a metabolic intermediate in polyamine biosynthesis in the thermophile. We therefore concluded that the *T. thermophilus* speB gene homolog codes for N\textsuperscript{3}-aminopropylagmatine ureohydrolase, and the speE gene homolog codes for agmatine aminopropyltransferase.

Although MOSB could not grow in minimum medium at 78 °C (Table III), the growth recovered when 250 μM spermidine was added to the medium. The polyamine composition of MOSB grown at 78 °C is shown in Fig. 7. A long polyamine, homocaldopentamine, was accumulated as the major component in these cells. A small amount of quaternary branched polyamine, tetrakis(3-aminopropyl)ammonium, was also present. N\textsuperscript{3}-Aminopropylagmatine was not produced under these conditions, suggesting that the accumulation of long polyamines represses its production. The finding that MOSB produced long and branched polyamines in medium supplemented with spermidine suggests that conversion of N\textsuperscript{3}-aminopropylagmatine to spermidine is an essential step in the synthesis of long and branched polyamines and that the speB gene product of *T. thermophilus* is responsible for the reaction.

To confirm that SpeE converts agmatine to N\textsuperscript{3}-aminopropylagmatine and that SpeB uses N\textsuperscript{3}-aminopropylagmatine as a substrate to produce spermidine, we purified SpeE and partially purified SpeB to perform in vitro enzymatic reactions. A typical purification procedure of SpeE was summarized in Table IV. SpeE was recovered from the soluble fraction and purified by an anion exchange chromatography and a hydrophobic interaction chromatography. Purity of SpeE by SDS-PAGE was shown in Fig. 8. A. The purified enzyme was significantly stable; little loss of activity was observed after storage at 4 °C in 50 mM sodium phosphate buffer (pH 7.0), 1 mM ammonium sulfate for at least a year. The kinetic parameters of SpeE were determined by Hanes-Woolf plot. Because dcSAM decompose at high temperature under alkaline conditions (24), reactions for obtaining kinetic parameters were performed at 37 °C (pH 9) with various concentrations of agmatine (0.5–2 mM) and 38 μM dcSAM. The enzymatic reaction was performed in one tube, and 100 μl of reaction mixture was sampled to stop reaction at 0.5, 1, 5, 10, 15, and 20 min. The *Km* value for agmatine was 0.37 μM. The *kcat* was 0.37 s\textsuperscript{-1} when agmatine was used as substrate. The partially purified SpeB migrated as a single band (36 kDa). The final preparation had a specific activity of 2.4 μmol/min/mg proteins, 51-fold that of the crude enzyme.

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Aminopropylagmatine was also converted to spermidine by SpeB (see Fig. 8C, traces e and f). SpeB could not utilize agmatine as a substrate (Fig. 8C, traces c and d). These results confirmed that SpeE converts agmatine to N₁-aminopropylagmatine, and SpeB converts N₁-aminopropylagmatine to spermidine.

**Fig. 5. GC and GC-MS analyses of polyamines in MOSB.** A, GC of an MOSB cell extract after derivatization to heptafluorobutyryl compounds and concentration. B, GC-MS analysis of the peak eluted at 9.32 min. Polyamines identified by GC are indicated in abbreviated forms expressing the numbers of methylene chain units. Parentheses represent a branched polyamine. 33, norspermidine; 34, spermidine; 3 (3H₄), N₁-aminopropylspermidine.
In the present study, we constructed an agmatine biosynthetic pathway using the genome sequence data of *T. thermophilus* to clarify the first step of polyamine biosynthesis. MOSB (speB::HTK strain), a speB gene homolog coding for ornithine decarboxylase, was expressed when cellular polyamines disappeared, as has been reported for *E. coli* lysine decarboxylase. In *E. coli*, expression of the lysine decarboxylase gene is suppressed by putrescine and spermidine (28). Similarly cadaverine may not be present in TTY1 (Fig. 4). It is worthy of note that only agmatine and cadaverine support the growth of the thermophile at lower temperature. Other polyamines, however, are essential for growth at higher temperatures. As shown in Fig. 7, when spermidine was added to the minimum medium, MOSB cells produced long and branched polyamines and were able to survive at 78 °C. Therefore, long and branched polyamines are essential for growth at over 75 °C. In the wild-type strain, cellular content of the yellow pigments, carotenoids, which are known as antioxidants, increase when the cells are grown at higher temperatures (29). We speculate that MOSB and MOSBE are unable to grow at over 75 °C because of the suppression of carotenoid synthesis (4).

