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Unique polyamines produced by an extreme thermophile, *Thermus thermophilus*

Review Article

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Summary. Recent research progress on polyamines in extreme thermophiles is reviewed. Extreme thermophiles produce two types of unique polyamines; one is longer polyamines such as caldopentamine and caldohexamine, and the other is branched polyamines such as tetrakis(3-aminopropyl)ammonium. The protein synthesis catalyzed by a cell-free extract of *Thermus thermophilus*, an extreme thermophile, required the presence of a polyamine and the highest activity was found in the presence of tetrakis(3-aminopropyl)ammonium. In vitro experiments, longer polyamines efficiently stabilized double stranded nucleic acids and a branched polyamine, tetrakis(3-aminropyl)ammonium, stabilized stem-and-loop structures. In *T. thermophilus*, polyamines are synthesized from arginine by a new metabolic pathway; arginine is converted to agmatine and then agmatine is aminopropylated to N^1-aminopropylagmatine which is converted to spermidine by an enzyme coded by a gene homologous to *speB* (a gene for agmatinase). In this new pathway spermidine is not synthesized from putrescine. Reverse genetic studies indicated that the unique polyamines are synthesized from spermidine.

Keywords: Archaeon – Caldopentamine – Cell-free protein synthesis – Hyperthermophile – Tetrakis(3-aminopropyl)ammonium

Abbreviations: Poly(U), polyuridilic acid; *speA*, gene coding for arginine decarboxylase; *speB*, gene coding for agmatine ureohydrolase; *speD*, gene coding for S-adenosylmethinine decarboxylase; *speE*, gene coding for spermidine synthase or spermine synthase; *speC*, gene coding for ornithine decarboxylase; T_m, melting temperature

Introduction: unique polyamines in extremely thermophilic organisms

Organisms which are able to grow at 80 °C or higher are called extreme thermophiles. *Thermus thermophilus* is an extremely thermophilic bacterium (not archaeon) isolated from a hot spring, and is capable of growing in a temperature range of 50–85 °C at neutral pH (Oshima and Imahori, 1974). Cells of *T. thermophilus* are long rods, non-motile, and non-spore forming. G + C content of the chromosomal DNA is about 70%. It is easy to carry out DNA manipulation experiments using *T. thermophile* as a host cell. The whole genome sequences were determined using two strains of *T. thermophilus*, strain HB8 and HB27. These two strains are quite similar to each other and a major difference between them is the existence of a plasmid pTT8 in the cells of strain HB8. A thermophilic phage YS40 is infectious to strain HB8, but not to HB27 (Sakaki and Oshima, 1975).

One remarkable feature of *T. thermophilus* is its cellular polyamine composition. The thermophile produces many unusual polyamines as shown in Fig. 1. The polyamines detected in the cells of *T. thermophilus* include standard polyamines; putrescine, spermidine and spermine. Homologs, analogs and structural isomers of the standard polyamines are also identified in the cells of the thermophile; examples are 1,3-diaminopropane (= analog of putrescine), *sym*-homospermidine, norspermidine (= caldine), thermine (= norspermine), thermospermine (= an isomer of spermine) and so on. In addition to these unusual polyamines, two types of unique polyamines were identified; one is long polyamines such as caldopentamine and caldohexamine, and the other is branched polyamines such as mitsubishine (= tris-(3-aminopropyl)amine) and tetrakis-(3-aminopropyl) ammonium (= N^4-bis(aminopropyl) spermidine) (Oshima, 1983; Hamana et al., 1991). These unique polyamines are abundant in the thermophile cells grown at higher temperatures such as 80 °C, but they are minor components in the cells grown at relatively lower

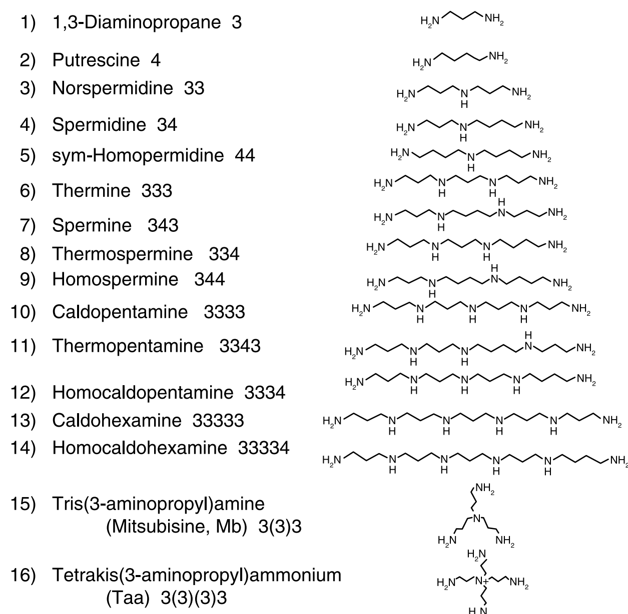

Fig. 1. Names and chemical structures of polyamines detected in the cells of *T. thermophilus*

temperature such as 65 °C (Oshima, 1989). This observation supports the hypothesis that long and/or branched polyamines are important for life at temperature extremes.