Both MOSB and MOSBE formed pale yellow colonies, although the parent strain, TTY1, forms a bright yellow colony. These observations suggest that one or more long and/or branched polyamines are required for the following: 1) gene transcription, 2) gene translation, or 3) enzymatic activity of the enzyme(s) involved in the dye synthesis. The intracellular content of the yellow pigments, carotenoids, which are known as antioxidants, increase when the cells are grown at higher temperatures (29).

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**DISCUSSION**

*T. thermophilus* produces no fewer than 16 polyamines (Fig. 9C), and its long and branched polyamines have been suggested to play important roles in thermophily (4, 8, 17). However, the starting point of polyamine biosynthesis of this bacterium remains to be elucidated. We could not find a gene homolog coding for ornithine decarboxylase (speC gene) in the *T. thermophilus* genome, even though Pantazaki et al. (25) have reported purification and characterization of ornithine decarboxylase of *T. thermophilus*. Some bacteria such as *Rhodopirellula baltica* and *Selenomonas ruminantium* contain a homolog of eukaryotic ornithine decarboxylase with dual specificity on lysine and ornithine (26, 27). We could not find a gene homolog coding for eukaryotic ornithine decarboxylase. We speculate two possible explanations for the discrepancy between the report for ornithine decarboxylase and genome sequence data of *T. thermophilus*: either the gene coding for ornithine decarboxylase has no homology to speC genes from other organisms, or an amino acid decarboxylase, such as arginine decarboxylase or lysine decarboxylase, has broad substrate specificity and accepts ornithine as a substrate.

**Phenotypes of Disruption Strains of Polyamine Biosynthetic Genes**—In the present study, we constructed an speB gene homolog-disruption mutant of *T. thermophilus* (MOSB) in order to clarify the first step of polyamine biosynthesis. MOSB exhibited significantly defective growth at 78 °C in minimum medium but normal growth at 70 °C (Table III and Fig. 3). Polyamines found in MOSB cells (Fig. 4) were cadaverine, agmatine, and *N*-aminopropylagmatline, compared with 16 or more found in TTY1. In addition, two larger unknown polyamines were found. Judging from their peak positions in HPLC, these compounds may be aminopropylated derivatives of *N*-aminopropylagmatline. Their identifications will be the subject of future studies. These observations indicate that *T. thermophilus* can grow at up to 75 °C with aminopropylagmatline and other aminopropylated derivatives that could substitute for long and branched polyamines in biochemical reactions at high temperature.

When speB and speE gene homologs of *T. thermophilus* were disrupted, MOSBE had defective growth even at 70 °C (Fig. 3). Agmatine accumulated in MOSBE cells, and the levels of *N*-aminopropylagmatline and other polyamines diminished (Fig. 4). A small amount of cadaverine was also detected for the first time in the cells of *T. thermophilus*. These observations suggest that a gene coding for lysine decarboxylase is present in the *T. thermophilus* chromosome and is suppressed under normal conditions but is expressed when cellular polyamines disappear, as has been reported for *E. coli* lysine decarboxylase. In *E. coli*, expression of the lysine decarboxylase gene is suppressed by putrescine and spermidine (28). Similarly cadaverine may not be present in TTY1 (Fig. 4). It is worthy of note that only agmatine and cadaverine support the growth of the thermophile at lower temperature. Other polyamines, however, are essential for growth at higher temperatures. As shown in Fig. 7, when spermidine was added to the minimum medium, MOSB cells produced long and branched polyamines and were able to survive at 78 °C. Therefore, long and branched polyamines are essential for growth at over 75 °C. In the wild-type strain, cellular content of these polyamines increased with the rise in the growth temperature (4).

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**N'-Aminopropylagmatline**—In the present study, we identified the major unknown polyamine accumulated in MOSB as *N*-aminopropylagmatline. A related polyamine in leech, *hirudinone* (G3–4N), was reported by Robin et al. (30). Chemically
N\textsuperscript{1}-aminopropylagmatine also appears in the literature; it was named N\textsuperscript{8}-guanylspermidine and is known to be a strong inhibitor of deoxyhypusine synthase (31). However, this is the first time that N\textsuperscript{1}-aminopropylagmatine has been found as a natural compound.

Enzymatic Activity of SpeE—When SpeE was incubated with agmatine and dcSAM, only a small amount of aminopropylagmatine was formed as shown in Fig. 8C, trace a. This is not due to low catalytic activity of SpeE protein but is probably due to instability of dcSAM under the conditions employed. The $k_{cat}$ value (0.37 s\textsuperscript{-1}) of the thermophile SpeE is by no means inferior to those of other organisms even at 37 °C (19, 32). The $K_m$ value for agmatine (0.77 mM at 37 °C) of the present thermophile aminopropyltransferase is smaller than the reported $K_m$ value for putrescine (20 mM at 37 °C) of Thermotoga maritima spermidine synthase (32). On the other hand, dcSAM is known to be unstable at alkaline pH (24). To reduce the rate of spontaneous degradation of dcSAM, the experiment shown in Fig. 8C, trace a was carried out at 37 °C. The reaction mixture was incubated overnight, but the reaction might terminate earlier due to shortage of dcSAM. In the thermophile cells, some mechanisms, such as coupling of reactions, may exist to prevent the degradation of dcSAM at high temperature.

Biosynthetic Pathway of Polyamines in T. thermophilus—Based on the intracellular polyamine composition of MOSB and MOSBE (Fig. 4), we conclude the following: 1) major polyamines are mainly derived from arginine rather than ornithine; 2) SpeE produces N\textsuperscript{1}-aminopropylagmatine from agmatine; and 3) SpeB converts N\textsuperscript{1}-aminopropylagmatine to spermidine.

### Table IV

| Step                  | Total protein | Specific activity (nmol/min/mg) | Total activity (μmol/min) | Purification | Yield (%) |
|-----------------------|---------------|---------------------------------|---------------------------|--------------|-----------|
| Cell-free extract     | 474.6         | 46.8                            | 22.2                      | 1            | 100       |
| 1st heat treatment    | 53.1          | 805.4                           | 42.8                      | 17           | 193       |
| 2nd heat treatment    | 52.3          | 836.9                           | 43.8                      | 18           | 197       |
| Hiprep QXL            | 16.6          | 1738.9                          | 28.8                      | 38           | 130       |
| Resource HIC Phe      | 9.7           | 2402.2                          | 23.2                      | 51           | 105       |

**FIG. 8. In vitro polyamine syntheses using purified SpeE and SpeB.** A, purification of recombinant SpeE. Samples were separated by SDS-PAGE on 12% gel. M, molecular marker (SDS-PAGE standard low, Bio-Rad); lane 1, total cell lysate; lane 2, 1st heat treatment; lane 3, 2nd heat treatment; lane 4, ammonium sulfate precipitation; lane 5, Hiprep QXL fraction; lane 6, Resource HIC PHE fraction. B, purification of recombinant SpeB. Samples were separated by SDS-PAGE on 9.4% gel. M, molecular marker; lane 1, supernatant after sonication; lane 2, supernatant of heat treatment; lane 3, 40% ammonium sulfate precipitation; lane 4, Hiprep QXL fraction. C, in vitro reactions of purified SpeE and SpeB. Trace a, polyamine composition after agmatine was incubated with SpeE and dcSAM. For catalytic activities of SpeB, substrates used are the products of SpeE enzymatic reaction (traces a and b), agmatine (traces c and d), or chemically synthesized N\textsuperscript{1}-aminopropylagmatine (traces e and f).
Aminopropylagmatine and a New Polyamine Biosynthetic Pathway

**A**

[Diagram illustrating the polyamine biosynthetic pathway for bacteria and plants.]