Interestingly, long polyamines and branched polyamines are also identified in the cells of extreme thermophiles (both bacterial and archaeal). Often these polyamines are major polyamine components in hyperthermophilic organisms. For example, hyperthermophilic bacterium, *Thermotoga maritima*, and hyperthermophilic archaeon, *Aeropyrum pernix*, produce long polyamines as well as the standard polyamines (Kneifel et al., 1986; Hamana et al., 1998). The quaternary amine is the major polyamine in the cells of hyperthermophilic bacterium, *Aquifex pyrophilum*, and hyperthermophilic archaeon, *Methonococcus jannaschii* (Kneifel et al., 1986; Hamana et al., 1999).

Though long and/or branched polyamines are found in cells of extreme thermophiles, these unique polyamines are not detected in moderate thermophiles. For instance, tetrakis(3-aminopropyl)ammonium which is the major polyamine in hyperthermophilic and methanogenic archaeon, *M. jannashii*, but the amine is not detected in moderately thermophilic and mesophilic methanogens, suggesting important roles of these unique polyamines in biochemical reactions at high temperatures. Hamana and Kato (2000) reported the presence of tetrakis(3-aminopropyl)ammonium in hot spring sulfur-turf bacterial mats, suggesting the existence of unidentified thermophile(s) which can produce the quaternary amines. However, there are some mysterious issues to the roles of unique polyamines at high temperatures. Some of extreme thermophiles do not produce either long or branched polyamines. An example is Sulfolobus species (an archaeal hyperthermophilic acidophile). The acido-thermophilic archaeon produces unusual polyamines such as caldine and thermine, but does not produce either longer polyamines than tetraamine or branched amines (Friedman and Oshima, 1989). Generally these unique polyamines are not produced by moderate thermophiles and mesophilic organisms, but rare exceptions have been reported. For example, some of aquatic plants produce branched polyamines along with homospermidine and thermospermine. Branched polyamines were also detected in seeds of legume (Hamana et al., 1992, 2000).

Polyamines are essential for protein synthesis at high temperature

In vitro protein synthesis at high temperature (65 °C or higher) using a cell-free extract of *T. thermophilus* requires the presence of polyamines (Ohno-Iwashita et al., 1975). The reaction at lower temperature such as 37 °C did not require the presence of polyamine though the activity was poor. At 65 °C or higher temperatures, no activity was detected in the absence of polyamine. The addition of a tetraamine or tetrakis(3-aminoproppyl)ammonium recovered the cell-free protein synthesis catalyzed by an extract of *T. thermophilus*, but the activity depended on polyamine added. Incorporation of phenylalanine to trichloroacetic acid insoluble fractions was much faster in the presence of spermine than that in the presence of thermine. The presence of polyamines is absolutely required and magnesium ion did not replace the roles of polyamines in the cell-free translation at 65 °C. When translation was carried out using a natural messenger such as MS2 RNA instead of polyuridylic acid (poly(U)), polyamines were also essential but a lesser effect was observed.

The role of polyamine is not prevention of thermal denaturation of protein(s) or nucleic acid(s) involved in the protein biosynthesis, since the full activity was observed in an experiment in which a polyamine was added after 5 min of incubation at 65 °C. Our detailed studies revealed that polyamines are essential for the formation of proper structure of the initiation complex between 30S ribosomal subunit, the messenger, and the initial aminoacyl-tRNA. Polyamines also enhanced the elongation step, but polyamines are not essential for this step. Similar enhancement by polyamine was recorded when in vitro protein synthesis was studied using

a *Sulfolobus acidocaldarius* cell-free extract and poly(U) (Friedman and Oshima, 1989).