**B**

[Diagram illustrating the proposed polyamine biosynthetic pathway of *T. thermophilus*.]

**C**

[Diagram showing the structures of polyamines. * represents polyamines that were identified in wild-type *T. thermophilus* cells.]

**Fig. 9.** Polyamine biosynthetic pathway. **A**, reported polyamine biosynthetic pathway for bacteria and plants. **B**, proposed polyamine biosynthetic pathway of *T. thermophilus*. **C**, structures of polyamines. * represents polyamines that were identified in wild-type *T. thermophilus* cells.
We confirmed that SpeB acts on N\(^2\)-aminopropylagmatine but not on agmatine (Fig. 8C, traces a–f). Sequence homology between "T. thermophilus" speB and speB of other organisms is not high as shown in Fig. 2, and this low similarity would be reflected in the different substrate specificity of the thermophile enzyme. It would be interesting to compare the tertiary structures of SpeB of T. thermophilus and speB gene products of other organisms that differ in substrate specificity. We are currently attempting to crystallize SpeB for structural analyses. The present results suggest the danger of utilizing only sequence homology for genome annotation.

Based on reverse genetic analyses, we propose a new polyamine biosynthetic pathway in T. thermophilus. As shown in Fig. 9B, biosynthesis of major polyamines in T. thermophilus starts only from arginine, which is decarboxylated to form agmatine. An aminopropyl group is added to agmatine by SpeE to form N\(^1\)-aminopropylagmatine. Finally, N\(^2\)-aminopropylagmatine is hydrolyzed to spermidine by SpeB. In this new polyamine biosynthetic pathway, spermidine is synthesized without the production of putrescine. It would be possible to find this polyamine pathway in other organisms.

As shown in Fig. 4, MOSB, which could not produce spermidine from N\(^2\)-aminopropylagmatine, failed to produce long and branched polyamines. Such polyamines were produced in MOSB cells only after addition of spermidine to the minimum medium (Fig. 7). Therefore, the conversion of N\(^2\)-aminopropylagmatine to spermidine is essential for production of long and branched polyamines in T. thermophilus.

Agmatine and aminopropylagmatine were not detected in wild-type strain (Fig. 4A). The formation of spermidine in wild-type cells may be so rapid that only trace amounts of these intermediates exist. In MOSB cells cultivated in the presence of spermidine, thermospermine should be present as a precursor of homocaldopentamine, but it was not detected by HPLC analysis (Fig. 7). Thermospermine has been identified in the cells of T. thermophilus, as well as homocaldopentamine and other long polyamines. In MOSB, it seems that the aminopropylagmatine pathway is unique in that putrescine is not produced directly from N\(^2\)-aminopropylagmatine, but not from putrescine. The pathway is unique in that putrescine is not involved in the biosynthesis of other polyamines. However, small amounts of putrescine, sym-homospermidine, and other polyamines containing aminobutyl groups are found in wild-type cells, suggesting that genes other than speB are also involved in polyamine metabolism of T. thermophilus. In this context, it is noteworthy that a homolog of a gene coding for deoxyhypusine synthase (33) (HB8, TT0337; HB27, TCC1205) is present in the T. thermophilus genome (both strains HB8 and HB27); and this gene might be involved in sym-homospermidine synthesis. However, not even a trace of sym-homospermidine was detected in MOSB, which accumulated N\(^2\)-aminopropylagmatine in the cells (Figs. 4 and 5). This is consistent with the fact that N\(^2\)-aminopropylagmatine acts as a deoxyhypusine inhibitor (31). To clarify the details of polyamine metabolism in T. thermophilus, further reverse genetic analyses as well as enzymatic investigations are necessary. In addition, detailed properties and structures of SpeB, SpeD, and SpeE are being studied in our laboratory. Structural data for SpeE have been deposited in the Protein Data Base (Protein Data Bank code 1UIR).

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