When poly(U) is used as a messenger, the highest activity was recorded in the presence of tetrakis(3-aminopropyl)ammonium (Uzawa et al., 1993). This conclusion was obtained when peptide bond formation was measured using phenylalanyl-tRNA^{Phe} as the substrate of the in vitro reaction. If incorporation of free phenylalanine guided by poly(U) was recorded, the highest activity was observed in the presence of the branched quaternary polyamine and one of the long, straight polyamines such as spermine or caldopentamine. This synergic effect was not observed if phenylalanyl-tRNA formation was done prior to the translation reaction in order to measure the rate of polypeptide formation.

Uzawa et al. (1994) found that tetrakis(3-aminopropyl) ammonium supports the polypeptide bond formation in the presence of poly(U) and phenylalanyl-tRNA^{Phe}, but the branched amine inhibits the phenylalanyl-tRNA formation reaction. The branched polyamine did not inhibit aminoacyl-tRNA synthesis of other amino acids; only Phenylalanyl-tRNA synthesis was inhibited. The inhibition was observed even when the thermophile tRNA or cell-free extract was replaced with the corresponding ones of *Escherichia coli*, but no inhibition was recorded when *E. coli* tRNA and *E. coli* cell-free extract were used. The inhibition was also found when yeast tRNA^{Phe} was used instead of *T. thermophilus* tRNA^{Phe}.

The addition of spermine relieved the inhibition by tetrakis(3-aminopropyl)ammonium. This observation explains why synergetic effects of the branched polyamine and long polyamine were observed in vitro polyphenylalanine synthesis from free phenylalanine. Molecular mechanisms of the inhibition reaction have not been clarified yet. Kawai et al. (unpublished) investigated the interaction of tetrakis(3-aminopropyl)ammonium with yeast tRNA^{Phe} using NMR. The preliminary results suggested that the branched polyamine interacts with some of nucleotide residues in D-loop of the tRNA. The branched polyamine may induce a conformational change of tRNA^{Phe} to an inactive form, and spermine competes with the branched amine.

Thermal protection of nucleic acids by the unique polyamines

It has been well known that polyamines can bind to nucleic acids, induce conformational change of nucleic acids and stabilize them (Minyat et al., 1978; Behe and Felsenfeld, 1981; Wemmer et al., 1985; Base and Marton, 1987; Hou et al., 2001; Vijayanathan et al., 2001). We systematically investigated the effects of unique polyamines produced by *T. thermophilus* in comparison with the standard polyamines (Terui et al., 2005). The raise of melting temperature (T_m) of a synthetic double-stranded oligodeoxyribonucleotide was measured using a differential scanning calorimeter.

When stability of the synthetic DNA is compared in the presence of a variety of linear polyamines, the T_m is proportional to the number of amino groups; that is, T_m is higher if a longer polyamine was added. We compared the stabilizing effects of homologous polyamines such as norspermidine, spermidine and *sym*-homospermidine, T_m is also proportional to the number of aminobutyl groups. T_m is higher in the presence of homocaldopentamine (containing one aminobutyl group) than in the presence of caldopentamine (consisting of only aminopropyl groups).

Many extreme thermophiles produce long polyamines such as caldopentamine and caldohexamine. These longer polyamines may effectively stabilize double stranded structures such as DNA and stem parts of RNAs in the thermophile cells at physiological temperatures. However, polyamines containing aminobutyl group(s) such as homocaldopentamine and homocaldohexamine are generally minor components of polyamines in extreme thermophiles, though our studies indicated that the aminobutyl containing polyamines are more effective to stabilize double stranded structures than polyamines consisted of only aminopropyl groups such as caldopentamine and caldohexamine.

Branched polyamines did not obey the empirical rules mentioned above; T_m in the presence of a branched polyamine was lower than that in the presence of its isomeric linear polyamine. For instance, T_m of the synthetic DNA oligomer in the presence of tetrakis(3-aminopropyl)ammonium was lower by 2.3 °C than that in the presence of caldopentamine though these two polyamines are structural isomers to each other, their chemical formulae are identical ($C_{12}H_{36}N_5$) and they consist of four aminopropyl groups.

When stability of a purified tRNA was examined, the highest T_m was recorded in the presence of tetrakis(3-aminopropyl)ammonium among the polyamines tested. We speculated that in the thermophile cells, long polyamines and branched polyamines share the responsibility of stabilizing nucleic acids; long polyamines stabilize double stranded DNA and stem parts of RNAs and branched polyamines stabilize loop parts of RNAs.

DNA depurination is thought to be one of the most serious causes of DNA damage even in mesophilic cells. Depurination reaction accelerates at high temperature,

and thus to suppress the reaction seems to be essential for extreme thermophiles. It has been observed that polyamines protect DNA from depurination (Andreev and Kaboev, 1982).

Terui et al. (unpublished) investigated the protection effects of unusual polyamines at high temperatures and found that longer polyamines prevent the reaction more effectively than shorter ones. Caldohexamine was the best polyamine to protect thermal depurination of DNA among polyamines tested. The quaternary amine, tetrakis(3-aminopropyl)ammonium, also protected DNA from thermal depurination, but at a slightly lesser degree than that in the presence of its structural isomer, caldopentamine.

Polyamine biosynthesis in *Thermus thermophilus*

Genome sequence of *T. thermophilus* has been completed and whole sequences of two strains, strain HB8 and HB27, are available (http://www.thermus.org/e_index.htm and http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org = nttt02). Our homology search revealed the presence of *speA* (arginine decarboxylase), *speB* (agmatine ureohydrolase), *speD* (SAM decarboxylase), and *speE* (spermidine synthase or spermine synthase) homologs in the chromosomal DNA of *T. thermophilus*. Homolog of *speC* (ornithine decarboxylase) is not found suggesting the lack of ornithine decarboxylase in *T. thermophilus*.

Figure 2A shows general metabolic pathways of polyamine biosynthesis in prokaryotes. Putrescine can be synthesized from either decarboxylation of ornithine or decarboxylation of arginine followed by hydrolysis with liberation of urea. Spermidine is synthesized by an aminopropyl transfer reaction to putrescine, and spermine is synthesized from spermidine by a similar reaction.

We knocked out genes homologous to *speA*, *B*, *D*, and *E*, and polyamine compositions of the knockout mutants were analyzed (Ohnuma et al., unpublished). All knockout mutants can grow at 70 °C, but not at 75 °C or higher. Colonies of the mutants are colorless though the wild type cells are bright yellow due to the presence of carotenoids in the membrane. All mutants could not produce unique polyamines, both long polyamines such as caldopentamine and caldohexamine, and branched polyamines such as tetrakis(3-aminopropyl)ammonium. The growth at 75 °C and pigment production of *speA* mutant were restored by the addition of spermidine, caldopentamine or the quaternary polyamine. These observations suggest that (1) spermidine is a key intermediate for the synthesis of unique polyamines, (2) unique polyamines are essential for the production of carotenoid dyes, and (3) unique polyamines are essential for the life at high temperature extremes. A possibility is that the physiological activity of the carotenoid dyes such as anti-oxidant activity is essential for the cells at 75 °C or higher temperatures.

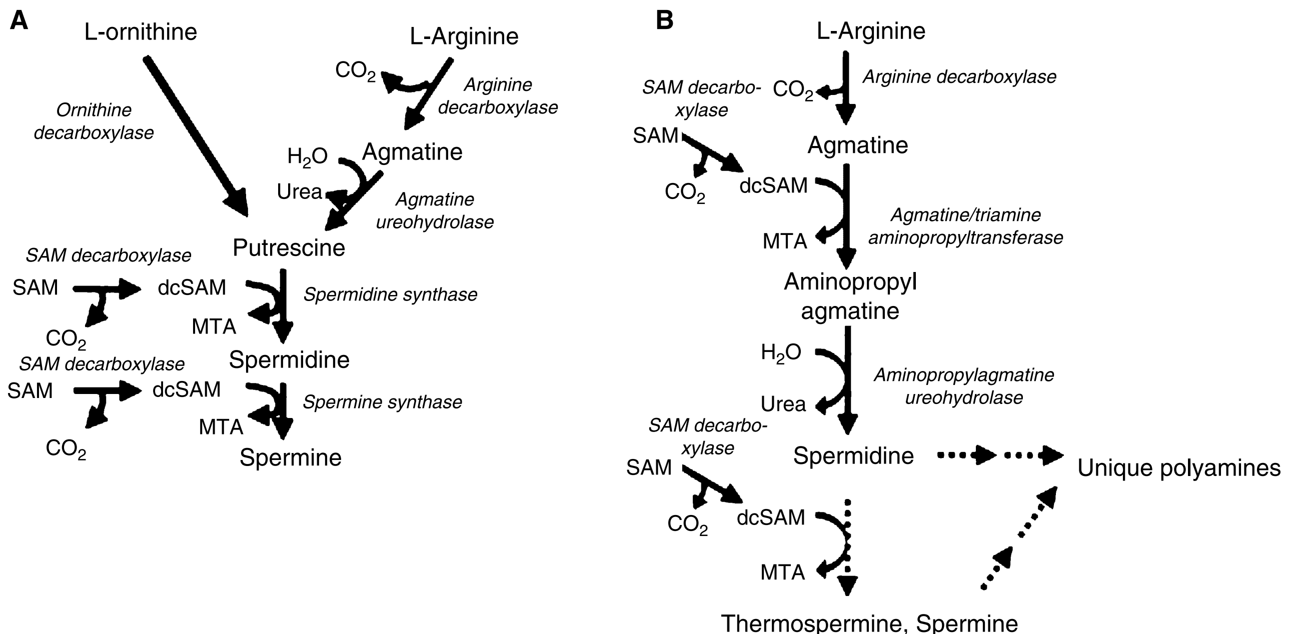

Fig. 2. Polyamine biosynthetic pathways. **A** General pathways found in prokaryotes. **B** New pathway found in *T. thermophilus*. *SAM* S-adenosymethionine, *deSAM* decarboxylated SAM, *MTA* methylthioadenosine. Each straight arrow indicates an enzyme reaction and the trivial name of the enzyme which catalyzes the reaction is shown along with the arrow using italics

The *speA* mutant did not produce polyamines except a small amount of diamines. The finding indicated that polyamines in *T. thermophilus* are synthesized from arginine, but not from ornithine (Ohnuma et al., 2005). We expected that *speB* mutant produces and accumulates agmatine, the product of arginine decarboxylase coded by *speA* gene. In contrast to our expectation, only small amount of agmatine was detected and instead an unknown polyamine was found in the cells of *speB* mutant cells. The unknown polyamine accumulated in the mutant cells was identified to be aminopropylagmatine indicating that agmatine was aminopropylated by the actions of *speD* and *speE* expression products. This speculation was proved by the observation that agmatine was accumulated in *speD* or *speE* mutant.

These reverse genetic studies suggested a new metabolic pathway for biosynthesis of polyamines in *T. thermophilus* and the pathway proposed is shown in Fig. 2B (Ohnuma et al., 2005). In this pathway, arginine is the starting material for polyamine synthesis in the extreme thermophile and is converted to agmatine by the action of speA. The second step is aminopropylation of agmatine and the product, aminopropylagmatine, is then converted to spermidine by the action of speB. In this metabolic pathway, spermidine is not synthesized from putrescine.

The proposed metabolic pathway shown in Fig. 2B has been supported by our enzymatic studies (Ohnuma et al., to be published). We purified speE (the expression product of *speE*) and the purified enzyme catalyzed aminopropylation of agmatine as expected. The enzyme also accepted spermidine and caldine (= norspermidine) as substrates, but not putrescine. The enzyme specificity also indicated that spermidine is not produced from putrescine in *T. thermophilus*. The enzyme coded by *T. thermophilus speB* gene did not accept agmatine as its substrate, but accepted aminopropylagmatine and converted it to spermidine. These enzymatic studies confirmed the pathway shown in Fig. 2B.

The new pathway may be not limited to *T. thermophilus*, but may exist in other organisms. Currently we are looking for the distribution of the pathway shown in Fig. 2B in other extremophiles.

Our investigation on biosynthesis of polyamines in *T. thermophilus* is still imperfect and many enigmas remain to be solved in future studies. Under standard conditions, the extreme thermophile produces putrescine though the amount is extremely small. The synthetic pathway of putrescine is not known. Double knockout mutant of *speD* and *speE* produced a large amount of putrescine and *sym*-homospermidine along with a relatively small amount of agmatine. At moment, it is hard to explain this finding.

Only one *speE* homolog was found in genome sequence of *T. thermophilus*. The expression product of the gene catalyzed aminopropyl transfer reaction, however the enzyme accepts only agmatine, spermidine, and caldine as substrates and produces aminopropylagmatine, spermine, and thermine, respectively. For biosynthesis of other polyamines, other aminopropyltransferase(s) are necessary. For instance, caldopentamine may be synthesized from thermine by aminopropyl transfer reaction. Since no other gene homologous to *speE* was found, the caldopentamine synthesizing enzyme should be an enzyme belonging to a different enzyme family from one containing spermidine synthase and spermine synthase. Likewise tetrakis(3-aminopropyl)ammonium may be derived from mitsubishine by aminopropyl transfer reaction. Nothing is known about tetrakis(3-aminopropyl)ammonium synthase.

Discussions and conclusions

Experimental results suggest the importance of unique polyamines in biochemical reactions such as protein biosynthesis and nucleic acid stabilization in extreme thermophiles. In *T. thermophilus*, it is revealed that polyamines are synthesized from arginine by a new pathway though genes and enzymes involved in the production of unique polyamines remain unsolved.

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